Synthesis, Purification and Characterization of 15-Lipoxygenase (15-LOX) Metabolites: Effects on Acute Lymphoblastic T-Cell Leukemia Cell line, (Jurkat)

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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August, 2008
Enrolment No. 04LAPH06

Dedicated to my Parents

University of Hyderabad
(A Central University established in 1974 by Act of Parliament)
HYDERABAD – 500 046, INDIA

DECLARATION

I hereby declare that the work embodied in this thesis entitled "***Synthesis, Purification and Characterization of 15-Lipoxygenase (15-LOX) Metabolites: Effects on Acute Lymphoblastic T-Cell Leukemia Cell line (Jurkat)"*** has been carried out by me under the supervision of Prof. P. Reddanna and this has not been submitted for any degree or diploma of any other University earlier.

Prof. P. Reddanna
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CERTIFICATE

This is to certify that ***Mr. K. Anil Kumar*** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph. D. ordinance of this University. We recommend his thesis "***Synthesis, Purification and Characterization of 15-Lipoxygenase (15-LOX) Metabolites: Effects on Acute Lymphoblastic T-Cell Leukemia Cell line (Jurkat)***" for submission for the degree of Doctor of Philosophy of this University**.**

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Acknowledgements

*I express my gratitude to my mentor **Prof. P. Reddanna** for providing me with an opportunity to work in his lab. I also thank him for all his unwavering support through out my doctoral discourse. I am grateful for his invaluable advice whenever needed while his unbounded optimism has kept my spirits high.*

*I would also like to express my thankfulness to my uncle **Dr. K. Subbarammaiah** – Cornell University, New York for his support and encouragement.*

*I am also indebted to the members of my doctoral committee**, Prof. S. Dayananda** and **Dr. Anita Jagota** for their valuable suggestions. I would especially thank **Prof. S. Dayananda,** Head, Department of Animal Sciences and former Head, **Prof. Aparna Dutta Gupta** for allowing me to use the facilities of the department.*

*I thank **Prof. A.S. Raghavendra**, Dean, School of Life Sciences and former dean **Prof. T. Suryanarayana** for allowing me to use the general facilities of the school.*

I thank Dr G.V. Reddy for his constant help during course of my Ph D, and also thankful to my seniors Dr. Maya, Dr. Sreenath, Dr. Subhashini, Dr. Mahipal, Dr. Aparna, Dr. Mallikarjun Reddy and Dr. Sreekanth for their cooperation and constant help throughout my work. My special thanks to my labmates Dr. Arunasree, Dr. Roy, for their extended help.

I thank my lab mates Sreedevi, Bharat, Smita, Chandra Mohan, Ramakrishna, Nishant, Aparoy, Praveen, Jyotsna, Chandrani for their cooperation and constant help throughout my work.

I thank G. Sreenivasulu and Pushpanjali for their help in cloning and activity assays.

I thank Dr. Krishnaveni and Dr. Sree Ramana of CDFD for their help during project work.

I wish to extend my thanks to all my friends who have helped me in many ways and made my stay at University of Hyderabad a pleasant and memorable one.

I take this opportunity to thank all the students of Prof. S. Dayananda for their timely help and constant support.

I thank Shiva Kumar, Balram, Nagesh and Shivaram for their co-operation throughout my doctoral work.

I wish to thank non teaching staff Ankineedu garu, Mr. Jagan, Babu Rao garu for their kind assistance and cooperation.

The financial assistance from DST-Dabur, University of Hyderabad and Shantha Biotechnics Pvt Ltd, Hyderabad is gratefully acknowledged.

The funding from DST-FIST, UGC-SAP, DBT, CSIR, for the department and the lab is highly acknowledged.

I wouldn't have achieved this without the constant support, love and encouragement given to me by my parents, grand parents, my brothers and sisters and other family members.

I finally thank almighty for every thing.

Anilkumar K.

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Abbreviations

AA	:	arachidonic acid
ALA	:	α-linolenic acid
Ac-DEVD-AFC	:	N-acetyl-(Asp-Glu-Val-Asp)-trifluoromethylcoumarin
Ac-IETD-AMC	:	N-acetyl-(Ile-Glu-Thr-Asp) -7-Amido-4-methylcoumarin
DCFH-DA	:	2', 7'-Dichlorofluorescein Diacetate
DPI	:	diphenylene iodonium
EDTA	:	ethylene diamine tetra acetic acid
FACS	:	fluorescence activated cell sorter
FBS	:	fetal bovine serum
GC-MS	:	gas chromatography-mass spectrometry
g	:	gram
h	:	hour(s)
15-(S)-HPETE	:	15-(S)-hydroperoxyeicosatetraenoic acid
15-(S)-HETE	:	15-(S)-hydroxyeicosatetraenoic acid
13-(S)-HPOTrE	:	13-(S)-hydroperoxyoctadecatrinoic acid
13-(S)-HOTrE	:	13-(S)-hydroxyoctadecatrienoic acid
13-(S)-HPODE	:	13-(S)-hydroperoxyoctadecadienoic acid
13-(S)-HODE	:	13-(S)-hydroxyoctadecadienoic acid
kDa	:	kilodalton
l	:	litre
LOX	:	lipoxygenase
mg	:	milligram
min	:	minute(s)
ml	:	millilitre
mM	:	millimolar
MTT	:	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]
NAC	:	N-acetyl cysteine
nm	:	nanometer(s)
NSAIDs	:	non-steroidal anti-inflammatory drugs
PAGE	:	polyacrylamide gel electrophoresis
PARP	:	poly(ADP)ribose polymerase
PBS	:	phosphate buffered saline
PI	:	propidium iodide
Tris	:	tris-(Hydroxymethyl) aminoethane
UV	:	ultraviolet
μM	:	micro molar
ºC	:	degree centigrade/degree celsius

Introduction

1. Introduction

1.1. Leukemias

Leukemias are neoplastic proliferations of immature cells of the hematopoietic system, which are characterized by arrested or abnormal differentiation. Leukemia cells rapidly divide and accumulate in the bone marrow. It is the replacement of the normal hematopoietic cells by the leukemic cells that results in the signs and symptoms of the disease. The consequences of such bone marrow failure may have effects on all three major cell lineages and cause anaemia, hemorrhage and infections.

Broadly, leukemias are classified into lymphogenic and myelogenic forms. Within each group a further subdivision into acute and chronic forms are recognized. Recent advances in molecular biology, genetics, and immunlology allow further precise subdivisions.

1.2. Acute lymphoblastic leukemia (ALL)

ALL is a blood cancer that affects the white blood cells. ALL is a spectrum of diseases comprised of several different subtypes, named for the cell type that is affected (B or T) and how abnormal the cell appears under a microscope. A person with ALL develops abnormal numbers of white blood cells rather quickly, giving the disease the name “acute”.

The white blood cell (WBC) count may be higher or lower than normal, but the WBCs that are being produced are immature and do not function well. Because WBCs are an important part of fighting infections, patients often have multiple infections that don’t respond to treatment before they are diagnosed. Some people will have low red blood cell or platelet counts because the overpopulation of WBCs crowds out these cells.

Acute leukemias are derived from primitive hematopoietic progenitor cells. Although they are rare cancers, accounting for less than 3% of all malignancies, they are the leading cause of death in persons younger than 35 years of age. The incidence of acute myelogenous leukemis (AML) is 2.5 per 100,000 persons; for acute lymphocytic leukemias (ALL) the incidence is 1.3 per 100,000. Both types have a slight male predominance.

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy of thymocytes characterized by high numbers of bone marrow and circulating blast cells, enlargement of mediastinal lymph nodes, and often central nervous system involvement. T-ALL accounts for approximately 15% of pediatric and 25% of adult ALL cases.

Several studies have examined the role of potential carcinogens in the environment, such as low-dose radiation, cigarette smoke, and electromagnetic radiation. Both ALL and AML risks increased after atomic bomb radiation exposure, but the actual occupational risks associated with low-dose are controversial. Although some reports indicated that paternal exposure to low-dose radiation resulted in an increased risk for subsequent children, this could not be confirmed. Cigarette smoking has been linked to leukemia in a dose-dependent manner, and 20% cases of AML may be attributable to smoking. Recently the carcinogenic effect of exposure to electromagnetic fields has raised a great deal of attention, but the actual risk of leukemia from exposure to commercial and residential power lines remains controversial.

Similar to other types of leukemias, T-ALL is caused by genetic alterations in hematopoietic precursor cells leading to a variety of changes, including loss of cell cycle control, unlimited self-renewal capacity, impaired differentiation, hyperproliferation and loss of sensitivity to death signals.

For most patients with acute leukemias, the cause of disease is unknown. A clear cause can be found in a few patients with a history of previous chemotherapy or radiation therapy. Although leukemias are acquired disorders, there seem to be

genetic and immunologic factors predisposing certain individuals to develop the disease. For example, several genetic syndromes such as Down's syndrome, Bloom's syndrome, Fanconi's anemia, and Ataxia-telangiectasia are associated with an increased risk of leukemias. Also, a high concordance for leukemia between identical twins has been observed.

The major chromosomal abnormalities in ALL are t(9;22)(q34;q11), t(12;21)(p13;q22), t(4;11)(q21;q23), t(1;19)(q23;p13) and 8q24 translocations and hyperdiploidy. Generally, hyperdiploidy, occurring most frequently in paediatric cases, is associated with good prognosis, while hypodiploidy confers a poor prognosis. Among structural chromosomal abnormalities, the t(9;22)(q34;q11) resulting in the BCR/ABL fusion protein, and rearrangements of the MLL gene, confer a poor prognosis in both children and adults, while t(12;21)(p13;q22), resulting in the TEL/AML1 fusion protein, and del (12p) confer a good prognosis. More recently, additional diagnostic and prognostic information has been gained from fluorescence in situ hybridization (FISH) and DNA microarray techniques.

1.2.1. Cytogenetic evaluation

Cytogenetic analysis of each ALL patient's cells has become an essential component of diagnosis prior to treatment. It has furthered our understanding of leukaemogenesis at molecular level. Specific and well-characterized recurring chromosomal abnormalities facilitate diagnosis, confirm subtype classification, and have major prognostic value for treatment planning. These cytogenetic abnormalities are acquired somatic (rather than germline) mutations that frequently result from translocations of chromosomal DNA, resulting in new (abnormal) protein products from the resultant fusion genes. It is assumed that the protein products from these fusion genes are responsible for the cellular dysregulation that leads to the malignant state. Deletions or loss of DNA may eliminate genes that have tumor suppressor

functions. Gains of additional chromosomes may lead to gene dosage effects that provide transformed cells with survival advantages.

Conventional cytogenetic analysis which requires dividing cells is technically difficult and can be time-consuming due to the presence of multiple abnormal cell lines and complex chromosomal banding patterns. Therefore, alternative diagnostic methods have been sought, including fluorescence in situ hubridization (FISH), in which labeled probes are hybridized to either metaphase chromosomes or interphase nuclei and then detected with fluorochromes. This method of analysis is more rapid, and in some cases more sensitive, than conventional cytogenetic analysis. Additionally, FISH can be used to study differentiated or non-dividing cells.

1.2.2. Molecular evaluation

Translocations that result in fusion genes are especially suited for analysis with reverse transcriptase PCR (RT-PCR), and then amplified by PCR using gene specific primers. Quantitative RT-PCR allows for quantification of minimal residual disease (MRD). A number of large prospective studies in paediatric ALL have demonstrated the independent prognostic significance of MRD detection (van Dongen et al., 1998; Cave et al., 1998), less is known about the significance of MRD detection in adult ALL.

DNA microarray data of 72 acute leukemia samples were successfully employed to correctly classify the samples into ALL and AML (Golub et al., 1999). In a more recent study, Armstrong et al (2002) used DNA microarray to analyse adult and paediatric leukaemia samples and suggested that mixed-lineage leukaemia (MLL) should be considered as a distinct subset of leukaemia based on a unique expression profile (Armstrong et al., 2002). In addition to their potential for diagnosis and classification, microarrays can be used for prognosis. Yeoh et al (2002) used DNA microarray to study the gene expression profile of 360 paediatric ALL patients. They found that distinct expression profiles identified known

prognostically significant ALL subtypes as well as a distinct expression profile for ALL patients who later developed therapy-related AML. In addition, within some genetic subgroups, they were able to identify patients who eventually failed therapy based on unique expression profiles.

1.2.3. Treatment of ALL

The treatment of acute lymphoblastic leukemia (ALL) varies according to one's age, general condition at diagnosis and the results of the cytogenetic testing. Standard therapy for ALL has not changed in approximately 15 years, for the current strategy has been very effective at curing adults. Treatment can be divided into four phases:

First phase -- induction chemotherapy

Second phase -- consolidation chemotherapy

Third phase -- maintenance chemotherapy

Fourth phase -- CNS prophylaxis

The initial two phases use intensive chemotherapy medications designed to kill cells that grow quickly, like leukemia cells. Therapy for ALL typically continues for two to three years. Complete remission is achieved in approximately 90 percent of patients, with 25 percent to 40 percent enjoying long-term survival. Approximately 5 percent of patients die of treatment-related complications during their initial therapy and another 5 percent fail to ever achieve an initial remission.

1.2.3.1. Chemotherapy

Treatment of ALL is usually urgent and needs to be given within days and sometimes the same day as the diagnosis is made. Normally, patients are treated with chemotherapy. The first phase in treatment, called induction chemotherapy, requires that patients remain in the hospital for approximately four weeks.

The most common drugs used for induction treatment of ALL are daunorubicin, vin-cristine, prednisone, asparaginase and sometimes cyclophosphamide (Cytoxan). Intensive supportive care accompanies the chemotherapy, including transfusion of red blood cells and platelets. Antibiotics are needed both preventatively and as treatment for both bacterial and fungal infections. The agent G-CSF (Neupogen) can be useful in reestablishing a normal white blood count. Although the likelihood of mouth sores and disruption of the intestinal tract is rare, complete but temporary hair loss does occur.

Once blood counts have returned to normal, a repeat bone marrow biopsy is performed to determine whether the patient has entered complete remission. A complete remission is achieved when the blood and bone marrow show no evidence of persistent leukemia and blood counts have returned to normal.

Consolidation chemotherapy typically includes multiple cycles of intensive chemotherapy given over a six to nine month period. Frequent hospitalizations are required and intensive supportive care is still needed, including red blood cell and platelet transfusions. Stem cell transplantation is not typically performed to treat ALL unless abnormal cytogenetics are present.

Once patients have completed intensive chemotherapy, they need to take oral chemotherapy pills for an additional 18 to 24 months. These oral chemotherapy pills are usually well-tolerated with only minimal side effects. Patients need to have their

blood tests checked once a month while taking chemotherapy pills. Most patients with ALL can return to work during maintenance therapy.

1.2.3.2. CNS Prophylaxis

ALL frequently can recur in the spinal fluid (the fluid that bathes the spinal column and brain). To prevent relapse at this location, chemotherapy must be infused directly into the fluid that bathes the spinal column. This is done by inserting a needle between the vertebrae of the lower back and infusing chemotherapy directly into the clear spinal fluid, which is called intrathecal chemotherapy. Patients are routinely given 6 to 12 injections of intrathecal chemotherapy to prevent recurrence of ALL. More injections may be necessary if leukemia cells are detected in the spinal fluid. Most people complete intrathecal therapy within two to four months of starting their treatment. Headaches and nausea are the most common side effects.

1.2.3.3. Stem Cell Transplantation

Stem cell transplantation, also called bone marrow transplantation, is only performed in patients who have abnormal cytogenetics, chromosome testing, or other high risk ALL features. Cytogenetics is the most important component of deciding whether or not a person should have a bone marrow transplant for ALL. Patients with the Philadelphia chromosome or with the translocation involving chromosomes 4 and 11 should go on to bone marrow transplantation.

1. 3. PUFAs and Cancer

At least one third of all human cancers may be associated with diet and influenced by life style and physical exercise (Doll and Peto, 1981). Dietary fat is thought to be one of the main risk factors on the basis of reports of positive correlations between dietary fat intake and increased risks of cancers of the breast, colon and prostate (Key, 1995; Lipworth, 1995; Potter, 1995). Although the role of

individual fatty acids in human cancer risk has hitherto been poorly investigated, some recent epidemiological and clear experimental data have now linked a high dietary intake of ω-6 polyunsaturated fatty acids (PUFAs) such as linoleic acid (LA) (C18:2), especially in association with a low intake of ω-3 PUFAs such as docosahexaenoic acid (DHA) (C22:6), to increased risks for cancers of the breast, colon and possibly, prostate. ω-6 PUFAs enchance tumourigenesis and metastasis in experimental animals by several mechanisms, ω-3 PUFAs can inhibit the growth of initiated cancer cells. This is also supported by results from clinical studies showing a reduction in intestinal hyperproliferation after consumption of fish oil-derived ω-3 PUFAs in subjects at elevated risk of colon cancer due to sporadic colonic adenomas (Anti et al., 1992, 2001).

It has been shown that lowering the quantity of fat as a proportion of total calories decreases the growth rate of human prostate cancer cells in mice (Wang et al., 1995). Further human prostate cancer cells in-vitro exhibit increased growth in the presence of (ω)-6 LA, where as long chain (ω)-3 docosahexaenoic acid (DHA) and Eicosa pentaenoic acid (EPA) inhibit tumorogenesis in these cell lines (Rose and Connolly, 1991).

PUFAs alone can regulate cancer cell growth and viability. PUFAs create oxidative stress in biological systems as they undergo lipid peroxidation, forming free redicals such as peroxyl and alkoxyl radicals. Although these lipid hydroperoxides are relatively short lived, their breakdown results in the formation of secondary products of lipid peroxidation (aldehydes such as malondialdehyde and the 4-hydroxyalkenals) that are longer lived and can attack a variety of cellular targets.

Low concentrations of these aldehydes regulate the cell cycle in ways that reduce the rate of cell proliferation. These effects include inhibiting the transition of cells from the G0 phase to the G1 phase, prolonging the G1 phase, slowing progression through the S phase by inhibiting the activity of DNA polymerases, inhibiting cell cycle progression through the restriction point, and causing arrest at

cell cycle check points (Esterbauer et al., 1991; Barrera et al., 2008). These effects that retard cell cycle progression will impact proliferating cells such as those in culture and those of certain animal tissues, including neoplasmas, bone marrow, and the intestinal epithelium. Whereas low- level PUFA- induced oxidative stress is cytostatic, higher levels of oxidative stress result in apoptosis, and still higher levels cause cellular necrosis (Baty et al., 2005; Vissers et al., 2001; Chandra et al., 2000) .

Many investigators have demonstrated that ω-6 and ω-3 PUFAs- including linoleic acid (LA), gamma-linoleic acid (GLA), dihommo-γ-linolenic acid (DGLA), arachidonic acid (AA), alpha- linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)-inhibit growth and are cytotoxic to cancer cells in-vitro (Shirota et al., 2005; Menendz et al., 2001; Albino et al., 2000; Hawkins et al., 1998; Lai et al., 1996; Das, 1991; Chow et al., 1989; Begin et al., 1988, 1985; Fujiwara et al., 1986), and these effects are associated with the production of lipid peroxides and aldehydes (Shirota et al., 2005; Hawkins et al., 1998; Das, 1991; Chow et al., 1989; Fujiwara et al., 1986) and that the cytotoxicity of the added PUFAs is reduced by the addition of antioxidants (Shirota et al., 2005; Hawkins et al., 1998; Das, 1991; Chow et al., 1989; Fujiwara et al., 1986). Studies on laboratory animals have also demonstrated that feeding a diet containing peroxidation products of fish oil (Hardman et al., 2001) reduces tumor growth, and that the effect is reduced by administering antioxidants (Hardman et al., 2001; Gonzalez et al., 1993). However, these effects in-vitro are observed at PUFA concentrations (30 μM and above in most studies) exceeding normal plasma free fatty acid (FFA) levels.

1. 4. PUFAs and Leukemia

Polyunsaturated fatty acids of the ω-3 family have over all suppressive effects, inhibiting lymphocyte proliferation, antibody and cytokine production, adhesion molecule expression, natural killer cell activity and triggering cell death. The ω-6 fatty acids have both inhibitory and stimulatory effects. Lymphocyte

activation is usually inhibited by fatty acids, particularly by PUFA and volatile fatty acids. Thus, the pretreatment of animals with butyrate was shown to inhibit T-cell proliferation to immobilized anti-CD3 monoclonal antibody (Saemann et al., 2002). ω-3 fatty acid-rich diets are associated with a lower percentage of activated T and B cells upon stimulation (Robinson et al., 2001).

