"EXPLORING ANTI-CANCER POTENTIAL OF OMEGA 3 FATTY ACIDS AND SELECTED MEDICINAL PLANTS"

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SUBMITTED BY

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Certificate

This is to certify that the work incorporated in the thesis entitled "**Exploring Anti-Cancer Potential of Omega 3 Fatty Acids and Selected Medicinal Plants**" for the degree of 'Doctor of Philosophy' in the subject of Microbiology under the faculty of Science has been carried out by Ms. Rashmi Anil Deshpande in the Department of Cell and Translational Research Laboratory, Interactive Research School for Health Affairs, Bharati Vidyapeeth Deemed University,Pune during the period from 2010 to 2015 under the guidance of Dr. Ruchika Kaul-Ghanekar.

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I hereby declare that the thesis entitled "**Exploring Anti-Cancer Potential of Omega 3 Fatty Acids and Selected Medicinal Plants**" submitted by me to Bharati Vidyapeeth University, Pune for the degree of Doctor of Philosophy (Ph.D.) in Microbiology under the faculty of Science is original piece of work carried out by me under the supervision of Dr. Ruchika Kaul-Ghanekar and Dr. P. K. Ranjekar. I further declare that it has not been submitted to this or any other university or Institution for the award of any degree or Diploma.

I also confirm that all the materials which I have borrowed from other sources and incorporated in this thesis are duly acknowledged. If any material is not duly acknowledged and found incorporated in this thesis, it is entirely my responsibility. I am fully aware of the implications of any such act which might have been committed by me advertently or inadvertently.

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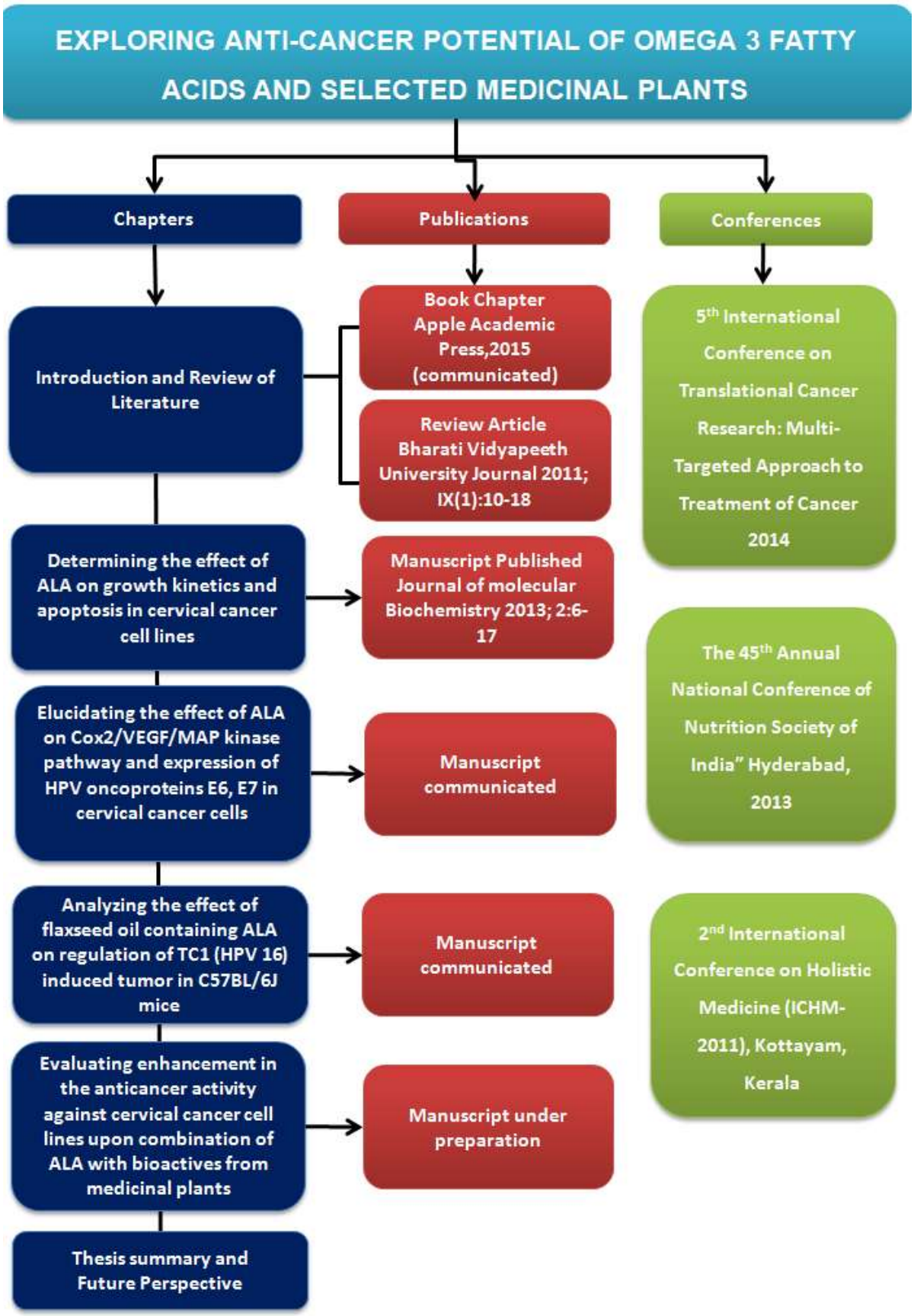
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Abbreviations and Symbols

AA	Arachidonic Acid
AAPH	2, 2‟ azobis- 2- methyl - propanimidamide
ALA	Alpha Linolenic Acid
ASR	Age Standardized incidence Rate
BAX	BCL2 Associated X protein
BCL- 2	B- Cell Lymphoma 2
BSA	Bovine Serum Albumin
CAM	Complementary and Alternative Medicine
CIN	Cervical Intraepithelial Neoplasia
CIS	Carcinoma in- Situ
COX-2	Cyclooxygenase-2
CT	Chemotherapy
DAF- FM	4- Amino- 5 Methylamino- 2,7 difluorofluorescein
DHA	Docosahexanoic Acid
DMEM	Dulbecco's Modified Eagles Medium
DMF	Dimethylformamide
dNTP	Deoxynucleotide triphophates
DTT	Dithiothreitol
ECM	Extracellular Matrix
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Eicosapentanoic Acid
ERK	Extracellular signal-Regulated Kinase
FAME	Fatty Acid Methyl Ester
FBS	Fetal Bovine Serum
FIGO	International Federation of Gynecology and Obstetrics
FITC	Fluorescein isothiocyanate
FO	Flax Oil
GLA	Gamma Linoleic Acid

HBSS	Hank‟s Balance Salt Solution
HEPES	Hydroxyethyl piperazinethanesulfonic acid
HER- 2	Human Epithelial Receptor- 2
HPV	Human Papilloma Virus
HR- HPV	High Risk Human Papilloma Virus
HRP	Horse Raddish Peroxidase
HSIL	High- grade Intraepithelial Lesion
HSV	Herpes Simplex Virus
IARC	International Agency for Research on Cancer
IFN – γ	Interferon - gamma
IL	Interleukin
JC- 1	5,5’,6,6’-tetrachloro-1,1’3,3’tetraethylenzamidazolocarbocyanin iodide
LA	Linoleic Acid
LPO	Lipid Peroxidation
LR- HPV	Low Risk Human Papilloma Virus
LSIL	Low- grade Squamous Intraepithelial Lesion
MAPK	Mitogen‑activated protein kinase
MMP- 2	Matrix Metalloproteinase- 2
MMuLV	Moloney Murine Leukemia Virus
MTT	4,5- dimethylthiazol - 2- yl - 2,5- diphenylthiazolium bromide
MUFA	Monounsaturated fatty acid
NCCS	National Centre for Cell Science
NF- kB	Nuclear Factor kappa- B
NK	Natural Killer
NO	Nitric Oxide
OD	Optical Density
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate Buffered Saline
PMSF	Phenylmethanesulfonyl fluoride
PUFA	Polyunsaturated Fatty Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute

RT- PCR	Reverse Transcription Polymerase Chain Reaction
RT	Radiotherapy
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SFA	Saturated Fatty Acid
TBARS	Thiobarbituric Acid Reactive Substances
TIMP	Tissue Inhibitor of Metalloproteinase
TMP	1, 1, 3, 3-tetraethoxypropane
VEGF	Vascular endothelial growth factor
$\Delta\Psi$	Mitochondrial Membrane Potential

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Chapter 1

Review of Literature

SECTION 1

Cervical Cancer and Associated Molecular Pathways

The review given in this chapter has been communicated in Book Chapter in Apple Academic Press, 2015

1.1.1. Cervical Cancer

Cancer of the cervix uteri is the fourth most common cancer among women worldwide (Bruni 2014). Human papilloma virus (HPV) is the main causative agent of cervical cancer. Almost 99.7 % of cervical cancer is associated with HPV infection. Globocan 2012 has reported the ratio of mortality to incidence to be around 50.3% (GLOBOCAN 2012) in cervical cancer. Mortality has been found to vary in different regions of the world, ranging from less than 2 per 100,000 women in Western Asia, to nearly 28 per 100,000 in East Africa. Chances of survival were found to be 70% in Europe and United States of America, 58% in Thailand, 42% in India, and 21% in sub-Saharan Africa.

1.1.2. Cervical cancer burden in India

Cervical cancer ranks as the second major female cancer and leading cause of mortality in India (Bruni 2014). It accounts for 17% of all cancer deaths among women aged between 30 to 69 years. The current incidence rates in India have been projected to increase to 225,000 by 2025. According to HPV and related disease report 2014, in India around 122,844 new cervical cancer cases were diagnosed annually and 67477 were reported to die from the disease. Indian women have been projected to face 2.8% cumulative lifetime risk and 1.7% cumulative death risk from cervical cancer (Bruni 2014). Around 38% of cases have been found to occur among women between the reproductive age of 15–49 years. The mortality due to cervical cancer in India was found to be substantially high relative to the world and South Asia region. In India, approximately 84.1% of invasive cervical cancers have been attributed to most common HPVs 16 or 18 (Table 1.1.1). Table 1.1.1 gives an estimate of burden of HPV associated cervical cancer among Indian women.

Table 1.1.1. Burden of cervical cancer due to HPV infection in India

Incidence of HPV 16/18 among women with	Rate (%)
Normal cytology	5.0
Low-grade cervical lesions (LSIL/CIN-1)	28.2
High-grade cervical lesions (HSIL/ CIN-2 / CIN-3 / CIS)	62.8
Cervical cancer	82.7

Source: *Bruni, L. ICO Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in the India Summary Report 2014-12-18.*

1.1.3. Human Papilloma Virus

Human papilloma virus (HPV) family has been reported to contain more than 100 types of viruses (Castellsagué *et al.,* 2008; Herrero *et al.,* 2003). Approximately 40 HPV types have been known to affect the genital area. Cutaneotropic viruses (HPVs 1, 4, 5, 8, 41, 48, 60, 63 and 65) have been isolated from the patients suffering from cutaneous lesions, warts, verruciform, epidermo-dysplasia, and also from some epithelial tumors (Castellsagué *et al.,* 2008). Another group of HPVs are mucosotropic (HPVs 6, 11, 13, 44, 55, 16, 31, 33, 35, 52, 58, 67, 18, 39, 45, 59, 68, 70, 26, 51, 69, 30, 53, 56, 66, 32, 42, 34, 64, 73, and 54) and they have also been identified in benign and malignant lesions of the genital tract in both the sexes (Castellsagué *et al.,* 2008). Occasionally, these viral types have been isolated from tissues and lesions of the oral cavity, oropharynx, larynx and esophagus. HPV presence in oral and head-neck cancers account for 5-11% and 11-25%, respectively (Ajay *et al.,*2012). Papillomaviruses have also been reported to be present in the colon (30%) and 90% of anal cancers (Damin *et al.,* 2013; Ajay *et al.,*2012). HPV has been classified as "low-risk" (wart-causing) or "high-risk"

according to their oncogenic potential. Low-risk (LR) HPV types include 6, 11, 42, 43, and 44 and high-risk (HR) types include 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70 (Ajay *et al.,*2012).

1.1.4. Structure of Human Papilloma Virus

HPVs belong to the Papovaviridae family and the genome consists of double-stranded, circular DNA of ~8 Kb size (Stanley *et al.,* 2007). It has an icosahedral capsid composed of 72 capsomers, which contain two capsid proteins, L1 and L2. The genome (Figure 1.1.1) has been divided into three regions: 1) **Early region**, consisting of genes encoding nonstructural proteins (E1, E2, E4, E5, E6, and E7), which are involved in viral replication and oncogenesis; 2) **Late region**, encoding L1 and L2 structural proteins for the viral capsid; and 3) **Upstream regulatory region (URR)**, also referred to as the long control region (LCR), containing a DNA replication origin, transcriptional regulatory sequence and one/more promoters, which regulate the expression of E6 and E7 oncoproteins (Doorbar *et al.,*2006).

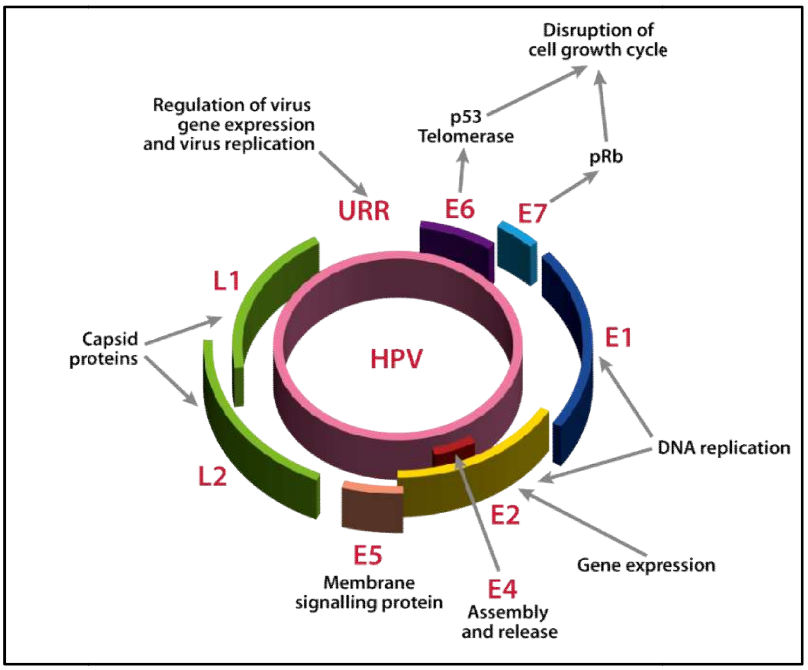

Figure 1.1.1. Schematic representation of the circular HPV DNA genome representing early and late genes.

Source: Stanley M Pathology and epidemiology of HPV infection in females. Gynecol Oncol. 2010 May; 117 (2 Suppl): S5-10.

1.1.5. HPV infection of cervix

HPV infects epithelial basal cells through mechanical micro-abrassions or by infecting the transformation zone, leading to rapid transition from a columnar to a squamous epithelium (**Phase 1**, Figure 1.1.2). The infected cells divides laterally.The hallmark of malignant transformation by HPV is the integration of the viral genome into the host cell genome. In order to maintain the viral DNA as an episome, early proteins E1, E2, E6 and E7, are actively expressed in infected cells (**Phase 2**, Figure 1.1.2) (Stanley *et al.,* 2007; Kines *et al.,* 2009).Further,amplification of viral genome (episome) begins in parabasal and squamous layer cells and requires expression of all viral early gene products including E4 and E5 and the late capside genes L1 and L2 (Kajitani *et al.,* 2012) (**Phase 3**, Figure 1.1.2).

HPV is non-lytic and remains within the epithelial cell. Episome is encapsidated in the nucleus of the upper layer epithelium with the help of L1 and L2 capsid proteins (**Phase 4**, Figure 1.1.2).These viral particles can infect new zones of epithelium or can be sexually transmitted. Functions of HPV oncoproteins in the virus life cycle have been summarized in Table 1.1.2.

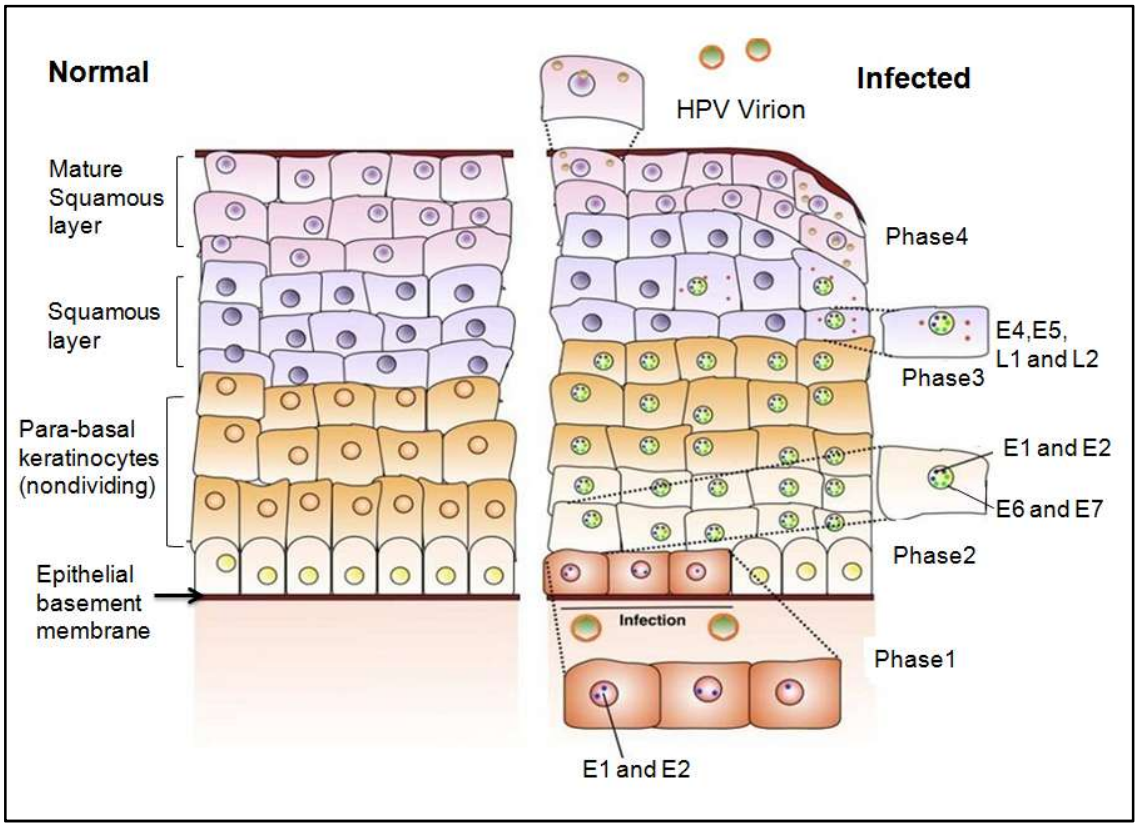

Figure 1.1.2. Events associated with transformation of normal cervical cells to cancerous phenotype after HPV infection.

Source: Molecular Events Towards Wnt Pathway Activation in Cervical Cancer: Changing the Balance on NKD/DVL Signals 2011. Book chapter "Human Papillomavirus and Related Diseases - From Bench to Bedside - Research aspects.

Table 1.1.2. Functions of HPV oncoproteins

Oncoprotein	Functions
E1	Recruits helicase, ATP-binding protein essential for viral DNA replication
E2	Viral transcription factor binds E1 to facilitate initiation of viral DNA replication important in genome encapsidation
E4	Interacts with cytoskeletal proteins, and involved in viral release at the epithelial cell layer surface
E5	Up-regulates growth factor receptors
E6	Binds to p53 and degrade it through ubiquitin-mediated pathway
E7	Associated with retinoblastoma protein and deregulates the G1/S checkpoint
L2	Minor capsid protein
L1	Major capsid protein

1.1.6. Molecular mechanisms affected by HPV infection during cervical cancer

A number of molecular pathways are modulated during cervical cancer. Some of these have been discussed below.

1.1.6.1. Lipid peroxidation and Nitric Oxide Release

Lipid peroxidation (LPO) plays an important role in the initiation and promotion of cancer (Banakar *et al.,* 2004). It causes oxidation of biomembranes as well as modulation of inter- and intracellular signaling networks resulting in changes in cell proliferation, differentiation and apoptosis. Excessive levels of malondialdehydes (MDA), a product of lipid peroxidation initiated by ROS, could alter the cellular function and lead to cancer (Barrera *et al.,* 2012). Thus, by measuring lipid peroxides as thiobarbituric acid reactive substance (TBARS) provides an indirect measure of the antioxidant deficit (Beevi *et al.,* 2004). Some earlier studies

have reported reduced antioxidant levels and increased LPO associated with cervical cancer patients (Agarwal *et al.,* 2009; Kim *et al.,* 2003).

Nitric oxide, a measure of RNS, is an endogenously produced free radical and involved in many physiological functions (Hofseth *et al.,* 2008, Hogg & Kalyanaraman 1999). The enzyme responsible for the conversion of L-arginine to NO is the nitric oxide synthase (NOS) that exists in three major isoforms; inducible (NOS II/iNOS), endothelial (NOS III/eNOS) and neuronal NOS (NOS I/nNOS).NO has been shown to play a dual role in cancer (Nakamura Y 2006). In cancer cells, increased NO generated by iNOS contributes to tumor angiogenesis by up-regulation of vascular endothelial growth factor (VEGF), which may increase metastasis (Fukumura *et al.,* 2006; Coulter *et al.,* 2008). NO has been reported to activate the p53 pathway by activating p53 which ultimately results into apoptosis. In cervical cancer patients, the presence of HR-HPV has been shown to be associated with increased release of NO and progression of malignancy (Beevi *et al.,* 2006; Hiraku *et al.,* 2007). Patients with cervical squamous cell carcinoma have shown increased plasma NO levels (Murta *et al.,* 2008; Wei *et al.,* 2011).

Thus, both oxidative and nitrosative stress are involved in the pathogenesis of cervical cancer.

1.1.6.2. Apoptosis and Cell Cycle

Apoptosis or programmed cell death is a tightly regulated process that plays an important role in development, homeostasis, and antiviral defense mechanism (Lowe*et al.,* 2000). Any defects in this process may lead to the development of malignant tumors (White *et al.,* 2006). Human papilloma virus has developed

strategies to block host mediated apoptosis; and to modulate the host cell machinery for viral gene expression and replication (Hay *et al.,* 2002; Benedict *et al.,* 2002; Moody *et al.,* 2007). One of the functions of HR-HPV E6 oncoprotein is the proteolytic inactivation of certain pro-apoptotic proteins such as p53 (Lagunas-Martínez *et al.,* 2010), Bak (Lagunas-Martínez *et al.,*2010), FADD (Lagunas-Martínez *et al.,* 2010), procaspase-8 (Filippova*et al.,* 2004), or c-Myc (Garnett *et al.,*2006) through ubiquitin proteasome pathway, which leads to inhibition of apoptosis. It has been reported that HPV-positive cervical cancer patients and cell lines display decreased expression of caspases (Aréchaga-Ocampo *et al.,* 2008).

p53 and Retinoblastoma (Rb) are tumor suppressor proteins that are required for regulation of cell growth (Tan *et al.,* 2012; Stanley *et al.,* 2007). HPV E6 and E7 oncoproteins deregulate the host cell growth cycle by binding and inactivating tumor suppressor proteins, cell cyclins, and cyclin dependent kinases (Fehrmann *et al.,* 2003). Binding of E6 and E7 to p53 and pRb, respectively, disrupts the normal function of these cellular proteins resulting into increased cell proliferation rate and genomic instability (Figure 1.1.3).

E6 targets the degradation of tumor suppressor protein p53 through ubiquitin dependent pathway (Shillitoe 2006). E6 binds with E6-associated protein (E6-AP), a cellular protein with known E3 ligase activity (Figure 1.1.3). This complex binds to p53, leading to its ubiqutinization and degradation by 26S proteasome. Degradation of p53 leads to loss of its tumor suppressor activity and subsequent inhibition of apoptosis (Nevins 2001). p21WAF1/CIP1, a downstream target of p53,is a cyclin-dependent kinase inhibitor whose expression is induced by p53 and it is required for the blockage of the cell cycle progression from G1 to S phase (Beskow *et al.,* 2009; Tan *et al.,* 2012). Due to degradation of p53 by HPV E6-E6AP complex, it is not

available for activation of p21 and this leads inhibition of G1 arrest resulting into progression of cell cycle leading to cell proliferation of cancer cells (Kim *et al.,*2005).

HPV E7 protein binds to the hypophosphorylated form of Rb protein (Wang *et al.,* 2001). This binding disrupts the complex between pRb and the cellular transcription factor E2F-1, resulting into its liberation that leads to the transcription of genes required for entry of cells into the S phase (Chinnam and Goodrich 2011)(Figure 1.1.3). E7 also increases transcription of cyclin E (CDK), leading to cell cycle progression and proliferation (Avcl 2012;Kim *et al.,* 2005). E7 interacts with p21 to abrogate p21-mediated inhibition of cyclin-E–CDK (cyclin dependent kinase) complex, thereby resulting into cell-cycle progression.

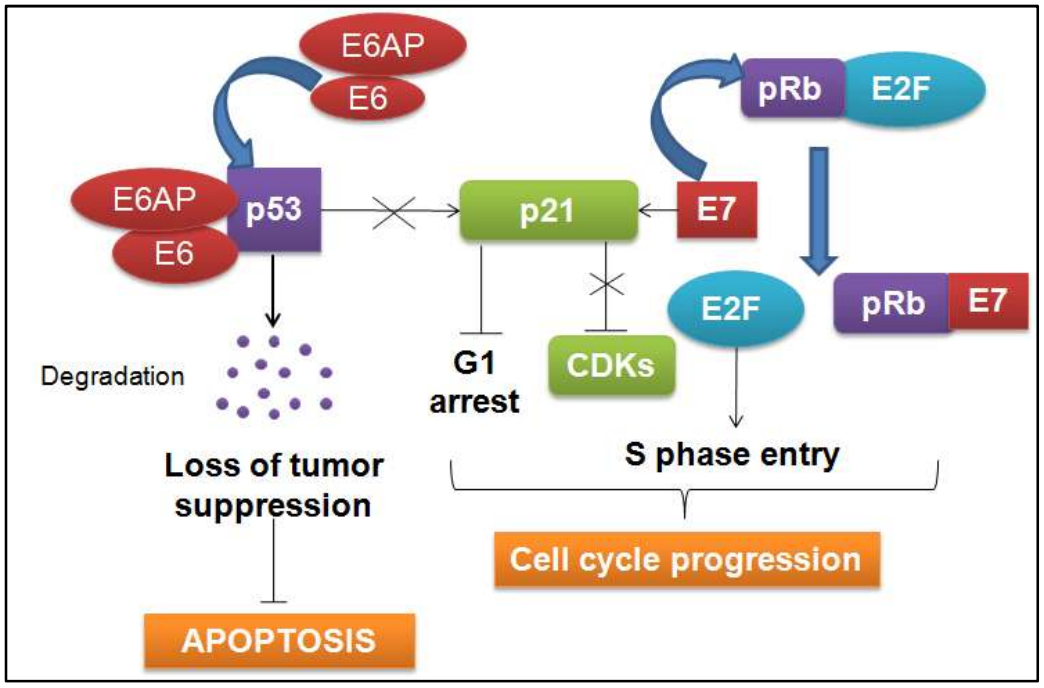

Figure 1.1.3. Mechanisms involved in HPV E6 and E7 mediated cervical cancer development. *HPV E6 binds to E6-AP and this complex binds to p53, which results in the ubiquitination and degradation of p53 leading to loss of tumor suppressor activity and inhibition of apoptosis. Binding of p53 with E6-E6AP complex results into loss of activation of p21 leading to progression of cell to G1 and S phase.E7 binds to Rb protein resulting into release of E2F and leading to entry of cells into the S phase.*

1.1.6.3. Angiogenesis

Angiogenesis (generation of new blood vessels)is an essential processin tissue development, reproduction, and wound healing (Wu *et al.,* 2005). It plays an important role in growth, progression, and metastasis of tumors. HIF1 α, VEGF, MMPs and HER2 are essential pro-angiogenic markers.

Hypoxia-Inducible Factor (HIF1α) is induced in response to hypoxic condition that is found in the tumor microenvironment (Jiabin *et al.,* 2008). Up-regulation of HIF1α results into increased expression of growth factors, including vascular endothelial growth factor (VEGF) (Wang *et al.,* 2014). HPV infection has been shown to increase expression of HIF-1α protein, indicative of poor prognosis and weak response to chemotherapeutic treatment in cervical cancer patients (Kim *et al.,*2011). HPV E6 has been shown to independently enhance induction of HIF1α in various experimental models (Jiabin *et al.,* 2008).

Vascular endothelial growth factor (VEGF) is an important growth factor involved in regulation of angiogenesis (Céline *et al.,* 2014). It has been shown to be upregulated in various cancers including cervical cancer. VEGF inhibitors block the growth of malignant cells by inhibiting blood vessel formation through interference with signaling pathways involved in proliferation and metastasis of tumors (Chien *et al.,* 2013). A recent clinical trial showed that inhibition of VEGF reduced resistance to radiation and drug therapy in colorectal, lung and breast cancer (Loges *et al.,* 2010). HPV contributed to angiogenesis in cervical cancer by upregulation of angiogenic factors including VEGF (Hammes *et al.,* 2008). Its up-regulation has been correlated with different stages of cervical cancer (CIN) lesions as well as invasive disease (Chien *et al.,* 2013).

Matrix metalloproteinases (MMPs) belong to a family of zinc proteases that have been found to be responsible for degradation of extracellular matrix, which is required for cell migration, metastasis and angiogenesis (Rajkumar *et al.,* 2011). Recent studies have indicated an association between MMP expression and HPVinfection in cervical cancer (Hagemann 2007). Elevated expression of MMP 2 (Ung *et al.,* 2002), 9 (Deryugina *et al.,* 2006), 13 (Boccardo *et al.,* 2010) and 15 (Boccardo *et al.,* 2010) has been reported in invasive cervical carcinomas. Increased levels of MMP-2 and MMP-9 were correlated with poor prognosis in cervical cancer patients (Song *et al.,* 2013). In addition, HPV 16 E6 and E7 have been shown to specifically up regulate MMP-2 and MT1-MMP, which promote cervical cancer invasion (Kaewprag *et al.,* 2013).