Many experiments performed with concanavalin A- or lipopolysaccharide-activated lymphocytes have shown that fatty acids, particularly PUFAs and volatile fatty acids, can inhibit cell proliferation by an eicosanoid-independent action (Rosa et al., 1995; Egami et al., 1993). It is also known that higher doses of these fatty acids can cause cell death via apoptosis or, depending on their concentration, necrosis. Because of these effects, patients with autoimmune diseases or chronic inflammation have been advised to eat PUFA-rich diets. It should be mentioned that a minimum amount of fatty acids is needed for optimum lymphocyte proliferation, as in the absence of fatty acids cells have impaired growth (Spieker–polet and Polet, 1981; Anel et al., 1990). Accordingly, it has been shown that lymphocyte DNA synthesis is stimulated by low and inhibited by high free fatty acid concentrations (Karsten et al., 1994).

The cytotoxic effect of PUFAs on lymphocytes or leukemia cells has long been known. However, it is not clear whether these effects are mediated by PUFAs themselves or their oxygenated metabolites. Higher doses of fatty acids preferentially cause necrosis, with a rapid loss of membrane integrity, lysosomal enzyme leakage and cell swelling. Both apoptosis and necrosis seem to be associated with oxidative stress, as they can be partially prevented by antioxidants such as tocopherol (Finstad et al., 2000). Recent studies have indicated that PUFAs mediate their effects through their oxygenated metabolites, collectively termed as “eicosanoids”. In fact, cyclooxygenase inhibitors and sometimes lipoxygenase inhibitors have been found to protect cells from PUFA-induced cytotoxicity (Koller et al., 1997).

As is the case for macrophages and neutrophils, fatty acids can also modulate lymphocyte cytokine production. Thus, dihomo-γ-linolenic acid and arachidonic acid inhibit the production of IL-2 by lymphocytes, an effect that cannot be abolished by cyclooxygenase inhibitors.

Palmitic acid enhances the release of IFN-γ by human lymphocytes, while diminishing TNF-α production (Anel et al., 1990). The same authors have shown that other saturated fatty acids are more potent than unsaturated fatty acids in modulating cytokine production, particularly in relation to IFN-γ. Lymphocytes treated with ω-3 fatty acids as well as with butyrate show decreased IFN- γ production (Purasiri et al, 1997; Dongona and Gullans, 1998).

Lymphocyte interaction with other cells, important for immune response regulation and cell migration, depends on the expression of many surface proteins, which can be modulated by fatty acids. Thus, animals fed diets rich in ω-3 fatty acids (fish oil) and ω-6 fatty acids (olive oil) have decreased expression of LFA-1, ICAM-1 (inter cellular adhesion molecule) and CD2 (Calder et al., 2006; Fernandes et al., 1994). Pretreatment of lymphocytes with DHA or EPA (ω-3) can reduce the expression of the adhesion molecules, L-selectin and LFA-1 (Khalfoun et al., 1996). The addition of DHA or EPA to adhesion assays involving endothelial cells and lymphocytes reduces cell interaction, which is associated with reduced expression of VCAM-1 (Khalfoun et al., 1996).

Docosahexaenoic acid increases the expression of Thy-1.2 (CD 90.2: Cluster of differentiation 90.2) and alters CD8 epitope expression, therefore potentially regulating T-cell activation. There is also regulation of CD44 and CD45 expression by fatty acids, particularly oleic acid, although the effect on CD45 can also occur by cell treatment with stearic acid. Oleic acid increases CD44 and CD45 capping, as has been confirmed by in vivo and in vitro studies (Peck et al., 1996). Fish oil-rich diets (ω-3 PUFA) are associated with higher TGF-β1 expression in T cells (Fernandes et al., 1994). This growth factor is implicated in immune response modulation.

A very important cell type that acts in natural immunity is the natural killer (NK) cell, which can identify and kill virus-infected or microbe-infected cells, as well as tumor cells. Regarding natural immunity against tumor cells, lymphokine-activated killer (LAK) cells that efficiently recognize and kill transformed cells, are also very important. As a rule, ω-3 PUFAs can inhibit NK cell activity. For instance, high fat diets, particularly those rich in omega-3 PUFAs, can lower NK and LAK cell activity, as well as the number of circulating cells (Robinson et al., 2000; Kelley et al., 1999). This inhibitory effect is also found in other experimental models, such as animals submitted to intravenous infusion with ω-3 PUFAs and human cells treated in culture with omega-3 PUFAs, that not only suppress NK, but also LAK cell activity (Purasiri et al., 1997). Oleic and palmitic acid-rich diets also seem to inhibit NK cell activity (Thies et al., 2001), whereas short-chain fatty acids, when delivered with parenteral nutrition, increase the cytotoxic activity of these cells (Pratt et al., 1996).

The immune response to tumors depends on the balance of T-helper and T-suppressor cells. It has been found that such a ratio is increased in patients with solid tumors submitted to diets rich in ω-3 PUFAs, mainly due to a decrease in suppressor T cells, indicating that these fatty acids could inhibit tumor growth (Gogas et al., 1995).

1.5. Eicosanoids

The term “eicosanoids” is used to embrace biologically active lipid mediators (C20 fatty acids and their metabolites), including prostaglandins, thromboxanes, leukotrienes and other oxygenated derivatives, which are produced primarily by three classes of enzymes- cyclooxygenases (COX-1 and COX-2), lipoxygenases (LOX) and cytochrome P450 dependent epoxygenases (Fig. 1).

The key precursor fatty acids are 8Cis, 11Cis, 14Cis-eicosatrienoic (dihomo-γ-linolenic or 20:3 (ω-6)); and 5Cis, 8Cis, 11Cis, 14Cis-eicosatetraenoic

(arachidonic or 20:4(ω-6)) and 5Cis, 8Cis, 11Cis, 14Cis, 17Cis-eicosapentaenoic (20:5(ω-3)) acids or EPA.

Of the precursor fatty acids, arachidonic acid has been by far the most studied, and it is special in many ways. It is a derived essential fatty acid in that it is synthesized in animals from linoleic acid, which is obtained from the diet. As a major component of phospholipids, and especially of phosphotidyl inositol, it is important for the integrity of cellular membranes. The four cis-double bonds mean that the molecule is highly flexible, and this helps to confer the correct degree of fluidity in the membranes.

Arachidonic acid has only rarely been encountered in higher plants, but it is a constituent of some algae, fungi, and moulds. In fungal infections of plants, it is known to elicit the production of plant defense compounds (phytoalexins), probably after conversion to oxygenated metabolites.

The oxygenated metabolites derived from arachidonic and related fatty acids are produced through a series of complex interrelated biosynthetic pathways sometimes termed the ‘arachidonate cascade’.

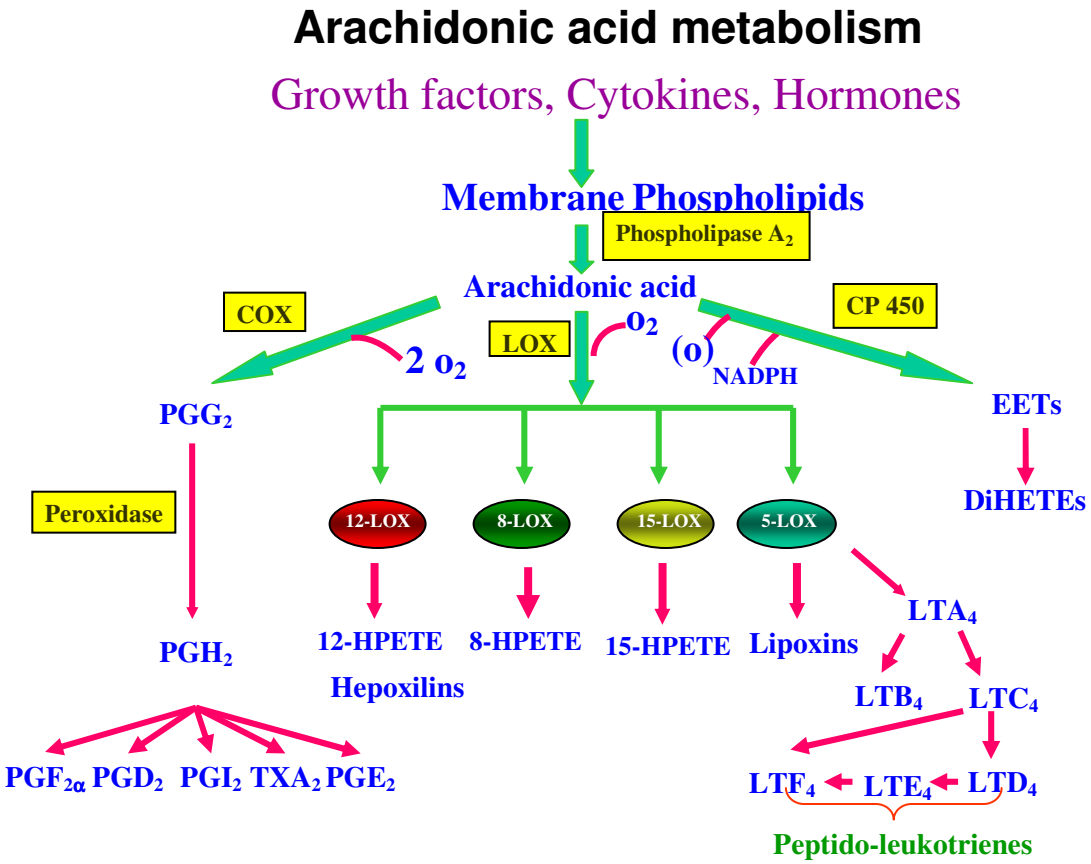

Fig. 1: Arachidonic acid metabolism

They are so numerous and have such a range of biological activities that they must provide a substantial component of the reason for the essentiality of the latter to the survival and well being of animals. Most of the arachidonic acid (AA) (and other polyunsaturated fatty acids) in animal tissues is in esterified form, mainly to phospholipids and phosphotidylinositol in particular. Once AA is released from membrane phospholipids by the action of phospholipases it will be acted upon by one of the three pathways (Cyclooxygenase, Lipoxygenase, Epoxygenase) to generate biologically active metabolites, collectively termed as "eicosanoids".

1.5.1. Cyclooxygenases (Prostaglandin Endoperoxide H Synthases)

Cyclooxygenase-1 and cyclooxygenase-2 (COX-1 and COX-2), more correctly termed prostaglandin endoperoxide H synthases–1 and –2 (PGHS-1 and PGHS-2), are key enzymes that catalyze the first committed step in the synthesis of prostanoids from fatty acid precursors. COX-1 is always present in tissues, while COX-2 is induced by appropriate physiological stimuli (cytokines, tumor promoters and growth factors). The two iso-enzymes have about 60% homology in their amino acids, and are very similar in structure. They are integral membrane proteins but are located on only one side of the bilayer. Both the enzymes catalyze the same two reactions at different sites. Thus each carries out a cyclooxygenase reaction in which two molecules of oxygen are added to arachidonic acid to form a bicyclic endoperoxide with a further hydroperoxy group at position 15, i.e. to form prostaglandin G2 (PGG2). These reactions occur at a hydrophobic channel in the center of the enzyme. The hydroperoxide is then reduced by a peroxidase reaction to form prostaglandin H2 (PGH2), a reaction that occurs at a heme-containing site on the surface of the same enzyme. PGH2 is a highly reactive intermediate that is the starting point for the synthesis of tissue specific prostanoids. Both iso-enzymes and the prostaglandin synthesis are inhibited by non-steroidal anti-inflammatory drugs (NSAIDS), such as aspirin (acetylsalicylic acid) and ibuprofen. Steroidal anti-inflammatory drugs at the level of transcription inhibit synthesis of COX-2. In addition, as the active site of COX-2 is bigger than that of COX-1, a number of drugs that specifically inhibit the action of COX-2 have been developed. These drugs are being used clinically to prevent cancer of the colon in addition to their well known use as analgesic and anti-inflammatory agents.

1.5.2. Lipoxygenases

The lipoxygenases are a group of dioxygenases involved in the insertion of one molecule of O2 at different sites of AA. The particular site of incorporation is

tissue and enzyme specific. Lipoxygenases catalyze the oxygenation of PUFAs containing a 1, 4-cis,cis-pentadiene system producing a 1-hydroperoxy -2,4-trans,cis-pentadiene product. Lipoxygenases are found widely in plants, fungi, and animals (Grechkin, 1998; Yamamoto et al., 1997; Funk, 1996; De Petrocellis, and Di Marzo 1994; Gerwick, 1994). The suitable substrates are polyunsaturated fatty acids containing a series of cis double bonds. These substrates are not present in most bacteria (cyanobacteria and some marine species) (Gerwick, and Bernart, 1993; Watanabe et al., 1997), and yeast also lack the necessary desaturases for their synthesis. In accord with the absence of substrate, there are no lipoxygenases in the yeast genome (*Saccharomyces cerevisiae*), and lipoxygenases are also absent from typical prokaryotes. There is no definitive account of a lipoxygenase in insects, although a typical arachidonic acid-derived lipoxygenase product (hydroxyeicosatetraenoic acid (HETE) is identified in the primitive insect *Thermobia domestica* (Ragab, 1991). There is a lipoxygenase in the unicellular *Chlorella* (Zimmerman, 1973) and a partial lipoxygenase cDNA sequence in the data bases from the slime molds, *Dictyostelium discoideum*. Higher plants contain multiple lipoxygenases with at least eight identified in soybean, *Glycine max*. In the mouse there are seven genes that express lipoxygenase proteins, and five homologues (and an expressed pseudo gene) are characterized in humans (Krieg et al., 1998; Boeglin et al., 1998; Sun et al., 1998). Lipoxygenases are expressed in some plant and animal tissues in high levels; they constitute a few percent of the protein in soybeans, and a 15-lipoxygenase (15-LOX) represents one of the main proteins besides hemoglobin in rabbit reticulocytes during anemia (Rapoport et al., 1979). Lipoxygenase expression may also be more subtle and low level, as in the cell-specific expression of specific isozymes in soybean leaves (Stephenson et al., 1998) or the discrete expression of distinct lipoxygenases in mammalian skin (Jisaka et al., 1997).

Nomenclature-In practical usage this is based on the specificity of the enzyme acting on its substrate, and although this can become slightly awkward, it conveys a simple and useful message. 12-LOX oxygenates arachidonic acid at carbon-12, and when

necessary, the stereo configuration is specified [12-(R)-LOX or 12-(S)-LOX]. The differing chain lengths of the most common substrates of plants (linoleate, linolenate, 18-carbon) and animals (arachidonate, 20-carbon) result in a plant 13-LOX corresponding to a mammalian 15- LOX; these particular lipoxygenases "count" the substrate carbons from the tail end of the chain, and both react oxygen at the ω-6 position. Complications can arise, for example, when there is more than one 12-LOX in the same species. To get around this problem, currently the mammalian 12-LOXs are named after the prototypical tissues of their occurrence (hence, the platelet, leukocyte, or epidermal type of 12-LOX) (Yamamoto et al., 1997). These are distinct enzymes by sequence, catalytic activities, and function. Some lipoxygenases may form a mixture of products, e.g. the mammalian reticulocyte type of lipoxygenase catalyzes C-12 and C-15 oxygenation, with the relative proportions varying among species. In rabbits and humans the major product is 15-HPETE, and hence the enzyme is designated a 15-LOX. The most closely related enzyme in the rat, mouse, pig, and cow is the leukocyte type of 12-LOX, an enzyme that catalyzes mainly C-12 oxygenation with some reaction also at C-15 (Yamamoto et al., 1997).

Lipoxygenases are a family of enzymes that can be characterized as non-heme iron proteins, which catalyze the abstraction of hydrogen atoms from a bis-allelic position of fatty acids while adding oxygen to generate hydroperoxide products. Animal lipoxygenases that utilize arachidonic acid as substrate are of great biological and medical relevance, because of the functions of the products in signaling or in inducing structural or metabolic changes in the cell. For example, they react with arachidonic acid per se to produce specific hydroperoxides and thence by downstream processing the plethora of eicosanoids, each with distinctive functions. However, they can also react directly with phospholipids in membranes to produce hydroperoxides that perturb the membrane structure. Hence, programmed structural changes in the cell can be induced, as in the maturation of red cells. In addition, the phospholipid hydroperoxides can stimulate the formation of secondary

products. Lipoxygenases can attack low-density lipoproteins directly with major implications for the onset of atherosclerosis.

1.5.2.1. The mechanism of the lipoxygenase reaction

In principle, the LOX reaction consists of three consecutive steps and all of them are stereochemically controlled.

1) Hydrogen abstraction:

The first step is hydrogen abstraction from a bis-allylic methylene to form a pentadienyl radical; this step is rate limiting for the overall LOX reaction (Rickert and Klinman, 1999). The LOXs select one of the bis-allelic methylene from polyunsaturated fatty acids for hydrogen removal (regioselectivity) and also select either the pro-R or the pro-S hydrogen (enantioselectivity).

2) Radical rearrangement:

In the second step, the pentadienyl radical is rearranged by electron redistribution either in the direction of the methyl terminus ([2]-rearrangement) or in the direction of the carboxyl terminus ([-2]-rearrangement) of the fatty acid. The positional specificity of the overall reaction depends on both the regiospecificity of hydrogen removal and the direction of radical rearrangement (Kuhn et al., 1986).

3) Oxygen insertion:

Molecular oxygen is introduced at either C-1 or C-4 of the rearranged pentadienyl radical system. This creates a new chiral center whose configuration (S- or R-) depends on the specificity of the LOX. For all LOXs investigated so far, hydrogen abstraction and oxygen insertion proceed in an antarafacial manner, meaning that hydrogen abstraction and oxygen insertion occur on opposite faces of the plane of the double bonds (Maas and Brash, 1983; Egmond et al., 1973).

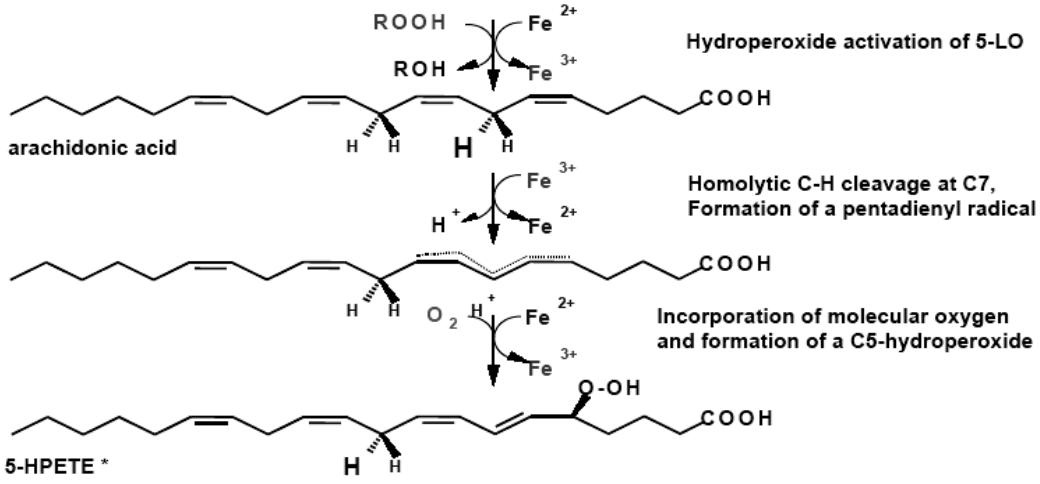

Fig. 2: Mechanism of lipoxygenase reaction

Four main enzyme types with positional specificities occur in animal tissues, i.e. 5-LOX, 8-LOX, 12-LOX, and 15-LOX. The positions at which these enzymes interact and the natures of the main products are illustrated in the figure below (Fig. 3). For example, in the action of 5-LOX, the first step is the abstraction of a hydrogen atom from carbon 7 by ferric hydroxide, involving a proton-coupled electron transfer in which the electron is transferred directly to the iron (III) to produce a substrate radical. The structure of this radical is uncertain, as are the details of the next steps in which an oxygen atom is added, and the cis-double bond in position 5 migrates to position 6 with a change to the trans-configuration leaving the hydroperoxyl moiety in position 5. The resulting product is 5S-hydroperoxy-6t, 8C, 11C, 14C-eicosatetraenoic acid (5-HPETE). 8-, 12- and 15-LOX operate in the same way to give analogous products.

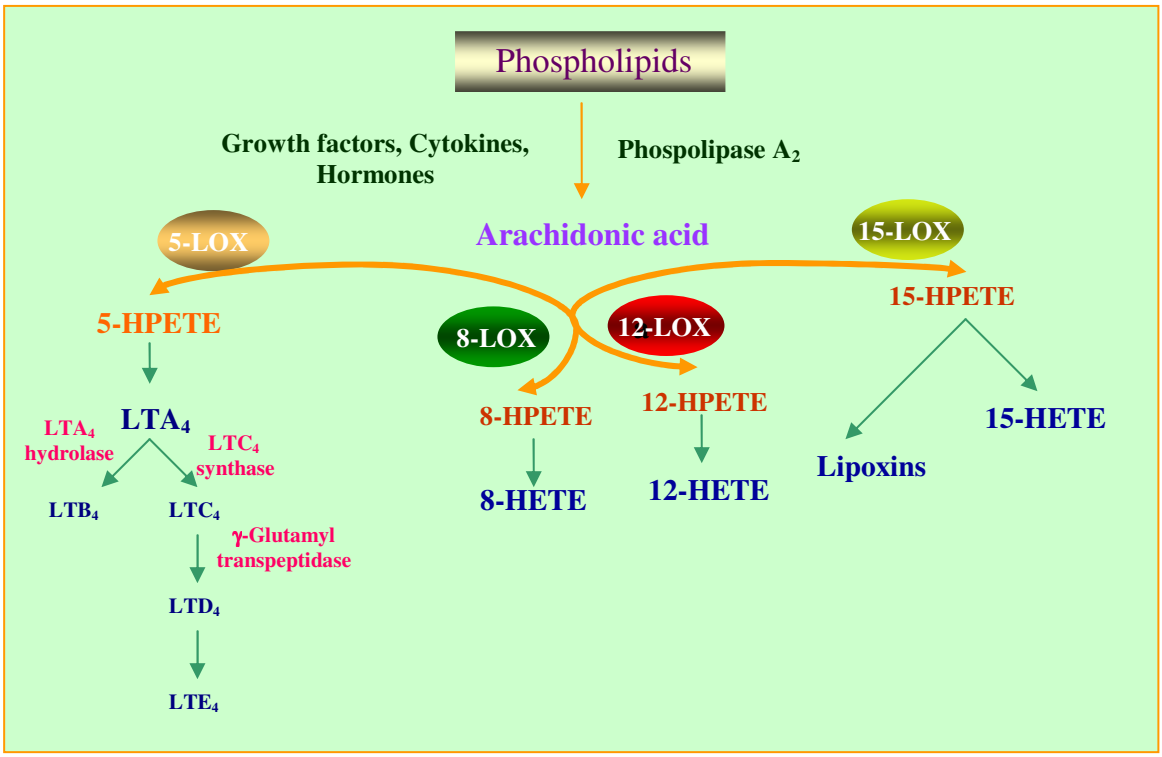

Fig. 3: Lipoxygenase pathways

15-LOX has a broader specificity and is able to oxidize linoleate to 13-hydroperoxyoctadecadienoate (and in part to the 9-isomer). It is also able to utilize arachidonate bound to phospholipids as a substrate, hence the interest in the role of the enzyme in membrane disruption and in disease states. Although the products generally have a hydroperoxide moiety in the S-configuration, some 12-lipoxygenases can produce the R-form. These are especially common in aquatic invertebrates, but are also present in mammalian skin. There is particular interest in 5-LOX as the product is the primary precursor for the leukotrienes, but all the other lipoxygenases interact with arachidonic acid to form products that give rise to eicosanoids with distinct biological properties. Also, these enzymes interact with the other essential polyunsaturated fatty acids of the ω-3 and ω-6 families to give comparable series of metabolites.

Within this family of enzymes, it is now believed that regiospecificity is regulated by the orientation and depth of substrate entry into the active site. Stereospecificity is apparently controlled by switching the position of oxygenation on the reacting pentadiene of the substrate at a single active moiety site, which is conserved as an alanine residue in S-lipoxygenases and a glycine residue in R-lipoxygenases.

Lipoxygenase reactions may initiate the synthesis of a signaling molecule or be involved in inducing structural or metabolic changes in the cell. For signaling, synthesis of a single fatty acid hydroperoxide is required. For inducing structural changes, synthesis of a particular product may not be so important as the ability to induce enzyme-catalyzed lipid peroxidation. Reflecting these different functions are lipoxygenases with different characteristics in catalysis.

1.5.3. 15-Lipoxygenase and cancer

The role of various LOXs in regulating carcinogenesis was very well documented. 5-LOX, 8-LOX and 12-LOX were shown to have a procarcinogenic role, whereas 15-LOX was shown to be anti-carcinogenic (Shureiqi, et al., 2001). 15-LOX exists in two isoforms, 15-LOX-1 and 15-LOX-2. While linoleic acid is the preferred substrate for 15-LOX-1, 15-LOX-2 oxygenates mainly arachidonic acid. Among these two isoforms, the role of 15-LOX-2 as anti-carcinogenic agent is established (Shureiqi, et al., 2001), the role of 15-LOX-1 in controlling carcinogenesis is still unclear.

12/15-LOXs have been implicated in carcinogenesis, but its overall function is not yet clear. 15-LOX-1 is expressed at low level in the colorectal carcinoma cell line, Caco-2 but is strongly induced when the cells were treated with sodium butyrate, an inhibitor of histone deacetylation (Kamitani et al., 2001; Kamitani et al., 1998).

The role of 15-LOX in cancer has been explored to some degree in the past decade. In human lung cancer, 15-LOX was found to be expressed at low percentage compared with normal tumours and most interestingly, was inversely correlated with tumour grade and cell proliferation (Gonzalez et al., 2004).

In prostate cancer, expression of 15-LOX-1 has been thought to be linked to cancer progression by upregulating IGFIR expression in prostate cancer cells (Kelavkar and Cohen, 2004). Human prostate tumours have elevated levels of 15-LOX-1 and data suggest that 15-LOX-1 may play a role in the development of prostate cancer. In prostate tumour models both 15-LOX-2 and its variant 15-LOX-2sv-b can suppress the growth of prostate tumour. The study also suggested that the inhibitory effect is independent of the arachidonic acid metabolites. 13-(S)-HODE (a 15-LOX-1 product) and 15-(S)-HETE (a 15-LOX-2 product) up-regulate or down-regulate, respectively, both the MAPK and Akt pathways after activation with IGF-1. This probably indicates that in tumours such as prostate cancer, the two isoforms of 15-LOX may have opposing effects (Kelavkar et al., 2001; Tang et al., 2002; Hsi et al., 2002).

In oesophageal cancer, 15-LOX-2 is expressed at low levels (Xu et al., 2003). Transfection of oesophageal cancer cells with the 15-LOX-2 results in growth inhibition. In colon tissue, 15-LOX-1 has been shown to be strongly stained in the normal epithelial cells. However, the staining was reduced and diffuses in pattern in tumour tissues and cells (Nixon et al., 2004). In addition, over-expression of 15-LOX-1 results in smaller tumours in in-vivo models (Nixon et al., 2004; Hsi et al., 2001). The 15-LOX metabolite, 15-(S)-HETE, was found to inhibit proliferation and induce apoptosis in colon cancer cells (Chen et al., 2003).

In breast cancer cell line BT-20, 15-LOX has been shown to be linked to the production of 13-HODE, an anti-cancer eicosanoid (Reddy et al., 1997). Furthermore, 15-LOX-1 has been indicated as a novel molecular target of

nonsteroidal anti-inflammatory drugs (NSAIDs) for inducing apoptosis in colorectal carcinogenesis (Shureiqi et al., 2000). This effect is likely to be via the PPAR-γ pathway, as blocking PPAR-γ results in inhibiting the action of 15-(S)-HETE (Shappell et al., 2001).

1.5.4. 15-Lipoxygenase and Leukemia

Leukemia results from genetic defects that lead to enhanced proliferation and survival of bone marrow-derived cells, disrupting homeostasis in the hematopoietic compartment. LOXs have been implicated in the pathogenesis of several disorders including asthma, allergy, inflammation, psoriasis and atherosclerosis. The relationship between LOXs and carcinogenesis is well established and recent studies show that various LOX pathways exist in a dynamic balance that shifts towards 5-, 8- and 12-LOXs and away from 15-LOX during carcinogenesis (Shureiqi et al 1999). The role of 15-LOX metabolites on leukemia was not extensively studied. Available studies indicate that 15-LOX metabolites are antiproliferative and are responsible for inducing apoptosis in various leukemic cells.

The metabolites of 15-LOX-1 (13-(S)-HPODE and 13-(S)-HODE) and 15-LOX-2 (15-(S)-HPETE and 15-(S)-HETE) were shown to inhibit the growth of chronic myeloid leukemia cell line K-562, mediating their effects through intrinsic pathway involving mitochondria (Mahipal et al., 2007).

1.6. Reactive oxygen species and Oxidative stress:

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell & Gutteridge, 1999). Reactive oxygen species (ROS) include all those reactive radical and non-radical oxygen species which are highly reactive entities and can readily participate in a variety of chemical/biochemical reactions. ROS can be formed in the heart, and other tissues, by several mechanisms; they can be produced by xanthine

oxidase (XO), NAD(P)H oxidase, cytochrome P450; by autooxidation of catecholamine; and by uncoupling of NO synthase (NOS) (Sawyer et al., 2002; Seshaiah et al., 2002; Griendling et al., 2000; Xia et al., 1998). NO · contains an unpaired electron, and under certain conditions can react with O2•– to form peroxynitrite (ONOO•–), a powerful oxidant. ROS formation in the heart can be induced by the action of cytokines and growth factors as well. Angiotensin II (ATII), PDGF, and TNF-α, for example, can induce H_2O_2 and O2•– formation via activation of the NAD(P)H oxidases (Sawyer et al., 2002; Seshaiah et al., 2002; Griendling et al., 2000; Xia et al., 1998). This NAD(P)H dependent pathway is best described in vascular smooth muscle cells but has also been documented in other cell types, including cardiomyocytes (Sauer et al., 2004; Heymes et al., 2003; Sabri et al., 2003; Bendall et al., 2002; Wu et al., 2000). A number of additional ligands have been associated with the induction of ROS, including several with particular relevance to the cardiovascular system (Thannickal and Fanburg, 2000).

1.6.1. Antioxidants protection against ROS

There are several cellular mechanisms that counter balance the production of ROS, including enzymatic and nonenzymatic pathways (Nordberg and Arner, 2001). Among the best-characterized enzymatic pathways are catalase and glutathione peroxidase, which coordinate the reduction of inorganic and organic peroxides, and the superoxide dismutases (SODs), which facilitate the formation of H_2O_2 from O2•– (de Haan et al., 2004; Kirkman et al., 1999; Ursini et al., 1995; Kirkman and Gaetani, 1984). Thioredoxin and thioredoxin reductase together form an additional enzymatic antioxidant and redox regulatory system that has been implicated in a wide variety of ROS-related processes (Nordberg and Arner, 2001). Thioredoxin and thioredoxin reductase can catalyze the regeneration of many antioxidant molecules, including ubiquinone (Q10), lipoic acid, and ascorbic acid, and as such constitute an important antioxidant defense against ROS. Deletion of thioredoxin reductase results

in development of heart abnormalities and in cardiac death secondary to a severe dilated cardiomyopathy (Conrad et al., 2004). Nonenzymatic mechanisms include intracellular antioxidants such as the vitamins E, C, and β-carotene (a precursor to vitamin A), ubiquinone, lipoic acid, and urate (Nordberg and Arner, 2001). They also include glutathione, which acts as a reducing substrate for the enzymatic activity of glutathione peroxidase

1.6.2. The biological significance of ROS

ROS have an important role in several important biological processes, including the oxidative burst reaction essential to phagocytes (Hensley et al., 2000). They are involved in a variety of cellular signaling pathways (Nishida et al., 2000), acting in some instances as second messengers downstream of specific ligands, including TGF-β1, PDGF, ATII, FGF-2, endothelin, and others (Griendling and FitzGerald, 2003; Machida et al., 2003; Sawyer et al., 2002; Thannickal and Fanburg, 2000). ROS are also involved in modulating the activity of specific transcription factors, including NF-κB and activator protein–1 (AP-1) (Sabri et al., 2003; Hirotani et al., 2002; Turpaev, 2002; Wu et al., 2002; Hsu et al., 2000). NF-κB, for example, becomes more transcriptionally active in response to the contribution of ROS to the degradation of I-κB, the inhibitory partner of NF-κB that sequesters it in the cytosol. Thus ROS can play an important role in modulating inflammation.

Perhaps the most widely recognized biological effects of ROS, however, are those that occur when cellular antioxidant defenses are overwhelmed and ROS react directly with cellular lipids, proteins, and DNA, causing cell damage and death (Suematsu et al., 2003; Hensley et al., 2000; Hemnani and Parihar, 1998; Davies, 1995). Lipid peroxidation, for example, is a well-characterized effect of ROS that results in damage to the cell membrane as well as to the membranes of cellular organelles (Rathore et al., 1998; Thollon et al., 1995). ROS can contribute to mutagenesis of DNA by inducing strand breaks, purine oxidation, and protein-DNA

cross-linking, and other ROS mediated alterations in chromatin structure may significantly affect gene expression (Rahman, 2003; Konar, 2003). Modification of proteins by ROS can cause inactivation of critical enzymes and can induce denaturation that renders proteins non functional (Lockwood, 2000; Stadtman and Levine, 2003). General aging and age-related alteration in the cardiovascular system have been attributed to the long-term cumulative effects of ROS, although the relative contribution of ROS to the aging process remains the subject of debate (Lakatta, 2003; Sinclair, 2002).

1.7. Cellular Death Pathways:

1.7.1. Necrosis, Apoptosis and Paraptosis

Programmed cell death is a major component of both normal development and disease (Bajl et al., 2001; Cecconi and Gruss, 2001; Clarke et al., 2001; Debatin, 2001; Dumont et al., 2001; Joaquin and Gollapudi, 2001; Mayr and Xu, 2001; Monk et al., 2001; Pru and Tilly, 2001 Domen, 2000; Gerber and Ferrara, 2000;). The roles of cell death during either embryogenesis or pathogenesis, the signals that induce or regulate this event, and the mechanisms of cell demise are common subjects that drive research in this field (Bortner and Cialowski, 2002; Buendia et al., 2001; Bursch, 2001; Henson et al., 2001; Leist and Jaattela, 2001; Zoring et al., 2001). The classical ultrastructural studies of Kerr and coworkers (Kerr et al., 1972) provided evidence that cells may undergo atleast two distinct types of cell death-necrosis or apoptosis. Necrosis results from physical injury, metabolic block, mutations, toxic substances etc and is not genetically controlled. It is characterized by cell swelling, mitochondrial dilation, and dissolution of other organelles, non-caspase proteolytic cascades depending on serine proteases calpains or cathepsins plasma membrane rupture and spillage of the cytoplasmic contents ultimately leading to inflammatory response.

In contrast, apoptosis is a genetically controlled process exhibiting a constellation of structural and functional changes including calcium flux, cytochrome *c* redistribution, caspase activation, loss of plasma membrane asymmetry, reduction in cell volume and selective proteolysis of a subset of cellular proteins. Chromatin condensation and nucleosomal DNA fragmentation ultimately lead into apoptotic bodies that are rapidly phagocytosed. Inhibition of the classical caspase dependent apoptotic pathway may lead to necrotic cell death suggesting that the same death stimulus can result in either apoptotic or necrotic cell death, depending on the availability of activated caspases. Paraptosis, a caspase independent programmed cell death that often exists in mammalian as well as non mammalian model systems in parallel with apoptosis, is distinct from later by the criteria of morphology, biochemistry as well as response to apoptotic inhibitors. Paraptosis is dependent on RNA and protein synthesis and its molecular mechanism is yet to be understood. Oncosis and magentosis are the other forms of non apoptotic Programmed cell death whose mechanisms are not clear.

1.7.2. Fas-mediated apoptosis

Higher organisms have developed several mechanisms of apoptosis to ensure the rapid and selective elimination of unwanted cells, one of which involves the interaction of cell surface Fas with its cognate ligand, FasL. The cell surface Fas (CD95/APO-1) is a 45 kD type I membrane protein, and Fas ligand (FasL) is a 37 to 40 kD type II membrane proteins that belong to tumor necrosis factor (TNF) receptor and ligand families (Suda et al., 1993; Itoh et al., 1991). Fas receptor contains three cysteine–rich extra cellular domains at the amino terminus, which are responsible for ligand binding, and an intercytoplasmic death domain (DD) of about 80 amino acids that is essential for transducing the apoptotic signal (Peter et al., 2003). Binding of FasL to Fas causes a higher-order aggregation of the receptor molecules and recruitment of the adoptor molecule (Holler et al., 2003).

Fas-associated death domain (FADD) via death domain (DD-DD) interactions. FADD also has another domain called the death, which in turn recruits

pro-caspase-8 (FLICE) and /or pro-caspase-10 to the receptor. The resulting multimeric protein complex is called the death-inducing signaling complex (DISC) and forms within seconds of receptor engagement (Peter et al., 2003).

At the DISC, pro-caspase-8 (and/or -10) is activated. Caspases are cysteine proteases that cleave their substrates at aspartic acid residues (Earnshaw et al., 1999). They are synthesized as relatively inactive zymogens called pro-caspases. Initiator caspases (e.g. caspases-8 and -9) are the first to be activated in response to a pro-apoptotic stimulus and initiate a cascade of increasing caspase activity by cleaving and activating effector caspases (e.g. caspases-3, -6, and -7). The effector caspases, in turn, selectively cleave a restricted set of target proteins and are responsible for the demise of the cell.

Effective formation of the DISC is required for Fas mediated apoptosis. Cells in which caspase-8 is activated at the DISC, leading to the rapid activation of caspase-3 and cell death, are known as type I cells. In some cells, however, DISC formation following Fas stimulation is strongly reduced. In these cells, known as type II cells, mitochondria play an essential role as signal amplifiers (Barnhart et al., 2003). This mitochondrial or intrinsic, apoptosis pathway is activated by caspase-8–mediated cleavage of the Bcl-2 family member Bid. Truncated Bid translocates to the mitochondria, where it can induce both the oliogomerization of pro-apoptotic Bax and /or Bak in the membrane and the release of pro-apoptotic molecules, including cytochrome c, from the mitochondrial intermembrane space. Cytochrome c can then associate with the scaffolding protein Apaf-1, dATP and activate pro-caspase-9 to caspase-9 which then activates caspase-3 resulting in cell death.

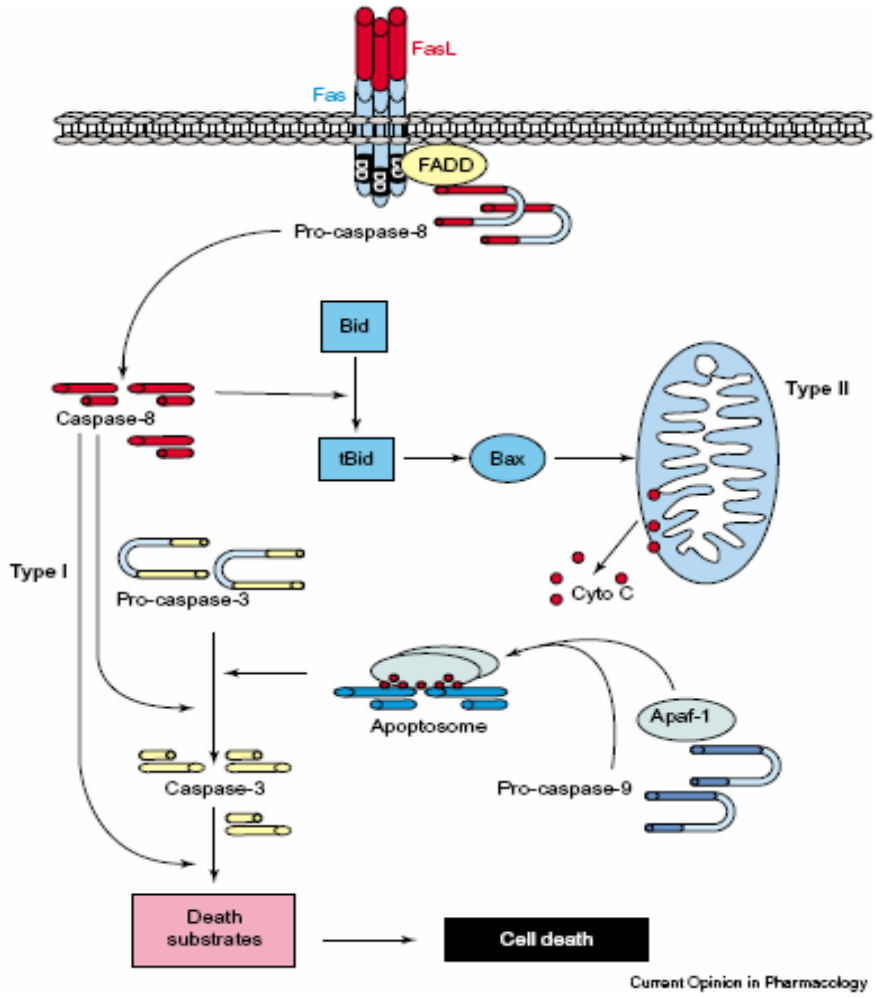

Fig. 4. Apoptotic pathways

Scope & Objectives

2.0. Scope and Objectives

Leukemias are neoplastic proliferations of immature cells of the hematopoietic system, which are characterized by arrested or abnormal differentiation. Leukemic cells rapidly divide and accumulate in the bone marrow. It is the replacement of the normal hematopoietic cells by the leukemic cells that results in the signs and symptoms of the disease. The consequences of such bone marrow failure may have effects on all three major cell lineages and cause anemia, hemorrhage and infections.