HER-2 receptor (also known as c-erbB-2) is a transmembrane receptor protein whose over-expression is linked with poor prognosis, metastatic potential, aggressive behavior and therapeutic resistance in cervical cancer (Gupta *et al.,* 2009; Minner 2010; Joseph *et al.,* 2015). Increased expression of Her2 has been reported to upregulate expression of MMP2, MMP9 and COX-2, that inturn leads to invasion of cancer cells to other organs (Johanna *et al.,* 2004; Wang *et al.,* 2010). Increased Her2 expression has been reported to positively correlate with poor prognosis and tumor aggressiveness in cervical carcinoma (Joseph *et al.,* 2015). Thus, HER2/neu oncogene is a potential therapeutic target in cervical cancer.

1.1.6.4. Inflammatory markers associated with cervical cancer

Numerous studies have shown relationship between inflammation and cancer (Young *et al.,* 2008). Chronic inflammation has been linked to different steps of cancer such as cellular transformation, survival, proliferation, invasion, angiogenesis,

and metastasis (Mantovani *et al.,* 2005; Coussens *et al.,* 2002). Different inflammatory mediators have been shown to be involved in cancer progression which includesinterleukins, NF-kB, COX-2, prostaglandin (PGE2), lipoxygenase, cytokines (TNFα) and chemokines.

NF-kB (nuclear factor kB) is a transcription factor found to mediate several pathways related to inflammation, cell survival, cell proliferation, invasion, angiogenesis, metastasis and chemo-resistance (Hoesel *et al.,* 2013). One of the studies revealed that, NF-kB precursors, p100, p105, p65 (Rel 65) were present in high amount in HPV-16 cervical carcinoma. Increased expression of p65 (Rel 65) leading to progression of cervical intraepithelial neoplasia lesion to cancer (Branca et al 2006). E6 and E7 oncoproteins have been reported to increase the activity of NF-kB (Dwarampudi *et al.,* 2013). Cervical cancer progression has been shown to be positively associated with the activation of NF-κB and different target genes, VEGF and MMPs which promote the proliferation and metastasis of cancer cells (Huang *et al.,*2009). NF-κB has also been shown to regulate COX-2 expression in cancer cells.

COX-2(Cyclooxygenase-2) is involved in inflammation, pathogenesis and progression of cancer by affecting cell proliferation, mitosis, cell adhesion, apoptosis, immune surveillance, and/or angiogenesis (Trappen *et al.,* 2002; Harris *et al.,* 2007). Various in vitro and *in vivo* studies have shown that COX-2 promoted expression of angiogenic factors VEGF and bFGF in cancer cells (Kim *et al.,* 2003; Sreekanth *et al.,* 2011). Earlier studies have reported increased expression of COX-2 in precancerous intraepithelial lesions in cervical cancer (Young *et al.,* 2008, Saldivar *et al.,* 2007).

1.1.6.5. ERK/MAP kinase pathway in cervical cancer

ERK/MAP kinase cascades consist of a core of three protein kinases that include extracellular receptor kinase ERK1/2, p38 kinase, and c-Jun (Raman *et al.,* 2007). ERK1/2 is a key member of the MAPK signaling pathway and can be activated by a variety of stimuli (Branca *et al.,* 2004). ERK/MAPK cascade is over-expressed upon activation of cellular oncogenes in many human carcinomas, including cervical cancer cell lines (Branca *et al.,* 2004). In addition, HR HPV and some low-risk HPV types have also shown to activate ERK/MAPK signaling pathway resulting into cell proliferation and cancer development (Kim *et al.,* 2006). HPVE5-mediated overexpression of VEGF has been shown to involve phosphorylation of EGFR, resulting into activation of MEK-extracellular signal-regulated kinase1/2 (ERK1/2) and phosphatidylinositol 3-kinase (PI3K)–Akt pathways (Kim *et al.,* 2006).

All these molecular mechanisms modulated during cervical carcinogenesis have been summarized in Figure 1.1.4.

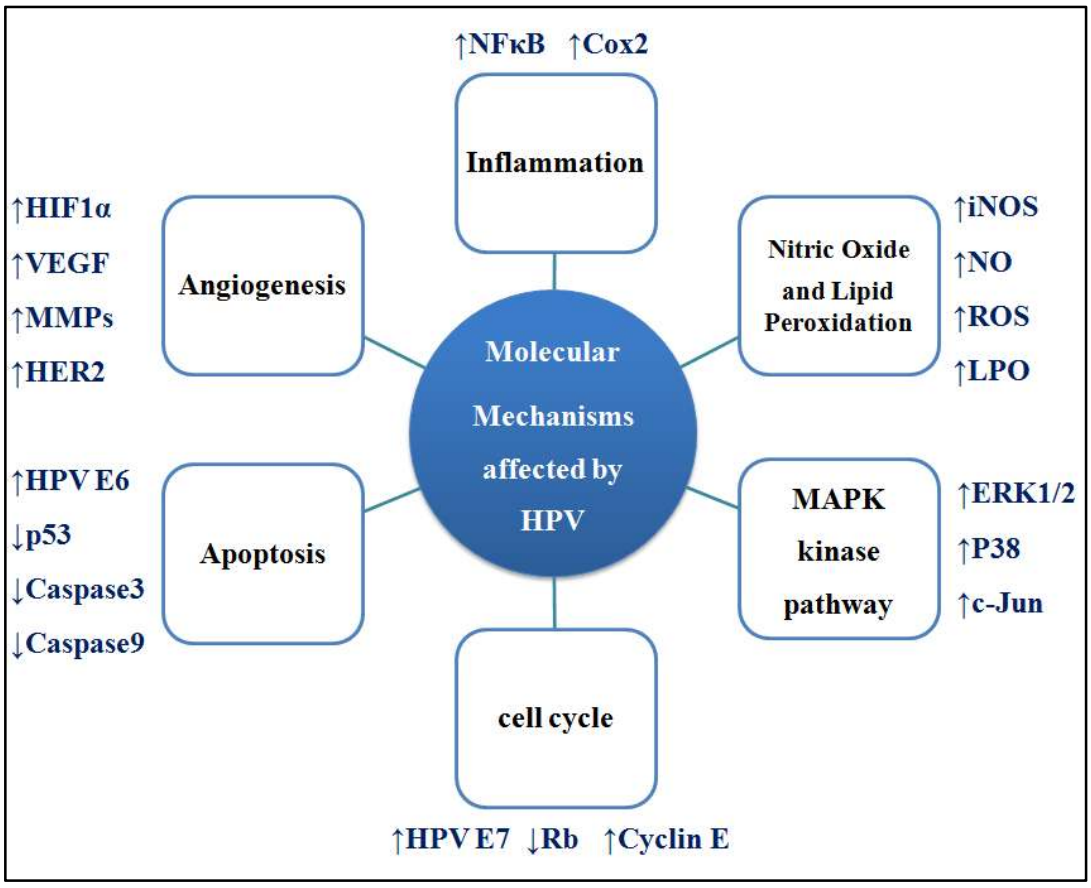

Figure 1.1.4. Molecular mechanisms modulated in cervical cancer.

1.1.7. Other risk factors

Besides HPV infection, there are many other risk factors that could lead to cervical cancer development. These include parity, early age of marriage, genital hygiene, promiscuity, long term use of oral contraceptives, smoking, immune suppression caused by HIV, infection with other sexually transmitted agents such as Chlamydia and poor nutrition (Atlanta 2013) (Figure 1.1.5.).

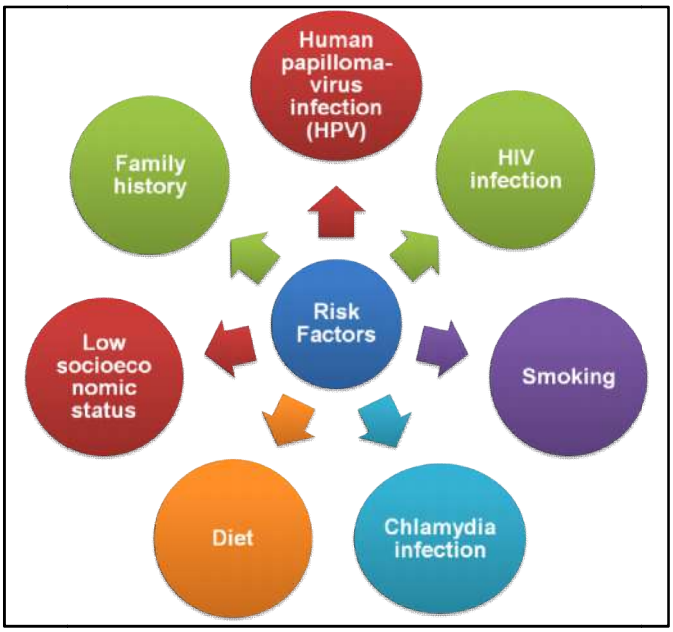

Figure 1.1.5. Risk factors for cervical cancer development.

1.1.8. Current Treatment/Preventive strategies for cervical cancer

Treatment of invasive cervical cancer generally includes radiation therapy or chemotherapy along with surgical removal of the primary tumor.

1.1.8.1. Surgery

Surgery is used for removing small tumor present in the cervix, especially when the patient has no signs of metastasis in the lymph node (Dueñas-González *et al.,* 2005). It is done by different methods such as conization or cone biopsy, cold-knife conization, loop electrosurgical excision procedure (LEEP), laser surgery, and hysterectomy depending upon the size of tumor.

1.1.8.2. Radiation therapy

Radiation therapy uses X-rays or other radiations (electrons, protons, neutrons, carbon ions, alpha particles, and beta particles) to kill the cancer cells (Halperin *et al.,* 2006). The external or internal radiation therapy could be given depending upon the type and stage of the cancer being treated. Radiation therapy couldbe combined with

surgery and chemotherapy (Rubin *et al.,* 2014).This type of radiation therapy causes less damage to healthy tissue near the tumor.

1.1.8.3. Chemotherapy

Chemotherapy includes drugs that stop the growth of cancer cells, either by killing the cells or by stopping them from dividing. Cisplatin is the commonly used chemotherapic drug to treat cervical cancer (Cornelio *et al.,* 2009). Other chemotherapic drugs such as carboplatin, platinol-AQ, nedaplatin, paclitaxel, gemcitabine, capecitabine, vinorelbine and topotecan are also used either alone or their combination (Marchiole *et al.,* 2011).

1.1.8.4. HPV vaccines

Prophylactic vaccines have been developed to prevent papillomavirus infection (Frazer 2004).There are two vaccines in the market that aim at preventing HPV-related cervical cancer that include Cervarix® (GlaxoSmithKline) and Gardasil® (Merck & Co). These vaccines protect against four types of HPVs 6, 11, 16, and 18. However, both vaccines are prophylactic and provide no benefit to individuals already infected with the virus (Zur 2008; Nigam *et al.,* 2014). Therefore, there is a need to develop therapies that can benefit people infected with the virus but have not yet developed advanced neoplasia.

1.1.9. Complementary and Alternative Medicine (CAM) therapies

Despite high treatment benefits, conventional therapies are associated withserious side effects. Radiation therapy related side effects include shortening and drying of vagina, symptoms of menopause; vaginal spotting and sometimes infertility. Chemotherapy related side effects usually include vomiting, nausea, myelosuppression, alopecia and gastrointestinal discomfort (Marchiole *et al.,* 2011).

To overcome these side effects, tremendous efforts are being focused towards inclusion of Complementary and Alternative Medicine (CAM) therapies as an adjunct to conventional treatment (Boon *et al.,* 2000). CAM includes medical interventions that are not part of conventional medicine and are being practiced either alone or in combination with conventional treatment methods (Molassiotis *et al.,* 2005). The National Center for Complementary and Alternative Medicine in USA has classified CAM therapies mainly into five groups namely, manipulative and body based therapies, mind-body interventions, energy therapy, alternative medical systems and biological based approaches (Figure 1.1.6) (Metcalfe *et al.,* 2010). Biological based therapies include use of herbs, dietary supplements or vitamins as well as traditional medicine system such as Chinese herbal medicines or Ayurveda.

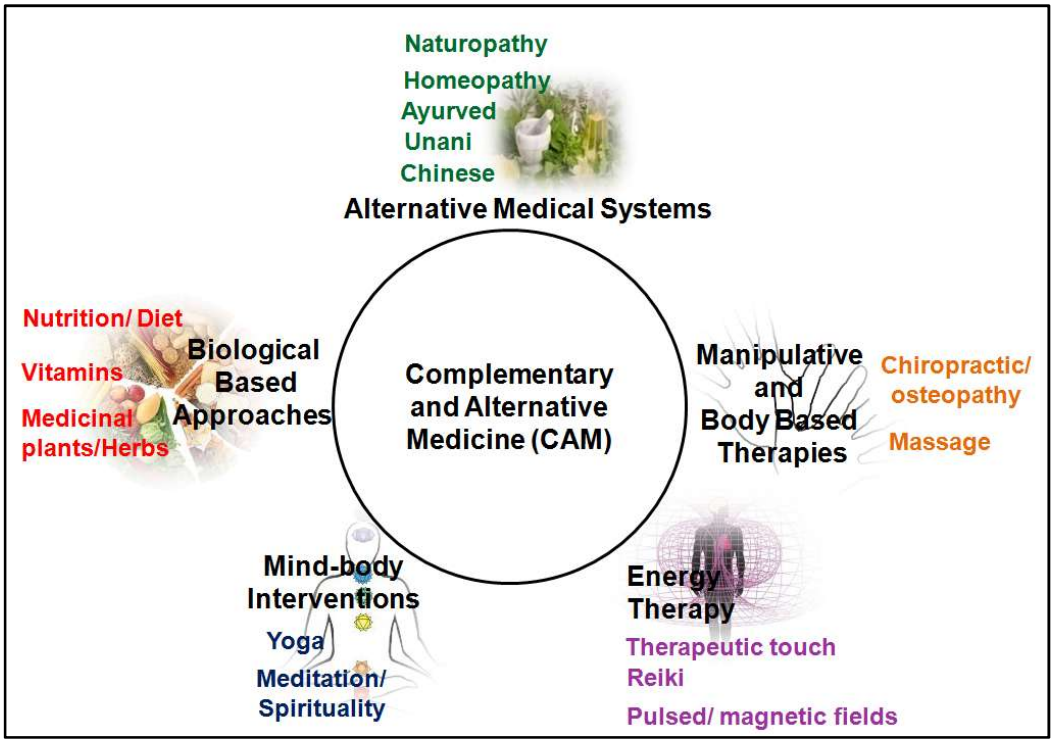

Figure 1.1.6. Complementary and Alternative Therapies.

SECTION 2

Literature on
Omega 3 fatty acid, Alpha Linolenic Acid

Review Article Published In

**Bharati Vidyapeeth University Journal 2011;
IX(1):10-18.**

1.2.1. Role of Nutrition in Health

Nutrition is an important aspect of our life that helps in growth, repair, and maintenance of our body (Khalil *et al.,* 2013). It is a key factor that governs the metabolic and physiological processes of the body. Diet exerts a profound effect on many aspects of health and disease (Jiménez-Chillarón *et al.,* 2012). Clinical and epidemiological studies have shown that change in the dietary pattern is one of the major risk factors involved in the development of diseases including obesity diabetes mellitus, cardiovascular disease (CVD), hypertension and stroke, as well as cancer (Jiménez-Chillarón *et al.,* 2012). Nutritional factors have been proposed to prevent progression from transient to persistent HPV infection and have been shown to act as efficient scavengers of free radicals and oxidants (García-Closas *et al.,* 2005).

1.2.2. Diet and Cancer Relationship

Association of diet with cancer etiology and prevention has been well established (Greenwald *et al.,* 2001; Sinha 2001). Risk of developing cancer has been shown to be directly proportional to intake of saturated fats and inversely proportional to intake of vegetables, fruits, whole grains, dietary fiber, micronutrients and certain types of unsaturated fats (e.g. n-3 fatty acids) (Osetti *et al.,* 2001; Ghosh *et al.,*2008) .

1.2.3. Fatty Acids

Fatty acids (FAs) are important macronutrients and are divided into saturated and unsaturated fatty acids (Rustan *et al.,* 2005) (Figure 1.2.1). Saturated fatty acids are long-chain carboxylic acids without carbon-carbon double bond. Unsaturated fatty acids are divided as a) monounsaturated FAs (MUFAs) that are non-essential FAs with one carbon–carbon double bond, and b) polyunsaturated fatty acids (PUFAs) that

are Essential FAs with two or more carbon–carbon double bonds (Rustan 2005). Essential fatty acids (EFAs) cannot be synthesized by human beings and therefore must be obtained from the diet. EFAs consist of a hydrocarbon chain of variable length, with a carboxyl group (COOH) at one end and a methyl (CH3) group at the other end (n- or omega end). There are two families of EFAs, n-6 (omega-6) and n-3 (omega-3), named according to the position of the first double bond from n-end or omega end. n-6 PUFAs include linoleic acid (LA), gamma linoleic acid (GLA) and arachidonic acid (AA). n-3 PUFAs consist of alpha linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoicacid (DHA) (Simopoulos 2002).

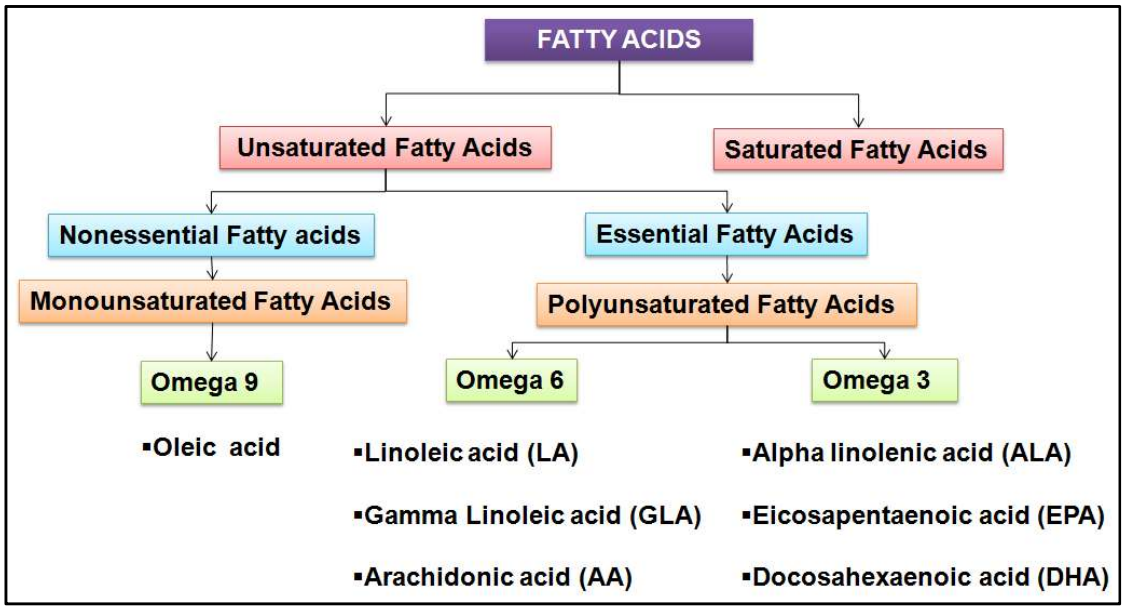

Figure 1.2.1. Classification of Fatty acids.

1.2.4. Role of Omega Fatty Acids in Human Health

1.2.4.1. Omega 6 Fatty Acids

Omega-6 fatty acids are carboxyalic acids containing C=C double bond at n-6 position from methyl end (Calder & Grimble2002). Excessive consumption of certain omega−6 fatty acids lead to production of omega−6 eicosanoids that have been found to be responsible for chronic conditions such as arthritis, inflammation and cancer (Simopoulos 2001; Donaldson *et al.,* 2004). Cancer promoting effects of n-6 PUFAs is due to the increase in levels of pro-inflammatory eicosanoids (prostaglandin E) which promote angiogenesis and hamper apoptosis (Murff *et al.,* 2011). Even though few reports suggest that n6 PUFAs such as LNA, Gamma Linolenic Acid (GLA) and AA induce apoptosis in cancer cells through generation of lipid peroxidation (Colquhoun *et al.,* 2001; Trombetta *et al.,* 2007) but they also kill the non-cancerous cells (Cao *et al.,* 2000; Prasad *et al.,*2010; Seegers *et al.,* 1997)

1.2.4.2. Omega 3 Fatty Acids

The health benefits of n-3 PUFAs were first explored by Norwegian Professor Notevarp during 1950s-1960s. Dietary fatty acids have been reported to be essential for cellular growth and metabolism, as well as for membrane structure and function (Lewis *et al.,* 2008; Reisman *et al.,* 2006). These FAs have been shown to modulate numerous processes such as brain and visual development, inflammatory reactions, thrombosis and carcinogenesis (Simopoulos 2002). A survey conducted in Greenland population showed less incidence of coronary heart disease due to low cholesterol and TAG levels that has been correlated with the dietary pattern involving high intake of seafood, a rich source of n-3 PUFAs (Simopoulos 2002). These fatty acids have been shown to inhibit the formation of atherosclerotic plaques by decreasing blood pressure

(Simopoulos 2002) and reducing the risk of sudden death (Mozaffarian & Wu 2011), cardiac arrhythmias and stroke (Allayee 2009; London *et al.,* 2007). n-3 PUFAs may also prevent different neurodegenerative disorders such as Alzheimer's disease (Bourre *et al.,* 2005) and inflammatory diseases including rheumatoid arthritis and inflammatory bowel disease (Calder 2002).

1.2.4.3. Anticancer effect of omega 3 fatty acids

Long chain omega 3 fatty acids, namely EPA and DHA have been studied for their anticancer potential in various cancers including breast cancer (Chamras *et al.,* 2002; Sun *et al.,* 2005; Schley *et al.,* 2005). Growth-inhibitory effects of these FAs depend upon specific signal transduction pathways (Chamras *et al.,*2002; Sun *et al.,* 2005; Schley *et al.,* 2005) such as upregulation of tumor suppressor proteins including p53 (Kikawa *et al.,* 2011), BRCA Jourdan *et al.,* 2007) $p16^{INK4A}$ (Tsujita-Kyutoku *et al.,* 2004) , SDC-1 (Sun *et al.,* 2008), p21 (Wu *et al.,* 2005), caspase-3 (Siddiqui *et al.,* 2001) and caspase-8 (Kang *et al.,*2010) and downregulation of cell cycle regulatory proteins including cyclin A, cyclin B1, cyclin-dependent kinase 1 and p-cyclin B1 (Barascu *et al.,* 2006). EPA and DHA have been shown to induce apoptosis in breast cancer cells through activation of caspase 8 and 3 (Barascu *et al.,* 2006). They have also been shown to down regulate the expression of anti-apoptotic protein Bcl-2 which tends to decrease the Bcl-2/Bax ratio in breast cancer cells (Barascu *et al.,* 2006). In presence of EPA and DHA, there is free radical production through lipid peroxidation mechanism that induces oxidative stress in cancer cells leading to activation of cell death pathway (Kang *et al.,* 2010). This process may lead to upregulation of tumor suppressors and/or down regulation of tumor activators/promoters, finally resulting in tumor retardation. EPA and DHA have been

found to inhibit the growth of cervical keratinocytes, immortalized with the oncogenic human papillomavirus (HPV) type 16 (Chajès *et al.,*1995). EPA and DHA inhibit metastasis, cell growth and invasion through suppression of MMPs/COX-2/VEGF and NF-kB in cervical cancer cells (Jing *et al.,* 2010). DHA has been shown to induce degradation of HPV E6/E7 oncoproteins by activating the ubiquitin–proteasome system (Jing *et al.,* 2014). EPA and DHA have been studied extensively for their anticancer properties, however, there are very few reports available on the anticancer activity of ALA in cervical cancer.

1.2.5. Alpha Linolenic Acid (ALA)

Alpha-linolenic acid (ALA 18:3), is an omega-3 (n-3) fatty acid. It is the shorter-chain carboxylic acid with 18-carbon chain and three double bonds (Stark *et al.,* 2008). The double bond is located at the 9, 12 and 15 position from the methyl end of the fatty acid chain, known as the n end. ALA is also called as *cis*-9, 12, 15-octadecatrienoic acid (Figure 1.2.2). ALA is the parent n3 fatty acid and gets converted into longer chain fatty acids EPA (20:5, ω3) and DHA (22:6, ω3). ALA has been reported to be beneficial in management of CVDs (Vijaimohan *et al.,* 2006), diabetes (Ihara-watanabe *et al.,* 1999), blood cholesterol levels (Rezaei & Heidarian 2013) and cancer (Colquhoun *et al.,* 2009).

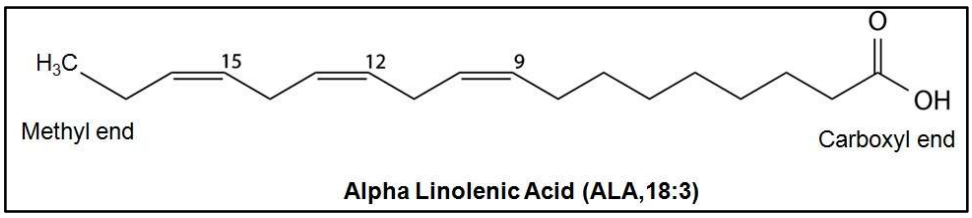

Figure 1.2.2. Structure of Alpha Linolenic Acid*. It is a short-chain fatty acid consisting of 3 double bonds at 9, 12 and 15 positions.*

1.2.5.1. Sources of ALA

ALA is found mostly in certain plant foods that include flaxseed, flaxseed oil, Perilla Chia seed, camellia oil, canola or rapeseed oil, soybean oil, soybean, walnuts, cloudberry, blueberry, almonds, peanuts, cashews, green leafy vegetables (spinach, brussels' sprouts, fenugreek and kale), and several legumes (Barceló-Coblijn and Murphy, 2009).

1.2.5.2. Conversion of ALA to EPA and DHA

ALA and LA get elongated and desaturated through a series of steps involving elongases and delta-5 (δ5) - and δ6 desaturases (Figure 1.2.3) (Burdge 2006). LA is desaturated to form gamma-linolenic acid (GLA, 18:3 omega-6) that in turn is elongated to form dihomo gamma linoleic acid (DGLA, 20:3 omega-6) and finally converted to AA (arachidonic acid, 20:4 omega-6). Similarly, ALA is converted to eicosapentaenoic acid (EPA, 20:5 omega-3) that is further converted to docosahexaenoicacid (DHA, 22:6 omega-3) (Burdge 2006).

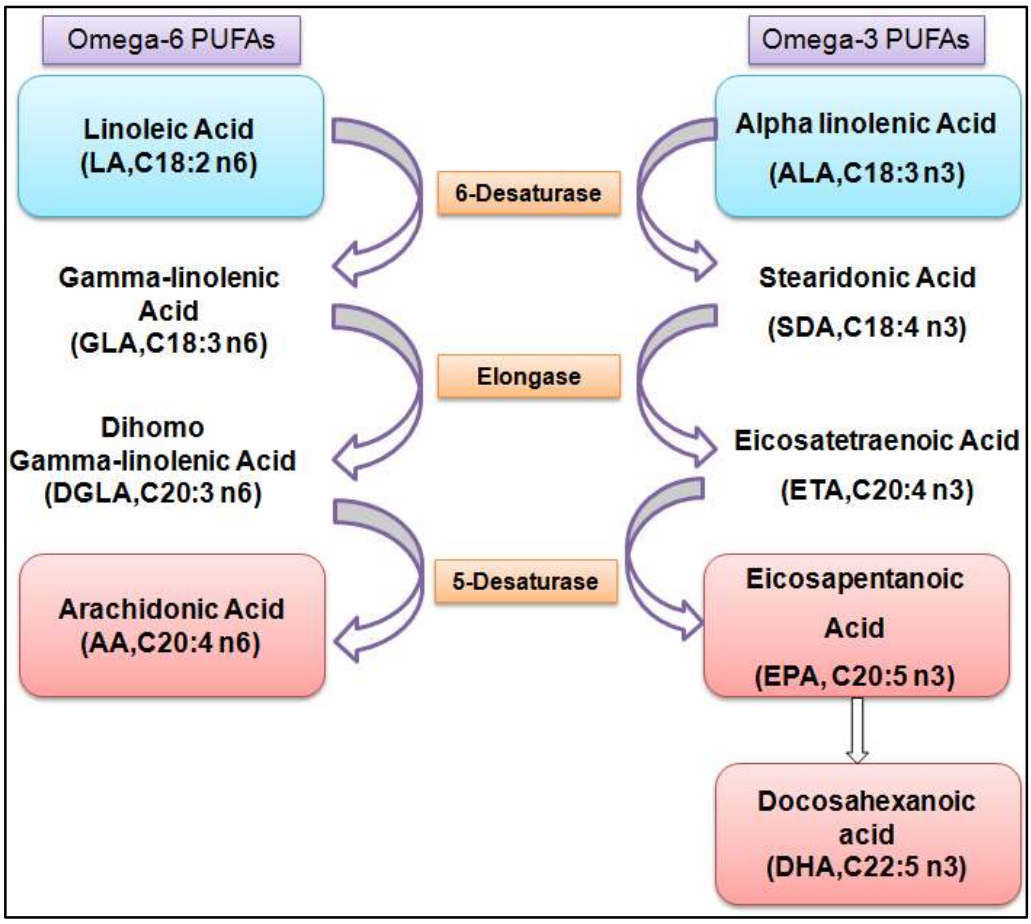

Figure 1.2.3. Biosynthesis of omega-6 and omega-3 PUFAs*. The conversion pathway involves elongase and desaturase enzymes for conversion of short chain to long chain omega fatty acids.*

1.2.5.3. Dietary trend in different countries

The average intake of ALA in European countries, USA and Canada has been shown to be in the range of 0.8 and 2.2 g/d (Burdge and Calder 2005). According to the British adult diet survey, the intake of ALA has been reported to be in the range of 1.4 g/d to 2.1 g/d in UK (Henderson *et al.,* 2004). There has also been a rapid increase in the consumption of foods rich in n-6 PUFAs and a decrease in the intake of n-3 PUFAs in Western societies during the past 150 years. This has resulted into increase in n-6 to n-3 ratio (n-6/n-3), ranging from 10-20/1 compared to 1/1 ratio found in the

ancestral diet which contained as much as ~5-6 g/day intake of n-3 PUFAs. Table 1.2.1 shows intake of ALA and LA in population in different countries.