T-cell lymphoblastic leukemia (T-ALL) is an aggressive malignancy of thymocytes characterized by high numbers of bone marrow and circulating blast cells, enlargement of mediastinal lymph nodes, and often central nervous system. T-ALL accounts for approximately 15% of pediatric and 25% of adult ALL cases. Similar to other types of leukemia, T-ALL is caused by genetic alterations in hematopoietic precursor cells leading to a variety of changes, including loss of cell cycle control, unlimited self-renewal capacity, impaired differentiation, hyperproliferation and loss of sensitivity to death signals.

Polyunsaturated fatty acids of the ω-3 family have over all suppressive effects, inhibiting lymphocyte proliferation, antibody and cytokine production, adhesion molecule expression, natural killer cell activity and triggering cell death. The ω-6 fatty acids have both inhibitory and stimulatory effects. The cytotoxic effect of PUFAs on lymphocytes or leukemia cell lines has long been known. However, it is not clear whether these effects are mediated by PUFAs themselves or their oxygenated metabolites. Higher doses of fatty acids preferentially cause necrosis, with a rapid loss of membrane integrity, lysosomal enzyme leakage and cell swelling. Both apoptosis and necrosis seem to be associated with oxidative stress, as they can be partially prevented by antioxidants such as tocopherol. Recent studies have indicated that PUFAs mediate their effects through their oxygenated metabolites, collectively termed as “eicosanoids”.

The term "eicosanoids" is used to embrace biologically active lipid mediators (C_{20} fatty acids and their metabolites), including prostaglandins, thromboxanes, leukotrienes and other oxygenated derivatives, which are produced primarily by three classes of enzymes- cyclooxygenases (COX-1 and COX-2), lipoxygenases (LOX) and cytochrome P450 dependent epoxygenases. Of the precursor fatty acids, arachidonic acid (AA) has been by far the most studied, and it is special in many ways. It is a derived essential fatty acid of ω-6 series in that it is synthesized in animals from linoleic acid (LA), which is obtained from the diet. α-linolenic acid (ALA), the essential fatty acid of ω-3 series, is also important as it is the precursor for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

The lipoxygenases are a group of dioxygenases involved in the insertion of one molecule of O_2 at different sites of AA. The particular site of incorporation is tissue and enzyme specific. Lipoxygenases catalyze the oxygenation of PUFAs containing a 1, 4-cis, cis-pentadiene system producing a 1-hydroperoxy-2, 4-trans, cis-pentadiene product. The role of various LOXs in regulating carcinogenesis was very well documented. 5-LOX, 8-LOX and 12-LOX were shown to have a procarcinogenic role; whereas 15-LOX was shown to be anti-carcinogenic. 15-LOX exists in two isoforms, 15-LOX-1 with preference to linoleic acid as the substrate and 15-LOX-2 with arachidonic acid as the preferred substrate. Among these two isoforms, the role of 15-LOX-2 as anti-carcinogenic agent is established and the role of 15-LOX-1 in controlling carcinogenesis is still unclear.

The specific objectives of the present study are:

- Biosynthesis, purification and characterization of 15-LOX metabolites.
- To check the antiproliferative effects of PUFAs and 15-LOX metabolites on acute lymphoblastic T-cell leukemia cell line (Jurkat).
- To understand the mechanisms underlying the anti-proliferative effects of 15-LOX metabolites [15-(S)-HPETE and 15-(S)-HETE] on acute lymphoblastic T-cell leukemia cell line (Jurkat).

Materials & Methods

3.0 Materials and Methods

3.1. Materials

Cell line used in this study, Jurkat (acute lymphoblastic T cell leukemia), was obtained from National Center for Cell Science (NCCS), Pune, India. Arachidonic acid (AA) and linoleic acid (LA), α-linolenic acid (ALA) were procured from Cayman Chemical Company (Ann Arbor, Michigan, U.S.A). Phosphate buffered saline (PBS), RPMI medium and fetal bovine serum (FBS) were purchased from GIBCO Ltd. (BRL Life Technologies, Inc., Grand Island, NY). MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], DAPI (4', 6- diamidino-2-phenylindole), proteinase K, RNase A, propidium iodide (PI), N-acetyl cysteine (NAC), reduced glutathione (GSH), catalase, soybean lipoxygenase and superoxide dismutase were from Sigma Chemical Co (St.Louis, MO). Nitrocellulose membrane was from Millipore (Bangalore, India). Mouse monoclonal antibody against cytochrome *c* was from Chemicon (California, USA). Bcl_2, Bax, Fas receptor antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Fas Ligand, Caspase-8, and Bid antibodies were from BD Biosciences, USA. DCFH-DA was purchased from Molecular Probes, USA. Diphenylene iodonium (DPI), verapamil and L-NMMA were procured from Calbiochem (San Diego, USA). All other chemicals, which have not been mentioned here, were of fine grade and high quality.

3.2. Biosynthesis, purification and characterization of 15-LOX-2 metabolites by employing recombinant green gram 15-LOX

In order to generate 15-LOX metabolites, 15-LOX gene from green gram is cloned as per the details given below:

3.2.1 Isolation of total RNA

Green gram seeds were soaked overnight in water and total RNA was prepared from the embryo using RNA preparation kit (Qiagen) following manufacturer's instructions. In brief, 100 mg of embryo was grinded in liquid nitrogen using mortar and pestle and suspended in 1ml of buffer RLT (supplied), which contains guanidine isothiocyanate and β-mercaptoethanol. After a brief vortexing, it was incubated at room temperature for 3-5 minutes. This tissue lysate was then transferred to QiaShredder column and centrifuged at 12000 rpm at room temperature for 2 min. QiaShredder removes cell debris and simultaneously homogenizes the lysate. The flow through was transferred to an RNase free microcentrifuge tube and 0.5 ml volume of absolute ethanol was added and mixed by pipetting. This sample containing precipitate, if any, was transferred to spin column and centrifuged at 12000 rpm at room temperature for 1 min. The flow through was discarded, and the column was washed twice with 700 µl of wash buffer containing ethanol by centrifuging at 12000 rpm at room temperature for 1 min. The column was then air dried to remove traces of alcohol, placed in new RNase free microcentrifuge tube and eluted with 50 µl of elution buffer. The quantity of RNA was estimated by UV absorption and integrity of RNA was checked by 1% (w/v) denaturing formaldehyde-agarose gel.

3.2.2. First strand cDNA synthesis

Using 1 µg of total RNA prepared from green gram embryo, reverse transcription was carried out with MMLV-reverse transcriptase (Superscript-III, Invitrogen) as per manufacturer's instructions. Reaction mixture consisted 1 µl of 500 µM oligo d(T), 2-5 µg of total RNA and 1µl of 10 mM dNTP mix and the volume was made up to 13 µl with nuclease free water in a 0.2 ml tube. The reaction mixture was incubated at 65 °C for 5 min in a PCR (PTC 100, MJ research) machine

and cooled on ice for 1 min. Contents were collected by brief centrifugation and to this 5 µl of first strand buffer, 1 µl of RNase inhibitor (40U), 1 µl of reverse transcriptase (200 U) were added and incubated at 50 °C for 1 hour. Finally inactivation was done by heating at 70 °C for 15 min and cDNA was stored in –20 °C until use.

3.2.3. RT-PCR amplification of cDNA homologous to 15-LOX gene

A set of degenerate primers were designed by aligning the existing sequences of legume 15-LOX to clone a partial cDNA fragment from green gram embryo. Using these degenerate primers, a cDNA was amplified by RT-PCR, cloned in pTZ57R vector and subsequently sequence was determined. The identity of amplified partial cDNA was checked by BLAST search.

3.2.4. 5′ and 3′ RACE

RNA ligase -mediated rapid amplification of cDNA ends (RLM-RACE) was done using Gene Racer kit (Invitrogen) to isolate 5′ and 3′ ends of cDNAs following manufacturer's protocol. Total RNA was prepared as described above and the integrity of RNA was checked on a 1% denaturing agarose gel.

- To dephosphorylate non-mRNA or truncated mRNA, 5 µg of total RNA was subjected to dephoshphorylation using calf intestinal phosphatase.
- Then RNA was purified by phenol chloroform extraction method.
- A decapping reaction was performed to remove the 5′ cap structure of mRNA using tobacco acid pyrophosphatase and the RNA was purified by phenol chloroform extraction.
- Decapped mRNA was ligated to the Gene Racer RNA oligo to the 5′ end using T4 RNA ligase and the RNA was purified by phenol chloroform extraction method.

- Then the mRNA was reverse transcribed with oligo (dT) primer using Superscript III reverse transcriptase.

To perform 3′ RACE, total RNA was reverse transcribed with Generacer oligo (dT) using Superscript III.

Using gene specific primers designed from partial cDNA of 15-LOX obtained by RT-PCR and adopter primers (listed in table, page 37), touchdown PCR was performed with following thermal cycling conditions mentioned below. The PCR amplified products were separated on 1.5% agarose gel and visualized by ethidium bromide. Subsequently, the RACE products were cloned into pTZ57R vectors and sequence was determined bi-directionally. The identity of 5′ and 3′ RACE products was determined by BLAST search.

Temperature	Time	Cycles
94°C	3 min	1
94°C	30 sec	5
72°C	1min/1kb DNA	
94°C	30 sec	5
70°C	30 sec	
72°C	1min/1kb DNA	
94°C	30sec	25
68°C	30sec	
72°C	1min/1kb DNA	
72°C	10 minutes	

3.2.5. Cloning of full-length cDNA of 15-LOX gene

cDNA fragments obtained by RT-PCR, 5′ and 3′ RACE were aligned and full-length cDNA was obtained. Using this full-length cDNA sequence, primers were designed to amplify ORF (opean reading frame). An RT-PCR reaction was carried out with ORF primers using *Pfu* (Biorad) and cloned into pTZ57R vector. The sequence was verified by both restriction analysis and sequencing.

3.2.6. *In silico* analysis

The cloned 15-LOX cDNA sequence was compared with other lipoxygenases by Clustal W multiple alignment (http/www.ebi.ac.uk). Phylogenetic analysis was done using Laser Gene software.

3.2.7. Expression of green gram 15-LOX gene in *E. coli*

15-LOX cDNA was amplified by PCR with *Pfu* and cloned in to expression vector pETBlue-2 and subsequently introduced into *E. coli* BL21 strain. The expression constructs were verified by restriction analysis and checked for cloning artifacts, if any by nucleotide sequencing. Expression of recombinant 15-LOX was carried out by inducing the cultures with 1mM IPTG at an O.D. of 0.4. The induction of recombinant protein was verified by 12% SDS-PAGE. One litre culture was harvested, suspended in 50 mM Tris-Cl buffer pH 8.0 and homogenized by sonication. Clear lysate was obtained by centrifuging at 15,000 rpm for 30 min.

Table. 1 List of primers used.

S.No.	Primer name	Sequence 5′ to 3′	Purpose
1.	F1	TGGTSYTGATGCSCAAGAATG	To amplify a fragment
2.	R1	ACTTRAGGRHBTGTTSRCCATC	To amplify a fragment
3.	Fs	GATGTGCGTGGACTCTATGATGG	To amplify a fragment
4.	R2	TAYAYCTCATCAGAWGCATG	To amplify a fragment
5.	5P	GCCAGCTCCCAAGGTTGGTAAGGA	5′ RACE
6.	5N	TGGTAAGGAAGTAACAATACCTTC	5′ RACE
7.	3P	GGTGGCTACATCCTTAACCGACCAACT	3′ RACE
8.	3N	GGTGGCTACATCCTTAACCGACCAACT	3′ RACE
9.	PET FW	GATGGTTTCAGGTGTGACAG	To amplify full length gene
10.	PET RF	ATGGAGATACTGTTAGGAATAC	To amplify full length gene

3.2.8. Purification and characterization of green gram LOX products on HPLC

The cell lysate obtained above was used as the 15-LOX enzyme source for preparation of AA metabolites. In a typical reaction of 100 ml, enzyme (50 µl of lysate) was incubated with 133 µM AA for 2-3 min. The reaction was arrested by acidification with 6 N HCl and the AA products were extracted into equal volume of organic solvent containing hexane: ether (1:1), the contents evaporated to dryness and redissolved in HPLC solvent.

3.3. Biosynthesis, purification and characterization of 15-LOX metabolites by employing Soybean lipoxygenase

The commercially procured soybean LOX was employed for the biosynthesis of hydroperoxides and hydroxides of AA [15-(S)-HPETE & 15-(S)-HETE] and LA [13-(S)-HPODE & 13-(S)-HODE] as per the method described earlier (Sailesh et al., 1994; Reddy et al., 1992)**.** Soybean LOX was incubated with either arachidonic acid or linoleic acid in 0.1 M Tris, pH 9.3 buffer for 3 min and the reaction was terminated by acidifying the reaction mixture to pH 3.0 with 6 N HCl. The products formed were extracted with equal volumes of hexane: ether (1:1) twice and the organic solvent was evaporated under inert conditions. The dried products were then dissolved in straight phase HPLC mobile phase consisting of hexane: propane-2-ol: acetic acid in 1000:15:1 ratio and separated on the straight phase preparatory HPLC system at a flow rate of 6 ml/min. The purity of the compounds was checked on analytical column at a flow rate of 1 ml/min. To obtain corresponding hydroxides, these products were dissolved in methanol and reduced with sodium borohydride ($NaBH_4$) for 2 min and then separated on HPLC as mentioned above.

The compounds were separated on straight phase HPLC (Shimadzu model equipped with SPD 6AV detector and CR4A chromatopac), using CLCSIL (25 x 0.4 cm) column and the solvent system mentioned above. The samples were monitored at 235 nm and peaks were analyzed on a Shimadzu 1601 model UV/VIS scanning spectrophotometer. Peaks showing conjugated diene spectra were collected and identified based on co-chromatography with the standards and GC-MS analysis.

3.4. GC-MS analysis

3.4.1. Preparation of N-Nitroso methyl urea

N-methyl urea (3.7 g, 50 mM), dissolved in 6.25 ml of conc. HCl (75 mM), was diluted with equal volume of water and cooled below 10 °C in ice cold water. A

cold solution of $NaNO_2$ (4.12 g in 6 ml of water) was added slowly drop wise (brown fumes were observed) while stirring with glass rod. After complete addition, the mixture was allowed to cool for an hour in ice cold water, filtered in the buckner flask and washed with 10 ml of cold water. The sample was then dried on the filter paper and left for air drying over night.

3.4.2. Preparation of Diazomethane

- 3 ml of 50% KOH (1.5 g) was taken into a 25 ml conical flask.
- 10 ml of diethyl ether was layered onto aqueous KOH and cooled on ice to 4 °C for 15 min.
- 1 g of N-nitrosomethyl urea was weighed and added to the above solution mixture pinch by pinch while continuously swirling the flask.
- The ether layer, which turned into yellow color after complete addition of N-Nitrosomethyl urea, was poured into a round bottom flask and the remaining aqueous KOH solution was discarded.
- The diazomethane was collected and moisture was removed by adding KOH pellets.
- Aluminum foil was wrapped around falcon tube and was kept at -20 °C.

3.5. Cell culture and treatment

The human T cell leukemia cell line (Jurkat) were grown in suspension in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 mg/ml streptomycin and 2 mM-glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were sub cultured twice each week, seeding at a density of about 2 x 10^5 cells/ml. For treatment, exponentially growing Jurkat cells were collected and resuspended in fresh culture medium with 1% FBS. The HPLC purified 15-LOX metabolites of AA [15-(S)-HPETE, 15-(S)-HETE], LA [13-(S)-HPODE, 13-(S)-HODE] and ALA

[13-(S)-HPOTrE, 13-(S)-HOTrE] dissolved in ethanol were used for the treatments. Cells incubated with ethanol served as the control. The final concentration of the vehicle never exceeded 0.1%. The effects of 15-(S)-HPETE and 15-(S)-HETE were standardized in terms of dosage (10 μM & 40 μM) and time (3 h & 6 h) of exposure and further studies were taken up by following the standardized conditions.

3.5.1. ***Inverted microscope analysis***

Jurkat cells (1 x 10^5) were treated with15-HPETE (10 μM) and 15-HETE (40 μM) for 3 hours and 6 hours respectively. Cells after treatment were observed for morphological changes under inverted microscope.

3.6. Cell viability and cytotoxicity

15-LOX metabolite-induced growth inhibitory effects were assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay as described (Mosmann, 1983). For the MTT assay, 5 x 10^3 exponentially growing cells were plated in 100 μl of the growth medium in the presence or absence of 1–20 μM hydroperoxy metabolite of 15-LOX [15-(S)-HPETE] and 1–80 μM hydroxy metabolite of 15-LOX [15-(S) - HETE] in 96 well plates and cultured at 37 °C in 5% CO_2 for 3–24 h. The cells were then incubated with 20 μl of MTT (5 mg/ml) at 37 °C for 4 h. After dissolving the crystals in a triplex solution containing 12% SDS, 5% isobutanol and 12 mM HCl, the plates were read in a microtiter plate reader at 570 nm. Each concentration was tested in three independent experiments run in four replicates. Standard errors of means were calculated and reported as % growth versus control. The concentration of the compound that inhibited cell growth by 50% (IC_{50}) was determined from cell survival plots.

3.7. DNA fragmentation assay

Jurkat cells were treated with 5 µM and 10 µM of 15-(S)-HPETE for 3 h and with 20 µM and 40 µM 15-(S)-HETE for 6 h and used for the isolation of the DNA. DNA laddering was detected by isolating fragmented DNA using the SDS/ Proteinase K/ RNase A extraction method, which allows the isolation of only fragmented DNA without contaminating genomic DNA (Hermann, 1994). Five million cells were pelleted, washed in cold PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2 % Triton X-100 for 20 min at 4 °C. After centrifugation at 14,000 g for 15 min, the supernatant was treated for 1 h at 37 °C with RNase A (0.5 mg/ml) and then with proteinase K (0.5 mg/ml) for 1 h at 50 °C. DNA was extracted with buffered phenol and precipitated with ethanol and DNA was resolved on 1% agarose gel in TBE (44.6 mM Tris, 44.5 mM boric acid and 1 mM EDTA). DNA fragmentation was visualized upon staining gel with ethidium bromide (0.5 mg/ml) and exposed to UV light.

3.8. Quantification of apoptosis by flow cytometry

To quantitate apoptosis, flow cytometric analysis using propidium iodide was performed. Cells that were less intensely stained than G1 cells (sub-G0/G1 cells) in flow cytometric histograms were considered apoptotic cells. The method for DNA labeling was done as described previously (Madhava Reddy et al., 2003) with minor modifications. After treatment, cells were prepared as single cell suspension in 200 µl PBS, fixed with 2 ml of ice-cold 70% ethanol, and maintained at 4 °C overnight. The cells were harvested by centrifugation at 500xg for 10 min, resuspended in 500 µl PBS supplemented with 0.1 % Triton X-100 and RNase A (50 µg/ml), incubated at 37 °C for 30 min, and stained with 50 µg/ml propidium iodide (PI) in the dark at 4 °C for 30 min. The red fluorescence of individual cells was measured with a FACS

Caliber flow cytometer (Becton Dickinson, San Jose, CA, USA). A minimum of 10,000 events were counted per sample.

3.9. Preparation of whole cell extracts and immunoblot analysis

The cell lysis was carried out based on the method described by Sambrook et al., (1989). To prepare the whole cell extract, cells were washed with PBS and suspended in a lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxy cholate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml leupeptin, 20 µg/ml aprotinin). After 30 min of shaking at 4 °C, the mixtures were centrifuged (10,000xg) for 10 min, and the supernatants were collected as the whole-cell extracts. The protein content was determined according to the Bradford method (Bradford, 1976). An equal amount of total cell lysate was resolved on 8-12 % SDS-PAGE gels along with protein molecular weight standards, and then transferred onto nitrocellulose membranes. Membranes were stained with 0.5% Ponceau *S* in 1% acetic acid to check the transfer. The membranes were blocked with 5% w/v nonfat dry milk and then incubated with the primary antibodies in 10 ml of antibody-diluted buffer (1X Tris-buffered saline and 0.05% Tween-20 with 5% milk) with gentle shaking at 4 °C for 8-12 h and then incubated with peroxidase conjugated secondary antibodies. Signals were detected by using peroxidase substrate TMB/H_2O_2. The blots were probed with β-actin antibodies to confirm equal loading.

3.10. Detection of cytochrome *c* release using Western blot analysis

After exposure to 10 µM 15-(S)-HPETE or 40 µM 15-(S)-HETE for various time periods, cells were collected and washed once with PBS and subsequently with buffer A (0.25 M sucrose, 30 mM Tris-HCl, pH 7.9, 1 mM EDTA). Cells were then resuspended in buffer A containing 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml

pepstatin, 1 mg/ml aprotinin and homogenized with a glass dounce homogeniser. After centrifugation for 10 min at 21,000xg, protein concentration of the supernatant was determined using the Bradford method. 30 µg of cytosolic protein extract was then used for Western blot analysis as described above. Cytochrome *c* was detected using the mouse monoclonal antibody directed against human cytochrome *c*.

3.11. Caspase-8 & Caspase-3 activity assays

After the stipulated treatments, $2x10^6$ cells were lysed in 100 µl of CHAPS lysis buffer by three to four freeze thaw cycles. The extracts were centrifuged at 12,000xg and resulting supernatants were used for the assay. The assay was performed according to the manufacturer's protocol (BD biosciences, USA). The assay buffer contained 20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% (w/ v) CHAPS, 10% sucrose. 50 µg of the protein and 8 µM of the substrate were added to 1 ml of the assay buffer and incubated for 1 h at 37 °C. Measurements were done on spectrofluorimeter with an excitation wavelength of 380 nm and an emission wavelength of 460 nm for caspase-8 and with an excitation wave length of 400 nm and emission wave length of 480-520 nm for caspase-3.

3.12. Measurement of reactive oxygen species (ROS)

ROS production upon treatment with 15-LOX metabolites was measured using the dye 2', 7'-Dichlorodihydrofluorescein diacetate (DCFHDA). DCFH-DA, a nonfluorescent cell-permeant compound, is cleaved by endogenous esterases once inside the cell and the de-esterified product becomes the fluorescent compound 2'7'-dichlorofluorescein upon oxidation by ROS (Bass et al., 1983; Cathcard et al., 1983). Cells were treated with either 10 µM 15-(S)-HPETE or 40 µM 15-(S)-HETE for various time periods. After specific time points of exposure, cells were incubated with 10 µM DPI at 37 °C for 15 min and then washed twice in PBS supplemented with 10 mM glucose. Data were collected using the data acquisition program CELL

Quest (Becton Dickinson, San Jose, CA). DCF data was collected with the following excitation and emission wavelengths: λ_{exc} = 488 nm, λ_{em} = 525 nm. 10,000 cells were analyzed per sample.

Statistics

The data represent mean $\pm$ S.E. from three independent experiments

Results

Chapter 1

4.0. Results

LOXs are widely distributed in animals and plants and the LOX products have a wide range of biological functions as diverse signal molecules, oxidants and modifiers of membrane structures.

LOXs are versatile catalysts because they are multifunctional enzymes, catalyzing atleast three different types of reactions: (1) dioxygenation of lipid substrates (dioxygenase reaction), (2) secondary conversion of hydroperoxy lipids (hydroperoxidase reaction) (Kuhn et al., 1991), and (3) formation of epoxy leukotrienes (leukotriene synthase reaction) (Shimadzu et al., 1984). However, mainly plant LOXs are used for biotechnological applications since they are more stable and exhibit higher catalytic rates than the mammalian LOXs.

In the present study also, 15-LOX gene was isolated from green gram genome, cloned into pET Blue-2 expression vector and the expressed protein was employed as the 15-LOX enzyme source for the biosynthesis of 15-LOX metabolites.

Green gram belongs to family fabaceae. It is an erect, bushy herb widely cultivated in warm regions of India, Indonesia and United States for forage and especially its edible seeds. Green gram is a tropical (or sub-tropical) crop, and requires warm temperatures (optimal at 30-35 °C). Loamy soil is best for green gram cultivation.

Kingdom : Plantae
Division : Mangoliphyta
Class : Mangoliopsida
Order : Fabales
Family : Fabaceae
Genus : *Vigna*
Species : *V. radiata*

Biosynthesis of 15-LOX metabolites

4.1. Cloning, expression of 15-LOX gene from green gram and biosynthesis of AA metabolites

4.1.1 Amplifications of cDNA fragments

Initially RNA was prepared from green gram embryo. cDNA was synthesized by employing RNA by RT-PCR. A 1.0 kb cDNA fragment was amplified by using Forward: TGGTSYTGATGCSCAAGAATG and Reverse: ACTTRAGGRHBTGTTSRCCATC primers (Fig. 5 a).

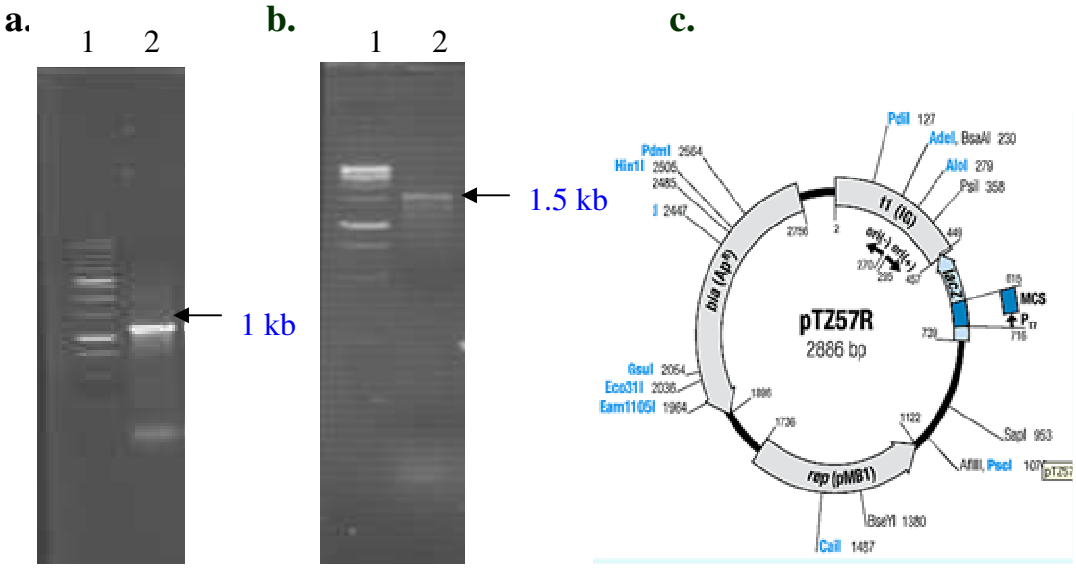

Fig. 5: Amplification of cDNA fragments of 15-LOX gene in green gram
a. Amplification of 1 kb fragment. lane 1. Molecular weight markers, lane 2. 1 kb fragment
b. Amplification of 1.5 kb fragment. lane 1. Molecular weight markers, lane 2. 1.5 kb fragment. **c.** Vector map of pTZ57R cloning vector.

PCR amplified product was cloned in pTZ57R vector and subsequently sequence was determined. The identity of this partial cDNA clone, when analyzed by BLAST search, showed sequence similarity with other legume 15-LOX sequences. After sequence confirmation, using it as a template 1.5 kb fragment was

amplified by using forward: GATGTGCGTGGACTCTATGATGG and Reverse: TAYAYCTCATCAGAWGCATG primers (Fig. 5 b). Upon initial confirmation of partial cDNA, the full-length cDNA for 15-LOX-2 was then isolated by 5´ and 3´ RACE procedures (Fig. 6).

4.1.2. 5′ and 3′ RACE

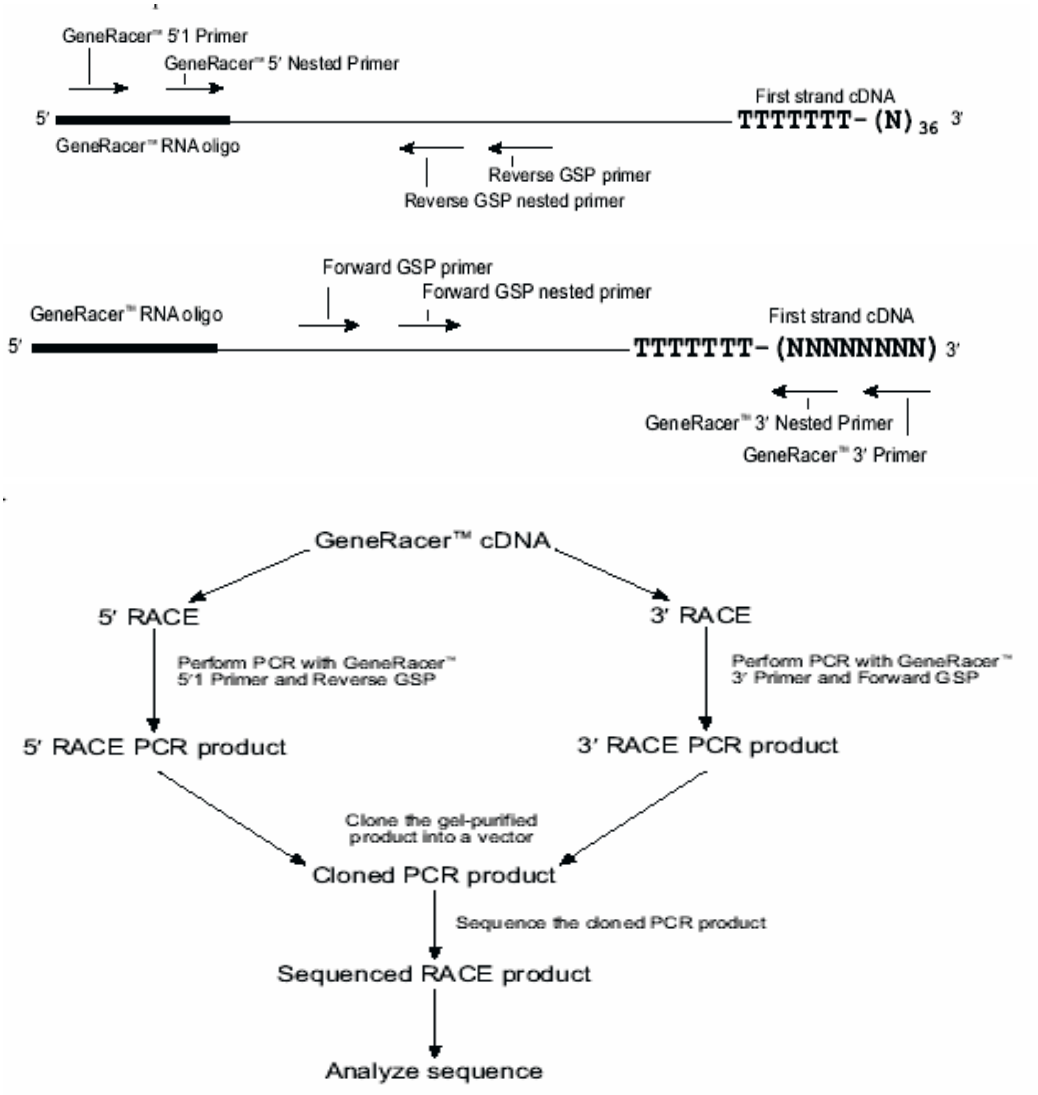

Fig. 6: Strategy employed for doing 5′ RACE and 3′ RACE

The full-length cDNA encoding 15-LOX was obtained by aligning the sequences from partial, 5′ and 3′ cDNA clones. The primers used for full length cDNA are PET FW: GATGGTTTCAGGTGTGACAG and PET RF ATGGAGATACTGTTAGGAATAC. The green gram 15-LOX cDNA was 2.601 kb with an open reading frame of 2.601, kb encoding a protein of 867 amino acids. The 5′ untranslated region (UTR) was of 41 nt where as the 3′ UTR was 166 nt long with two potential polyadenylation signals (Fig. 8).

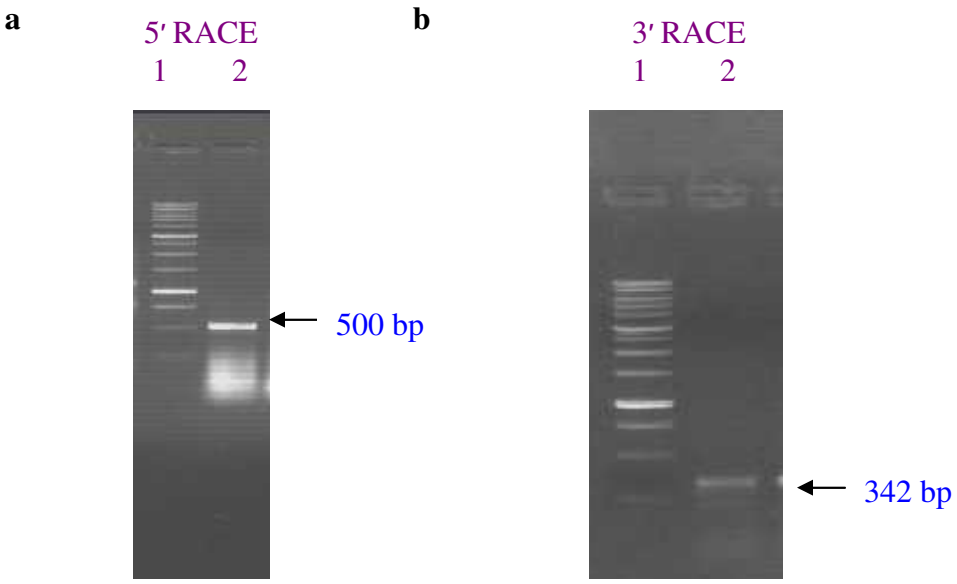

Fig. 7: 5′ and 3′ RACE.
a. 5′ RACE of 500 bp fragment. Lane 1: Molecular weight markers Lane 2: 500 bp fragment
b. 3′ RACE of 342 bp fragment. Lane 1: Molecular weight markers Lane 2: 342 bp fragment

TTGGACACTGACATGGACTGAAGGAGTAGAAATTTCTAAAG

atgttttcaggtgtgacaggtctcatcaaccgggggcagaagctgaagggaacagtggtg
M F S G V T G L I N R G Q K L K G T V V
ttgatgcgcaagaatgtgttggatatcaatgctctcacttctgctcaaagtcccaccggc
L M R K N V L D I N A L T S A Q S P T G
atcatcggtggcgcaatcggtgttgtgggtggtgtcatcggcaccaccgttgacactctc
I I G G A I G V V G G V I G T T V D T L
acttccttttctcggacgatctgtggctctcaggttgatcagtgccactgctgctgatgcg
T S F L G R S V A L R L I S A T A A D A
tctggaaaaggaaaggtgggaaaacaaacatatttggaaggtattgttacttccttacca
S G K G K V G K Q T Y L E G I V T S L P
accttgggagctggccagtctgcattcgatattcattttgaatgggacagtgacatggga
T L G A G Q S A F D I H F E W D S D M G
attcctggagccttttacattgagaatttcatgcaagttgaattcttccttgtgagtttg
I P G A F Y I E N F M Q V E F F L V S L
actcttgaagatattcctaaccacggaaccatacactttgtttgcaactcatggatttac
T L E D I P N H G T I H F V C N S W I Y
aacgacaaaaagtacaaatctgatcgcattttctttgccaacaaggcataccttccaagt
N D K K Y K S D R I F F A N K A Y L P S
gaaacaccaggcccactggtgaagtatagagaagaagaattaaagactttgagaggagat
E T P G P L V K Y R E E E L K T L R G D
ggaacaggagagcgcaaagaacatgaaagaatctatgactatgatgtctacaatgatttg
G T G E R K E H E R I Y D Y D V Y N D L
ggcgatcctgactccaatgccagattggcccgtccagttcttggaggatccacgttgcct
G D P D S N A R L A R P V L G G S T L P
tatcctcgcaggggaagaaccgggaagaaaaccaacatgaaagatcctaaaagtgagagt
Y P R R G R T G K K T N M K D P K S E S
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R S D S V Y L P R D E S F G H L K S S D
ttccttgtttatattcttaaatctgcatctcaaaatgtgatacctcaattacaatctgca
F L V Y I L K S A S Q N V I P Q L Q S A
cttagactacaattcaaccaacctgagtttactagctttgatgatgtgcgtggactctat
L R L Q F N Q P E F T S F D D V R G L Y
gatggtggaattaagttgcctactgatgcacttagcaagattagtcctataccattgttc
D G G I K L P T D A L S K I S P I P L F
agtgaactatttcgtactgatggagaacaggttcttaagtttccaccacctaaagtaatt

```
 S  E  L  F  R  T  D  G  E  Q  V  L  K  F  P  P  P  K  V  I
caagtggaccagtctgcatggatgactgatgaagagcttgcaagagagatgattgctggt
 Q  V  D  Q  S  A  W  M  T  D  E  E  L  A  R  E  M  I  A  G
gtgaatcctcatatcattacaagacttcaggagtttccgcctaagagcaagctagatagc
 V  N  P  H  I  I  T  R  L  Q  E  F  P  P  K  S  K  L  D  S
cagctctatggtgataataccagtacaattacaagagagcacttggagcctaacttgggt
 Q  L  Y  G  D  N  T  S  T  I  T  R  E  H  L  E  P  N  L  G
gggctaactgtagaacaggctatccaaaacaacagactcttcatactagatcaccatgac
 G  L  T  V  E  Q  A  I  Q  N  N  R  L  F  I  L  D  H  H  D
acacttattccatatttgagacgaatcaatgcaacagagacaaaggcctatgctactagg
 T  L  I  P  Y  L  R  R  I  N  A  T  E  T  K  A  Y  A  T  R
accatcattttcttacaagataatggaacattaaagccattggctattgagttaagtaaa
 T  I  I  F  L  Q  D  N  G  T  L  K  P  L  A  I  E  L  S  K
ccacatcctcagggtgataattttggtcctattagcaatgtttatcttccggcaaaccaa
 P  H  P  Q  G  D  N  F  G  P  I  S  N  V  Y  L  P  A  N  Q
ggagttgaagcttatatttggctacttgcaaaggcttatgtgatcgtaaatgactcttgt
 G  V  E  A  Y  I  W  L  L  A  K  A  Y  V  I  V  N  D  S  C
tatcaccaacttgtcggccattggttaaacactcatgcagttgttgagccattcgtgata

 Y  H  Q  L  V  G  H  W  L  N  T  H  A  V  V  E  P  F  V  I
gcaacaaacaggcatctcagtgtggttcaccctattcacaaacttcttcttccacattat
 A  T  N  R  H  L  S  V  V  H  P  I  H  K  L  L  L  P  H  Y
cgtgacacaatgaacatcaatgcacttgcaaggaatgtcttggtcaatgcagagggtatc
 R  D  T  M  N  I  N  A  L  A  R  N  V  L  V  N  A  E  G  I
atagaatcaactttcttgtggggaagctattctttagaaatgtctgcagttgtatacaag
 I  E  S  T  F  L  W  G  S  Y  S  L  E  M  S  A  V  V  Y  K
gattgggttttccctgagcaagcactacctgcagatcttcttaagagaggtgtggctgtt
 D  W  V  F  P  E  Q  A  L  P  A  D  L  L  K  R  G  V  A  V
aaggattcatctgctccacacggccttcgtcttctaatagaggactatccttatgctgct
 K  D  S  S  A  P  H  G  L  R  L  L  I  E  D  Y  P  Y  A  A
gatggactagagatatgggccaccatcaagtcatgggtgcaagaatatgtgtctttctac
 D  G  L  E  I  W  A  T  I  K  S  W  V  Q  E  Y  V  S  F  Y
tacaagtctgatgctgcaattggacaagatattgaactccaagccttttggaaagaactt
 Y  K  S  D  A  A  I  G  Q  D  I  E  L  Q  A  F  W  K  E  L
gtgcaagttggtcatgtgacaagaaaaatgagccatggtgggaaatggcaaactcgtgaa
 V  Q  V  G  H  V  T  R  K  M  S  H  G  G  K  W  Q  T  R  E
```

gagttgattgaatcttgcaccacactgatatggactgcttcagcccttcatgcagctgtg

E L I E S C T T L I W T A S A L H A A V

aatttcggacagtatccatatggtggctacatccttaaccgaccaactcttagtaggcaa

N F G Q Y P Y G G Y I L N R P T L S R Q

ttcatgcctgagattggatctcctgagtatgctgagcttgccaagaaccctgaaaaggtg

F M P E I G S P E Y A E L A K N P E K V

tacttgaaaacaatcacaggaaagagtgatgcccttaaagacctgaccattatagaagtg

Y L K T I T G K S D A L K D L T I I E V

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L S R H A S D E L Y L G E R D G G E F W

acttatgataaagagccattggaggccttcaagaggtttggaaagaggttggcagaaatt

T Y D K E P L E A F K R F G K R L A E I

gagcaaaagctcatccaaagaaacagtgatgagactctgaggaaccgatatggtccagtg

E Q K L I Q R N S D E T L R N R Y G P V

aagatgccttacacattgctctatccttcaagtgaggaaggcttgactttcagaggtatt

K M P Y T L L Y P S S E E G L T F R G I

cctaacagtatctccatctaaAGAGGTTTATGAGTATTTTGCATTCTGCATTCT

P N S I S I *

GAATAAAGAAAGAAAAGGGAGTTTAATTCCTTCTTTCCCCTCAGTATGAGAGTTTGTAT

TGTGTATGTTTGATGTTCTGAATTAATAAAATTCTGCATTTCTACTGTGTAAAAAAAAA

AAAAAAAAAAAAAAA

Fig. 8: Translational map of green gram 15-LOX. The small letters indicate the nucleotide sequence. The capital letters indicate the corresponding amino acid sequence. The letters in black colour indicate the ORF region. The 5′ UTR and 3′ UTR regions are represented in blue colour. *-stop codon.