Table 1.2.1. Fatty acid intake in population

Fatty acid intake in various population (g/day)				
	Linoleic acid (LA)	**Alpha Linolenic Acid (ALA)**	**LA:ALA ratio**	**References**
Belgium	12.8	1.4	9.1	Simopoulos 2011
Denmark	9.0	2.1	4.3	Simopoulos 2011
France	8.1	0.5	11.6	Simopoulos 2011
Germany	8.0	0.7	11.4	FAO 2010
Netherlands	13.2	1.2	11	FAO 2010
Italy	14.5	0.8	18.1	Burdge 2006
Spain	21.6	0.8	27	Burdge 2006
UK	14.4	1.4	10.3	Henderson *et al.,* 2004
Australia	9.9	1.2	8.3	Henderson *et al.,* 2004
USA	16	1.3	12.3	Burdge & Calder 2005
Norway	8.8	1.2	7.3	Burdge & Calder 2005
Japan	10.7	1.6	6.6	Burdge & Calder 2005
India	9.9	1.6	5-6	Pella 2003

1.2.5.4. Literature survey: Anticancer activity of Alpha Linolenic Acid

Previous studies on ALA have been predominantly undertaken in breast cancer. ALA was reported to induce apoptosis in breast cancer cell line MCF-7 through activation of caspase 8 and 3 with the release of cytochrome *c* and degradation of PARP (Kim *et al.,* 2009). It was also shown to down regulate the expression of anti-apoptotic protein Bcl-2 with decrease in Bcl-2/Bax ratio in breast cancer cells (Kim *et al.,* 2009). ALA was reported to decrease PGE2 production

(Horia *et al.,* 2005) as well as invasive potential of MDA-MB-231 cell line through decrease in the expression of COX-2 (Horia *et al.,* 2005). ALA also increased lipid peroxidation that leads to decrease in breast cancer risk (Chajès *et al.,* 2000).

ALA has been shown to exhibit cytotoxic activity in different cell lines which include SP 2/0 mouse myeloma cells and human cervical cancer cell line HeLa (Sagar *et al* 1995). However, the anticancer activity of ALA and its underlying mechanisms were unknown in HeLa cells (Sagar *et al.,* 1995). In one of the clinical study, it was found that the process of cervical carcinogenesis was prevalent in essential fatty acid deficiency (EFAD) which includes alpha linolenic acid (ALA) and Linoleic acid (LA) (Engelbrecht *et al.,* 1998). This EFAD in cancer cells may result in defective cellular mechanisms, since these fatty acids are associated with biochemical events such as lipid peroxidation, signal transduction and immune responses (Engelbrecht *et al.,* 1998).

Preclinical studies in rodent model (nude mouse and rat) have shown that consumption of flaxseed oil, canola oil (Hardman *et al.,* 2007) or mistol seed oil (Muñoz *et al.,* 1995) containing 50% 10% and 25% ALA, respectively, slowed the growth of breast cancer. Animal studies have demonstrated that dietary supplementation with flaxseed oil inhibited growth of breast, colorectal and other cancers (Mason *et al*., 2013; Nagel *et al.,* 2012). *In vivo* studies in nude mice suggest that flaxseed oil decreased VEGF, and reduced tumor growth and metastasis. In athymic mice, a combination of dietary flax oil (FO) (8%) with Trastuzumab (TRAS) treatment, it was observed that flax oil did not interfere with TRAS but enhanced its tumor-reducing effects (Mason *et al.,* 2010). Moreover, combination of flax seed oil and TRAS modulated HER2 signaling through Akt and MAPK pathways, leading to reduced cell proliferation and increased apoptosis in BT474

cells and significantly lowered tumor growth in athymic mice (Mason *et al.,* 2013). In one of the studies, consumption of walnuts significantly decreased the growth rate of the implanted human breast cancer MDAMB231 tumors implanted in nude mice (Hardman *et al.,* 2008). In another study, dietary ALA reduced COX-2 expression and increased apoptosis in hepatoma cells implanted in ACI/T rat (Nagel *et al.,* 2012). Table 1.2.2 summarizes the reported anticancer activity of ALA in different types of cancer.

Table 1.2.2. Anticancer activity of ALA in different cancers

Cancer type	Source of ALA	Study type	Mechanism of Action	References
Breast	Flaxseed oil	In vitro (BT-474) and *In vivo*	Flaxseed oil in combination with trastuzumab enhanced effectiveness of TRAS; modulated Akt and MAPK pathways; reduced cell proliferation; induced apoptosis and reduced tumor growth in athymic mice	Mason JK et al 2013
Breast	Flaxseed sprouts	In vitro (MCF-7, MDA-MB-231)	Flaxseed induced apoptosis and inhibited cancer cell growth, demonstrating its anti-proliferative effect in breast cancer cells	Lee J *et al.,* 2012
Breast	Flaxseed	*In vivo*	Flaxseed enhanced tumor-reducing effect of trastuzumab, decreased cell proliferation and induced apoptosis in athymic mice	Mason 2010
Breast	Pure form of ALA	In vitro (MCF-7)	ALA induced sub-G1 arrest in MCF-& cells and decreased ratio of Bcl-2/Bax by 50%. ALA activated caspase-3 by release of cytochrome c leading to apoptosis	Kim *et al* 2009
Breast	Pure form of ALA	In vitro (MCF-7)	Conjugation of doxorubucin with ALA increased the uptake of doxorubicin, inhibited cell growth and induced apoptosis	Huan *et al* 2009
Breast	Dietary walnut	*In vivo*	Dietary walnut (source of ALA) decreased growth of implanted MDA-MB 231 cells in nude mice	Hardman *et al* 2008
Breast	Flaxseed	In vitro (MCF-7)	flaxseed alone or in combination with tamoxifen inhibited breast tumor growth in ovariectomized athymic mice	Chen et al 2007

Cancer type	Source of ALA	Study type	Mechanism of Action	References
Breast	Pure form of ALA	In vitro (SKBr-3, BT-474)	ALA reduced the expression of HER2- oncoprotein and decreased growth of breast cancer cells	Menéndez *et al* 2006
Breast	Flaxseed	*In vivo*	Flax seed inhibited tumor metastasis in nude mice injected with MDA –MB 435	Chen *et al* 2006
Breast	Pure form of ALA	In vitro (MDAMB 231)	ALA reduced cell viability and decreased PGE2 and COX-2 production in MDAMB 231	Horia *et al* 2005
Breast	Pure form of ALA	In vitro (MDA-MB-231, SK-Br3, T47-D MCF-7)	ALA synergistically increased chemo-sensitivity to paclitaxel	Menendez *et al* 2004
Breast	Flaxseed	*In vivo*	Flaxseed modulated tumor biomarkers of estrogen and growth factor signaling pathways in MCF-7 induced tumor in Balb/c nude mice	Power 2008
Breast	Flaxseed	*In vivo*	Flaxseed decreased VEGF thereby metastasis in MDA-MB435 induced athymic nude mice	Dabrosin *et al* 2002
Cervical	Pure form of ALA	In vitro (HeLa)	ALA showed dose dependant decrease in the growth of cervical cancer cells	Sagar *et al* 1995

Cancer type	Source of ALA	Study type	Mechanism of Action	References
Cervical	Pure form of ALA	In vitro (HeLa)	Pre-incubation of DHA, GLA, EPA and ALA enhanced the uptake of vincristine and decreased cell proliferation	Das et al 1998
Colon	Pure form of ALA	In vitro (LT97,HT29)	ALA inhibited growth of human colon cell lines	Habermann *et al* 2009
Colon	Pure form of ALA	Invitro (LT97, HT29)	ALA inhibited cell proliferation, adhesion and invasion in both human and mouse colon cancer cell lines	Moon et al 2014
Colorectal	Walnut	*In vivo*	Dietary walnuts inhibited colorectal cancer growth by suppressing angiogenesis in HT-29 injected nude mice	Nagel *et al* 2012
Bladder	Vegetable and other sources of ALA	Clinical	Increased intake of ALA reduced risk of bladder cancer in patients	Brinkman *et al.,* 2011
Esophageal	Pure form of ALA	In vitro (OE19, OE33)	ALA significantly down-regulated cell proliferation, adhesion and/or migration and up-regulated tumor suppressor genes (p53, p21,and p27) in esophageal cancer cell lines	Moon et al 2014
Prostate	Flaxseed	Clinical	Study failed to confirm an association between dietary ALA intake and prostate cancer risk in patients	Carleton *et al.,* 2013

Cancer type	Source of ALA	Study type	Mechanism of Action	References
Prostate	Flaxseed	Clinical	Increased dietary intakes of ALA may increase the risk of advanced prostate cancer in patients	Leitzmann 2004
Prostate	Pure form of ALA	In vitro (DU-145)	ALA suppressed cell growth of prostate cancer cells	Motaung *et al.,* 1999

SECTION 3

Literature on Medicinal Plants

Book Chapter communicated in Apple Academic Press, 2015

1.3.1.1. Medicinal plants

Medicinal plants form an important component of CAM approach. They have been used from time immemorial in the treatment of various disorders including cancer.

1.3.1.2. Medicinal plants as anticancer drug candidates

The advances in the medicinal plant studies have resulted in discovery of various plant extracts, fractions and pure compounds as potential anticancer drug candidates (Dias 2012). More than 60% of the drugs that are in the market are derived from natural sources including plants, marine organisms and microorganisms (Kaur 2012). The natural products from plants contain secondary metabolites (PSMs) such as alkaloids, flavonoids, phenolics, tannins, anthraquinones and so on, which exhibit various biological properties including anticancer activity (Baikar 2010, Sakarkar 2011). Many plant based anticancer agents including taxol, vincristine, vinblastine and topotecan have been in clinical use for a long time all over the world (Kaur R 2012).

To identify lead anticancer compounds for prevention and treatment of cervical cancer, efforts should be made to understand the signaling pathways that get altered during the cancer development. During the last decade, various studies have been carried out to investigate several molecular targets in cervical cancer (Dwarampudi 2013). In conventional therapy, targeted therapies act by inhibiting a specific molecule involved in a pathway, thereby interfering either with proliferation or survival and in effect hindering the malignant transformation by inducing cell death or regulating cell cycle (Topcul 2014). The drug resistance is a major hurdle in targeted approaches which can be overcome by combination treatments with natural

products (Wang 2014; Heer 2013). In this context, medicinal plants have been reported to exhibit significant potential because of their ability to interact with different molecular pathways that regulate cell cycle, apoptosis, angiogenesis and metastasis (Yadav 2010; Chen 2012). Various medicinal plants that have been reported to target these major signaling pathways in cervical cancer have been tabulated in Table 1.3.1.

Table 1.3.1. Medicinal plants/herbal bioactives that target various signaling pathways in cervical cancer

Medicinal plant/ Herbal Bioactives	Cell lines used	Mechanism of Action	References
Boswellia serrata	SiHa HeLa	Generated nitric oxide and reactive oxygen species; up regulated the expression of p53/p21/PUMA;, decreased the expression of PI3K/pAkt ERK1/2, NF-kB; loss of cytochrome c; activated caspase 3 and induced apoptosis	Bhushan S,2009
Berberine aquifolium	SiHa HeLa C33A	Decreased cell viability; reduced E6 and E7 levels and concomitantly increased p53 and Rb expression; induced apoptosis through caspase 3 activation	Mahata *et al.,*2011
Cinnamomum cassia	SiHa, HeLa	Decreased cell viability; induced apoptosis through mitochondrial membrane potential disruption and increased calcium release; decreased migration with reduction in expression of Her2 andMMP2proteins	Koppikar et al 2010
Citrus grandis	HeLa	Downregulated Bcl-2 expression; activated caspases and degraded PARP protein leading to apoptosis	Kim *et al.,* 2010
Corallina pilulifera	HeLa	Induced apoptosis through mitochondria-dependent pathway and downregulated DNA topoisomerase IIa gene expression	Kwon *et al.,* 2007
Cordyceps pruinosa	HeLa	Decreased Bcl-2 protein; increased Bax protein;induced release of cytochrome-c; activated caspases-3 and -9 resulting in apoptosis	Kim *et al.,* 2010

Medicinal plant/ Herbal Bioactives	Cell lines used	Mechanism of Action	References
Cremanthodium humile	HeLa	Generated ROS; released cytochrome c; activated caspase-3, 7 and 9, and induced apoptosis	Li *et al.,* 2007
Crocus sativus	HeLa	Induced sub-G1 phase arrest and ROS production leading to apoptosis	Tavakkol-Afshari *et al.,* 2008
Curcuma longa	CaSki, SiHa, HeLa	Reduced expression of E6 and E7; restored p53 and rb proteins; down-regulated VEGF, COX-2 and EGFR expression	Lim, C. *et al.,2010* Diane *et al.,*2011
Duchesnea indica	HeLa C33A U14	Upregulated and translocation of Bax to mitochondria; downregulated Bcl-2 expression; released cytochrome c and induced apoptosis; induced S-phase arrest; decreased PCNA and Ki-67 expression; Reduced tumor weight and increased survival rate	Peng *et al.,* 2009
Epigallocatechin gallate (Camellia sinensis)	HeLa Me180	Inhibited growth; induced cell cycle arrest and induced apoptosis	Sah 2004 Zhang 2006
Ficus hirta	HeLa	Inhibited cell viability; induced morphology changes and increasedsub-G1phase	Zeng *et al.,* 2012
Nigella sativa	HeLa	Regulated expression of pro- and anti-apoptotic genes and exhibited immune-modulatory activity	Shafi *et al.,* 2009

Medicinal plant/ Herbal Bioactives	Cell lines used	Mechanism of Action	References
Ficus religiosa	SiHa HeLa C33A	Decreased cell viability; induced cell cycle arrest in SiHa; induced release of cytochrome c; activation of caspase-3; induced apoptosis in HeLa; decreased cell migration; restored expression ofp53 and Rb with decreased expression of HPV E6 and E7	Choudhari *et al.,* 2013
Mangifera indica	HeLa	Increased sub-G1 phase population and induced apoptosis; downregulatedBcl-2 expression; activated caspase and degraded PARP protein	Kim *et al.,* 2010
Pinellia pedatisecta	CaSki HeLa	Decreased HPV E6 mRNA and protein expression; increased caspase-8 and 3; Bax; p53 and p21 mRNAs as well as proteins; decreased Bcl-2 mRNA and protein resulting into apoptosis	Li *et al.,* 2007
Pinus massoniana	HeLa	Decreased migration rate; induced cell cycle arrest; apoptosis; increased Bax; downregulated Bcl-2 expression; activated caspase-9 and -3	Wu *et al.,* 2011
Pterocarpus santalinus	HeLa	Induced release of cytochrome c; activated caspases-9 and 3 and degraded PARP	Kwon *et al.,* 2006
Solanum nigrum	U 14	Modulated immune response of tumor-bearing mice; caused G0/G1 phase arrest and induced apoptosis	Li*et al.,* 2008

Medicinal plant/ Herbal Bioactives	Cell lines used	Mechanism of Action	References
Withaferin A	CaSki	Downregulated expression of HPV E6 and E7 oncoproteins; increased levels of p53;p21(cip1/waf1); caused G(2)/M cell cycle arrest; decreased the levels of STAT3 and its phosphorylation at Tyr(705) and Ser(727) and altered expression levels of p53-mediated apoptotic markers-Bcl2, Bax, caspase-3 and cleaved PARP.	Munagala 2011
Wogonin	SiHa CaSki C33A	Increased p53 and pRb with reduction of E6 and E7 expressions; decreased mitochondrial membrane potential; activated caspase-3; caspase-9 and induced apoptosis	Kim 2013

Thesis Organization

1.4.1.1. Genesis of Thesis

Human papillomaviruses (HPV) play an important role in the development of cervical cancer, which is a leading cause of cancer death worldwide (Castellsagué 2008). Despite advanced anticancer therapies, the issues of low survival rates, drug associated side effects and recurrence still persist (Aggarwal 2006). In recent years, herbal medicines and nutraceuticals are being investigated for their use as an adjunct to conventional therapies to manage the debilitating side effects associated with the latter (Ali 2006; Seifried 2003).

Numerous clinical and epidemiological studies have shown that greater intake of long-chain n-3 fatty acids, namely eicosapentaenoic acid (EPA) and docosahexaenoicacid (DHA) reduced mortality in all types of diseases, including cancer (Penny 2002). However, the availability of these fatty acids is limited to fish and other marine products only and it has been questioned whether sufficient resources exist to provide these fatty acids in the amounts needed worldwide (Campos 2008). Alpha linolenic acid, an essential n-3 fatty acid, is abundantly found in plant foods that include seeds (rapeseed, flaxseed, perilla seed) and seed oils (Flaxseed oil, soybean and canola oil); beans (soybeans, kidney beans) and nuts (walnuts) and green leafy vegetables (fenugreek, broccoli) and vegetable oils (Stark 2008). Thus, intake of ALA (from plant origin sources) is 5–10 times higher than that of n-3 fatty acids (from fish sources) and it could be proposed as a viable alternative to fish oil (Campos 2008). However, the potential benefits of ALA in human diseases are inadequately defined and this is exactly the theme of the present work, which revolves around exploring the role of ALA in cervical cancer. We have also tried to evaluate whether ALA together with medicinal plants/herbal bioactives could exhibit increased anticancer activity. Medicinal plants are composed of a

plethora of bioactive phytochemicals having anticancer activity. Various clinical trials have evaluated the potential of phytochemicals in the management of cervical cancer.

1.4.1.2. AIM

The broad aim of this thesis was to evaluate the anticancer potential of ALA in cervical cancer. We have also analyzed whether ALA on combination with herbal bioactives could induce increased anticancer effect in cervical cancer cells

1.4.1.3. OBJECTIVES

- To determine the role of ALA on growth kinetics and apoptosis in cervical cancer cell lines SiHa (HPV16 positive) and HeLa (HPV18 positive)
- To evaluate the effect of ALA on COX2/VEGF/MAP kinase pathway and HPV oncoproteins E6, E7 in cervical cancer cell lines
- To analyze the effect of flaxseed oil rich in ALA,on TC1 (HPV 16 positive) induced tumor growth in C57BL/6 mice model
- To evaluate whether combination of ALA and herbal bioactives could induce enhanced anticancer activity in cervical cancer cells

1.4.1.4. Thesis organization

The present thesis has utilized different studies to analyze the anticancer potential of ALA that includes effect on cell viability; nitric oxide release; induction of lipid peroxidation and apoptosis; regulation of inflammatory markers (NFκB, COX-2); expression of tumor regulatory proteins (p53, Rb); cell migration; angiogenic markers (MMPs, VEGF); MAPK pathway (ERK, c-Jun, p38) and tumor

retardation in TC1 induced tumors in C57BL/6J mice. The entire thesis has been organized into five chapters and the contents of each chapter have been summarized below

Chapter 1- Introduction and Review of Literature

Chapter1 encompasses general background, facts and figures of cervical cancer, its etiology, the major pathways modulated during cancer progression and current therapies for treatment. It includes the importance of diet with a special emphasis on Alpha linolenic acid (an omega 3 fatty acid).The chapter also includes on how medicinal plants modulate different signaling pathways in cervical cancer.

Chapter 2- Determining the effect of alpha-linolenic acid (ALA) on growth kinetics and apoptosis in cervical cancer cell lines

In this chapter, the anti cancer potential of Alpha linolenic acid was evaluated in cell lines. This chapter includes cellular and molecular level experiments on human cervical cancer cell lines, SiHa (HPV 16) and HeLa (HPV 18). The chapter describes the effect of ALA on cell growth kinetics as well as different mechanisms through which it induces apoptosis in cervical cancer cells.

Chapter 3- Evaluating the effect of ALA on COX-2/VEGF/MAP kinase pathway and expression of HPV oncoproteins E6, E7 in cervical cancer cell lines

This chapter evaluates the effect of ALA on expression of HPV oncoproteins and HPV mediated pathogenesis in cervical cancer cell lines. This chapter also demonstrates the effect of ALA on cell migration and angiogenic markers (MMPs and VEGF) through which it regulates growth kinetics.

Chapter 4- Analyzing the effect of flaxseed oil containing ALA on regulation of TC1 (HPV 16) induced tumors in C57BL/6J mice

This chapter evaluates the anticancer potential of ALA at *in vivo* level in TC1 induced tumors in C57BL/6J mouse model. Here, flax oil was used as a main source of ALA and its effect on tumor retardation and other mechanisms were studied.

Chapter 5- Evaluating enhancement in the anticancer activity against cervical cancer cell lines upon combination of ALA with bioactives from medicinal plants

This chapter illustrates whether combination of ALA with herbal bioactives such as Cinnamaldehyde (*Cinnamomum* spp), andrographolide (*Andrographis paniculata*), Curcumin (Turmeric*, Curcuma longa*) could result in increased anticancer activityin cervical cancer cells.

CHAPTER 2

Determining the effect of Alpha-linolenic acid (ALA) on growth kinetics and apoptosis in cervical cancer cell lines

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2.1. Introduction

In the present chapter, we have analyzed the effect of the essential fatty acid, alpha linolenic acid (ALA), on nitric oxide release as well as induction of lipid peroxidation in cervical (SiHa and HeLa) cancer cell lines. ALA-treated cells showed a dose-dependent decrease in cell viability in cervical cancer cell lines without affecting the viability of non-cancerous transformed HEK 293 cells. Further, ALA altered the growth kinetics of cervical cancer cells in a dose dependent manner. Cells treated with ALA demonstrated a significant reduction in the nitric oxide (NO) release with a simultaneous increase in lipid peroxidation (LPO). This was followed by a decrease in the mitochondrial membrane potential as well as activation of caspase-3 leading to apoptosis. Thus, ALA regulated the growth of cancer cells through induction of lipid peroxidation and modulation of nitric oxide release resulting into apoptosis.

2.2. Materials and Methods

2.2.1. Reagents

Tissue culture plasticware was purchased from BD Bio-sciences, CA, and USA. Alpha linolenic acid, fatty acid-free bovine serum albumin (BSA) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylthiazoliumbromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagles Medium (DMEM), Penicillin and streptomycin were obtained from Gibco BRL, USA. Fetal bovine serum was purchased from Moregate Biotech, Australia. L-Glutamine, BHT and TBA were obtained from Himedia Corporation, Mumbai, India. Sulfanilamide was

purchased from Qualigens, N-[1-napthyl] ethylenediamine (NEDD), was purchased from SRL and TCA was purchased from Merck.

2.2.2. Cell culture

The human cervical cancer (SiHa and HeLa) cell lines as well as human embryonic kidney cell line (HEK293) used in the study were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in DMEM containing 2 mM L-glutamine supplemented with 10% fetal bovine serum and 100 U/ml of penicillin-streptomycin. The cells were incubated in a humidified 5% CO_2 incubator at 37°C.

2.2.3. Conjugation of Alpha linolenic acid with BSA

ALA was reconstituted in 200 µL of ethanol. For conjugation, ALA (10mM) was added to fatty acid-free bovine serum albumin (BSA) (3mM) to obtain ~ 3:1 ratio of ALA:BSA (Mahadik *et al.,* 1996). The conjugated omega fatty acids were incubated at 37˚C for 30 min in CO_2 incubator and stored at -20˚C and before use they were diluted to the required concentration with 10% DMEM.

2.2.4. Cell Viability Assay

The cell viability was determined by MTT assay in cervical cancer cell lines in presence of different concentrations of ALA and compared with non-cancerous transformed cell line, HEK 293 and HaCaT. The cells were seeded at a density of 1×10^5 cells/ml density in 96-well plates (TPP, Europe/Switzerland) and grown for 24 h.ALA was added at different concentrations: (0-320µM) for 24, 48 and 72 h. The MTT solution (5 mg/ml) was added to each well and the cells were cultured for

another 4 h at 37°C in 5% CO_2 incubator. The formazan crystals formed were dissolved in 90 µl of SDS-DMF (20% SDS in 50% DMF) (Singh *et al.,* 2009). After 15 min, the amount of colored formazan derivative was determined by measuring optical density (OD) at 570 nm using the ELISA micro plate reader (Bio-Rad, Hercules, CA)

2.2.5. Cell proliferation

Proliferation of HeLa and SiHa cells was determined by trypan blue dye exclusion method. Briefly, the cells were seeded at a density of 1×10^5 cells/ml in 24-well plate in triplicates and were incubated for 24 h at 37^{o}C. After 24 hr, the cells were treated with different concentrations of ALA (0-320 µM/ml). The cells were harvested andcounted for viability using trypan blue dye exclusion method.

2.2.6. Soft agar assay

The soft agar assay was performed as described previously to determine long time survival of tumorigenic cancer cells after treatments. Briefly, HeLa and SiHa cells (5×10^3 cells/ml) treated with different concentrations of ALA (0-80 µM/ml) were suspended in culture medium containing 0.35% agarose mixed and plated at room temperature for 20 min over a previously gelled layer of 0.5% agarose in culture mediumin 6-well plates. After incubation for two weeks, the colonies were counted in 10 different fields using an Axiovert 200 M microscope (Carl Zeiss, Germany) and the average value was calculated.

2.2.7. Nitric Oxide assay

The concentration of NO was indirectly determined in culture supernatants as nitrite, a major stable product of NO. The cervical cancer cell lines were seeded at density of $1x10^5$ cells/ml in 96-well plates (TPP, Europe/Switzerland) for 24 h and then incubated with different concentrations of ALA (0-80 µM) for different time intervals (24, 48 and 72 h). The NO levels were estimated by Griess reaction (Udenigwe *et al.,* 2009). Briefly, 100 µL of culture supernatant was mixed with equal volume of Griess reagent [1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine in 5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 540 nm was measured with the ELISA micro plate reader (Bio-Rad, Hercules, and CA). Nitric oxide concentration was determined using sodium nitrite ($NaNO_2$) as a standard.

2.2.8. RT-PCR

The total cellular RNA from control as well as cells treated with different concentrations of ALA (0-80 µM) was extracted by a one-step acid guanidine isothiocyanate-phenol method using TRI reagent (Invitrogen). RNA was precipitated with isopropanol and the concentration was estimated using Nanodrop instrument (Eppendorff BioPhotometer plus). Ten microgram of total RNA was used for each RT-PCR reaction. Fifty units of Moloney murine leukemia virus reverse transcriptase (MMuLV) (Bangalore Genei, Bangalore, India) was added in a typical 50 µl reaction (10 µg RNA, 5X first-strand buffer, 1 mM DTT, 2.5 mM dNTPs, 50 ng/µl random primers and 15 U/µl RNAse i) and incubated for 1 h at 40°C followed by incubation at 95°C for 5 min. The purified cDNA template was amplified using different sets of primers. The primers used were β-actin-F: 5'-taccactggcatcgtgatg-gact-3'; β-actin-R:

5’-tttctgcatcctgtcggaaat-3'; iNOS-F: 5'-cagataagtgacataagtga-3'; iNOS-R: 5'-ctatctttgttgttgtccttg-3'. PCR was performed in 25 µl volume in which 1X PCR buffer, 2.5 mM dNTPs, 1.5 mM $MgCl_2$, 1 U of Taq polymerase and 100 ng of the specific primers were added. A brief initial denaturation at 95°C for 5 min was followed by 35 cycles with the following steps: 95°C for 1 min, annealing at 42°C for 1 min and extension at 72°C for 1 min. RT-PCR products were then separated on a 1.2% agarose gel and visualized by staining with ethidium bromide. The intensities of the bands corresponding to the RT-PCR products were quantified using phosphor imager (Alpha Imager, Alpha Innotech) and normalized with respect to the β-actin product.

2.2.9. Lipid peroxidation assay (Thiobarbituric Acid Reactive Substance)

The generation of thiobarbituric acid reactive substances (TBARS) was measured following a published protocol (Ding *et al.,* 2006) with minor modifications. Briefly, the cells were seeded at density of $4x10^5$ cells/ml in 6-well plates (TPP, Europe/Switzerland) and were grown for 24 h followed by treatment with different concentrations of ALA (0-80 µM). After 24 h of ALA treatment, cells were harvested and resuspended in 120 µl of 1X PBS. The cells were homogenized using micro-pestle on ice for 10 min and then centrifuged at 10,000 rpm for 10 min. To the homogenized samples, 100 mM butylated hydroxytoluene (1.5 µL), 15% Trichloroacetic acid (50 µL), 0.25 mM butylated hydroxytoluene (50 µL), 0.375% thiobarbituric acid (50 µL), and 8.5% SDS (20 µL) were added and the samples vortexed for 5 min. This mixture was incubated at 80^0C for 120 min and the reaction was stopped by cooling on ice for 10 min. The samples were centrifuged at 10,000 rpm for 10 min and the supernatant from each tube was transferred to a 96-well plate. The optical density was measured at 540 nm using the ELISA plate reader (Bio-Rad,

Hercules, and CA). Thiobarbituric acid reactive substance (TBARs) was calculated using 1, 1, 3, 3-tetraethoxypropane (TMP) as a standard. The resulting TBARs values were normalized by the protein concentration of each sample that was estimated by Bradford reagent (Bio-Rad Laboratories Inc, CA, and USA).

2.2.10. Mitochondrial membrane potential

The cervical cancer cell lines were seeded at density 1×10^5 cells/ml in a black 96-well plate and incubated at 37^0C in CO_2 incubator. Next day, the cells were treated with different concentrations of ALA (0-80 μM) and the cells were incubated in CO_2 incubator at 37°C for 24 h. The following day, the medium was removed and the cells were washed with 1X PBS and incubated with 2.5 μg/ml JC-1 staining solution (Sigma-Aldrich, St. Louis, MO) for 1 h in dark (Wang *et al.,* 2009). Fluorescence readings were measured using Fluostar Omega microplate reader (BMG Labtech) at 520 nm for JC-1 monomers and at 590 nm for JC-1 aggregates.