The green gram 15-LOX has all the signature domains including PLAT/LH2 domain that binds to lipid molecules and active site domain. ClustalW multiple alignment demonstrated that all these domains are highly conserved with other plant lipoxygenases (Fig. 9).

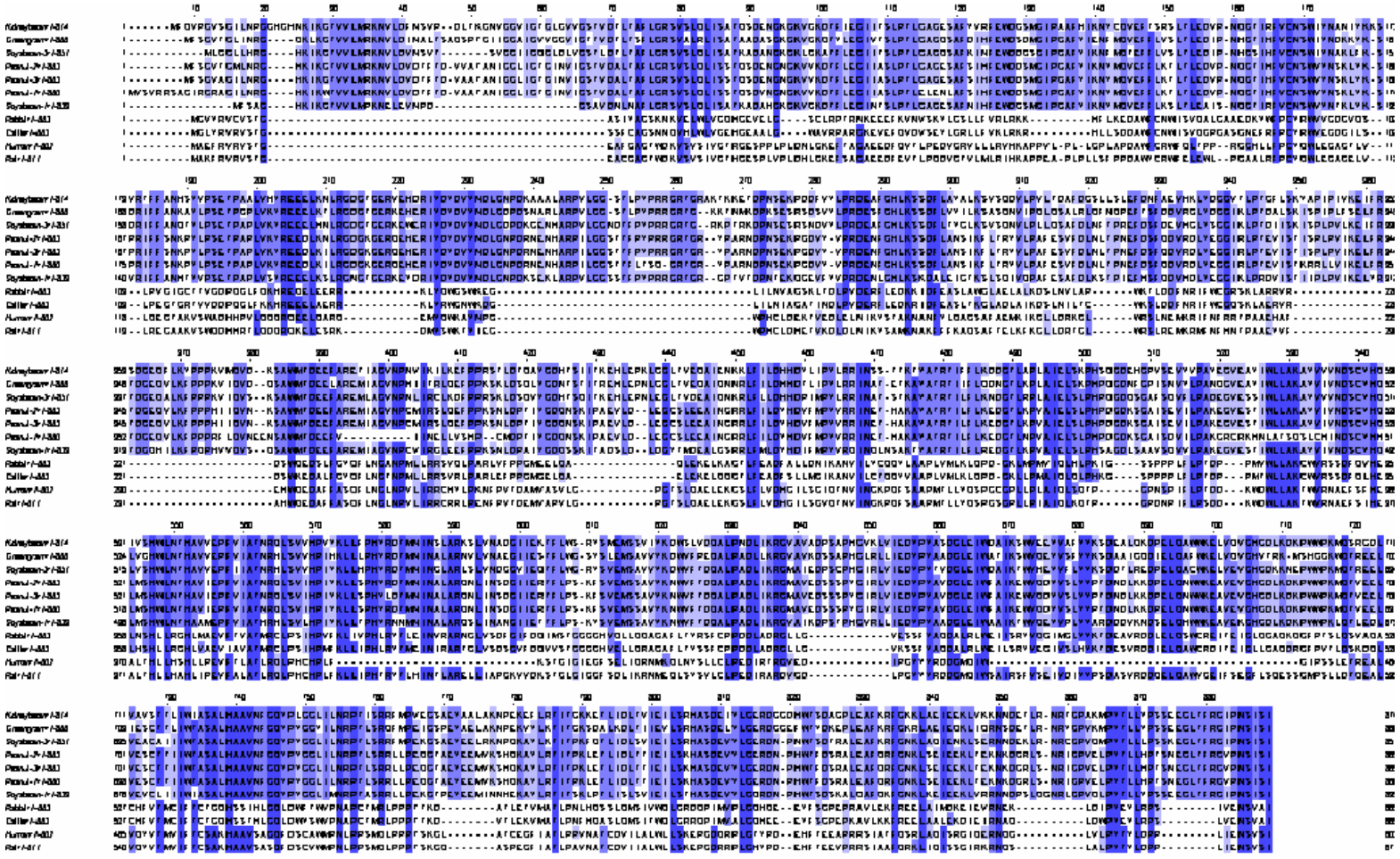


Fig. 9: ClustalW multiple alignment showing the conserved regions among various lipoxygenases. The ClustalW multiple alignment was done for green gram lipoxygenase along with other 15-lipoxygenases from various sources. The fig above shows the conserved regions among 15-lipoxygenases.

Phylogenetic analysis of green gram 15-LOX revealed a 70% homology to that of soybean LOX, where as it showed only 18.7 % homology with that of the human LOX at nucleotide level (Fig. 11) and at protein level it showed 73% homology with soybean LOX and 13% homology with humans (Fig. 12).

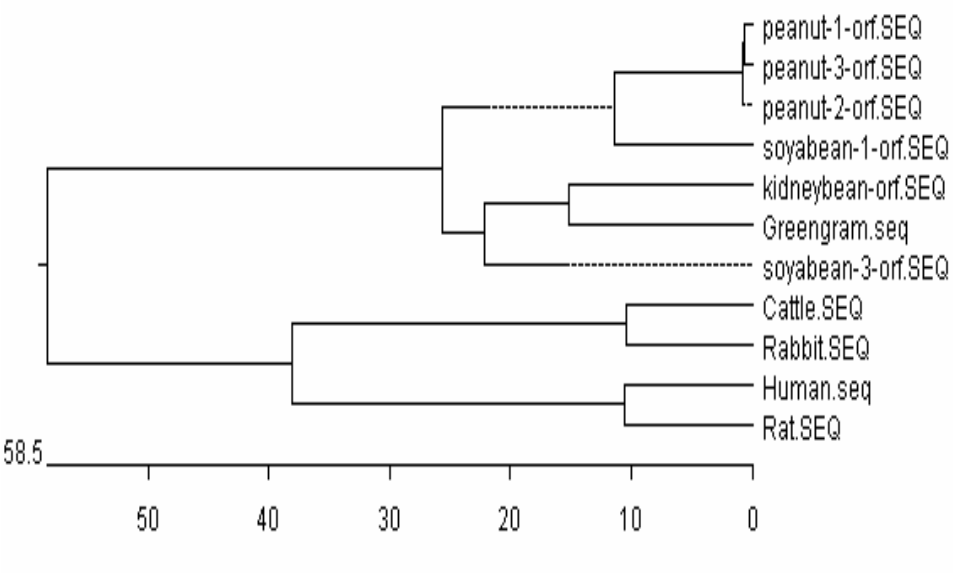


Fig. 10: Phylogenetic tree showing the homology of 15-lipoxygenases from various sources.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11		
1		36.8	36.9	80.0	21.1	19.7	19.4	20.0	21.1	21.6	20.9	1	Cattle.SEQ
2	75.3		78.8	36.5	19.7	21.3	20.6	20.8	21.3	20.0	18.7	2	Human.seq
3	79.8	21.1		37.9	24.2	24.2	24.7	24.6	26.2	23.1	22.1	3	Rat.SEQ
4	20.9	71.8	77.5		21.8	19.2	19.1	18.8	19.7	21.0	19.8	4	Rabbit.SEQ
5	112.6	116.0	111.7	118.4		57.5	66.1	65.8	65.4	69.2	69.6	5	kidneybean-orf.SEQ
6	102.4	107.5	103.5	102.0	36.0		94.2	94.6	71.4	65.4	62.1	6	peanut-1-orf.SEQ
7	101.5	106.2	103.0	100.8	34.7	1.6		99.5	75.9	70.0	66.3	7	peanut-2-orf.SEQ
8	102.6	106.7	103.1	101.7	35.1	1.2	0.5		75.6	69.8	66.0	8	peanut-3-orf.SEQ
9	105.0	106.3	104.6	104.3	34.1	23.5	22.3	22.5		70.5	62.7	9	soyabean-1-orf.SEQ
10	108.0	111.6	104.7	107.7	30.8	31.5	30.4	30.6	30.5		70.4	10	soyabean-3-orf.SEQ
11	118.9	116.1	107.1	116.4	30.2	36.9	35.3	35.4	37.0	27.4		11	Greengram.seq
	1	2	3	4	5	6	7	8	9	10	11		

Fig. 11: Percent identity of green gram LOX with other 15-lipoxygenases at nucleotide level.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11		
1		32.2	34.5	78.2	17.0	16.0	17.9	17.8	16.9	17.9	17.9	1	Cattle.SEQ
2	114.9		79.3	30.8	14.6	12.6	12.6	13.4	13.3	14.8	13.1	2	Human.seq
3	119.8	22.1		32.7	17.3	18.0	18.3	18.1	17.7	18.3	17.0	3	Rat.SEQ
4	25.7	124.8	127.7		17.2	16.6	17.0	16.7	16.9	17.2	17.8	4	Rabbit.SEQ
5	193.7	198.0	188.8	201.0		60.5	66.2	66.1	63.9	71.9	70.1	5	kidneybean-orf.SEQ
6	190.9	198.0	197.0	199.0	47.6		90.8	91.2	66.5	66.2	59.7	6	peanut-1-orf.SEQ
7	186.5	192.8	188.4	194.8	39.5	6.4		99.3	75.5	73.0	66.2	7	peanut-2-orf.SEQ
8	186.5	191.3	188.4	194.8	39.7	6.0	0.6		75.2	72.7	66.0	8	peanut-3-orf.SEQ
9	186.9	190.7	182.5	193.9	41.4	34.7	26.7	26.9		69.3	63.5	9	soyabean-1-orf.SEQ
10	172.7	181.6	178.8	189.1	31.7	38.0	31.2	31.5	33.8		73.3	10	soyabean-3-orf.SEQ
11	183.3	188.3	187.7	196.9	34.6	49.5	40.5	40.9	43.9	30.1		11	Greengram.seq
	1	2	3	4	5	6	7	8	9	10	11		

. **Fig. 12: Percent identity of green gram LOX with other 15-lipoxygenases at protein level.**

4.1.3 Expression of full length gene

The ORF encoding green gram 15-LOX was cloned into the *E. coli* expression vector pETBlue-2 (Fig. 13).

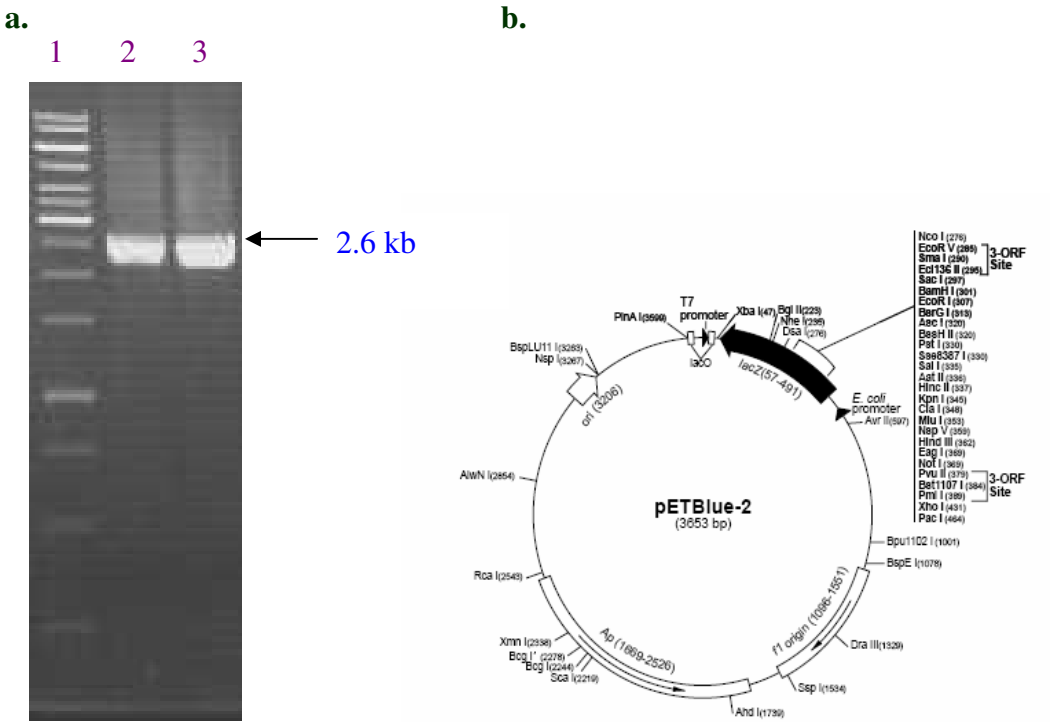


Fig. 13: Amplification of green gram 15-lipoxygenase ORF region.
a. Lane 1: Molecular weight markers. Lane 2&3 represents amplified green gram 15-lipoxygenase ORF region of 2.6 kb fragment.
b. Vector map of pET Blue-2 expression vector.

The green gram recombinant 15-LOX expressed in *E.coli* was about 100 kDa (Fig.14 a) and the identity of the expressed protein was confirmed by immunoblot analysis with anti-His antibody (Fig. 14 b).

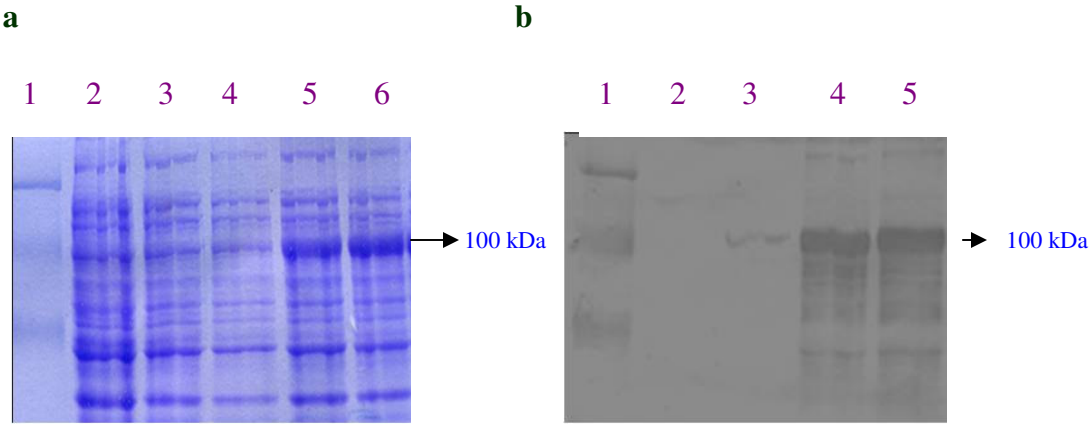


Fig. 14: Expression profile of green gram 15-LOX fragment.

a. SDS-gel profile: Lane 1: Protein ladder, lane 2: BL21-DE-3 cells, lane 3: pETBlue-2 alone transformed cells, lane 4: Uninduced Recombinant pETBlue-2 transformed cells, lane 5: Recombinant pETBlue-2 post induction (1 h), lane 6: Recombinant pETBlue-2 post induction (2 h).

b. Immunoblot analysis**:** Lane 1: Protein ladder, lane 2: BL21-DE-3 cell lysate, lane 3: Uninduced Recombinant pETBlue-2 transformed cell lysate, lane 4: Recombinant pETBlue-2 post induction (1 h), lane 5: Recombinant pETBlue-2 post induction (2 h)

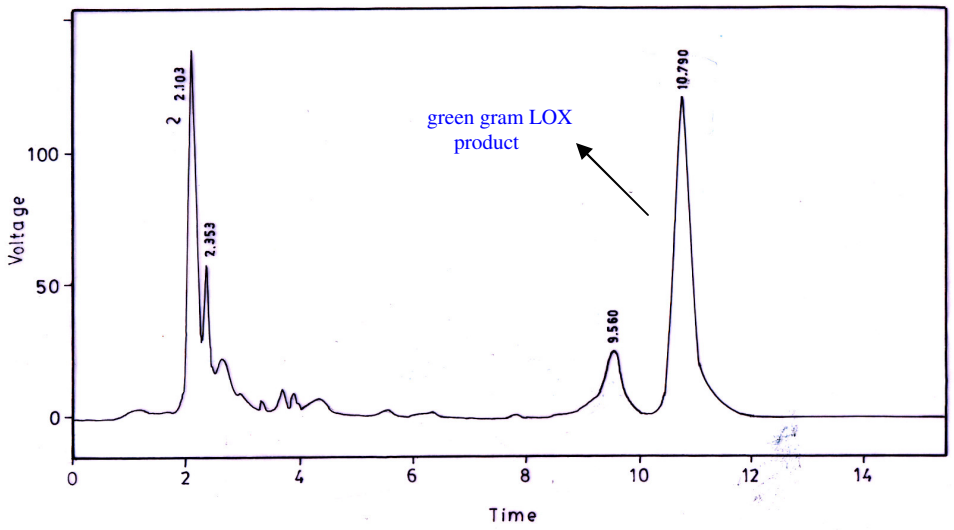
For the enzymatic characterization of green gram recombinant 15-LOX, *E. coli* cell lysates containing over expressed protein were taken as the 15-LOX enzyme source.

4.1.4. Analysis of green gram 15-LOX products:

The activity of the recombinant protein was confirmed upon reaction with arachidonic acid as substrate and separating the hydroperoxy metabolites by straight phase HPLC. The wave length spectrum of the hydroperoxy product was seen on spectrophotometer. The product showing absorption maximum at 235 nm wave length was collected and then characterized by recording uv/vis wave length scanning and co-chromatography with standard. As shown in Fig. 15 a, the

prominent peak with RT 10.79 min showed typical conjugated diene spectrum (Fig. 15 b). This peak was collected and then co-chromatographed with the standard 15-(S)-HPETE on reverse phase HPLC (Fig. 16).

a



b

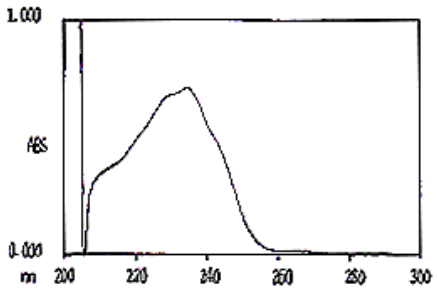


Fig. 15: SP-HPLC analysis of green gram LOX products.

a. The products obtained from green gram LOX with arachidonic acid substrate were extracted into hexane: ether (1:1) evaporated and dissolved in SP-HPLC mobile phase [hexane: isopropanol: acetic acid (1000:15:1)] and separated at the flow rate of 1 ml/min.

b. The wave length scan of the product with RT-10.79 min in Fig. 15 a. showing absorption maximum at 235 nm.

The hydroperoxy product when subjected to co-chromatography by reverse phase HPLC along with standard 15-(S)-HPETE from soybean LOX, the two products were eluted with different retention times (Fig. 16). In reverse phase

HPLC analysis the retention time of standard 15-(S)-HPETE was 11.497 min whereas the retention time of product from recombinant enzyme was 16.933 min. These studies reveal that the recombinant protein is not 15-LOX and needs to be further characterized. To identify the LOX metabolite formed by the recombinant 15-LOX expressed in *E.coli*, LC-MS analysis of the peak with RT 10.79 (Fig. 15 a) was performed. The fragments obtained (Fig. 17) were quite different from the standard 15-(S)-HPETE (Fig. 21). These results suggest that the recombinant 15-LOX expressed in *E.coli* is not a 15-LOX.

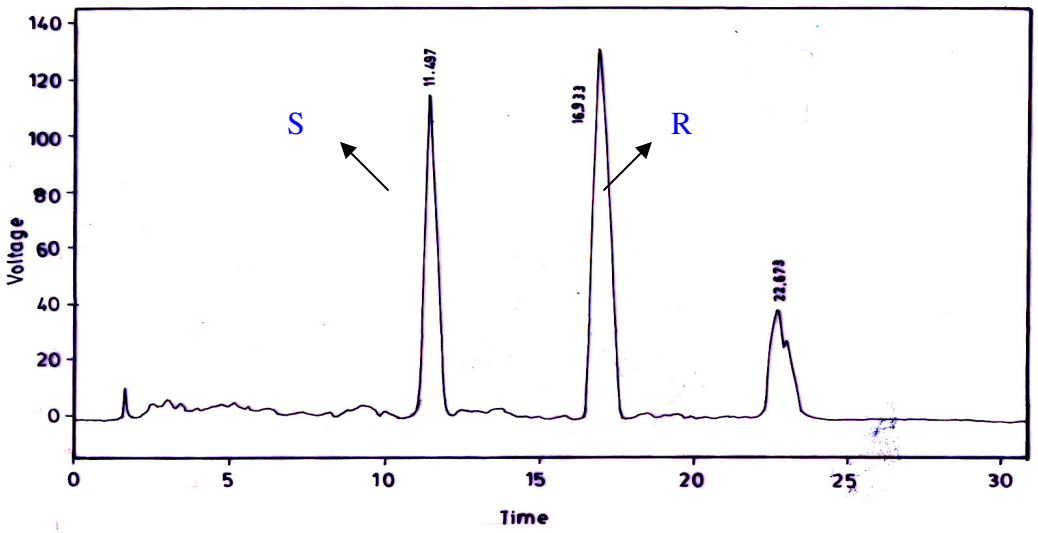


Fig. 16: Reverse phase HPLC separation of green gram LOX products along with standard 15-(S)-HPETE. The spectrum showing the difference in retention times of the products obtained from soybean LOX ((RT: 11.4) and green gram LOX (RT: 16.9) when separated on Reverse phase HPLC. 'S' in the fig indicates standard 15-(S)-HPETE from soybean LOX and 'R' indicates product from recombinant enzyme.

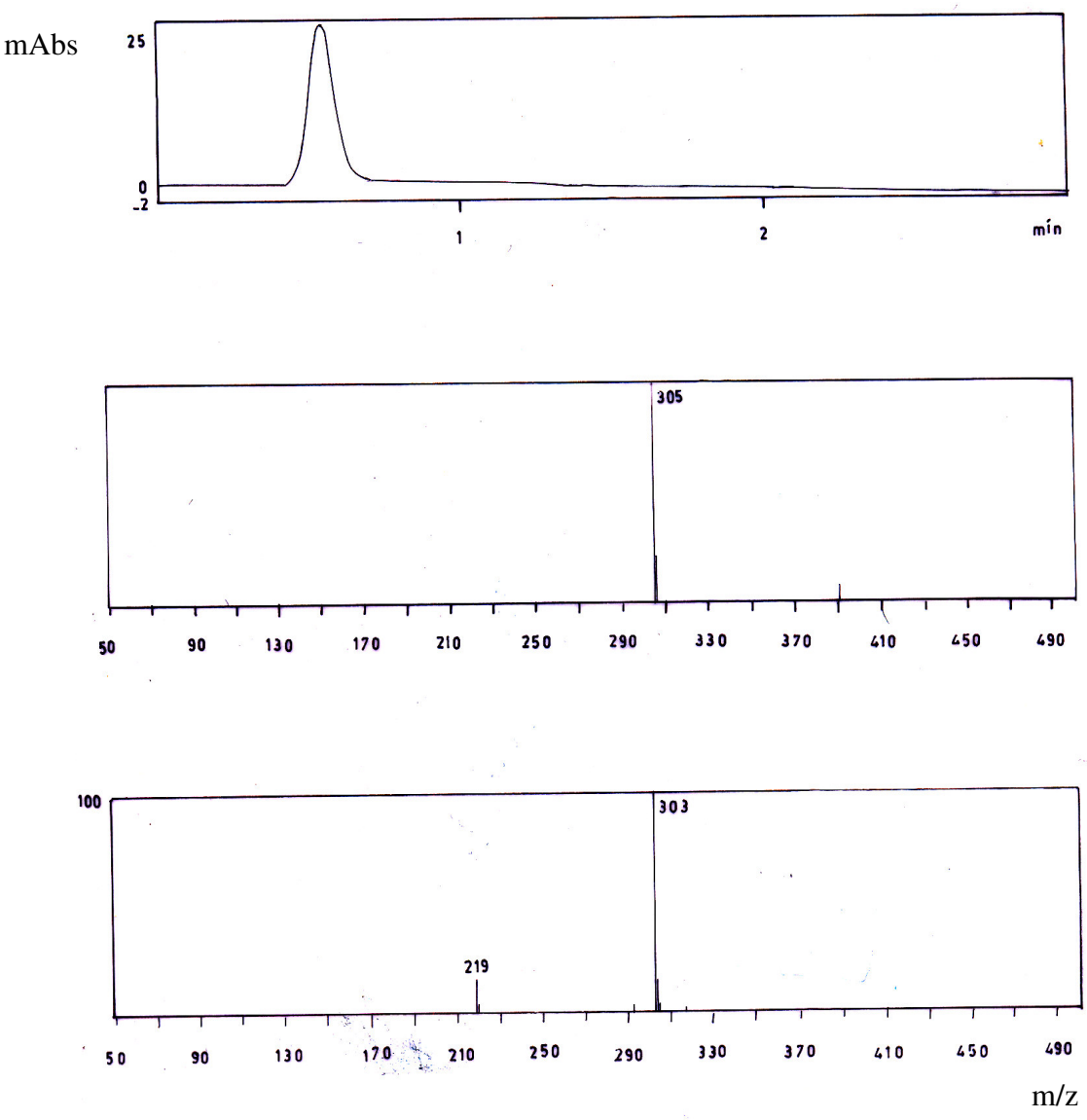


Fig. 17: LC-MS analysis of green gram LOX products. LC-MS analysis of green gram LOX product showed characteristic 305 and 303 fragments in positive and negative mode respectively.

4.2. Biosynthesis, purification and characterization of 15-LOX metabolites employing soybean LOX

15-LOX metabolites were synthesized by treating commercially available soybean LOX with AA, LA and ALA as described in methodology. The products were separated on HPLC and were characterized on GC-MS. The effects of these products were seen on acute lymphoblastic T-cell leukemia cell line-Jurkat.

4.2.1. Separation of products on HPLC

The products were extracted and separated as per the procedure described in the methodology. The products were initially separated on straight phase HPLC employing preparative silica column at a flow rate of 6 ml/min (Fig. 18), and later the purity was checked on analytical column at a flow rate of 1 ml/min. The retention times of hydroperoxy compound-15-(S)-HPETE and hydroxy compound-15-(S)-HETE was 22.42, and 14.80 min respectively when the flow rate was 1ml/min (Fig. 19 a). Similarly the retention times of 13-(S)-HPODE, 13-(S)-HODE, 13-(S)-HPOTrE, and 13-(S)-HOTrE were 9.41, 7.41, 9.59 and 7.73 min when the flow rate was 1ml/min (Fig. 19 b & c).

The absorption spectra of the compounds when recorded on UV-VIS spectrophotometer, gave characteristic conjugated diene spectra with a peak around 235 nm (insets in Fig. 19 a, b & c). The products were stored in aliquots under nitrogen gas and kept at -80 °C and used as and when required. The compounds thus separated on SP-HPLC were initially identified based on the order of elution and earlier reports on the specificity of soybean LOX (Kiran Kumar et al., 1993). Further confirmation was made based on GC-MS and LC-MS analysis of the metabolites.

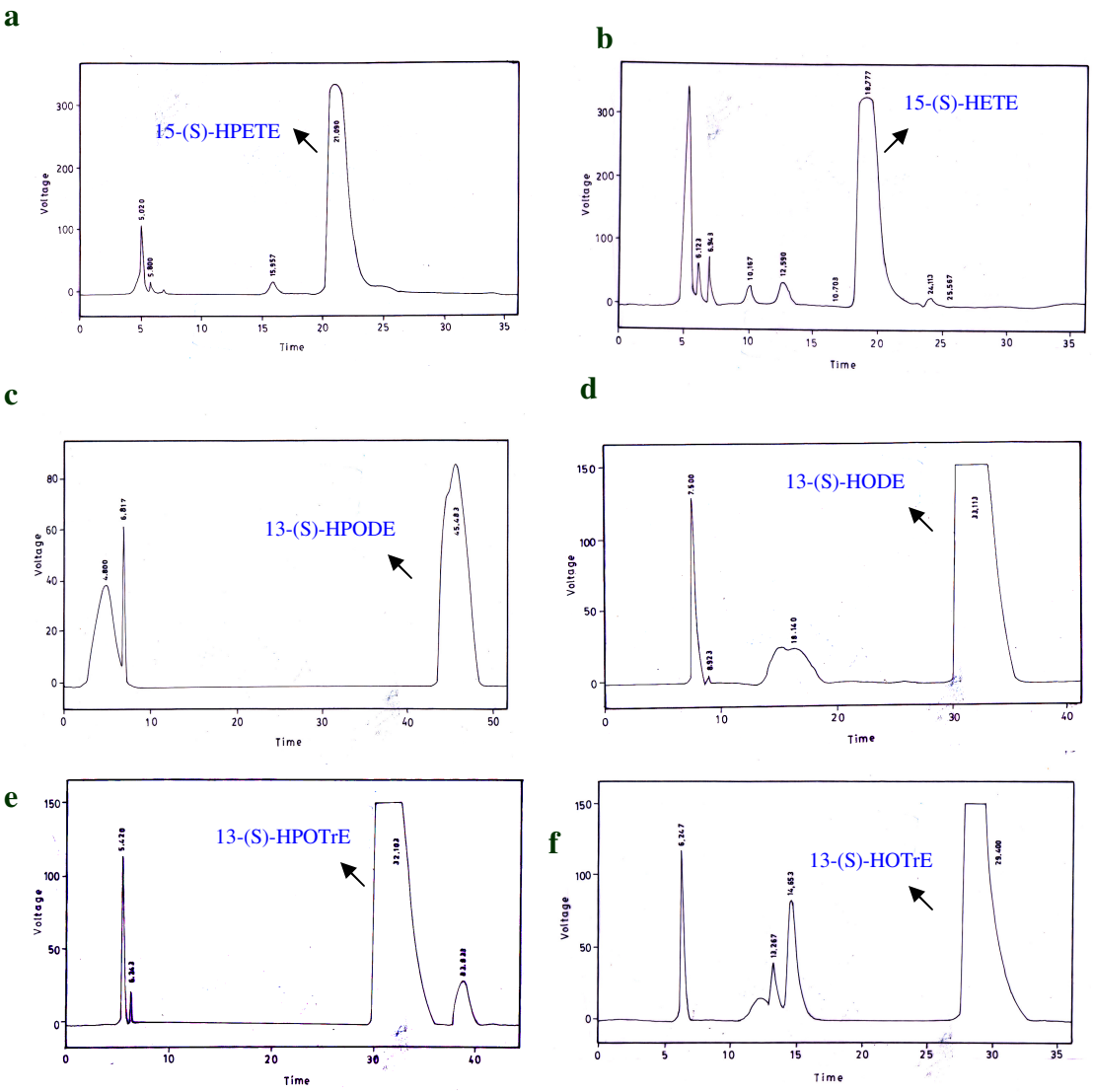


Fig. 18: SP-preparatory HPLC chromatograms of 15-LOX metabolites synthesized by employing Soybean LOX. The products were synthesized using AA, LA and ALA as substrates and commercially available soybean LOX as enzyme source. The products were separated on straight phase preparatory HPLC with mobile phase of hexane: isopropanol:acetic acid (1000:15:1) with a flow rate of 6 ml/min. **a**. 15-(S)-HPETE **b.** 15-(S)-HETE **c.** 13-(S)-HPODE **d.** 13-(S)-HODE **e.** 13-(S)-HPOTrE **f.** 13-(S)-HOTrE

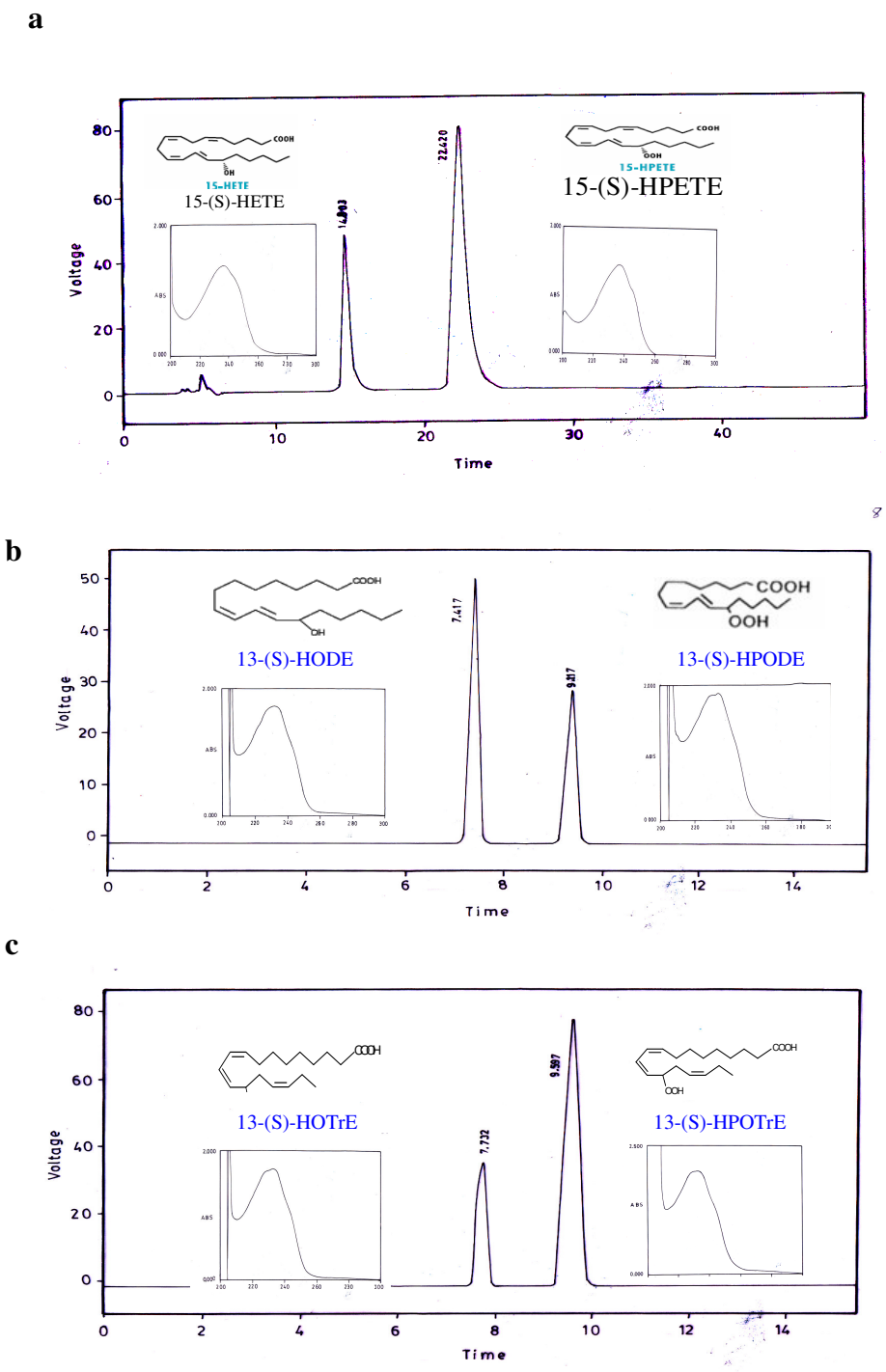


Fig. 19: HPLC chromatograms of 15-LOX metabolites. The products were synthesized using AA, LA and ALA as substrates and commercially available soybean LOX as enzyme source. The products were separated on straight phase analytical HPLC with mobile phase of hexane: isopropanol:acetic acid (1000:15:1) with a flow rate of 1 ml/min. **a.** 15-(S)-HPETE + 15-(S)-HETE **b.** 13-(S)-HPODE + 13-(S)-HODE **c.** 13-(S)-HPOTrE + 13-(S)-HOTrE

4.2.2 GC-MS and LC-MS Characterization of 15-LOX metabolites:

The peak with RT 14.80 min (Fig. 19 a), initially identified as 15-HETE was derivatized (methylated and silylated) before GC-MS analysis. The compound showed characteristic fragmentation pattern of 15-HETE (Fig. 20). The major fragments detected were 225, 316, 335, 391 and 406 (M^+). These fragments confirm that the peak with RT 14.8 min (Fig. 19 a) is 15-HETE. As soybean LOX is known to generate only metabolites of 'S' stereospecificity, the products generated in the present study were considered as 15-(S)-HPETE and 15-(S)-HETE.

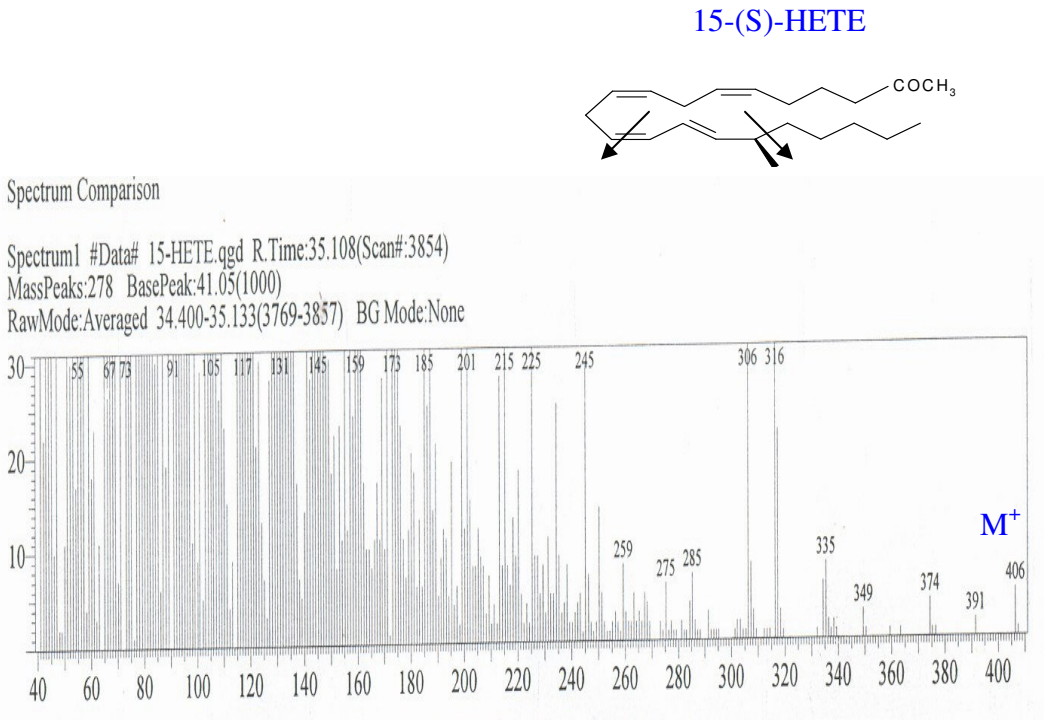


Fig. 20: GC-MS analysis of the peak with RT 14.80 min (Fig. 19 a) after methylation and silylation. Based on the fragmentation pattern, the compound is identified as 15-HETE.