2.2.11. Immunoblotting

Cell extracts were prepared from control as well as cells treated with different concentrations of ALA (0-80 μM). Briefly, the cell pellets were resuspended in 40 μl lysis buffer containing 50 mM Tris (pH 7.4), 5 mM EDTA, 0.5% NP40, 50 mM NaF, 1 mM DTT, 0.1 mM PMSF, 0.5μg/ml leupeptin (Pro-pure Amersco, Solon, USA), 1 μg/ml pepstatin (Amresco, Solon, USA), 150 mM NaCl, 0.5μg/ml aprotinin (Amersco, Solon, USA) and protease inhibitor cocktail (Roche, UK) and incubated on ice for 1 h with intermittent mixing. The extract was centrifuged for 20 min at 4°C at 12000 rpm. The protein was estimated using Bradford reagent (Biorad Laboratories Inc, CA, USA). Equal amount of protein was loaded on a 10% SDS-polyacrylamide

gel and transferred electrophoretically to Amersham Hybond-P PVDF membrane (GE Healthcare, UK) in sodium phosphate buffer (pH 6.8). The membrane was blocked in 5% BSA in TBST and incubated at room temperature for 1 h with rabbit polyclonal antibody for caspase 3 and mouse monoclonal antibody for tubulin (Santacruz, CA, USA) at 1:500 and 1:2000 dilutions respectively. The membrane was washed in TBST and incubated with donkey anti-rabbit IgG HRP conjugate at 1:5000 (for caspase) and donkey antimouse IgG HRP conjugate at 1:3000 (for tubulin) dilutions. Proteins were visualized using a chemiluminescence kit (Amersham ECL Advance western blotting detection kit, GE Healthcare, UK) and densitometric analysis of X-ray films was performed on Alpha Imager using Alpha Ease FC software, Alpha Innotech.

2.2.12. Statistical analysis

All the experiments were performed in triplicates and repeated at least three times and the data has been presented as mean ± SD. Statistical analysis was conducted with the Graph Pad 4 prism program using one-way ANOVA. The p values used for comparisons were < 0.05.

2.3. Results

2.3.1. ALA altered the cell viability of cervical cancer cell lines

Omega 3 fatty acids including, ALA, have been known to inhibit the growth of cancer cells (Kim *et al.,* 2009; Horia and Watkins, 2005; Das *et al.,* 1998; Sagar and Das, 1995). In the present report, we have treated both cervical cancer cell lines (SiHa and HeLa) with different concentrations (0-320 μM) of ALA for 24, 48 and 72 h. In SiHa, ALA was found to decrease the viability after 160 μM dose at 24 h

($p<0.0001$). However, after incubating the cells with ALA for 48 h and 72 h, the viability started decreasing at 20 µM and 40 µM, respectively (Figure 2.1 A). In HeLa, ALA decreased the cell viability after 40 and 160 µM at 24 and 48h, respectively. However, at 72h, there was a significant decrease in viability at a lower dose of 20 µM (Figure 2.1 B). IC_{50} values of ALA for SiHa and HeLa were 1531 and 1571 µM respectively. These results suggested that cervical cancer cells responded to ALA treatment at higher exposure times (beyond 24h) at lower concentrations (Figure 2.1 C). Interestingly, in HEK 293 cells and HaCaT (non-cancerous), ALA did not show any toxicity up till 320 µM dose implying that it was specific only for the cancerous cell lines.

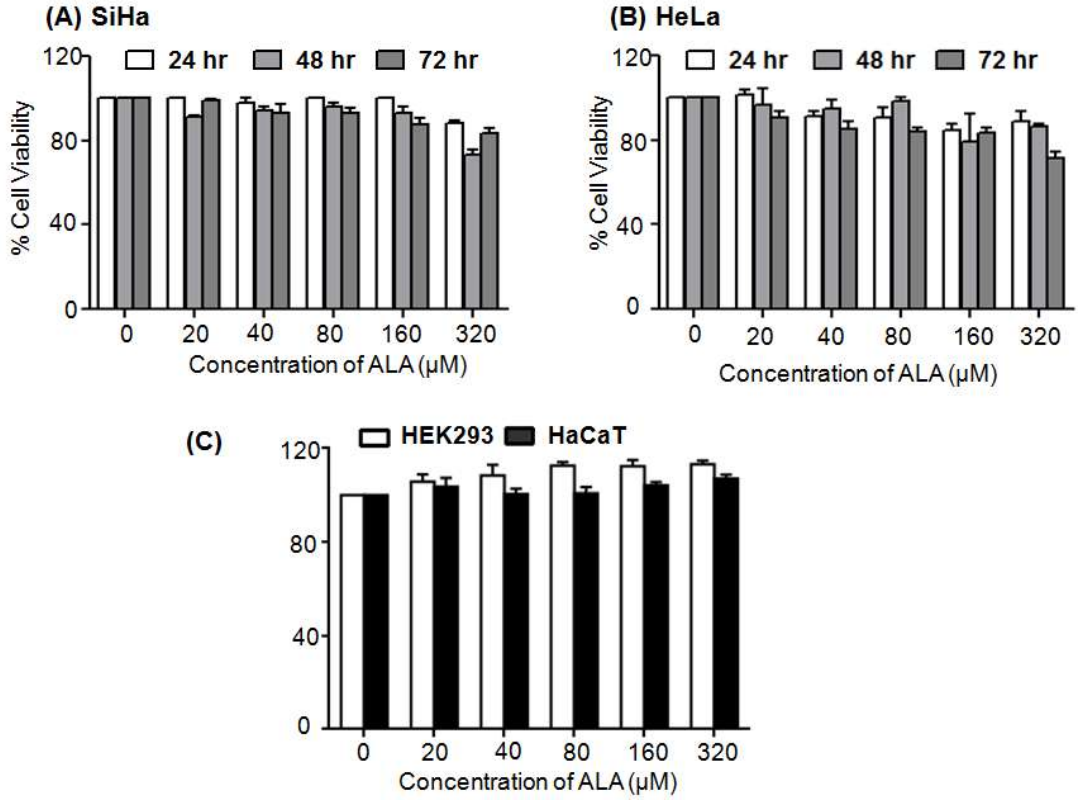


Figure 2.1. ALA altered the viability of the cervical cancer cell lines. *ALA altered the cell viability of cervical cancer cells SiHa (A) and HeLa (B) cells at 24, 48 and 72 h. ALA was nontoxic to noncancerous HEK293 and HaCaT cells (C). All the data have been presented as mean ± SEM of three independent experiments. $p < 0.05$ indicates statistically significant differences compared to the control untreated group.*

2.3.2. ALA altered growth kinetics of cervical cancer

To check the effect of ALA on growth kinetics of cervical cells were treated with different concentrations of ALA (0-80 μM/ml) and were grown for 24, 48 and 72 h. It was observed that ALA decreased the growth kinetics of the cells in a dose- and time-dependent manner. In SiHa, ALA decreased the cell growth at 80 μM/ml concentration by ~2 ($p = 0.008$), 2.4 ($p = 0.001$) and 3.05-fold ($p = 0.053$) at 24, 48 and 72 h, respectively, compared to the untreated control cells (Figure 2.2 A). Similarly, in HeLa, ALA decreased the cell proliferation by 2.25 ($p=0.001$), 1.68 ($p = 0.010$) and 2.33-fold ($p = 0.001$) at 24, 48 and 72 h, respectively, at 80 μM/ml concentration compared to the untreated control cells (Figure 2.2 B). This was further supported by a dose dependent decrease in the number of soft agarcolonies in both the cervical cancer cell lines (Figure 2.2 C). At 80 μM/ml concentration of ALA, there was ~ 2.8 fold ($p= 0.0032$) and ~3.01fold ($p= 0.0003$) decrease in number of colonies in HeLa and SiHa, respectively, compared to their respective untreated control cells (Figure 2.2 C). Thus, ALA regulated the growth kinetics of cervical cancer cells in a significant manner.

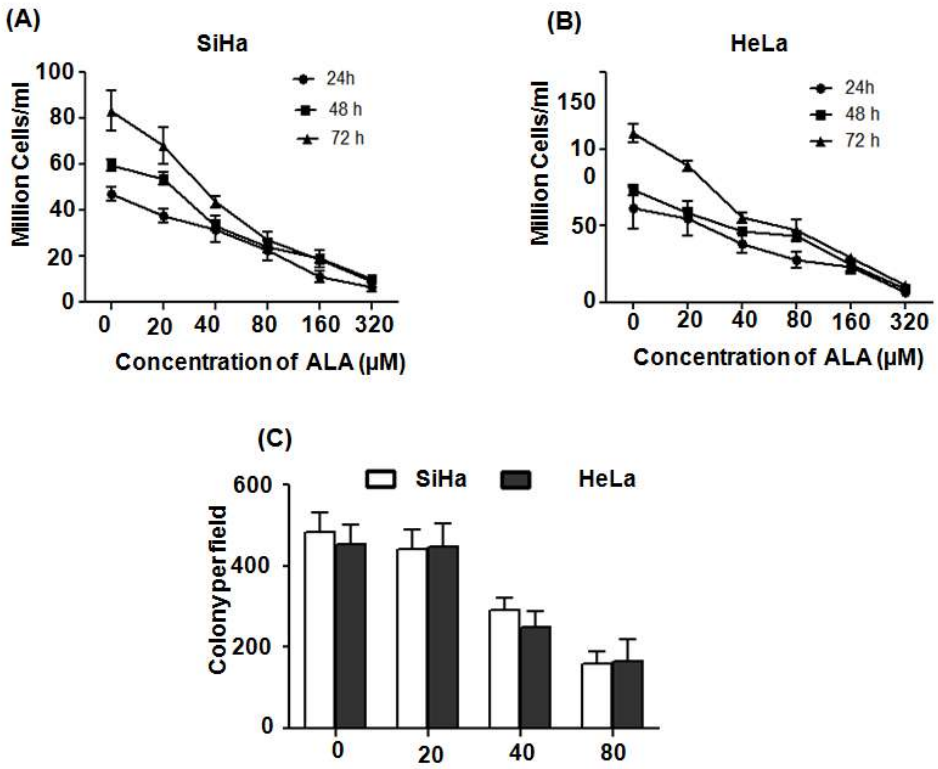


Figure 2.2 ALA regulated the growth kinetics of cervical cancer cells. *SiHa (A) and HeLa (B) were treated with ALA (0-80 µM/ml) for 24, 48 and 72 h and the number of viable cells were counted using the trypan blue dye exclusion method. Data represent mean ± SD of three independent experiments (C) Both SiHa and HeLa ($5x10^3$) were grownalong with ALA (0-80 µM/ml) in soft agar for two weeks. Colonies were counted from at least 10 different areas and the average of each has been plotted. The data represented mean ± SD of five independent experiments.*

2.3.3 ALA decreased the nitric oxide levels

Nitric oxide plays a dual role in cancer wherein it can either promote or suppress cancer (Crowell *et al.,* 2003; Lechner *et al.,* 2005). To test the effect of ALA on nitric oxide levels,cervical cancer cell lines were treated with different concentrations of ALA (0-80 µM) at different time intervals (24-72h). ALA significantly reduced the levels of NO by ~2.6 and 2.7-folds ($p<0.0001$) in SiHa at 40 and 80 µM ALA concentrations, respectively (Figure 2.3 A). On the other hand, in HeLa, the NO levels were reduced by ~1.7 and 1.8-folds ($p=0.0010$), respectively, at

40 and 80 µM concentration in 24 h (Figure 2.3 B). Here also, after treating the cells with ALA for 48 and 72h, the trend in the decrease in NO was similar to that observed at 24h, however, it was more significantly reduced in 24h treatment. Interestingly, at 24 h HeLa showed a significant decrease in NO levels compared to SiHa as observed by using fluorescent dye DAF-FM (Figure 2.3 C).

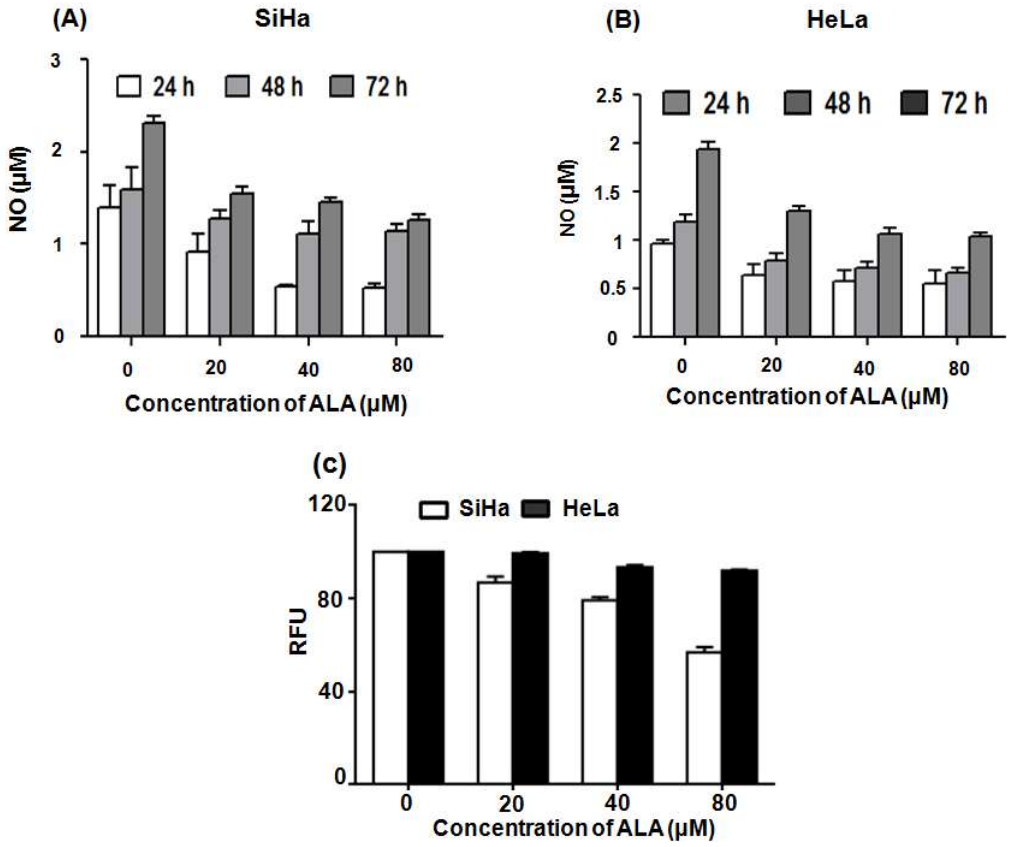


Figure 2.3. ALA reduced nitric oxide levels in cervical cancer cell lines.*The cells were treated with different concentrations of ALA (0-80 µM) for 24, 48 and 72 h and the nitric oxide release was measured in SiHa (A) and HeLa (B) cells by Griess reaction The cervical cancer cells were analyzed for intracellular nitric oxide levels by using DAF-FM fluorescence dye at 24 h (C). The data was analyzed by MARS data analysis software 2.10R3 (BMG Labtech). Values have been represented as mean ± SEM of five independent experiments, each conducted in triplicates.* $^{*}p < 0.05$ *indicates statistically significant differences compared to the untreated control cells.*

The decrease in NO was supported by a corresponding decrease in iNOS expression at mRNA level in a dose-dependent manner compared to the untreated control cells in both in SiHa (Figure 2.4 A) and HeLa (Figure 2.4 B). Densitometric analysis of iNOS expression was performed using phosphorimager.

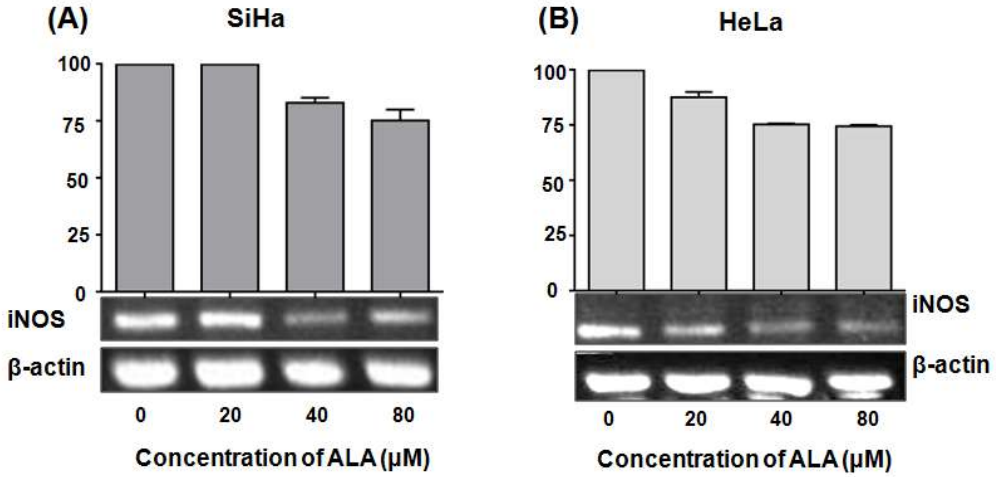


Figure 2.4 ALA reduced iNOS levels in cervical cancer cell lines. *ALA reduced iNOS expression at mRNA level in SiHa (A) and HeLa (B). β-actin was used as the loading control. Densitometric analysis of iNOS expression has been shown. Values have been represented as mean ± SEM of five independent experiments, each conducted in triplicates.* $^{*}p < 0.05$ *indicates statistically significant differences compared to the untreated control cells.*

2.3.4. ALA increased the lipid peroxidation in cervical cancer cells

Since ALA decreased the nitric oxide release and NO is known to either inhibit or promote lipid peroxidation (LPO) (Miles *et al.,* 1996; Cauwels*et al* 2005), we analyzed the effect of ALA on lipid peroxidation in cervical cancer cell lines. It was observed that ALA increased LPO at all the doses in cervical cancer cells, the increase being more significant at 40 μM dose. In SiHa, ALA showed a dose-dependent increase in LPO wherein at 80 μM concentration, there was ~2.6-fold increase in LPO was observed. However, in HeLa, there was a dose-dependent

increase in LPO till 40 μM ALA treatment, the latter showing wherein there was ~2.2-fold (p=0.0017) increase (Figure 2.5).

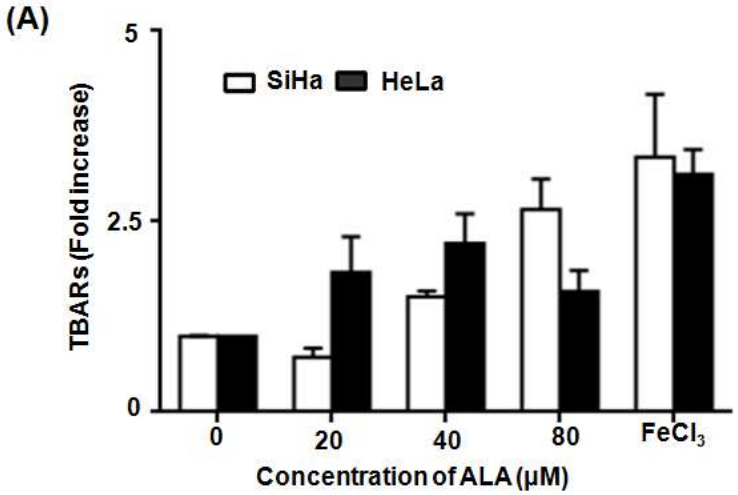


Figure 2.5 ALA increased lipid peroxidation in cervical cancer cells.*ALA induced lipid peroxidation in cervical cancer cells that has been shown by TBARs assay. TBARS values were normalized by the protein concentration of each sample estimated by Bradford reagent. Values have been represented as mean ± SEM of five independent experiments. *p<0.001 indicates statistically significant differences compared to $FeCl_3$ control.*

2.3.5. ALA induced apoptosis in cervical cancer cells

The loss of mitochondrial membrane potential is the hallmark of apoptosis (Wang *et al.,* 2009). Since ALA decreased NO and increased LPO, we wanted to analyze whether ALA induced apoptosis in the cell. Thus, we evaluated the effect of ALA on mitochondrial membrane potential in cervical cancer cells (Figure 2.6 A). ALA significantly reduced ($p<0.0001$) the mitochondrial membrane potential in SiHa, in a dose-dependent manner that correlated with a corresponding increase in caspase 3 expression (Figure 2.6 B). In HeLa, the decrease in mitochondrial membrane potential was observed more significantly at 20 μM ALA followed by a significant dose-dependent increase in caspase-3 expression. These results suggested that ALA induced apoptosis in cervical cancer cells (Figure 2.6 C). Densitometric analysis of

caspase-3 expression was performed by using phosphorimager (Figure 2.6 D). All these results suggested that ALA induced apoptosis in cervical cancer cell lines through activation of caspase-3 and decrease in mitochondrial membrane potential.

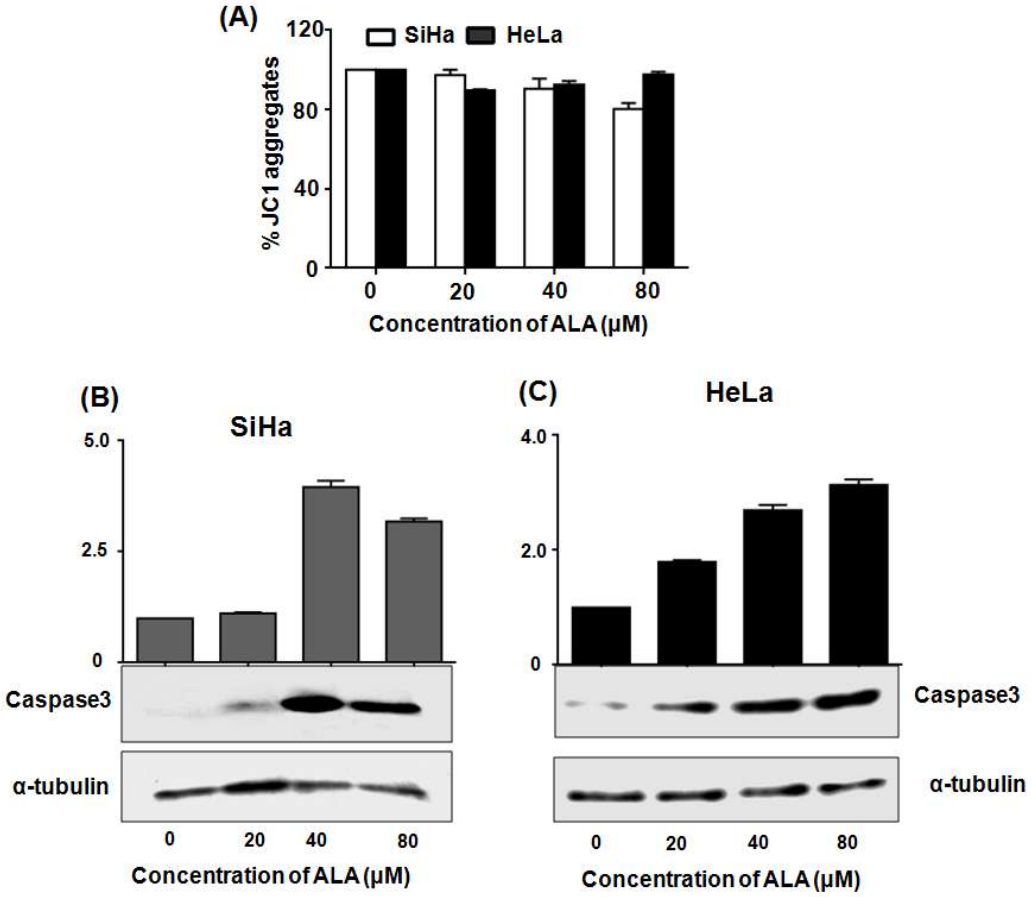


Figure 2.6 ALA induced apoptosis in cervical cancer cells. *ALA decreased the mitochondrial potential in cervical cancer cells SiHa and HeLa (A) and the data was analyzed by MARS data analysis software 2.10R3 (BMG Labtech). Caspase-3 (17/21 kDa) expression was determined in ALA treated SiHa (B) and HeLa (C) cell lines. The histograms depict densitometric analysis of western blots of caspase 3. Values have been represented as mean ± SEM of three independent experiments $p<0.001$ indicate statistically significant differences compared to the untreated control cells.*

2.4. Discussion

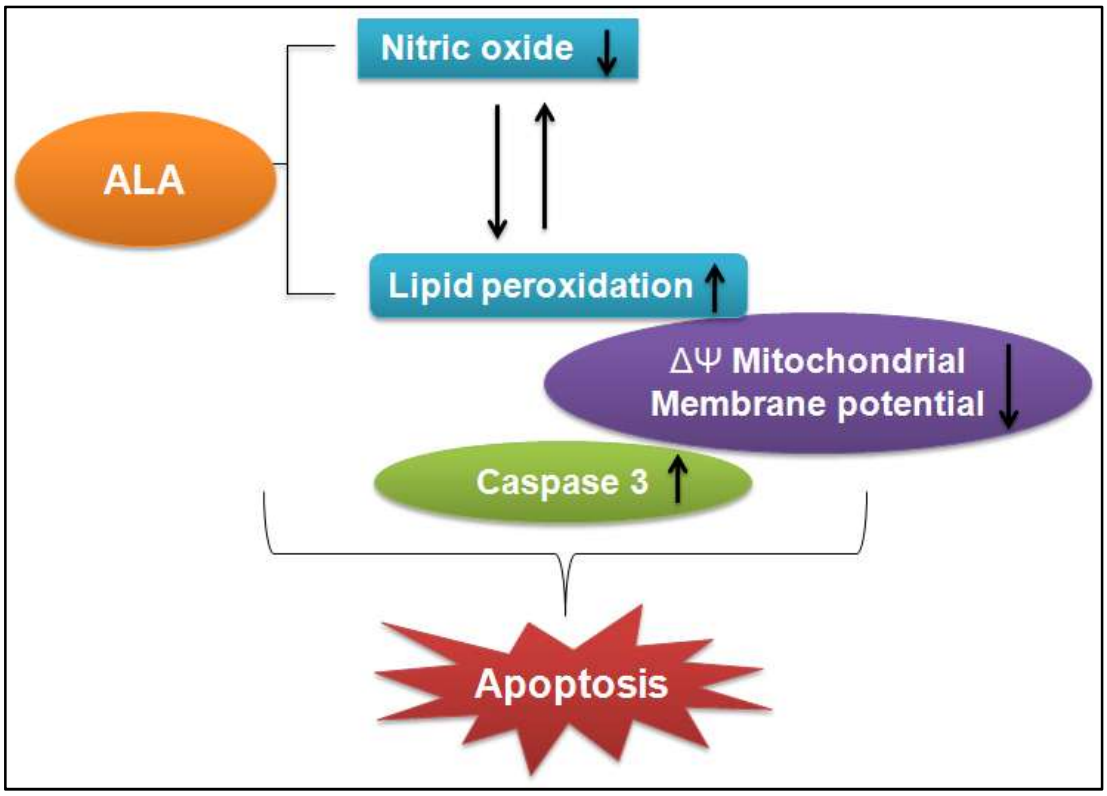
In the current study, we have analyzed the effect of ALA on modulation of growth of cervical cancer cells in terms of regulation of nitric oxide and lipid

peroxidation. ALA decreased the nitric oxide levels in the cervicalcancer cells. It is well-known that high levels of NO have both genotoxic and angiogenic properties (Nakamura *et al.,* 2006). Increased NO production catalyzed by iNOS enzyme in tumor cells, plays a critical role in tumor angiogenesis, cancer progression and metastasis (Narayanan *et al.,* 2003). ALA has been reported to decrease iNOS expression in LPS-stimulated macrophage cell line RAW 264.7 (Udenigwe *et al.,* 2009; Ren *et al.,* 2007). Our results showed that ALA not only decreased the iNOS expression at mRNA level but also reduced the intracellular levels of NO in cervical cancer cells. Thus, decrease in NO in both the cancer cell lines by ALA suggested its antineoplastic potential.

PUFAs have been shown to initiate free radical generation as well as lipid peroxide products selectively in tumor cells compared to the normal cells (Sun *et al.,* 2012; Das 2002). Several studies with ALA have shown that it increased lipid peroxidation in breast cancer cells (Menéndez *et al.,* 2001; Pardini 2006). Moreover, an inverse correlation has been reported between lipid peroxidation and cell proliferation (Das 2002). In this line, we found that ALA increased the lipid peroxidation with a simultaneous decrease in cell proliferation in the cervical cancer cell lines. Recently, it was demonstrated that peroxidized products of n-3 PUFAs suppress iNOS induction and NO production in peroxidation-dependent manner (Araki *et al.,* 2011). Thus, the observed decrease in NO in both the types of cervical cancer cell lines may be partly due to NO suppression by peroxidized products of ALA. A strong association between decreased NO levels and increased lipid peroxidation has been reported in several papers. For example, it was found that patients suffering from fibromyalagia had higher serum levels of TBARS (particularly, malondialdehyde) and lower levels of nitrite compared to the control

groups (Ozgocmen *et al.,* 2006).Another report has shown that a decrease in the level of NO in rats treated with alloxan-induced diabetes was associated with increased level of lipid peroxides (KrishnaMohan and Das, 2001). Thus, increase in lipid peroxides may lead to increased free-radical generation that may inactivate NO resulting into its low levels.

Mitochondria play an important role during apoptosis (Wang *et al.,* 2009). Reactive oxygen species can directly activate the mitochondrial permeability transition and result in loss of mitochondrial membrane potential ($\Delta\Psi$), which results into release of cytochrome c (cyt c) and activation of caspase pathway (Sun *et al.,* 2012; Cao *et al.,* 2010; Lee *et al.,* 2008; Kim *et al.,* 2005). ALA reduced the nitric oxide levels and increased lipid peroxidation in cervical cancer cells that could be responsible for the observed apoptosis (Scheme 2.1). Conversely, increased nitric oxide has been reported to inhibit lipid peroxidation by scavenging lipid peroxyl radicals (Hogg and Kalyanaraman*,* 1999). Increased NO has also been shown to prevent activation of caspase 3 resulting into inhibition of apoptosis (Zhou *et al.,* 2005; Maejima *et al.,* 2005; Mahidhara *et al.,* 2003; Kim YM *et al* 1997). Thus, the increased LPO in presence of ALA leads to decrease in NO resulting into disruption of mitochondrial membrane potential and activation of caspase 3 resulting into apoptosis of the cells (Scheme 2.1). Taken together, our data suggests that ALA regulated the growth of cervical cancer cells through regulation of lipid peroxidation as well as nitric oxide generation that may have lead to apoptosis.



Scheme 2.1. A proposed model for the mode of action of ALA.

2.5. Conclusion

Omega 3 fatty acids are known to possess anticancer effects through various mechanisms, one of them being through generation of free radicals and lipid peroxidation (Sun *et al.,* 2012). Most of the research work till date has discussed the significance of EPA and DHA in cancer with very less data reported on ALA. Our paper has tried to delineate the anticancer properties of ALA in terms of its potential to regulate lipid peroxidation as well as nitric oxide generation that in turn results into control of carcinogenesis process. However, more studies are required in future to elucidate the role of PUFAs in governing the inter-relationship between the nitric oxide and lipid peroxidation status of cells for regulation of cancer growth.

Chapter 3

Elucidating the effect of ALA on COX2/VEGF/MAP kinase pathway and expression of HPV oncoproteins E6/E7 in cervical cancer cells

Manuscript communicated

3.1. Introduction

In the earlier chapter, we have reported that ALA regulated cell viability and induced apoptosis in cervical cancer cell lines, SiHa and HeLa. In the present chapter, we found that ALA significantly decreased the cell migration, which was accompanied by decrease in the expression of VEGF, MMP-2 and MMP-9 proteins. ALA reduced the expression of phosphorylated p38, pERK1/2, c-JUN, NFκB and COX-2 proteins significantly. Interestingly, ALA disrupted the expression of HPV E6 and E7 oncoproteins resulting into restoration of expression of tumor suppressor proteins, p53 and Rb. These data suggest the therapeutic potential of ALA in cervical cancer.

3.2. Materials and Methods

3.2.1. Reagents

Alpha linolenic acid and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagles Medium (DMEM), Penicillin and streptomycin were obtained from Gibco BRL, USA. Fetal bovine serum was purchased from SAF (USA). L-Glutamine was obtained from Himedia Corporation, Mumbai, India. Primary antibody against p53 (DO-1), Her-2 (F-11), pRb (C-15), ppRb (SER 807/811), HPV16 E6/18 E6 (C1P5), HPV16 E7 (ED17), HPV18 E7(N-19) and alpha tubulin (B-7) and secondary antimouse, antirabbit antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).Tissue culture plasticware was purchased from BD Biosciences(CA, USA) and Axygen Scientific Inc (CA, USA). All other common reagents were procured from Qualigens Fine Chemicals (Mumbai, India)

3.2.2. Cell culture

The cervical cancer cell lines (SiHa and HeLa) used in the study was obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in DMEM containing 2 mM L-glutamine supplemented with 10% fetal bovine serum and 20 U/mL penicillin, 20µg/mL streptomycin and incubated in a humidified 5% CO_2 incubator at 37°C.

3.2.3. Cell migration

Cell migration was analyzed by wound healing assay as described previously (Wani *et al.,* 2014). Both SiHa and HeLa cells were seeded at a density of 4×10^5 cells/ml/well in 24-well plates and were allowed to adhere overnight at 37°C in 5% CO_2 incubator. A linear artificial wound was made by scratching the monolayer of cells after serum starvation for 6 h with a 10µl pipette tip. The cell debris was removed by washing the monolayer with 1X PBS followed by addition of complete medium with or without ALA (0–80 µM) and the wound was allowed to heal for 24 h at 37°C in 5% CO_2 incubator. The images for 0 h and 16 h were captured with the help of Axiovert 200 M microscope. The average extent of % open wound area was evaluated by measuring the width of the wound by T-Scratch software (Gebäck *et al.,* 2009).

3.2.4. Gelatin Zymography

The activity of MMP-2 and 9 in the conditioned medium was determined by gelatin zymography as described previously (Koppikar *et al.,* 2010).Briefly, SiHa and HeLa cells were seeded at a density of 4×10^5cells/ml/well in 6-well plates and allowed to adhere overnight at 37°C in 5% CO_2 incubator. Next day, the cells were

treated with various concentrations of ALA (0–80 μM) prepared in serum-free medium and incubated for 24 h. The following day, the culture medium was collected and centrifuged at 14,000 rpm for 20 min at 4^0C to remove the cellular debris. The conditioned medium from control cells and those treated with ALA was collected and concentrated in Centricon YM-30 tubes (Millipore, MA). The samples containing an equal amount of total protein were mixed with sample buffer (2% SDS, 25% glycerol, 0.1% bromophenol blue and 60 mM Tris-HCl pH 6.8) and subjected under non-reducing conditions on to 7.5% SDS-polyacrylamide gel containing gelatin (0.5 mg/ml). Following electrophoresis, the gel was washed with 0.25% Triton X-100 and incubated over night at 37^0C in buffer containing 150 mM NaCl, 100 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5, 1% Triton X- 100, 0.02% NaN_3. The gel was stained with 0.1% Coomassie Brilliant blue R-250 in 40% isopropanol and destained in 7% acetic acid. Gelatinolytic activity was detected as unstained bands against blue background. The quantification of bands in control and treated samples was performed by densitometric analysis on Image Lab™ Software, Gel Doc™ EZ System, Bio-Rad.

3.2.5. Immunoblotting

Cell extracts were prepared from control and those treated with different concentrations (0-80 μM) of ALA. Briefly, the cell pellets were resuspended in 40 μl lysis buffer containing 50 mM Tris (pH 7.4), 5 mM EDTA, 0.5% NP40, 50 mM NaF, 1 mM DTT, 0.1 mM PMSF, 0.5μg/ml leupeptin (Pro-pure Amersco, Solon, USA), 1 μg/ml pepstatin (Amresco, Solon, USA), 150 mM NaCl, 0.5μg/ml aprotinin (Amersco, Solon, USA) and protease inhibitor cocktail (Roche, Lewes, UK) and incubated on ice for 1 h with intermittent mixing. The extract was centrifuged for 20 min at 4°C at 12000 rpm. The protein was estimated using Bradford reagent (Biorad

Laboratories Inc, CA, USA). Equal amount of protein was loaded on a 10% SDS-polyacrylamide gel and transferred electrophoretically to Amersham Hybond-P PVDF membrane (GE Healthcare, UK) in sodium phosphate buffer (pH 6.8). The membrane was blocked in 5% BSA in TBST and incubated at 4^0C overnight with primary antibody against pERK1/2, ERK1/2, NFκB, COX-2, C-Jun, pp38 , p38, p53, Her-2, pRb, ppRb, HPV16 /18 E6, HPV16 E7, HPV18 E7 or alpha-tubulin (Santacruz, CA, USA) at a 1:500 dilution. The membrane was washed in TBST and incubated with secondary IgG HRP conjugate at 1:5000 dilution. Proteins were visualized with a chemiluminescence kit (Clarity™ Western ECL substrate, Bio-Rad,US) and densitometry analysis was performed on scanned immunoblot images using the ImageJ gel analysis tool (Wayne Rasband, National Institutes of Health, USA, http://imagej.nih.gov/ij).

3.2.6. Cellular fatty acid analysis

Cervical cancer cell lines were plated at 8×10^5 cells/well seeding density in 6 well plates and were incubated in 10% DMEM supplemented with ALA (0-80 μM) for 24 h. After incubation, cells were harvested by centrifugation, and the pellets were rinsed twice with 1X PBS. The procedure for fatty acid analysis used in our study was adapted from the original method (Horia & Watkins 2009). Briefly, cell pellets were mixed with 4 mL of Methanol/HCl/BHT (94.7:5.3:0.0005, v/v/w) in a 15-ml screw cap vial. The vials were sealed and incubated at 80ºC for 2 h and then cooled on ice for 30 min. The total fatty acid methyl esters were extracted by adding 2 mL hexane (2N), and the layers were separated by centrifugation in a swinging rotor at 3000 rpm for 15 min at room temperature. The hexane layers were carefully removed and collected in a separate vial. The hexane extract was completely dried by

passing argon gas and stored at -20˚C until analyzed. The methylated fatty acids were re-suspended in 100 µL of chloroform, and 1µL was injected in GC column. Helium was used as carrier gas at 1 mL/min. Oven temperature was held at 150ºC for 10 min, programmed to rise from 150 to 220 ºC at10ºC/min, and at 220 ºC for 10 min. The detector temperature was 275ºC, and the injector temperature was 240 ºC. The column was calibrated by injecting the standard fatty acid mixture in approximately equal proportion. The data was recorded and the peaks were identified as per the retention time of the standard fatty acids (Sigma, USA) run under the identical conditions.

3.2.7. Statistical analysis

All the experiments were performed in triplicates and repeated at least three times and the data has been presented as mean ± SD. Statistical analysis was conducted with the Graph Pad 5 prism program using one-way ANOVA. The p values used for comparisons were < 0.05.

3.3. Results

3.3.1. ALA reduced cell migration and decreased the expression of MMPs and VEGF protein

To analyze the effect of ALA on cell migration, we performed the wound healing assay in SiHa and HeLa. After 16 h, the untreated SiHa and HeLa cells covered up 94 and 92 % of the wound, respectively (Figure 3.1A and B, respectively), whereas cells treated with 80 µM concentration of ALA, covered up the wound by 60% ($p<0.001$) and 68 ($p<0.001$), respectively (Figure 3.1C).

At 80 µM ALA treatment, the expression of MMP-9 and 2 were down-regulated ($p \leq 0.001$) by ~ 1.4 and ~ 2.5-folds, respectively, in SiHa (Figure 3.1D) and ~1.6 and ~ 3.3-folds in HeLa, respectively (Figure 3.1 E).

Interestingly, ALA decreased the expression of VEGF and HER2 oncoproteins in a dose-dependent manner in both SiHa and HeLa. In SiHa, at 80 µM of ALA, ~ 8 and 7-fold decrease in the expression of VEGF and Her2 proteins, respectively, was observed (Figure 3.1 F). On the other hand, in HeLa, ~ 3- and ~3.5 folds decrease in the expression of VEGF and HER2, respectively, was observed at 80 µM ALA (Figure 3.1G). α-tubulin was used as a loading control. These results suggested that ALA reduced cell migration and angiogensis markers in cervical cancer cells.

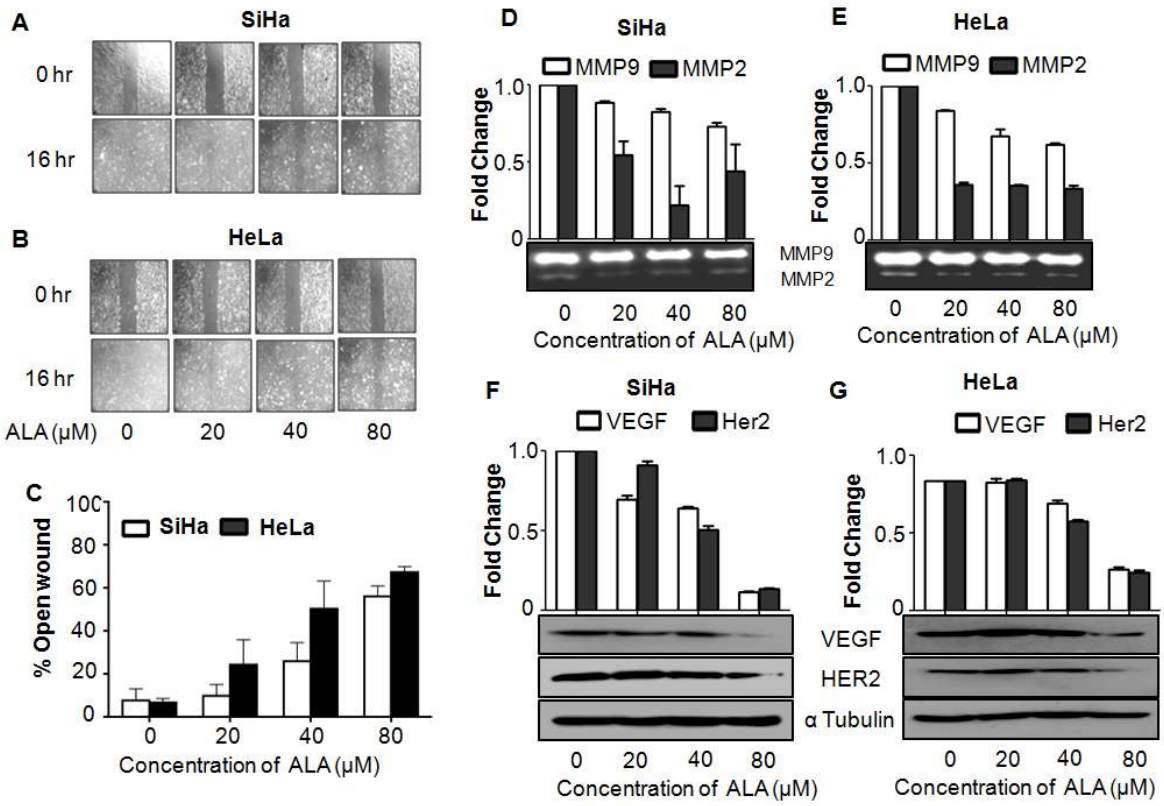


Figure 3.1. ALA reduced cell migration and decreased the expression of MMPs and VEGF proteins. *ALA reduced migration of SiHa (A) and HeLa (B) cells. The upper panel of each figure shows the wound made at 0 h and the lower panel shows the migration of cells after 16h. Graphical representation (C) of wound closure in SiHa and HeLa at 16 h after ALA treatment. Gelatin zymography showed downregulation of MMP-9 and MMP-2 expression in SiHa (D) and HeLa (E) after treatment with ALA with their corresponding densitometric analysis. ALA decreased VEGF and Her-2 expression in SiHa (F) and HeLa (G). The bands were quantified by densitometry using ImageJ 1.44p. Values have been represented as mean±SD of three independent experiments with $p<0.0001$, indicating statistically significant differences compared to the untreated control cells.*

3.3.2. ALA regulated NFκB and COX-2 expression

We analyzed the effect of ALA on pro-inflammatory markers COX-2 and NFκB. In SiHa, 80 μM ALA decreased the expression of NF-κB and COX-2 by ~2.5- and ~2.4-folds respectively (Figure 3.2 A). In HeLa, 80 μM ALA decreased COX-2 and NF-κB expression by ~3.1- and ~ 4.7- folds, respectively (Figure 3.2 B). α-tubulin

was used as a loading control. Thus, ALA regulated the expression of pro-inflammatory markers in cervical cancer cells.

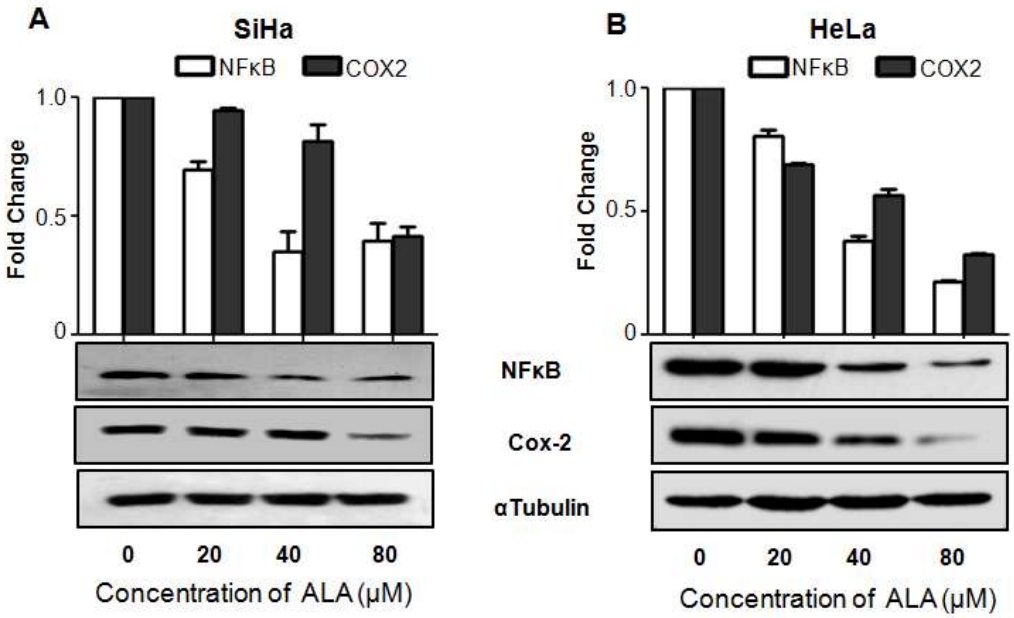


Figure 3.2 ALA regulated NFκB and* COX-2 *expression in cervical cancer cells. *ALA treatment reduced NFκB and* COX-2 *expression in SiHa (A) and HeLa (B) as observed by western blot analysis. The bands were quantified by densitometry using ImageJ 1.44p.The values have been represented as mean±SD of three independent experiments at $p<0.001$, indicating statistically significant differences compared to the untreated control cells.*

3.3.3. ALA reduced the expression of phosphorylated MAPKs and c-Jun

The effect of ALA on MAPKs that include the extracellular signal regulated kinase (ERK), c-Jun and p38 were investigated. The expression of pERK1/2 was decreased upto ~10- and 1.8- fold in SiHa and HeLa, respectively, without any effect on its total ERK1/2 at 80 μM of ALA. Similarly, in SiHa (Figure 3.3 A) and HeLa (Figure 3.3 B), at 80 μM of ALA, the expression of phosphoraylated p38 was decreased upto ~1.75 and 5– fold, respectively compared untreated control, without any alteration in the expression of total p38. Interestingly, at 80 μM of ALA, the expression of c-Jun decreased upto ~ 2-fold in SiHa and ~ 3-folds in HeLa (Figure 3.3

C). These results showed that ALA regulated the MAPK pathway in cervical cancer cells.

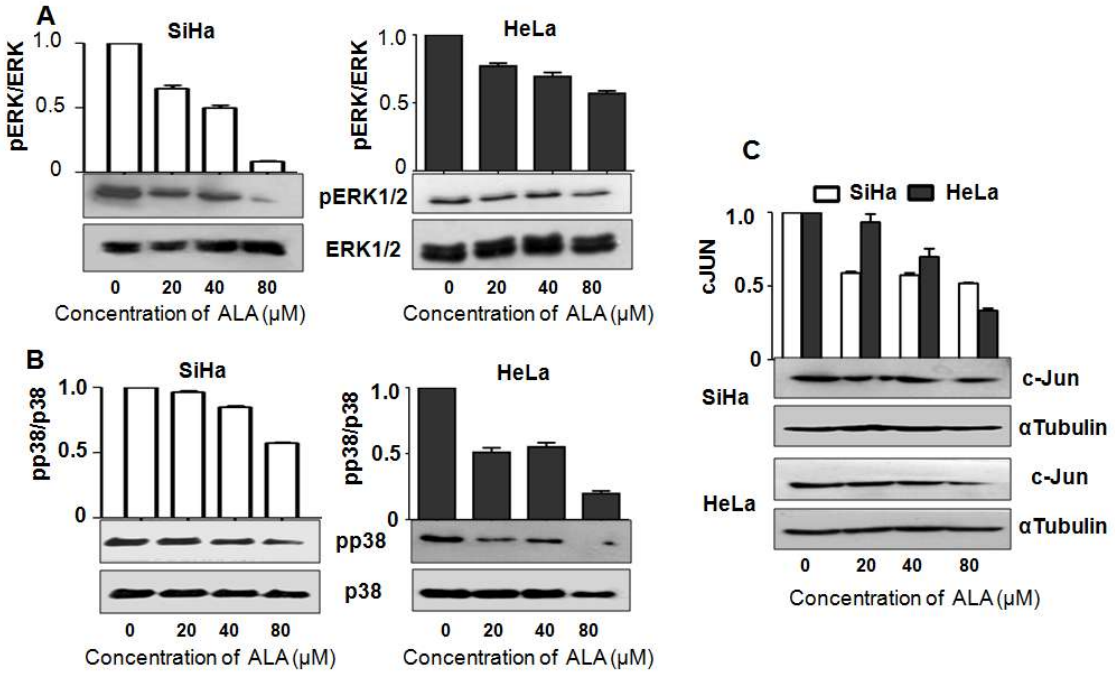


Figure 3.3. ALA reduced the expression of phosphoryled MAPKs and c-Jun *ALA decreased the phosphorylated pERK (upper panel) in SiHa and HeLa (A) without any effect total ERK. ALA decreased the expression of pp38 in SiHa and HeLa without any effect on total p38 (B). The expression of c-Jun expression was reduced after treatment with ALA in SiHa and HeLa (C). Expressions were determined by Western blotting .The bands were quantified by densitometry using ImageJ 1.44p.The values have been represented as mean±SD of three independent experiments at $p<0.001$, indicating statistically significant differences compared to the untreated control cells.*

3.3.4. ALA modulated the expression of p53 and Rb proteins

The effect of ALA on the expression of p53, Rb and pRb was analyzed in the cells. In SiHa, ~4 and ~3-fold increase in the expressions of p53 and Rb respectively, was observed at 80 μM of ALA (Figure 3.4 A). At the same concentration of ALA, in HeLa there was ~2.1 and 1.5-fold increase in the expressions of p53 and Rb, respectively, compared to the untreated control cells (Figure 3.4 B). The expression of Phospho Rb (pRb) was decreased in both SiHa (Figure 3.4 A) and HeLa (Figure 3.4

B). α-tubulin was used as a loading control. Thus, ALA modulated the expression of tumor regulatory markers in cervical cancer cells.

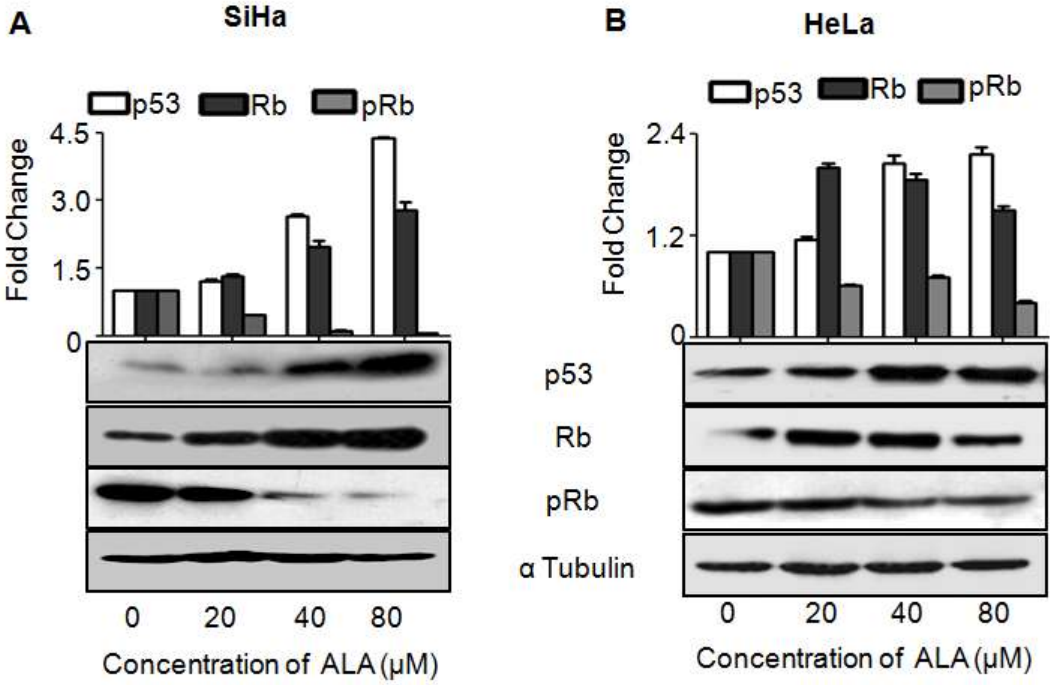


Figure 3.4. ALA modulated the expression of tumor regulatory proteins.*ALA increased the expression of tumor regulatory proteins p53, pRb and decreased expression of ppRb in SiHa (A) and HeLa (B).The bands were quantified by densitometry using Image J 1.44p.The values have been represented as mean±SD of three independent experiments at p<0.001, indicating statistically significant differences compared to the untreated control cells.*

3.3.5. ALA reduced the expression of viral oncoproteins E6 and E7

The expression of E6 and E7 proteins was investigated in ALA treated and untreated cells. ALA significantly reduced the expression of E6 and E7 oncoproteins in both SiHa and HeLa. At 80 μM concentration of ALA, the expression of E6 and E7 proteins was decreased by ~ 4- and 5-folds, respectively, in SiHa (Figure 3.5 A) and by ~ 3.3- and 3.5 -folds, respectively, in HeLa (Figure 3.5 B) compared to the untreated control cells. α-tubulin was used as a loading control. These results suggested the potential of ALA as a therapeutic agent that could regulate the cervical cancer.

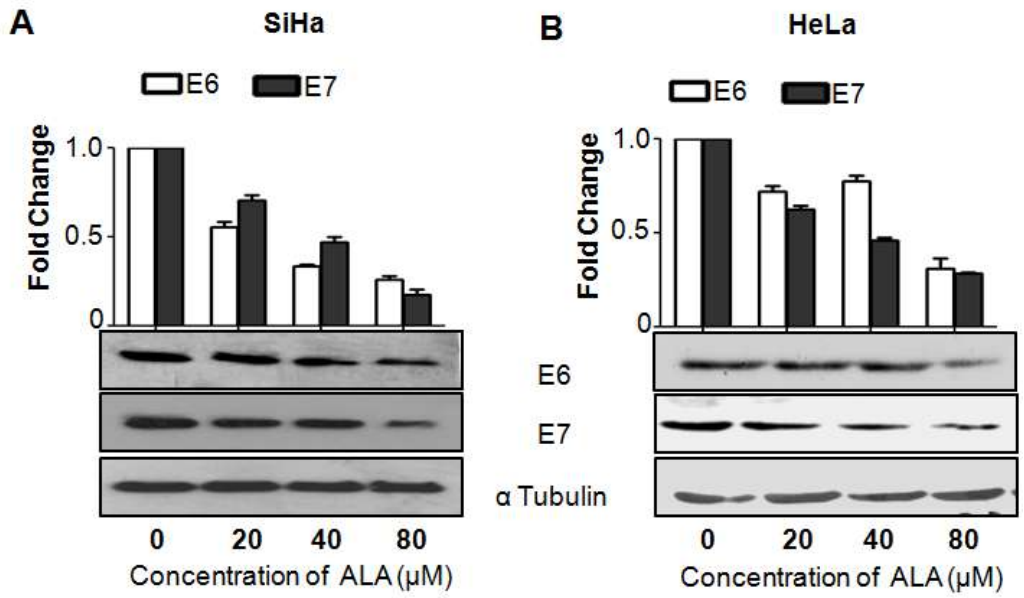


Figure 3.5. ALA reduced the expression of viral oncoproteins E6 and E7. *The expression of E6 and E7 oncoproteins was determined in SiHa (A) and HeLa (B) by western blotting. Alpha-tubulin was used as a loading control. The bands were quantified by densitometry using ImageJ 1.44p.The values have been represented as mean±SD of three independent experiments at $p<0.001$, indicating statistically significant differences compared to the untreated control cells.*

3.3.6. Effect of ALA on cellular fatty acid profiles

To detect whether treatment with ALA induced any specific changes in the fatty acid composition of the cells, we analyzed the fatty acid profile in the total lipid extract of the cells. Tables 3.1 and 3.2 refer to the relative percentage of the PUFA in the lipid fractions of SiHa and HeLa, respectively, after treatment with different concentrations of ALA.

Both SiHa and HeLa showed significant uptake of ALA. At 80 µM of ALA, there was ~3.1- fold increase in ALA in SiHa. Further, there was an increase in the content of EPA and DHA by ~16 and 2.3-fold, respectively in SiHa at 80 µM of ALA (Table 3.1). Total omega 6 FA (LA and AA) were found to get decrease decreased after ALA treatment. At 80 µM concentration, ALA decreased LA and AA by ~ 5-

and ~4.3- fold respectively, in SiHa (Table 3.1). In SiHa, the total percentage of saturated fatty Acids (SFA) was found to significantly decrease compared to the untreated control cells. However, total percentage of MUFA did not change significantly compared to the untreated control cells in SiHa.

In HeLa, at 80 μM of ALA, there was 2.6-fold increase in ALA and ~19 and 2.4-fold increase in the content of EPA and DHA, respectively (Table 3.2). At 80 μM of ALA, there was decrease in level of LA and AA by ~ 6- and 3-fold respectively, compared to untreated control in HeLa cells.

In HeLa, there was non-significant decrease in the total percentage of saturated fatty Acids (SFA) and total MUFAs was compared to the untreated control cells.

Interestingly, increase in EPA was more compared to DHA and ALA in both SiHa and HeLa. Both the cell lines showed efficient incorporation of ALA. These results showed that, both the cervical cancer cell lines converted ALA efficiently into EPA and to a small extent into DHA.

In SiHa and HeLa, omega 3FA content was increased in a dose dependant manner with a concomitant decrease in omega 6 FAs. Total PUFAs increased after ALA treatment in both the cell lines.

Table 3.1 Relative Fatty acid composition of SiHa cells treated with ALA (0-80 µM)

Fatty acid	Concentration of ALA (µM)			
	0	**20**	**40**	**80**
Omega 3 FAs				
ALA(18:3n-3)	0.88±0.30	1.95±0.021	2.89±0.38	4.57±1.6
DPA(22:5n-3)	3.44±2.21	3.05±0.3	2.08±0.445	1.94±0.5
EPA (20:5n-3)	3.59±2.44	35.5±2.08	43.81±19.4	60.4±3.16
DHA (22:6n-3)	2.17±1.19	2.9±0.21	3.67±0.825	5.1±1.52
Omega 6 FAs				
LA (18:2n-6)	6.9±2.7	4.5±1.5	1.6±1.4	1.3±1.10
GLA (18:3n-6)	2.42±2.16	0.38±0.36	0.42±0.32	0.18±0.17
DGLA (20:3n-6)	1.19±0.2	0.96±0.13	1.13±0.22	0.42±0.33
AA (20:4n-6)	30.1±8.48	12.0±1.06	11.4±2.23	7.02±5.41
SFA				
MA (14:0)	2.29±0.68	1.66±0.64	1.69±1.40	1.34±1.19
PAM(16:0)	19.5±5.61	12.4±4.14	3.77±1.69	2.9±0.76
SA(18:0)	18.2±0.40	12.3±1.82	7.70±5.71	6.0±4.23
MUFA				
MYRO (14:1 n-5)	1.33±0.45	1.15±0.50	0.21±0.09	0.23±0.11
PAMO (16:1 n-7)	2.63±1.14	1.63±0.33	3.5±1.58	2.23±0.97
OA(18:1 n-9)	4.3±1.19	4.86±0.85	3.777±2.53	3.13±0.88
NA(24:1n-9)	1.16±0.44	2.46±1.12	3.93±0.11	2.93±1.38
PUFAs	**36.1±2.3**	**42.5±4.35**	**63.8±13.4**	**81.0±9.6**
n-3 PUFAs	**10.3±6.28**	**43.9±2.52**	**60.8±13.16**	**70.9±1.8**
n-6 PUFAs	**40.7±13.24**	**18±3.1**	**14.6±0.26**	**9.02±4.81**

Table 3.2. Relative Fatty acid composition of HeLa treated with ALA (0-80 µM)

Fatty acid	Concentration of ALA (µM)			
	0	20	40	80
Omega 3 FAs				
ALA(18:3n-3)	0.54±0.06	1.09±0.91	1.89±1.24	3.92±0.82
DPA(22:5n-3)	1.8±1.3	2.27±0.93	1.62±0.79	2.60±1.93
EPA (20:5n-3)	1.43±1.1	9.50±6.187	53±0.19	57.1±3.14
DHA (22:6n-3)	1.75±0.17	1.64±0.80	2.04±0.03	3.91±0.15
Omega 6 FAs				
LA (18:2n-6)	9.6±3.19	2.09±0.76	2.5±2.2	1.5±1.54
GLA (18:3n-6)	1.1±0.69	0.14±0.03	1.2±0.92	0.67±0.28
DGLA (20:3n-6)	3.05±2.4	1.29±0.38	1.1±0.33	2.2±1.7
AA (20:4n-6)	23.17±3.4	16.25±5.17	10.54±2.4	7.92±4.9
SFA				
MA (14:0)	3.43±2.63	1.28±1.09	1.275±1.03	0.85±0.31
PAM(16:0)	16.08±0.50	4.28±1.67	5.26±1.259	4.53±4.32
SA(18:0)	16.14±1.52	8.02±6.11	6.81±3.35	7.89±6.37
MUFA				
MYRO (14:1 n-5)	2.115±1.308	0.23±0.25	0.35±0.134	0.44±0.35
PAMO (16:1 n-7)	1.78±0.56	0.42±0.12	0.38±0.084	0.40±0.38
OA(18:1 n-9)	13.36±10.56	11.67±9.42	7.35±3.012	7.52±5.671
NA(24:1n-9)	7.37±5.58	2.99±2.90	3.03±2.29	2.56±1.79
PUFAs	36.1±2.3	42.5±4.35	63.8±13.4	81.0±9.6
n-3 PUFAs	5.67±0.42	16.06±5.36	48.6±15.66	63.4±0.32
n-6 PUFAs	35.8±9.5	22.25±7.01	15.2±5.679	16.1±8.421

SFA indicates total saturated fatty acids (MA+PAM+SA); MUFA (MYRO+ PAMO + OLA+NA), total polyunsaturated fatty acids (n6 + n3 PUFA), n3 PUFAs (ALA+DPA+EPA+DHA), n6 PUFAs (LA+GLA+DGLA+AA). Data has been expressed as relative % of total fatty acids with mean±SEM. *p<0.05, **p<0.01 and ***p<0.001 indicate statistically significant differences between the treated and untreated control group and "ns" indicates non-significant results.

3.4. Discussion

Cervical cancer progression depends on critical steps such as cell proliferation, invasion and migration (Morandell 2012; Domenico 2012; Kim 2006; Behren 2005). In this report, we analyzed the effect of ALA on migration, angiogenesis, inflammation, and expression of tumor markers and HPV oncoproteins (E6 and E7) in cervical cancer cell lines.

ALA decreased the expression of VEGF whose over-expression has been correlated with increased vasculature development in tumorigenesis (Ueda 2002). ALA also reduced the expression of MMP2 and MMP9 that have been reported to be involved in connective tissue degradation, tumor-induced angiogenesis, and cell migration in cancer (Huang 2009). Both VEGF and MMPs play important role in cancer cell angiogenesis and metastasis (Brooks 2010).ALA decreased the expression of HER2/neu oncogene in both the cervical cancer cell lines, which has been reported to be frequently amplified in cervical cancer and has been considered as a potent therapeutic target (Choudhari 2013; Shen 2008).

ALA decreased the expression of NFκB and COX-2 in both SiHa and HeLa. In neoplastic cervical epithelial cells, HPV oncogenes have been shown to induce the inflammation by elevating expression of NF-κB and COX-2 proteins (Subbaramaiah 2007). NF-κB has been reported to activate COX-2, that inturn promotes further inflammatory cascades in cervical cancer (James 2006; Kim 2009; Gagnon 2004).

ALA decreased the expression of phosphorylated ERK1/2 and p38 without affecting the total respective proteins (Branca 2004). ERK1/2 is a key member of MAPK signaling pathway and can be activated by a variety of stimuli (Roberts 2007). Phosphorylated forms of ERK have been shown to be involved in cell proliferation, cell cycle, adhesion and invasion, as well as metastasis (Roberts 2007; Olson 2004).

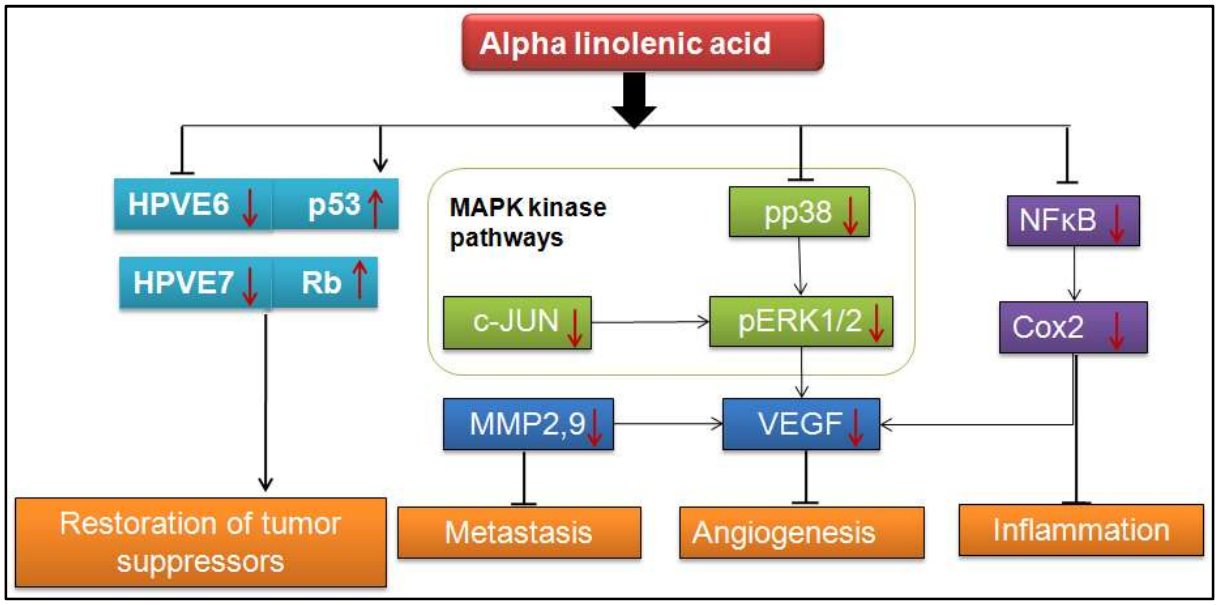
ALA decreased the expression of c-Jun in both the cervical cancer cell swhose levels are therewise increased in transformed cells and malignant tissues. HPV oncogenes have been reported to activate ERK1/2 and induce expression of c-Jun, which is necessary for cell proliferation (Yamaguchi 2005; Weiss 2004). Thus, ALA regulated c-Jun pathway through decreasing of its expression.

ALA increased the expression of tumor suppressor proteins p53 and Rb and interestingly, decreased expression of HPV oncoproteins E6 and E7 which are known to degrade these tumor suppressor proteins (Jing *et al.,* 2014; Yeung 2011). The down regulation of E6 and E7 oncoproteins by ALA might be responsible to restore tumor suppressor functions of p53 and pRb proteins, respectively (Scheme 3.1).

After treatment of cervical cancer cells with ALA, the percentage of n6-fatty acids (LA, AA) was found to decrease with simultaneous increase in n3 fatty acids. There was a significant increase in EPA indicating that cells had efficiently converted ALA to EPA. It has been previously reported that ALA treated MDAMB231 showed increase in EPA levels (Horia 2005). Few reports have shown that HeLa cells, when treated with GLA, show decreased AA content with increase in amount of EPA and DHA (Sagar 1995). The decrease in ARA with a concomitant increase in EPA could be responsible for decreased expression of inflammatory markers found in ALA treated cells. Arachidonic acid acts as a substrate for cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, and also mediates the inflammatory processes leading to activation of transcription factors such as nuclear factor kappa B (NFκB) (Calder 2002; Calder 2006).

In summary, our data indicated that ALA modulated the expression of COX-2, NF-κB, MMPs, VEGF, c-Jun, phosphorylated ERK1/2 and p38 in cervical cancer cells. The possible signaling pathways modulated by ALA in cervical cancer

have been depicted in Scheme 1. Cervical cancer affects various pathways that include inflammation, MAPK kinase and angiogenesis (Behren 2005; Domenico 2012). ALA reduced the expression of HPV E6, E7, NFκB, COX-2 with simultaneous increase in the expression of p53 and Rb. ALA also decreased the downstream targets of MAPK pathway (ERK, p38), thereby suppressing cell growth and cancer development. Thus, ALA could be explored for its therapeutic potential to decrease inflammation, angiogenesis and metastasis in cervical cancer.



Scheme 3.1. Signaling pathways modulated by ALA in cervical cancer.

CHAPTER 4

Analyzing the effect of flaxseed oil containing ALA on regulation of TC1 (HPV 16) induced tumors in C57BL/6J mice

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Introduction

Our previous studies indicated that ALA induced apoptosis and reduced expression of HPV oncoproteins in cervical cancer cell lines. Flaxseed contains high concentration of ALA (57% of total fatty acids) and has been reported to inhibit growth of various cancers including breast, colon and prostate (Wang *et al.,* 2005; Thompson *et al.,* 2005; Williams & Verghese 2007; Carleton *et al.,* 2013). Several *in vivo* studies suggest that flax oil reduced breast cancer growth and metastasis (Chen *et al.,* 2007; Chen *et al.,* 2006). However, there is no data available on the anticancer activity of flax oil supplementation in cervical cancer model of TC-1 induced tumor in C57BL/6J mice.

In the present chapter, we have evaluated the potential of dietary flax oil (FO) rich in ALA to reduce tumor growth in TC1 induced tumors in C57BL/6J mice. After oral supplementation with FO, tumor growth was significantly reduced in mice that was comparable to control drug cisplatin (Cis). Interestingly, in mice treated with a combination of FO and Cis (Cis+FO), there was enhanced reduction in tumor volume. FO and Cis+FO significantly increased the plasma antioxidant levels in mice compared to either tumor control or cisplatin treated group. There was a significant increase in lipid peroxidation in tumor tissue of FO and Cis+FO group compared to either tumor control (TC) or Cis treated group. On the other hand, in liver tissue, FO and Cis+FO decreased LPO compared to either TC or Cis treated group. FO and Cis+FO significantly modulated immune response in mice by increasing Th1 cytokine, IFN- γ with a subsequent decrease in Th2 cytokine, IL- 4. Further, Cis, FO and Cis+FO decreased the expressions of HPV E6 and E7 oncoproteins significantly. These results suggest that flax oil rich in ALA could reduce the tumor volume and

could reduce the side effects associated with cisplatin. Thus, intake of ALA from dietary sources could help in the management of cervical cancer.

4.2. Materials and Methods

4.2.1. Reagents

Flax oil was purchased from Inlife Health Care, Hyderabad. Cisplatin was procured from MP Biomedicals. Fluorescein sodium salt, AAPH (2, 2'-azobis-2-methyl-propanimidamide, dihydrochloride), and Troloxwere purchased from Sigma-Aldrich (St. Louis, MO, USA).Mouse Th1/Th2 ELISA Ready-SET-Go kit was purchased from eBioscience (CA, USA). All other common reagents were procured from Qualigen fine chemicals (Mumbai, India). TBA, BHT and TCA were purchased from Merck.

4.2.2. Cell culture

TC-1 cell line, a kind gift from Dr. T. C. Wu, John-Hopkins University, Baltimore MD USA, was cultured in RPMI (Gibco, NY, USA). Cervical cancer cell lines, SiHa and HeLa, used for viability assay were purchased from National Centre for Cell Science (NCCS), Pune, India and were grown in DMEM (Sigma-Aldrich, St. Louis, MO, USA). Both RPMI and DMEM were supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 100 U/ml of penicillin-streptomycin. The cells were incubated in a humidified 5% CO_2 incubator at 37°C.

4.2.3. Experimental design for *in vivo* study

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) constituted in accordance with the rules and guidelines of the

Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA), India (Approved protocol no-CPCSEA/2014-15/1396; institutional approval no – CPCSEA/258). Animals were procured from the National Institute of Nutrition (NIN), Hyderabad and acclimatized for 7-15 days at 22 ± 3°C with relative humidity 30-70 %, and 12 hours light and 12 hours dark light cycle in separate cages. Animals were fed with diet pellet (Nutrivet, Pune) and sterilized water ad libitum. After acclimatization, TC-1 cells were injected in the right flank of each C57BL/6J mouse at a density of 5×10^6 cells/ml (Taguchi *et al.,* 2014).On 6^{th} day, palpable tumors were observed that were measured by using vernier caliper. The animals were divided into six groups, each consisting of six mice. **Group I**: No tumor control group (NTC) animals received distilled water orally as a vehicle for 21 days; **Group II**: Tumor control group (TC) animals received distilled water orally as a vehicle for 15 days; **Group III**: Cisplatin (Cis) treated group animals received cisplatin (4 mg/kg, i.v.) on 1^{st}, 5^{th} and 9^{th} day; **Group IV**: Flax oil (FO) supplementation group animals receiving 200 µl FO orally for 15 days; **Group V**: Flax oil(FO) supplementation group animals receiving 400 µl FO orally for 15 days and **Group VI**: Flax oil + cisplatin group (Cis+FO) animals received flax oil (400 µl) orally for 15 days and were also treated with cisplatin (4 mg/kg, i.v.) on 1^{st}, 5^{th} and 9^{th} day. All groups were fed on normal diet. After every 3 days, food consumption, body weights and tumor sizes were recorded. Tumor volume was calculated using the formula:

$$\text{Tumor volume } (\text{mm}^3) = \frac{1}{2} * shortest\ diameter^2 * largest\ diameter$$

The percent tumor growth inhibition in treated groups was calculated as follows:

% Tumor inhibition

$$= \left[\frac{\text{Av. tumor volume of control group} - \text{Av. tumor volume of test group}}{\text{Av. tumor volume of control group}}\right] * 100$$

Blood was withdrawn on fifteenth day from each mouse by retro-orbital puncture and plasma was separated for analyzing antioxidant capacity and cytokine levels. After completion of treatment, tumors were excised and preserved in Trizol for further studies.

4.2.4. Immunoblotting

Frozen tumor tissue was homogenized and used for RNA isolation. RNA was extracted from the samples according to manufacturer's instructions (Invitrogen). Protein was isolated according to manufacturer's instructions from Trizol (Invitrogen). Protein pellet in the tubes was dissolved in 1% SDS by keeping it in waterbath at 50^0C for 15 min followed by sonication for 10 sec cycle according to Simoes et al 2013.

4.2.5. Analysis of Th1 and Th2 cytokine levels in plasma

The levels Th1 (IFN-γ) and Th2 **(**IL-4) cytokines, in serum samples from C57BL/6Jmice were determinedfollowing the manufactures protocol from by the commercially available Th1/Th2 ELISA-Ready-Set-Go kit (eBioscience, San Diego, CA, USA). All the assays were performed in triplicates. Absorbance was measured at 450 nm with FLUOstar Omega microplate reader (CA, USA) (Budhu *et al.,* 2006).

4.2.6. Determination of total antioxidant capacity in plasma by ORAC assay

The Oxygen Radical Absorbance Capacity was determined in the plasma samples. Briefly, blood samples were collected from C57BL/6J mice of treatment groups (Cis, FO200, FO400, Cis+FO) as well as NTC and TC groups. Blood was centrifuged at 3500 g for 15 min to separate out the plasma (Choudhari *et al.,* 2013). Twenty five microlitre plasma was mixed with 150 µl of freshly prepared fluorescein solution (10 nM), and incubated for 30 min at 37°C. To this, 25µl of AAPH substrate (500 mM) was added and the fluorescence measurements were recorded for 150 min at excitation and emission wavelengths of 485 and 520 nm, respectively, using BMG Fluostar omega microplate reader. Trolox, a water-soluble analogue of vitamin E, was used as an antioxidant standard. A standard calibration curve was made for Trolox at 0, 0.78, 1.56, 3.125 and 6.25 µM concentrations following the manufacturer's instructions. The final ORAC values were calculated by using MARS Data analysis software 2.10R3 (BMG Labtech).

4.2.7. Determination lipid peroxidation activity in liver and tumor tissue

The generation of thiobarbituric acid reactive substances (TBARS) was measured following a published protocol (Jangale *et al.,* 2013). In brief, 10% w/v liver tissue homogenate was prepared in 1X PBS. A reaction mixture was prepared by taking 0.5 ml of liver or tumor homogenate and 1 ml of 0.15 M KCl and incubated at 37°C for 30 min. After incubation, 2 ml ice-cold 0.25 N HCl [containing 15% tricholroacetic acid (TCA) and 0.38% thiobarbituric acid (TBA)] and 0.2 ml of 0.05% of butylated hydroxy toluene (BHT) were added to the mixture. The mixture was then heated for 60 min at 80°C. After cooling to room temperature, the reaction mixture

was centrifuged at 5000 g for 15 min. The absorbance of collected supernatant was measured at 540 nm using a Fluostar Omega microplate reader (BMG Labtech). TBARS levels were calculated using a standard curve plotted against absorbance at 540 nm of various concentrations of 1, 1, 3, 3-tetramethoxypropane (TMP).

4.2.8. Fatty acid analysis in tumor tissue

Tumors (50 mg), excised from treated as well as untreated mice, were homogenized.The homogenized tumors were mixed with 4 mL of Methanol/HCl/BHT (94.7:5.3:0.0005, v/v/w) in a 15-ml screw cap vial. The vials were sealed and incubated at 80ºC for 2 h and then cooled on ice for 30 min. The total fatty acid methyl esters were extracted by adding 2 ml hexane (2N), and the layers were separated by centrifugation in a swinging rotor at 3000 rpm for 15 min at room temperature. The hexane layers were carefully removed and collected in separate vials. The hexane extract was completely dried by passing argon gas and stored at -20ºC until further use. The methylated fatty acids were re-suspended in 100 µl of chloroform, and 1 µl was injected in GC column. Helium was used as the carrier gas at 1 ml/min. Oven temperature was held at 150ºC for 10 min, programmed to rise from 150 to 220ºC at 10ºC/min, and at 220ºC for 10 min. The detector temperature was 275ºC, and the injector temperature was 240ºC. The column was calibrated by injecting the standard fatty acid mixture in approximately equal proportions. The peaks were identified as per the retention time of the standard fatty acids (Sigma, USA) run under the identical conditions.

4.3. Results

ALA in combination with cisplatin reduced the viability of TC1 cell line

In previous experiments, ALA showed good results at 80 μM concentrations in cervical cancer cell lines and thus this concentration of ALA was used in further experiments. SiHa, HeLa and TC-1 cell lines were treated with different doses of either cisplatin or ALA or combination of cisplatin and ALA (Cis+ALA) (Figure 4.1). The latter was done to test whether ALA could enhance the effectiveness of cisplatin in reducing the cell viability at lower doses. IC_{50} values of cisplatin in TC1 were found to be 14.57 μM and interestingly, after combination with ALA, it was reduced to 6.85 μM (Figure 4.1, Table 4.1). Cis+ALA reduced IC_{50} value from 15 μM to12 μM in SiHa, and from 16.6 μM to 13 μMin HeLa (Figure 4.1, Table 4.1). Cis+ALA significantly decreased the viability of all the cell lines. Based on these encouraging in vitro results, we used combination of cisplatin and ALA in one of the treatment groups in vivo (Group VI; Cis+Flax Oil) to evaluate whether this combination could enhance the drug responsiveness and reduce the side effects of cisplatin through modulation of immune system.

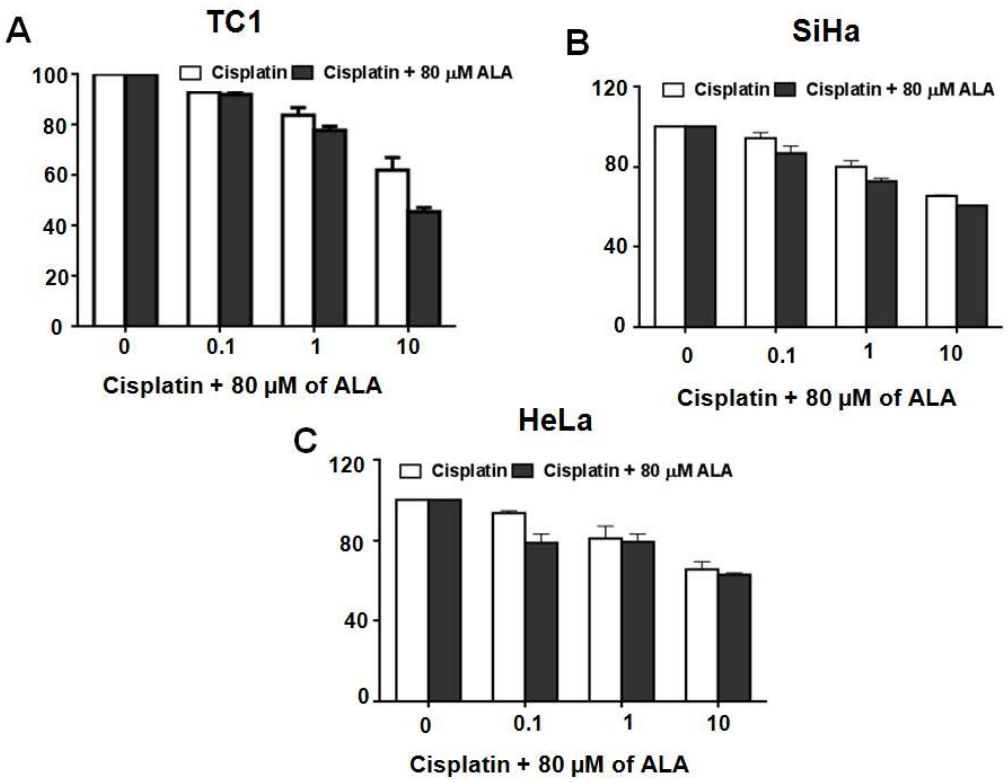

Figure 4.1. Combination of ALA and cisplatin reduced the viability of cancer cells. *TC1 (A), SiHa (B) and HeLa (C) cell lines were treated with different doses of cisplatin (0, 0.1, 1 and 10 μM) with or without 80 μM of ALA, for 24 h. After addition of 80 μM of ALA, there was decrease in IC_{50} values. All the data have been presented as mean ± SEM of three independent experiments.*$p < 0.05$ indicated statistically significant differences compared to the tumor control group.*

Table 4.1. IC_{50} value of cisplatin in combination with ALA in TC1, SiHa and HeLa

Treatment	IC_{50} value (μM)		
	TC1	SiHa	HeLa
ALA	1959	1531	1761
Cisplatin	14.57	15	16.6
Cisplatin+80 μM ALA	6.85	12	13

4.3.1. Flax oil (ALA) reduced tumor growth in mice

For analyzing the anti-cancer potential of ALA, TC1 tumor induced C57BL/6J mouse model was used. TC-1 cell line was a kind gift from Dr. T. C. Wu, John-Hopkins University, Baltimore MD USA. This cell line has been derived from a primary lung epithelial cell from C57BL/6J mice and immortalized using HPV 16 E6/E7 and cHas-ras (Taguchi *et al.,* 2014). Sub-cutaneous tumors of TC1 were induced in mice by injecting of 5×10^6 viable cells in the right flank of each mouse. Six days later, when subcutaneous tumors were formed, tumor-bearing mice were orally given 200 and 400 µL of Flax oil (FO) for 15 consecutive days. Mice from all the groups showed 100% survival till the end of the experiment (Figure 4.2 A).Tumor control (TC) mice had a tumor volume of 539.2±71.3 mm^3, while mice receiving cisplatin, 200 and 400 µl of FO showed tumor volumes of 187.7±72.1 mm^3 ($p<0.001$), 394.3±105 mm^3 (NS) and 244.8±74mm^3 ($p < 0.05$), respectively (Figure 4.2 B). Mice that were given combination of cisplatin (4 mg/kg) and 400 µl FO (Cis+FO) showed ~ 132.5±16.3 mm^3 tumor volume ($p < 0.05$) (Figure 4.2 B). The retardation in tumor volume in 200 and 400 µl of FO treated animals was found to be ~26 and ~50 % respectively compared to TC. On the other hand, cisplatin treatment (4 mg/kg i.v.) resulted into ~65% decrease in tumor volume compared to the tumor control. The mice treated with Cis+FO showed ~75 % significant decrease in tumor volume.

On evaluating the weight of excised tumors from mice, reduction in tumor weight was found in Cis, FO 200, FO 400and Cis+FO groups compared to TC. However, reduction in tumor weight of Cis+FO was similar to that of Cis treated group (Figure 4.2 C). Tumor weight was significantly reduced by ~ 3.4 ($p<0.05$), 1.71($p<0.05$), 2.5 ($p<0.05$) and 4-fold ($p<0.05$) in Cis, FO 200, FO 400 and Cis+FO,

respectively (Figure 4.2 C). Supplementation of FO and Cis+FO significantly reduced tumor growth in mice.

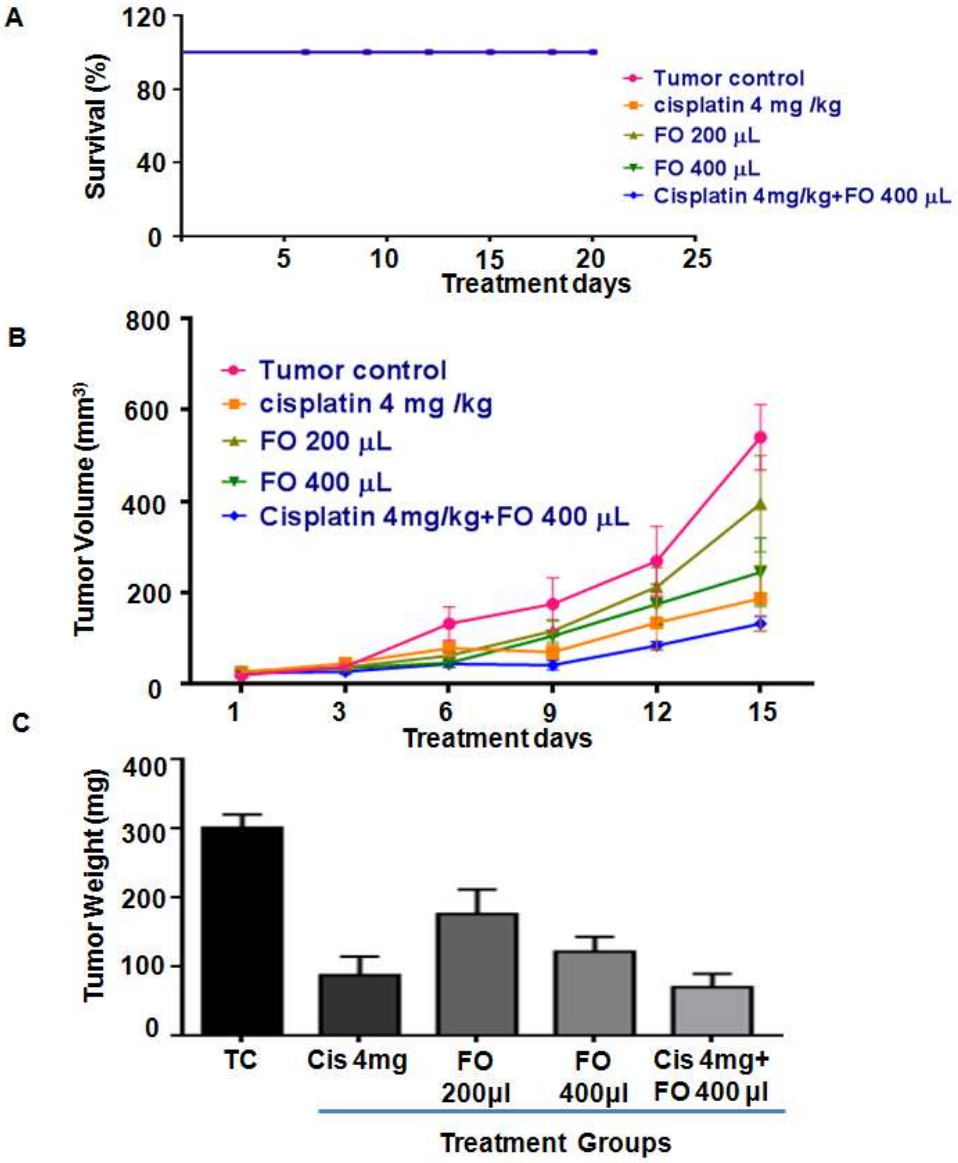

Figure 4.2. Flax Oil reduced tumor growth in TC1 mouse melanoma model. *Survival rate (A) of all the mice treated and untreated animals has been plotted. The tumor volume (B) and tumor weight of excised tumor (C) from all the groups has been shown. The data represented means±SEM of mice from each group. Comparisons were done between the tumor control group and mice treated with cisplatin, Flax oil 200,400 and FO+Cis treated group by Dunnet's multiple and Holm-Sidak's multiple comparisons test.*

4.3.2. Effect of flax oil supplementation on body and relative organ weights of C57BL/6Jmice

The effect of flax oil on body weights and relative organ weights of animals was determined (Table 4.2 and 4.3). The body weight of animals did not change upon treatment with either cisplatin or FO 200 or FO 400 or Cis+FO compared to NTC or TC (Table 4.2). The relative organ weights of the mice treated either with Cis or FO 200 or FO 400 or Cis+FO also didn't show any statistically significant difference ($p>0.05$) compared to either NTC or TC mice. These results indicated that oral supplementation of flax oil was well tolerated by mice with no adverse effects.

Table 4.2. Body weights of C57BL/6J mice

Treatment days	Body weight of study animal (in grams)					
	No Tumor Control (NTC)	**Tumor Control (TC)**	**Cisplatin (4 mg/kg) (Cis)**	**Flax oil 200 µl (FO200)**	**Flax oil 400 µl (FO400**	**Cisplatin (4mg/kg) + FO400µl (Cis+FO)**
1	20.75±2.2	20.5±1.9	21.25±1.7	21.0±1.7	20.16±1.3	20.1±0.91
3	20.84±2.7	21.0±2.3	20.66±2.1	21.5±1.7	20.08±1.4	20.3±1.04
6	20.33±2.5	21.25±2.8	20.75±2.4	21.25±1.8	20.08±1.6	20.0±0.93
9	20.16±2.4	21.16±1.8	20.91±1.9	21.16±1.7	20.16±2.0	19.4±1.37
12	19.83±2.1	21.33±1.7	21.25±2.1	21.5±1.6	20.25±2.1	19.4±1.32
14	19.66±2.5	22.16±2.0	21.66±2.0	21.41±1.9	19.83±2.0	19.2±1.25

Table 4.3. Relative organ weights of C57BL/6J mice

Organ weights of study animals (in grams)					
Groups	**Thymus**	**Heart**	**Lung**	**Liver**	**Spleen**
NTC	0.05±0.01	0.1±0.02	0.17±0.03	0.77±0.15	0.08±0.03
TC	0.05±0.02	0.10±0.01	0.17±0.01	1.2±0.13	0.14±0.03
Cis	0.29±0.25	0.25±0.325	0.19±0.01	1.0±0.16	0.10±0.03
FO200	0.033±0.01	0.08±0.010	0.15±0.01	0.85±0.057	0.21±0.28
FO400	0.054±0.009	0.10±0.009	0.16±0.02	0.98±0.170	0.080±0.01
Cis+FO	0.049±0.01	0.23±0.31	0.22±0.11	0.80±0.112	0.09±0.01

4.3.3. Effect of flax oil supplementation on the expression of HPV oncoproteins E6 and E7 in TC-1 tumor induced mice

We next evaluated whether flax oil had any effect on the expression of viral oncoproteins E6 and E7.Total protein was extracted from TC-1 tumors and analyzed for expression of E6 and E7. FO decreased the expression of E6 protein by ~6, 1.5, 1.9 and 2-fold, respectively, in cisplatin, FO 200, FO 400 µl and Cis+FO compared to tumor control (Figure 4.3 A). Similarly, expression of E7 was decreased by ~ 1.9-, 1.25-, 1.8- and 2.2-fold, respectively in cisplatin, FO 200, FO 400 and Cis+FO compared to the TC (Figure 4.3 B).These results suggest that flax oil supplementation significantly decreased the expression of HPV E6 and E7 oncoproteins.

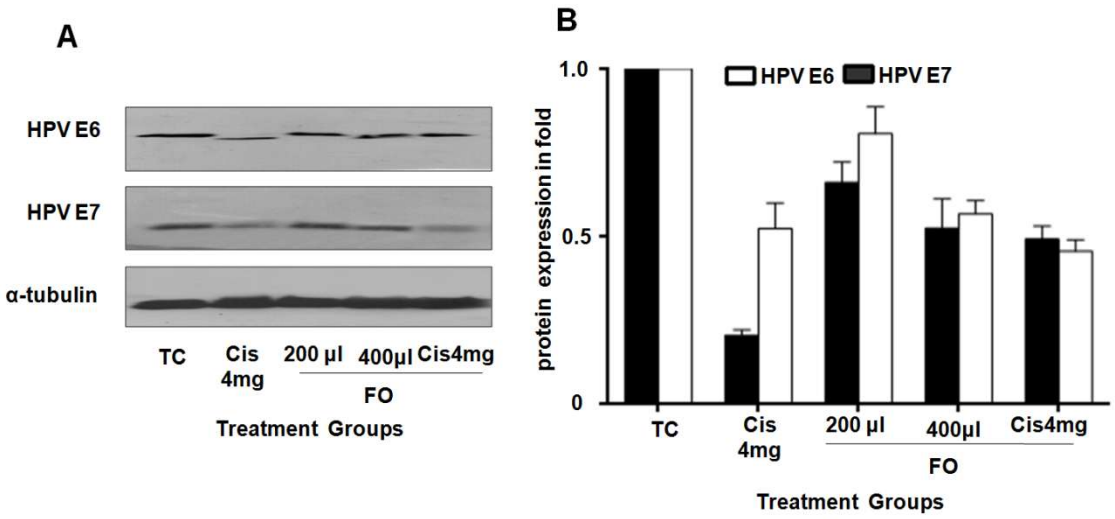

Figure 4.3. Effect of flax oil, cisplatin and combination of cisplatin with FO on expression of HPV E6 and E7 of TC1 induced Tumor mice model. *Expression of HPV E6 and E7 was evaluated in tumor isolated from all treatment and control groups (A). Tubulin was used as a loading control. Densitometric analysis of the western blot showing fold change in E6 and E7 protein levels (B). The bands were quantified by densitometry using ImageJ 1.44p.The values have been represented as mean±SD of three independent experiments at $p<0.001$, indicating statistically significant differences compared to the tumor control.*

4.3.4. Flax oil treatment modulated IL-4 and IFN-γ (Th1/Th2) cytokines in plasma

We further evaluated whether flax oil exhibited immunomodulatory potential by measuring the plasma levels of Th1 (IFN-γ) and Th2 (IL-4) cytokines, in mice. IFN-γ was significantly decreased by ~ 3.4, 5, 4.6 and 2-fold in TC ,Cis, FO 200 and CiS+FO, respectively, compared to NTC except for FO400 treated mice that showed ~ 1.4 increase in IFN-γ level. On the other hand, CiS+FO showed ~1.6 and 2.5-fold ($p=0.0455$) increase in IFN-γ levels, respectively, compared to TC or Cis group (Figure 4.4 A).

Similarly, TC and all treatment groups showed increase in IL-4 level compared to NTC. In TC, Cis, FO 200, FO 400 and Cis+FO mice, there was ~2.1, 2.9, 1.9, 1.4 and 1.5-fold increase in IL-4 levels, respectively, compared to NTC. On the other hand, FO 400 and Cis+ FO treated group showed decrease in the IL-4 levels by ~1.6-fold ($p=0.0056$) and ~1.5-fold ($p=0.0134$), respectively, compared to cisplatin treated mice (Figure 4.4 B). These observations showed that flax oil as well as its combination with cisplatin activated Th1/Th2 cytokine response in mice compared to cisplatin alone.

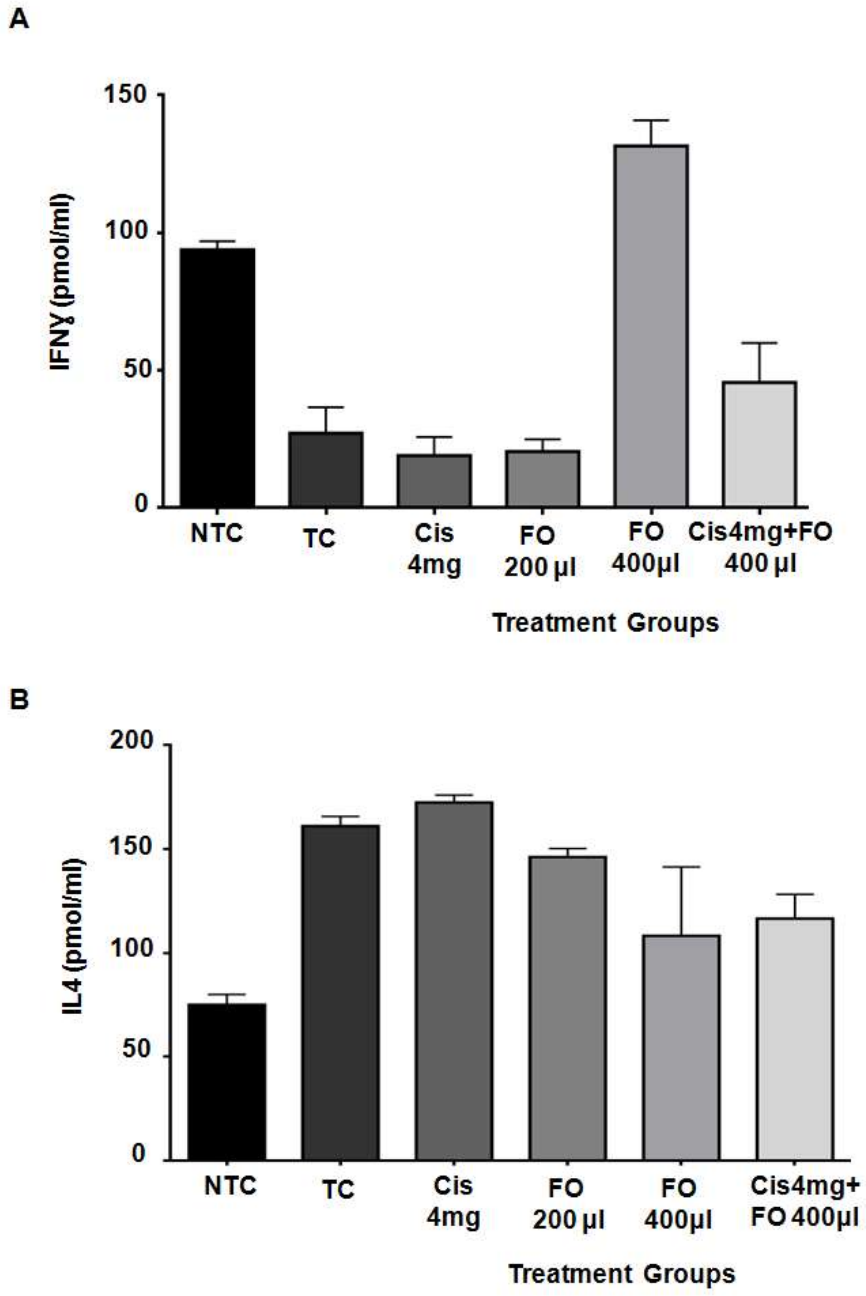

Figure 4.4. Flax oil, cisplatin and Cis+FO modulated Th1/Th2 cytokine levels.*Plasma cytokine levels of IFN- γ (A) and IL-4 (B) were analyzed. The data represented means±SEM of mice from each group. Comparisons were done between no tumor control group, tumor control group, cisplatin group and each individual treated group by Dunnet's multiple and Holm-Sidak's multiple comparisons test.*

4.3.5. Plasma Antioxidant Capacity

ORAC assay was done to evaluate total antioxidant capacity of plasma isolated from blood samples of mice. Trolox was used as a standard. Mice treated with 200 and 400 µl flax oil exhibited ~16.8 (p=0.0045) and 20 % (p=0.002) increase in plasma antioxidant capacity, respectively, compared to TC group (Figure 4.5). Mice treated with Cis and Cis+FO showed ~ 14% and ~16.6 % (p=0.0101) increase, respectively, in antioxidant capacity compared to the TC group (Fig 4.5). The results suggested that mice treated with FO or Cis+FO showed a considerable increase in plasma antioxidant level compared to either tumor control or Cis group, the increase being more significant in FO 400 group.

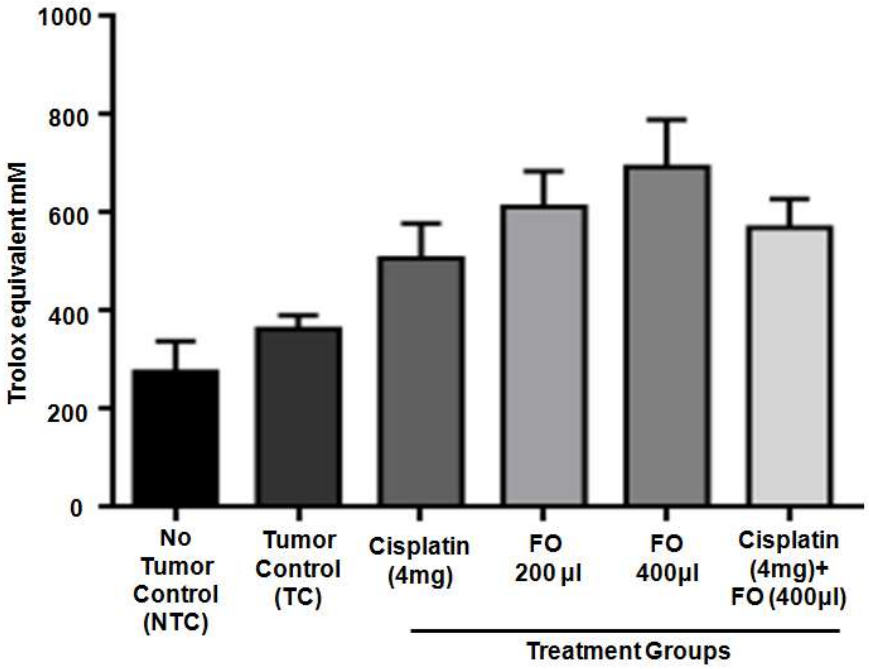

Figure 4.5 Effect of Flax oil, cisplatin and Cis+FO on plasma antioxidant levels in mice. *ORAC assay was used to measure the antioxidant levels from the plasma isolated from blood of mice. The data represented mean±SEM from mice in each group. p value was calculated using Dunnet's multiple and Holm-Sidak's multiple comparisons test.*

4.3.6. Flax oil modulated LPO in tumor tissue and liver homogenates

We further analyzed the effects of Cis, FO and Cis+FO on lipid peroxidation in the tumor tissue and liver homogenates by TBARs assay. In tumor tissue, a significant increase in TBARs levels was observed in Cis+FO group compared to either TC or Cis group (Figure 4.6 A). The increase in TBARs was found to be ~ 1.5, 1.7, 2 and 3- folds in Cis, FO 200, FO 400 and Cis+FO groups, respectively, compared to TC group.

On the other hand, in liver homogenates, there was ~ 1.4 and 1.6-fold increase in TBARs level in TC and Cis group compared to other groups, the increase being more significant in Cis group. Conversely, the level of TBARs were decreased significantly ($p=0.0052$) by ~1.4, 2 and 1.3-fold in FO 200,FO 400 and Cis+FO,respectively compared to TC or Cis group. The decrease in TBARs in Cis+FO group suggested that with Flax oil decreased the oxidative stress in normal liver cells that must have been induced by cisplatin treatment.

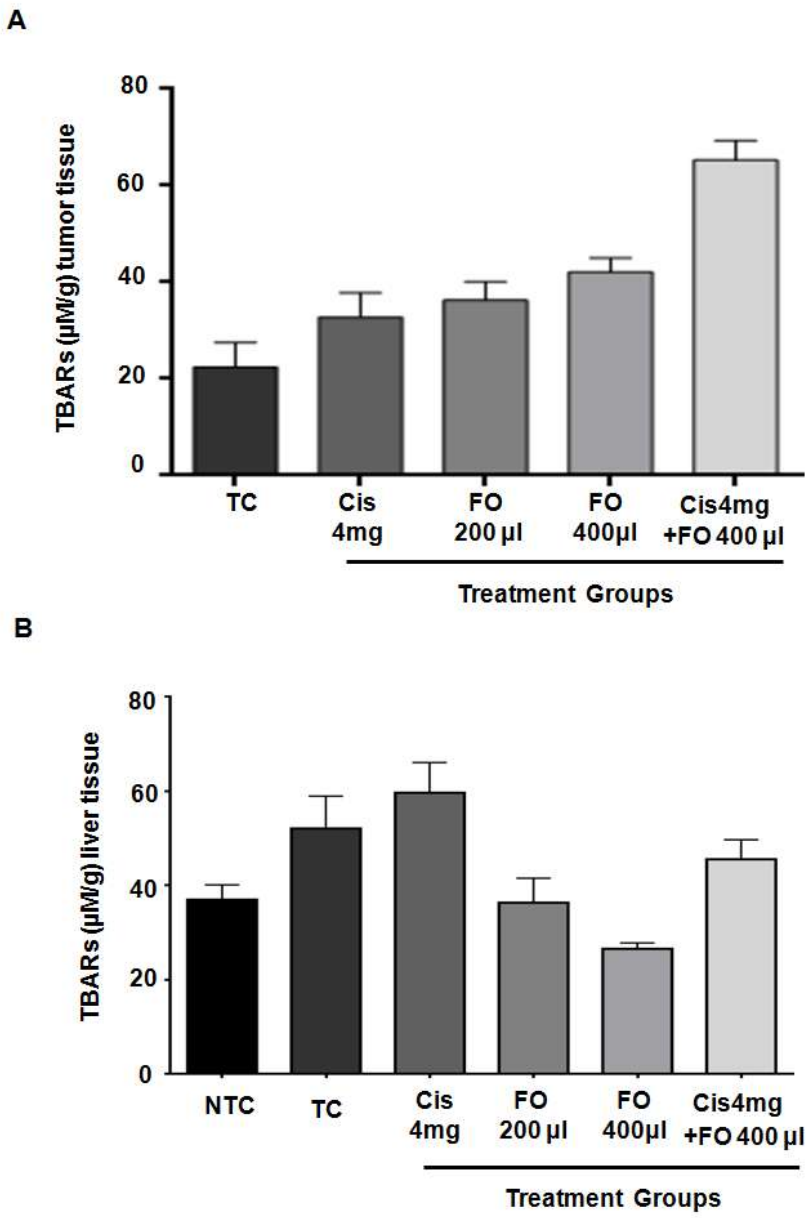

Figure 4.6. Effect of Flax oil, cisplatin and Cis+FO on TBARs levels in the tumor tissue and liver tissues of mice. *TBARs assay in tumor tissue (A) and in liver homogenate (B). The data represented means±SEM of tumor tissues from mice in each group. Comparisons were done between the NTC, TC, Cis groups and each individual groups by Dunnet's multiple and Holm-Sidak's multiple comparisons test.*

4.3.7. Fatty acid analysis of tumor tissue

We next analyzed the fatty acid composition of flax oil and pelleted diet fed to the animals during experiment. The pelleted diet contained 9.6 and 12.5 % of LA and

AA per 100 mg of diet respectively (Table 4.3). The diet also contained saturated and monounsaturated fatty acid such as Palmitic acid, Stearic acid and oleic acid. Interestingly, the pelleted diet contained less omega 3 fatty acids such as ALA, EPA and DHA. On the other hand, animals treated with flax oil contained 54, 13 and 19% of ALA, LA and oleic acid, respectively (Table 4.4).

To detect whether different treatments (cisplatin, flax oil and CiS+FO) induced any specific changes in the fatty acid composition of the tumor cells, we analyzed the fatty acid profile of the total lipid extract of the tumor tissue (Table 4.4). Tumor control group showed high amount of n6 fatty acids(LA and AA)and low amounts of n3 fatty acids (ALA, EPA and DHA). The saturated fatty acids such as Palmitic acid and stearic acid level were more in tumor control (Table 4.4).

Cisplatin and FO200 treatment showed increase in the ALA level whereas in FO 400 and Cis+FO treated group, there was significant increase in ALA content. Cis treated group showed no change in EPA level whereas, FO 200,400 and Cis+FO group showed ~8.6,11 and 7.5-fold increase in EPA content, respectively, compared to TC group. DHA level was increased by ~ 1.8, 2.2, 3.6 and 3.1-fold in Cis, FO 200,400 and Cis+FO, respectively compared to tumor control (Table 4.4).

Interestingly, compared to TC group, in Cis, FO 200,400 and Cis+FO showed ~ 1.35, 1.24, 1.3 and 1.6-fold increase, respectively, in the amount of LA as well as ~2,2.5,12.1 and 12-fold, respectively, decrease in AA. No change was observed in the level of other n6 fatty acids such a GLA and DGLA in all treatment groups compared TC group (Table 4.5).

In all the treatment groups, the level of saturated fatty acids such as palmitic acid and stearic acid was decreased with no change in the level of myristic acid compared to TC.

Monounsaturated fatty acid such as oleic acid was increased in all the treatment groups compared to TC group. These results suggested that increased in intake of flax oil increases levels of omega 3 fatty acids with concomitant decrease in omega 6 fatty acids.

Table 4.4. Relative Fatty acid composition of diet and flax oil fed to animals during experiment

Fatty acid	Diet (mg/100 mg fatty acids)	Flax oil Capsule (mg/100 mg fatty acids)
Omega 3 FAs		
ALA(18:3n-3)	0.08	54.43
DPA(22:5n-3)	0.29	0.05
EPA (20:5n-3)	0.23	0.08
DHA (22:6n-3)	0.04	0.02
Omega 6 FAs		
LA (18:2n-6)	9.6	13.72
GLA (18:3n-6)	0.5	0.13
DGLA (20:3n-6)	0.09	0.14
AA (20:4n-6)	12.5	0.17
SFA		
MA (14:0)	1.09	0.05
PAM(16:0)	25.4	6.15
SA(18:0)	23.9	5.75
MUFA		
MYRO (14:1 n-5)	0.18	0.02
PAMO (16:1 n-7)	3.91	0.04
OA(18:1 n-9)	21.3	19.22
NA(24:1n-9)	0.89	0.01
n-3 PUFAs	0.64	54.58
n-6 PUFAs	22.69	14.16

Table 4.5. Relative Fatty acid composition in tumor cells treated with Cisplatin, FO 200 and 400 and Cis+FO

Fatty acids	Treatment groups				
	TC	Cisplatin	FO200	FO400	Cis+FO
Omega 3 FAs					
ALA(18:3n-3)	0.05±0.01	0.67±0.19	2.3±0.40	6.54±3.05	7.40±1.24
DPA(22:5n-3)	0.40±0.08	0.47±0.15	3.45±0.29	4.48±1.10	3.02±0.79
EPA (20:5n-3)	0.92±0.88	1.74±0.38	2.1±0.32	3.33±0.21	2.93±0.56
DHA (22:6n-3)	0.29±0.03	0.77±0.61	2.95±0.26	3.24±0.52	2.42±0.57
Omega 6 FAs					
LA (18:2n-6)	9.53±0.17	12.87±1.53	11.84±0.96	13.06±0.77	15.8±0.69
GLA (18:3n-6)	11.92±0.29	5.92±1.09	4.61±0.40	0.93±0.15	0.91±0.47
DGLA (20:3n-6)	0.39±0.03	0.21±0.01	0.14±0.05	0.26±0.07	0.15±0.04
AA (20:4n-6)	0.84±0.22	0.60±0.22	0.65±0.23	0.36±0.20	0.45±0.19
SFA					
MA (14:0)	1.07±0.19	1.81±0.60	1.06±0.16	1.31±0.25	1.03±0.07
PAM(16:0)	24.28±1.21	24.14±1.68	21.34±1.16	21.07±0.95	19.83±0.81
SA(18:0)	22.82±0.58	19.27±2.78	20.01±0.75	20.12±1.42	18.65±3.66
MUFA					
MYRO (14:1 n-5)	0.81±0.64	0.15±0.03	0.31±0.19	0.13±0.02	0.17±0.03
PAMO (16:1 n-7)	3.83±0.29	4.24±0.61	3.46±0.23	3.25±0.39	2.85±0.19
OA(18:1 n-9)	21.77±1.26	26.86±1.55	23.07±0.86	25.00±1.14	27.87±3.11
NA(24:1n-9)	0.71±0.03	0.28±0.12	0.16±0.04	0.19±0.07	0.18±0.07
PUFAs	24.72±0.87	23.25±1.13	28.05±2.05	28.64±1.61	32.26±2.34
n-3 PUFAs	2.04±0.71	3.65±1.06	10.80±0.81	12.06±0.97	13.42±0.84
n-6 PUFAs	22.69±0.28	19.60±0.26	17.25±1.42	16.58±1.46	16.93±0.97

SFA indicates total saturated fatty acids (MA+PAM+SA); MUFA (MYRO+ PAMO + OLA+NA), total polyunsaturated fatty acids (n6 + n3 PUFA), n3 PUFAs (ALA+DPA+EPA+DHA), n6 PUFAs (LA+GLA+DGLA+AA). Data has been expressed as relative % of total fatty acids with mean±SEM. *p<0.05, **p<0.01 and ***p<0.001 indicate statistically significant differences between the treated and untreated control group and "ns" indicates non-significant results.

4.4. Discussion

Various epidemiological studies have shown that distinct dietary patterns as well as specific dietary components could alter the risk of cancer, thereby emphasizing the importance of nutrition in cancer management (Aggarwal 2006). In vitro and *in vivo* studies are excellent tools to provide evidence regarding the association of diet with cancer (Donaldson 2004). We analyzed the effect of ALA in combination with cisplatin in vitro and found that combination increased the effectiveness of cisplatin at lower doses in SiHa, HeLa and TC1cell lines. Similarly, ALA enhances effectiveness of chemotherapeutic drugs such as doxorubicin (Huan 2009) and paclitaxel (Menendez 2001) in breast cancer cell line MCF-7 and MDAMB 231.

To check the anticancer effect of ALA at *in vivo* level we have orally gavaged dietary flaxseed oil (a rich source of ALA) alone or in combination with cisplatin, on TC 1 induced tumors in C57BL/6J mice. By gas chromatography we confirmed that flax oil contained 266 mg ALA (~54 %), 62 mg LA (13%) and 102 mg oleic acid (19%).Oral supplementation of FO makes sure that exact amount of dose goes to each animal. On the other hand pelleted diet takes into account average amount of feed consumed by one group of animals and thus the results become ambiguous. In most of *in vivo* studies, flax oil has been incorporated in the diet. There are only few reports are available on oral supplementation of FO in breast cancer and other disease model such as oxidative stress model (Wabha 2013; Bhatia 2007).

The supplementation of mice with FO did not induce any harmful effects such as mortality or weight loss.FO 400 and Cis+ FO significantly reduced tumor volume in C57BL/6 mice. Some studies have shown that flax oil reduced tumor growth in nude mice implanted with breast cancer cell lines MCF-7 or MDAMB231 (Power *et*

al 2008; Chen *et al* 2006; Dabrosin *et al* 2002). Flax oil when used in combination with anticancer drugs tamoxifen and trastuzumab has been shown to decrease tumor growth more significantly in nude mice (Mason *et al.,* 2015; Chen *et al* 2007). Thus, FO could be used as an adjunct to chemotherapy regimen.

Flax oil significantly reduced the expression of HPV E6 and E7 oncoproteins. In one of the reports, DHA has been shown to decrease the expression of HPV oncoproteins in SiHa and HeLa (Jing *et al.,* 2014). Other reports have suggested that fish oil when given in diet decreased expression of HPV E6 and E7 at mRNA level in TC1 induced mice (Taguchi *et al.,* 2014). Thus, we are for the first time reporting the decrease of HPV E6 and E7 oncoproteins with supplementation of flax oil in TC1 model.

Flax oil significantly increased Th1 cytokine (IFN-γ) level with a concomitant decrease in Th-2 cytokine (IL-4). Immune system plays a major role in defense against tumor growth. IFN-γ is produced by activated T cells and natural killer (NK) cells that enhance cellular immune responses by increasing T-cell cytotoxicity and NK cell activity (Kordi-Tamandani 2008.) Certain reports suggest that increased activity of IFN-γ reduced growth and expression of E6 and E7 oncoproteins of HPV-16 and 18 transfected immortalized keratinocytes (Scott 2001; Kim. 2000). On the other hand, IL-4 acts as a natural antagonist that is known to suppress IFN-γ (Boccardo 2010). IL-4 is immunoinhibitory and has been shown to induce tumor growth (Bhairavabhotla 2007). Expression of IL-4 has been reported to be high in HPV infected patients (Bermúdez-Morales 2011). Thus, flax oil not only reduced the tumor volume but also activated of Th1 immune system, whose down-regulation otherwise worsens the severity of cancer.

The plasma antioxidant capacity of mice was increased after treatment with FO and Cis+FO compared to TC or Cis treated group these results are supported by earlier studies that have shown increase in plasma antioxidant capacity after dietary supplementation of walnut (rich in ALA) in nude mice implanted with MDAMB231 breast cancer cell line (Hardman *et al* 2008). FO and Cis+FO effectively decreased hepatic LPO and increased tumor LPO compared to TC or Cis groups whereas cisplatin alone increased LPO levels in both liver and tumor tissue. This corroborated with earlier studies showing that omega 3 fatty acids induced LPO in cancer cells but not in normal cells (DAS 2002). In oxidative stress induced rat model as well asSwiss albino mice, liver lipid peroxidation was reduced upon flax oil treatment (Wabha 2013; Bhatia 2007). In diabetes, it has been shown that dietary supplementation of flax oil decreased liver lipid peroxidation (Jangale *et al.,* 2013; Chavan *et al.,* 2013). Previous studies have demonstrated an association between reduced antioxidant levels and increased lipid peroxidation in cervical cancer patients (Maha I 1999). Cisplatin has been known to decrease the antioxidant level in cancer patients (Ajith *et al.,* 2007; Naqshbandi 2012; Weijl 2000). The hepatoprotective activity of flax oil observed in our experiments may be due to its antioxidant effect. Thus, diet rich in antioxidants may benefit cancer patients by compensating for their antioxidant depletion and may also enhance the chemotherapy response with minimum side effects (Tarlovsky 2013).

Flax oil treatment increased levels of ALA and EPA significantly with reduction in AA levels. The level of LA and oleic acid were found to be increased in all the treatment groups because both normal diet and FO contained LA and oleic acid. The significant increase in ALA and EPA in Cis+FO group could be responsible for inducing more lipid peroxidation in tumor tissue.

All these data suggested that FO exhibited the potential to reduce the side effects such as hepatotoxicity associated with cisplatin and could be used as an adjunct to chemotherapy.

4.5. Conclusion

In cancer patients, chemotherapy leads to impairment of antioxidant levels resulting into oxidative stress that leads to damage of not only the cancerous cells but also the normal cells. Thus, supplementation of Flax oil a rich source of ALA to the cervical cancer patients undergoing chemotherapy may be beneficial as it may decrease the side effects of chemotherapy through modulation of immune system. This study emphasizes the role of ALA as an effective nutraceutical with therapeutic potential in cervical cancer management.

CHAPTER 5

Evaluating enhancement in the anticancer activity against cervical cancer cell lines upon combination of ALA with bioactives from medicinal plants

Manuscript Under preparation

5.1. Introduction

A large number of herbs and plants, traditionally used in managing various gynecological disorders, have been found to possess significant anticancer activity. In recent years, herbal medicines and nutraceuticals have been investigated for their use as adjuvant to conventional therapies to address the debilitating side effects associated with them (Molassiotis *et al.,* 2005). Plants, herbs and nutraceuticals have been increasingly accepted as one of the main sources of chemopreventive drugs (Debas *et al.,* 2004). We have also reported the anticancer activity of *Cinnamomum cassia* (cinnamon) and *Ficus religiosa* in cervical cancer cell lines SiHa and HeLa. Researchers have identified bioactive components in medicinal plants and studied their mechanisms of action. Various herbal bioactives with anticancer activity include paclitaxel isolated from (Pacific yew tree, *Taxus brevifolia*) *(*Nikolic *2011)*, cinnamaldehyde (Cinnamomum spp) (Singh 2007), andrographolide (*Andrographis paniculata*)(Chun *et al.,* 2010), curcumin (Turmeric, Curcuma longa)(Tan *et al.,* 2011), berberin (*Berberis*) (Tan *et al.,* 2011), wogonin (Chinese medicinal herb, *Scutellaria baicalensis*) (Tan *et al.,* 2011), and many more.

Cinnamaldehyde is a major bioactive component of the *cinnamon cassia* and *cinnamon zeylanicum* that is a common spice used as a culinary all over the world. Cinnamon has been prescribed innative Ayurvedic system for flu, indigestion, mouth washes and gynaecological ailments (Ravindran *et al.,* 2003). Cinnamaldehyde has been reported to exhibit diverse biological functions including anticancer activity (Koppikar *et al.,* 2010; Singh *et al.,* 2009; Kwon *et al.,* 1998). However, its therapeutic efficiency is limited due to its low water solubility (Stammati *et al.,* 1999) and thus large doses may be required to obtain the desired response.

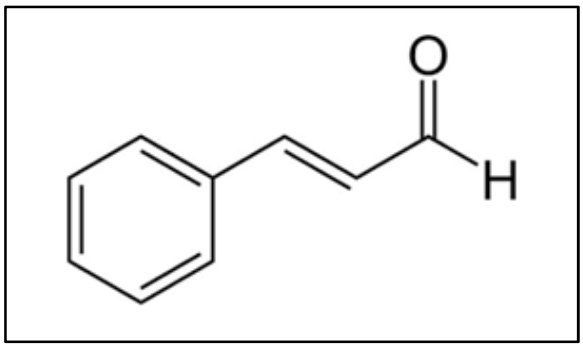

Figure 5.1. Structure of Cinnamaldehyde

Curcumin (CU 1,7-bis (4-hydroxy 3-methoxy phenyl)-1,6-heptadiene-3,5-dione) is a polyphenolic compound extracted from turmeric(*Curcuma longa*), a rhizomatous herbaceous perennial plant of the ginger family, Zingiberaceae (Anand *et al.,* 2008). Turmeric has been used traditionally for thousands of years as a remedy for stomach and liver ailments, as a topical agent to heal sores and as an antimicrobial agent (Prasad 2011).Curcumin has been used for centuries throughout Asia as a food additive, cosmetic, and traditional herbal medicine because of its various biological activities. Curcumin has been shown to exhibit anticancer activity in different cancer cell lines and animal models (Tan *et al.,* 2011). The main drawback restricting the widespread use of CU as an anticancer drug is its limited solubility in aqueous solutions and its inherent instability in alkaline medium, which in turn limits its bioavailability.

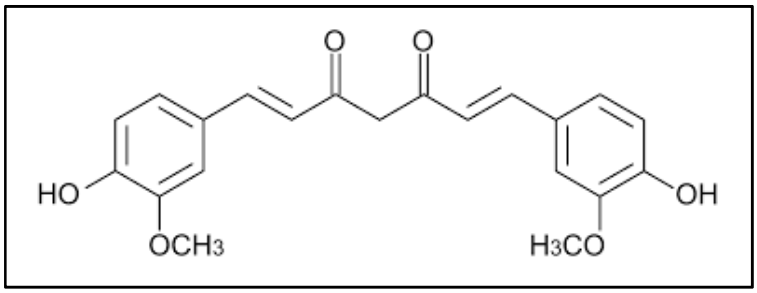

Figure 5.2 Structure of Curcumin

Andrographolide, a diterpenoid lactone and a major bioactive of *Andrographis paniculata* has a long history of therapeutic usage in medicine (Varma *et al.,* 2011). *Andrographis paniculata*, found mainly in China and India, is a medicinal herb. It has been effectively used in the treatment of liver disorders, bowel complaints of children, colic pain, common cold and upper respiratory tract infection (Chun *et al.,* 2010). Andrographolide has been reported to exhibit anti-oxidant, anti-bacterial (Suebsasana *et al.,* 2009), anti-viral (Hen *et al.,* 2009), anti-diabetic (Zhang *et al.,* 2009), anti-inflammatory and anti-tumor activities (Jada *et al.,* 2008).

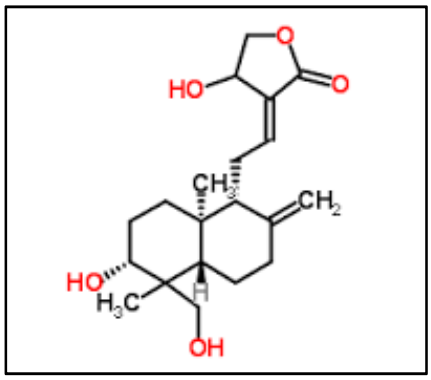

Figure 5.3. Structure of andrographolide

The source of ALA is flax plant and this fact prompted us to study whether combination of ALA with herbal bioactives having known anticancer activity, could induce enhanced anticancer effect in cervical cancer cell lines. Thus, herbal bioactives with proven anticancer activity that were used included curcumin, cinnamaldehyde and andrographolide. Since ALA showed good results at 80 µM concentration in our previous experiments, we used the same concentration in our further experiments. After combination with 80 µM concentration of ALA, IC_{50} values of all the herbal bioactives were significantly reduced. Interestingly, andrographolide showed more significant decrease in IC_{50} value in SiHa and HeLa cells. On the other hand, in non-cancerous cell line,

HaCaT, combination of 80 μM of ALA with different concentrations of andrographolide, increased its IC_{50} value from 141.12 μM to 365 μM. This important finding revealed that ALA protected non-cancerous cells on one hand and reduced the viability of cancerous cells on the other hand. These preliminary data suggested that ALA if combined with herbal bioactives could increase their effectiveness at lower doses in cancer cells.

5.2. Materials and Methods

5.2.1. Reagents

Tissue culture plasticware was purchased from BD Bio-sciences, CA, and USA. Alpha linolenic acid, curcumin, cinnamaldehyde, andrographolide, fatty acid-free bovine serum albumin (BSA), fetal bovine serum (FBS) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylthiazoliumbromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagles Medium (DMEM), Penicillin and streptomycin were obtained from Gibco BRL, USA. L-Glutamine was obtained from Himedia Corporation, Mumbai, India.

5.2.2. Cell Viability Assay

The cell viability was determined by MTT assay in cervical cancer cell lines (SiHa and HeLa) in presence of different concentrations of herbal bioactives (cinnamaldehyde, curcumin and andrographolide) and combination of these drugs with different concentration of ALA compared with non-cancerous transformed cell line, HaCaT. The cells were seeded at a density of 1×10^5 cells/ml density in 96-well plates (TPP, Europe/Switzerland) and grown for 24 h. The cells were treated with different concentrations of herbal bioactives and/or their combination with ALA (0-

160 µM cinnamaldehyde, 0-160 µM cinnamaldehyde+ 20/40/80µM ALA, 0-160 µMcurcumin, 0-160 µM curcumin+ 80 µM ALA, 0-160 µM andrographolideand 0-160 µM andrographolide+ 80 µM ALA). After 24 h, MTT solution (5 mg/ml) was added to each well and the cells were incubated for another 4 h at 37°C in 5% CO_2 incubator. The formazan crystals formed were dissolved in 90 µl of SDS-DMF (20% SDS in 50% DMF) (Singh *et al.,* 2009). After 15 min, the amount of colored formazan derivative was determined by measuring optical density (OD) at 570 nm using the ELISA micro plate reader (Bio-Rad, Hercules, CA). The percentage viability was calculated as:

% Viability = [OD of treated cells/ OD of control cells] x 100

5.2.3. Cell proliferation

Proliferation of HeLa and SiHa cells was determined by trypan blue dye exclusion method. Briefly, the cells were seeded at a density of 1×10^5 cells/ml in 24-well plate in triplicates and were incubated for 24 h at 37^{o}C. After 24 hr, the cells were treated with different concentrations of andrographolide (0-320 µM) alone and combination of 80 µM of ALA. The cells were harvested and counted for viability using trypan blue dye exclusion method.

5.3. Results

5.3.1. ALA reduced IC_{50} value of Cinnamaldehyde in cervical cancer cells.

We evaluated the effect of different concentrations of either cinnamaldehyde (0-80µM) alone or its combination with different concentrations of ALA on the viability of SiHacells. It was observed that upon combination with 20, 40 and 80 µM concentrations of ALA, IC_{50} value of cinnamaldehyde was reduced from 136 µM to

120,116 and 84 μM, respectively, in SiHa (Table 5.1). Upon combination with 80 μM of ALA, there was a marked decrease in IC_{50} value of cinnamaldehyde. In our further experiments we used only 80 μM of ALA in combination studies. We observed that HeLa cells, when treated with a combination of 80 μM of ALA and different concentrations (0-80 μM) of cinnamaldehyde, showed a marked decrease in IC_{50} value from 163 to 133 μM (Table 5.2).

Table 5.1.Percent viability of SiHa cells treated with ALA,Cinnamaldehyde or their combinations.

Concentration (µM)	% viability of SiHa				
	ALA	Cinnam-aldehyde	Combination of Cinnamaldehyde with		
			20 µM ALA	40 µM ALA	80 µM ALA
0	100±0	100±0	100±0	100±0	100±0
10	99.3±0.27	99.3±0.27	93.4±2.64	90.5±0.89	85.2±0.78
20	94.8±1.57	94.2±3.0	89.3±1.27	89.5±0.86	81.2±0.37
40	92.8±0.29	87.0±1.15	87.0±1.79	85.9±1.84	70.8±4.46
80	92.0±0.55	82.9±1.38	84.8±2.03	81.3±4.65	63.6±0.39
160	89.59±1.61	37.13±5.51	7.51±3.89	9.57±2.36	33.53±4.44
IC_{50} value	1531	136	120	116	84

Table 5.2 Percent viability of HeLa cells treated with either ALA, Cinnamaldehyde or their combinations.

Concentration (µM)	% viability of HeLa		
	ALA	Cinnamaldehyde	Cinnamaldehyde +80 µM ALA
0	100±0	100±0	100±0
10	99.30±0.27	98.62±4.90	94.58±2.51
20	99.9±0.27	96.14±5.88	86.98±2.29
40	97.66±2.64	88.87±9.34	83.74±2.0
80	95.99±1.49	77.65±2.63	68.02±5.86
160	87.37±1.49	38.10±4.56	35.43±2.85
IC_{50} value	1571	163	133

5.3.2. ALA reduced IC_{50} value of Curcumin in cervical cancer cells.

SiHa cells were treated with either ALA or curcumin or combination of different concentrations of curcumin (0-80 µM) and ALA (20, 40 and 80 µM). It was found that at 20, 40 and 80 µM concentration of ALA, IC_{50} value of curcumin was reduced from 36 µM to 19, 18 and 15 µM (Table 5.3), respectively. At 80 µM of ALA, the combination with curcumin induced more significant decrease in cell viability. Similarly in HeLa cells, IC_{50} value of curcumin was reduced from 83 to 66 µM, after combination with 80 µM of ALA (Table 5.4).

Table 5.3. Percent viability of SiHa cells treated with either ALA, Curcumin or their combinations.

Concentration (µM)	% viability of SiHa				
	ALA	Curcumin	Combination of Curcumin with		
			20 µM ALA	40 µM ALA	80 µM ALA
0	100±0	100±0	100±0	100±0	100±0
10	99.3±0.27	79.5±1.70	74.6±7.18	69.9±4.79	76.2±3.97
20	94.8±1.57	65.9±6.14	55.7±1.14	47.6±4.11	44.7±2.91
40	92.8±0.29	39.4±3.26	24.8±0.99	26.9±1.83	37.6±1.19
80	92.0±0.55	15.5±0.38	10.5±0.24	5.1±1.08	0.7±0.14
160	89.59±1.61	0.31±0.11	1.04±0.12	0.98±0.11	0.0±0.01
IC_{50} value	1531	36	19	18	15

Table 5.4. Percent viability of HeLa cells treated with ALA, Curcumin and their combinations.

Concentration (μM)	% viability of HeLa		
	ALA	Curcumin	Curcumin +80 μM ALA
0	100±0	100±0	100±0
10	99.30±0.27	94.18±2.11	91.29±1.34
20	99.9±0.27	90.57±2.54	84.69±3.16
40	97.66±2.64	86.95±1.11	81.17±1.44
80	95.99±1.49	55.64±1.81	43.78±3.36
160	87.37±1.49	3.51±0.22	2.35±0.60
IC_{50} value	1571	83	66

5.3.3. ALA reduced IC_{50} value of Andrographolide in cervical cancer cells.

SiHa and HeLa cells were treated with either ALA or andrographolide or combination of different concentrations of andrographolide (0-320 μM) and 80μM of ALA. In SiHa, IC_{50} value of andrographolide was reduced from 134.21 to 45 μM after combination with 80 μM ALA (Table 5.5). In HeLa, andrographolide alone showed IC_{50} value of 197 μM, which was decreased to ~ 115 μM after addition of 80 μM ALA (Table 5.6).We performed similar experiments in non-cancerous HaCaT cell line that showed a high IC_{50} value of andrographolide at 141.12 μM. Interestingly, after combining andrographolide with 80 μM ALA, IC_{50} value was above 160 μM (Table 5.7). This important finding showed that ALA protected non-cancerous cells from getting killed by andrographolide and targeted only cancerous cells.

Table 5.5. Percent viability of SiHa cells treated with ALA, andrographolide and their combinations.

Concentration (µM)	% viability of SiHa		
	ALA	Andrographolide	Andrographolide +80 µM ALA
0	100±0	100±0	100±0
10	99.30±0.27	104±4.60	85.77±1.13
20	94.89±1.57	104±4.32	73.07±1.12
40	92.88±0.29	99.6±0.99	68.64±0.64
80	92.0±0.55	92.7±3.29	34.79±0.56
160	88.97±1.29	24.04±4.51	1.58±0.50
IC_{50} value	1531	134	45

Table 5.6. Percent viability of HeLa cells treated with ALA, andrographolide and their combinations.

Concentration (µM)	% viability of HeLa		
	ALA	Andrographolide	Andrographolide +80 µM ALA
0	100±0	100±0	100±0
20	99.30±0.27	102.0±2.65	92.28±1.51
40	99.9±0.27	93.92±1.45	75.86±2.01
80	97.66±2.64	89.7±7.12	54.82±6.46
160	87.37±1.49	63.76±7.04	33.89±6.21
320	8177±1.49	4.19±0.38	1.39±0.36
IC_{50} value	1571	197	115

Table 5.7. Percent viability of HaCaT cells treated with ALA, andrographolide and their combinations.

Concentration (µM)	% viability of HaCaT		
	ALA	Andrographolide	Andrographolide +80 µM ALA
0	100±0	100±0	100±0
10	106.9±3.15	100±4.60	114±8.6
20	98.75±2.98	111±4.32	126±5.17
40	97.4±1.5	67±0.99	124±8.45
80	104.0±2.3	25±3.29	91±1.13
160	104±1.07	17.7±4.51	81±3.45
IC_{50} value	2000	141	>160

5.3.4. ALA and andrographolide altered cell growth

To check the effect of combination of ALA and andrographolide on growth kinetics, SiHa and HeLa cells were treated with different concentrations of andrographolide (0-320 μM) alone and in combination with 80 μM of ALA. It was observed that andrographolide decreased the growth kinetics of the both the cell lines in a dose- and time-dependent manner. In SiHa, 80 μM concentration of andrographolide decreased the cell growth by ~4.7 ($p = 0.008$), 6.11 ($p = 0.001$) and 4.28-fold ($p = 0.053$) at 24, 48 and 72 h, respectively, compared to the untreated control cells (Figure 5.1.A). Combination of 80 μM ALA with 80 μM andrographolide decreased the cell growth by ~7.51 ($p = 0.008$), 10 ($p = 0.001$) and 8.6-fold ($p = 0.053$) at 24, 48 and 72 h, respectively, compared to the untreated control cells (Figure 5.1.B). Only andrographolide was more effective till 48 h where addition of ALA leads to decrease in cell growth till 72 h. On the other hand, in HeLa, 80 μM concentration of andrographolide decreased the cell proliferation by 5.2 ($p=0.001$), 11 ($p = 0.010$) and 3.63-fold ($p = 0.001$) at 24, 48 and 72 h, respectively, as compared to the untreated control cells (Figure 5.1.C). Combination of 80 μM ALA with 80 μM andrographolide decreased the cell proliferation by 2.9 ($p=0.001$), 13 ($p = 0.010$) and 16.2-fold ($p = 0.001$) at 24, 48 and 72 h, respectively, at 80 μM/ml concentration compared to the untreated control cells (Figure 5.1.D). Thus, Combination of andrographolide with ALA regulated the growth kinetics of cervical cancer cells in a significant manner.

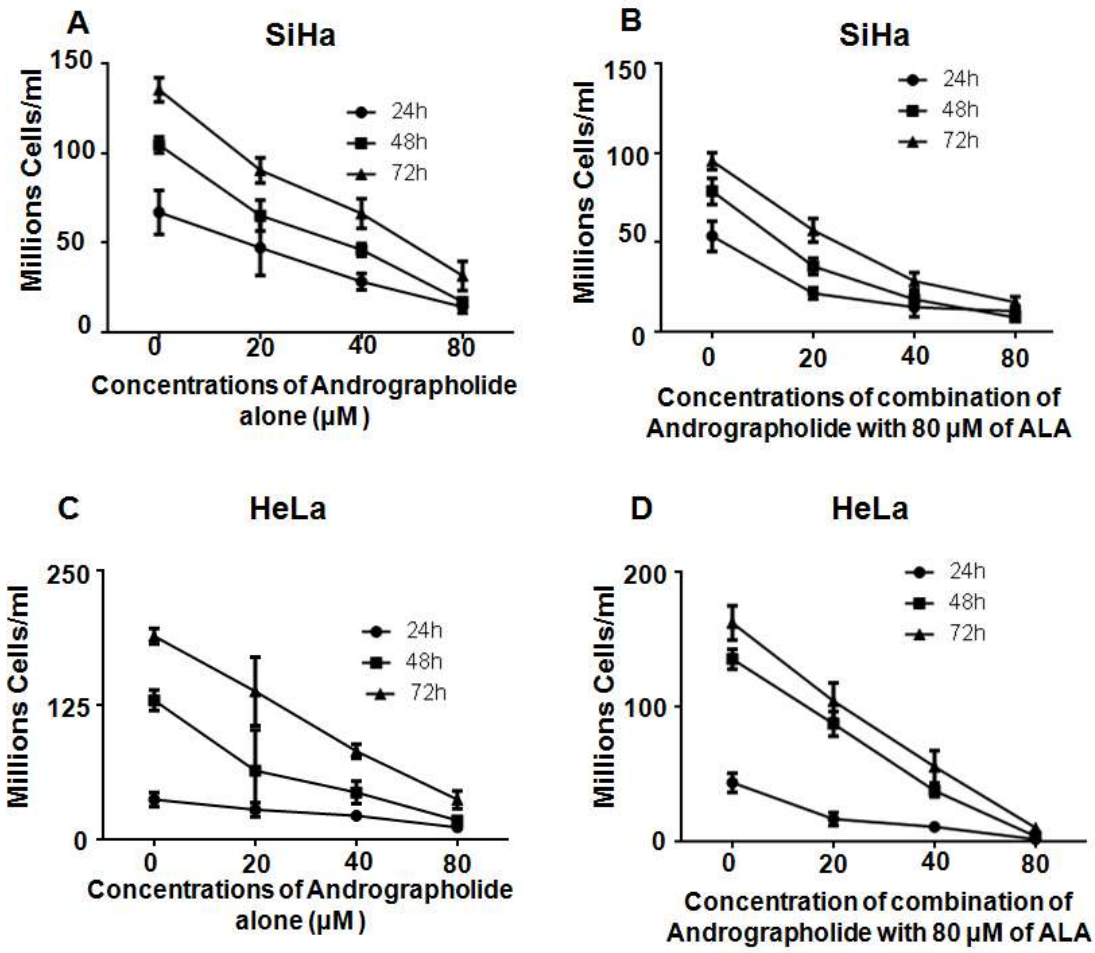

Figure 5.4 Combination andrographolide and 80µM of ALA altered cell growth. *SiHa (A and B) and HeLa (C and D) treated with different concentration of Andrographolide and 80µM of ALA. All the data are presented as means ± SEM of three independent experiments. *$p < 0.05$ indicated statistically significant differences compared to the control untreated group.*

5.4. Discussion

Several studies have demonstrated that polyunsaturated fatty acids (all omega 3 and omega 6 fatty acids) may help in enhancing the cytotoxicity of several antineoplastic agents as well as the anticancer effects of radiotherapy (Altenburg *et al.,* 2011). After combination with 80 µM concentration of ALA, IC_{50} values of cinnamaldehyde, curcumin and andrographolide were significantly reduced showing that combination of ALA and herbal bioactives could enhance their

effectiveness at lower doses. Out of three bioactives andrographolide showed more reduction in cell viability and thus could have a significant therapeutic potential. A similar observation has been reported where DHA in combination with curcumin had synergistic effect on breast cancer cell lines (Altenburg *et al.,* 2011). These effects were possibly mediated by incorporation of ALA, EPA and DHA into cancer cell membranes thus altering the physical and functional properties (Pardini 2006). Increase in percentage of polyunsaturated fatty acids has been known to increases membrane fluidity which inturn increases drug transport and diffusion (Altenburg *et al.,* 2011).

In earlier chapter we have shown that ALA increased IC_{50} value of cisplatin and here we show that it increased IC_{50} value of herbal bioactives, thereby, increasing their effectiveness in cancer cells. Thus ALA could be used either with chemotherapeutic drugs or with herbal bioactives to increase their effectiveness. Since andrographolide proved more promising compared to other bioactives, further studies could be done to delineate the underlying molecular mechanisms of its anticancer activity both in vitro and *in vivo*.

THESIS SUMMARY AND FUTURE PERSPECTIVES

Thesis Summary

Present thesis focuses on analyzing the anticancer potential of alpha linolenic acid (ALA) in cervical cancer at in vitro and *in vivo* levels. ALA is beneficial for the management of various diseases including CVDs, diabetes, blood cholesterol levels and cancer. However; its role in cervical cancer needs to be studied in detail.

To begin with, effect of ALA on the viability of cervical cancer cell lines (SiHa and HeLa) was studied and compared with that of noncancerous (HaCaT and HEK293) cell lines. ALA showed a dose-dependent decrease in the viability of cervical cancer cell lines (SiHa and HeLa) without affecting the viability of non-cancerous transformed HEK 293 and HaCaT cell lines. Cell based assays were undertaken wherein it was found that ALA decreased the growth kinetics of cervical cancer cell lines in a dose and time dependent manner. Further, we checked the molecular mechanism governing the anticancer activity of ALA and it was through regulation of NO release, induction of lipid peroxidation leading to apoptosis through caspase-3 activation. We also analyzed the effect of ALA on the markers involved in other signaling pathways in cervical cancer besides apoptosis such as angiogenesis, inflammation and tumor regulatory pathways. ALA reduced the expression of angiogenic markers VEGF and MMPs; inflammatory markers COX-2and NFκB and HPV oncoproteins E6 and E7 with concomitant increase in tumor regulatory markers p53 and Rb.

After demonstrating the role of ALA at in vitro level, we were interested in confirming the anticancer activity of ALA at *in vivo* level. We mimicked the cervical cancer model by injecting TC1 cell line containing HPV 16 E6 and E7

oncoproteins in C57/BL6J mice.In this experiment, flax oil as a source of ALA wasgiven orally to mice. Flax oil treatment was given either alone or in combination with a well-known anticancer drug cisplatin used in chemotherapy for cervical cancer patients. The objective behind combining flax oil with cisplatin was to find out whether flax oil could reduce side effects of chemotherapy such as decreased immunity and increased oxidative stress. Flax oil and its combination with cisplatin increased the expression of immunomodulatory cytokines such as Th1 (IFNγ) and Th2 (IL 4) and decreased oxidative stress by reducing liver peroxidation. We also checked the effect of combination of ALA and cisplatin in cervical cancer cell lines. There was decrease in IC_{50} value of cisplatin upon combination with ALA. We also evaluated the effect of anticancer herbal bioactives in combination with ALA on cervical cancer cell lines.We used herbal bioactives curcumin, cinnamaldehyde and andrographolide. IC_{50} values of all herbal bioactives were reduced and out of the three bioactives, andrographolide showed more decrease in IC_{50} value.

These results suggested that ALA was an effective nutraceutical and simple modifications in our routine diet could help in the management of cervical cancer along with other health benefits.

Thesis highlights

1. **ALA reduced growth kinetics and induced apoptosis in cervical cancer cell lines**

- ALA showed a dose-dependent decrease in cell viability in cervical cancer cell lines (SiHa and HeLa) without affecting the viability of non-cancerous transformed HEK 293 and HaCaT cell lines
- ALA decreased the growth kinetics of cervical cancer cell lines in a dose and time dependent manner
- ALA regulated the growth of cervical cancer cell lines through regulation of NO release and induction of lipid peroxidation leading to apoptosis through caspase-3 activation

2. **ALA reduced expression ofCOX2/VEGF/MAP kinase pathway and HPV oncoproteins E6, E7 in cervical cancer cell lines**

- ALA reduced expression of angiogenic markers VEGF and MMPs
- ALA reduced expression of inflammatory markers COX-2 and NFκB
- ALA decreased expression of HPV oncoproteins E6 and E7 with concomitant increase in tumor regulatory markers p53 and Rb

3. **ALA in the form of flaxseed oil reduced tumor growth in TC1 (HPV 16 positive) induced tumor growth in C57BL/6 mice**

- Flax oil (FO) alone and in combination with cisplatin (Cis) reduced more the tumor growth compared to cisplatin in TC1 induced tumor in C57BL/6J mice
- FO and Cis+FO increased plasma antioxidant levels

- FO and Cis+FOincreased lipid peroxidation in tumor tissue with concomitant decrease in liver lipid peroxidation
- FO and Cis+FO increased IFNγ level and decreased levels of IL-4 leading to immunomodulation in mice.
- FO and Cis+FO decreased expression of HPV oncoproteins E6 and E7

4. **ALA increased anticancer activity of andrographolide in cervical cancer cell lines**

- ALA decreased IC_{50} value of herbal bioactives curcumin, cinnamaldehyde and andrographolide
- More decrease in IC_{50} value was observed in combination of ALA with andrographolide

Original finding in this thesis

The following observations are being reported for the first time in this thesis

- Demonstration of anticancer activity of Alpha linolenic acid and analyzing the underlying molecular mechanism through which ALA act on cervical cancer cells at invitro and *in vivo* level
- Alpha linolenic acid decreased Nitric oxide level and increase in lipid peroxidation in cervical cancer cells which lead to apoptosis by capsase activation
- Alpha linolenic acid reduced the expression of HPV oncoproteins E6 and E7 in cervical cancer cells and inhibited migration through suppression of COX-2/VEGF/MAP kinase pathway

- Elucidation of the positive effects of oral supplementation of flax oil in TC1 (HPV16 positive) induced tumor model where flax oil in combination with cisplatin reduced not only tumor volume but also decreased side effects of cisplatin by increasing plamsa antioxidant level and decreasing in liver lipid peroxidation
- Combination of ALA with andrographolide, an active component of a medicinal plant enhancedcytotoxic effect in cervical cancer cells

Future perspectives

- Effect of ALA could be studied at epigenetic level, both invitro and *in vivo*
- Effect of flax oil supplementation could be tested in cervical patients undergoing chemotherapy in terms of quality of life (QoL) and other biochemical parameters

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Publications

Publications from the present study

Research articles published/communicated

1. **Rashmi Deshpande**, Prakash Mansara, Snehal Suryavanshi, Ruchika Kaul-Ghanekar. Alpha-linolenic acid regulates the growth of breast and cervical cancer cell lines through regulation of NO release and induction of lipid peroxidation. 2013. Journal of Molecular Biochemistry.

2. Prakash Mansara, **Rashmi Deshpande**, Ruchika Kaul-Ghanekar, Prabhakar Ranjekar. Role of Omega-3 fatty acids in the Prevention of Breast Cancer: *In vitro*, *In vivo* and Clinical studies. Bharati Vidyapeeth University Journal 2011, IX (1):10-18.

3. **Rashmi Deshpande**, Prakash Mansara and Ruchika Kaul-Ghanekar* Alpha linolenic acid regulates COX2/VEGF/MAP kinase pathway and decreases the expression of HPV oncoproteins E6/E7 through restoration of p53 and Rb in human cervical cancer cells (communicated)

4. **Rashmi Deshpande**, Kavita Shinde and Ruchika Kaul-Ghanekar* **Flaxseed oil containing Alpha Linolenic Acid (ALA) reduces tumor growth in TC1 induced in C57BL/6J mice.** (Communicated)

5. Savita Pandita, **Rashmi Deshpande**, Shama Aphale,Dr.Ruchika Kaul-Ghanekar Role Of Medicinal Plants In Targeting Important Signaling Pathways In Cervical Cancer Book Chapter communicated in Apple Academic Press, 2015

Other Publication

1. Prakash Mansara, **Rashmi Deshpande**, Ruchika Kaul-Ghanekar. Differential ratios of omega fatty acids modulate growth, lipid peroxidation, NO release and expression of tumor regulatory MARBPs in breast cancer (communicated)

2. Prakash Mansara, Mrunal Ketkar, **Rashmi Deshpande**, Amol Chaudhary, Kavita Shinde, Ruchika Kaul-Ghanekar,Improvement in antioxidant status of chemotherapy undergoing breast cancer patients supplemented with omega-3 fatty acids- 5 cases (communicated)

3. Snehal A Suryavanshi, Prerna Raina, **Rashmi Deshpande**, Ruchika Kaul-Ghanekar.*Nordostachys Jatamansi* root extracts alters growth kinetics and modulates p53 expression in neuroblastoma cells.