LC-MS analysis was done for the products 15-(S)-HPETE, 15-(S)-HETE, 13-(S)-HPODE, 13-(S)-HPODE, 13-(S)-HODE, 13-(S)-HPOTrE and 13-(S)-HOTrE. The characteristic fragments obtained confirm the products (Fig. 21- 26).

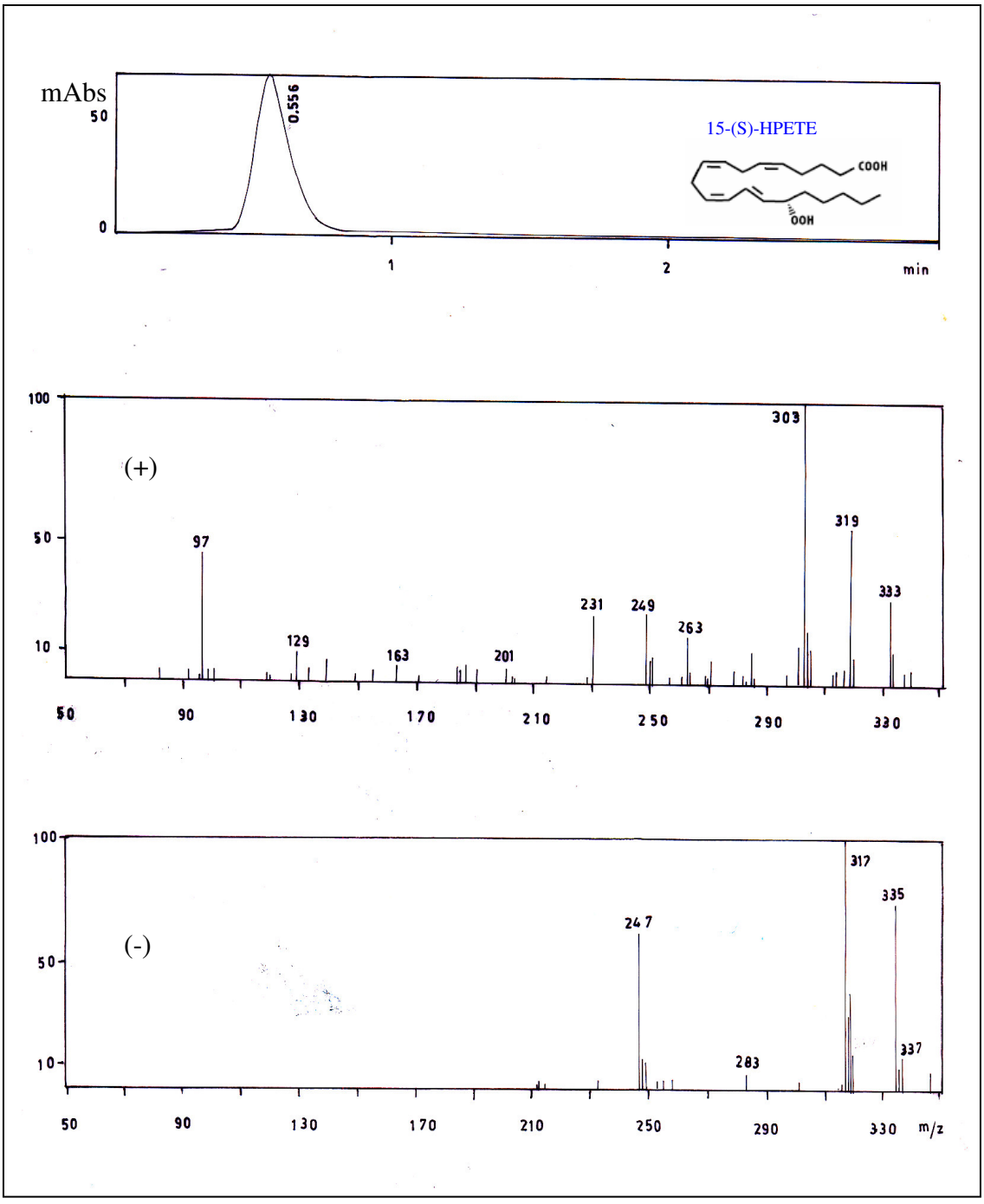


Fig. 21: LC-MS analysis of 15-(S)-HPETE. LC-MS analysis of 15-(S)-HPETE showed characteristic 249, 319, 333 fragments in positive mode and 247, 317 and 335 fragments in negative mode.

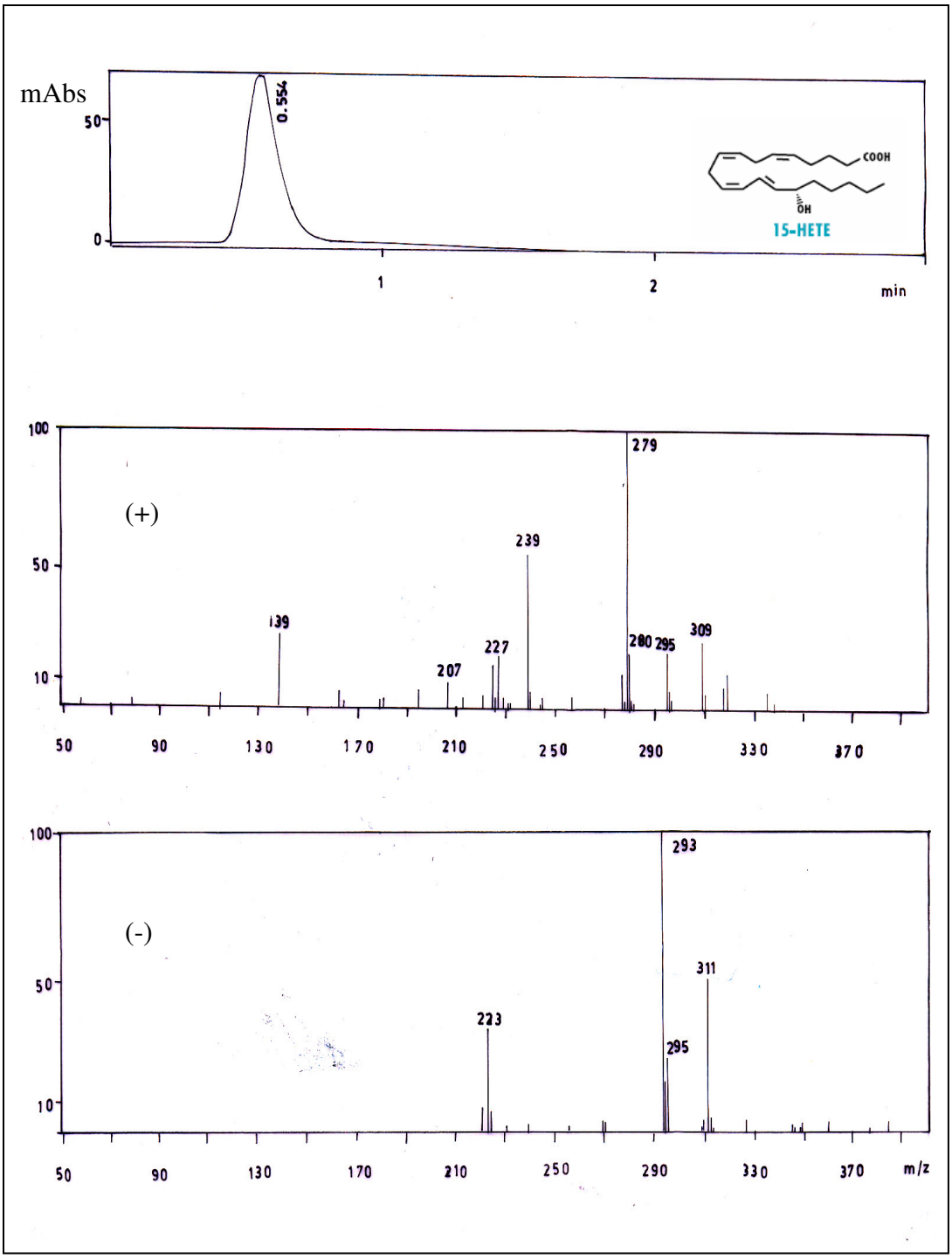


Fig. 22: LC-MS analysis of 15-(S)-HETE. LC-MS analysis of 15-(S)-HETE showed characteristic 239, 279 fragments in positive mode and 223, 293, 295, 311 fragments in negative mode.

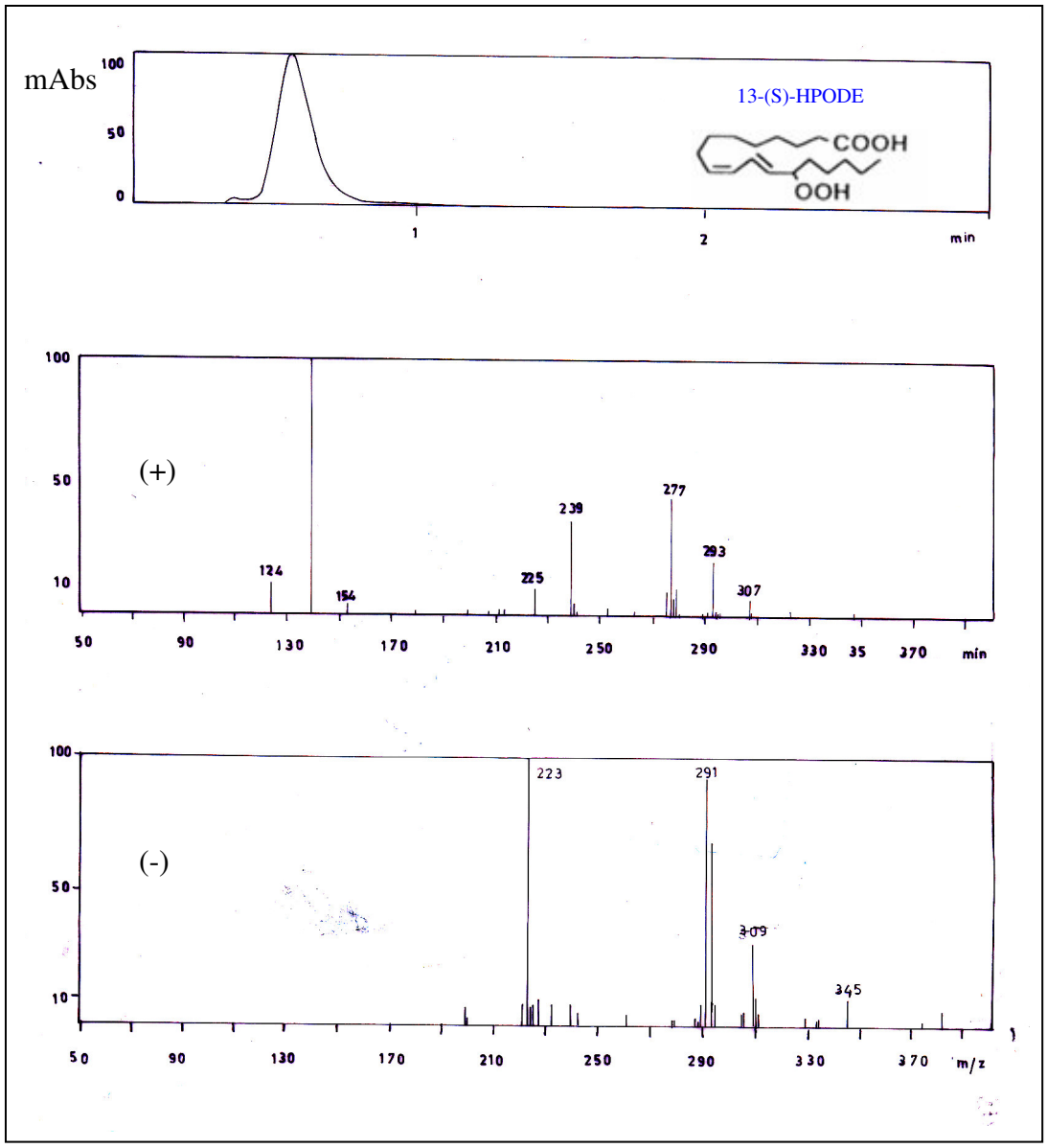


Fig. 23: LC-MS analysis of 13-(S)-HPODE. LC-MS analysis of 13-(S)-HPODE showed characteristic 225, 293, 307 fragments in positive mode and 223, 291, 309 fragments in negative mode.

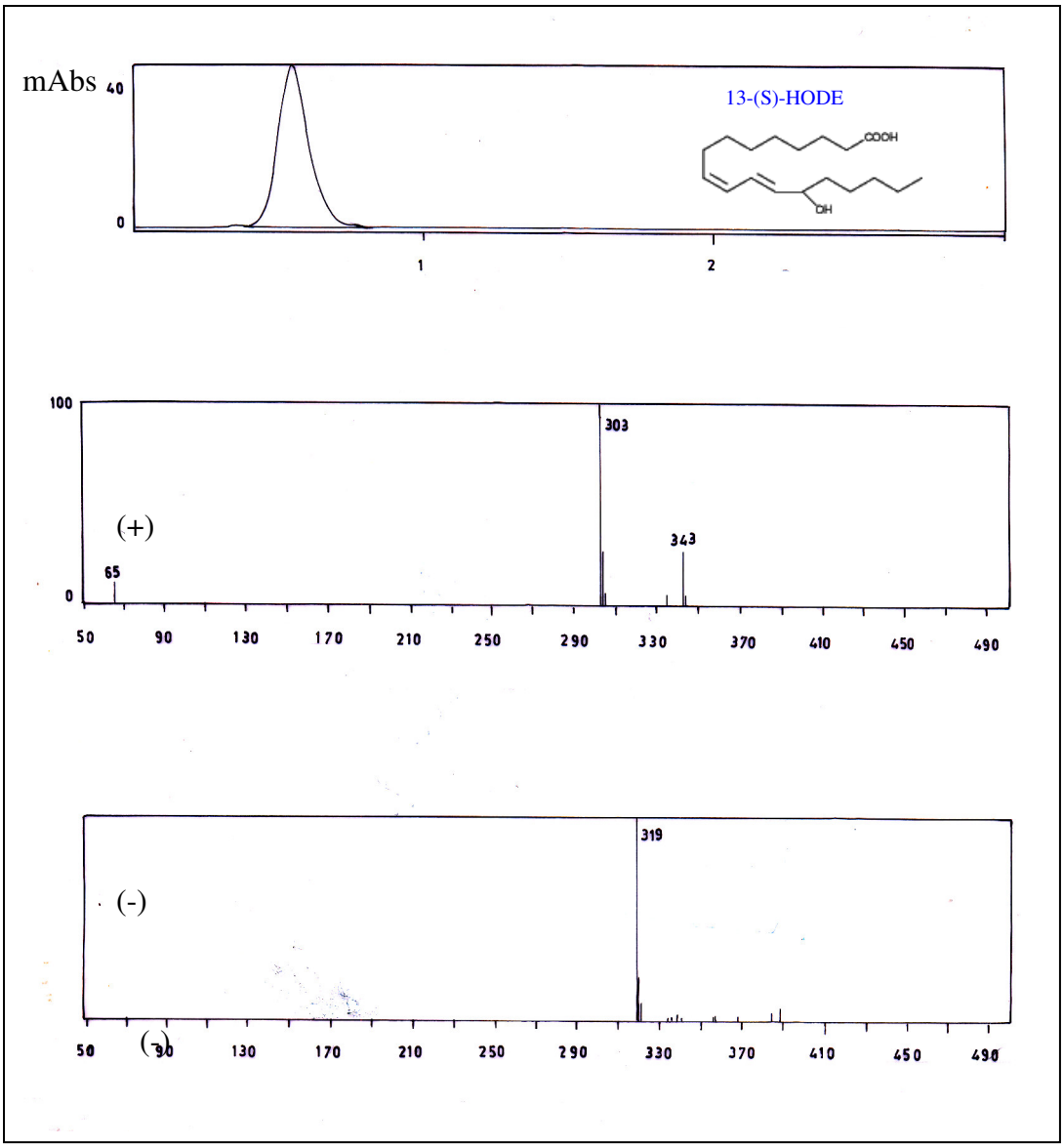


Fig. 24: LC-MS analysis of 13-(S)-HODE. LC-MS analysis of 13-(S)-HODE showed characteristic 303, 343 fragments in positive mode and 319 fragment in negative mode.

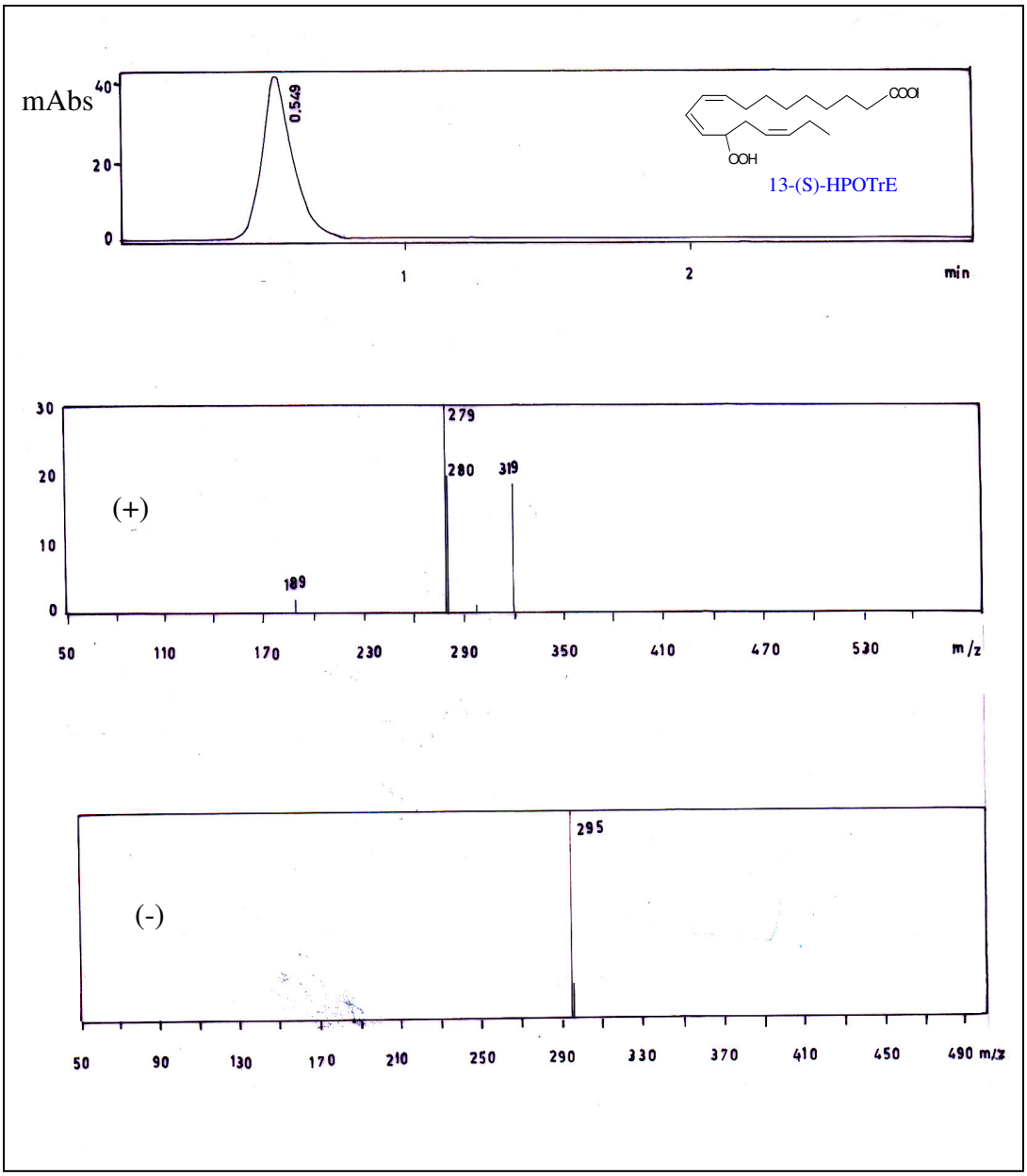


Fig. 25: LC-MS analysis of 13-(S)-HPOTrE. LC-MS analysis of 13-(S)-HPOTrE showed characteristic 279, 280, 319 fragments in positive mode and 295 fragment in negative mode.

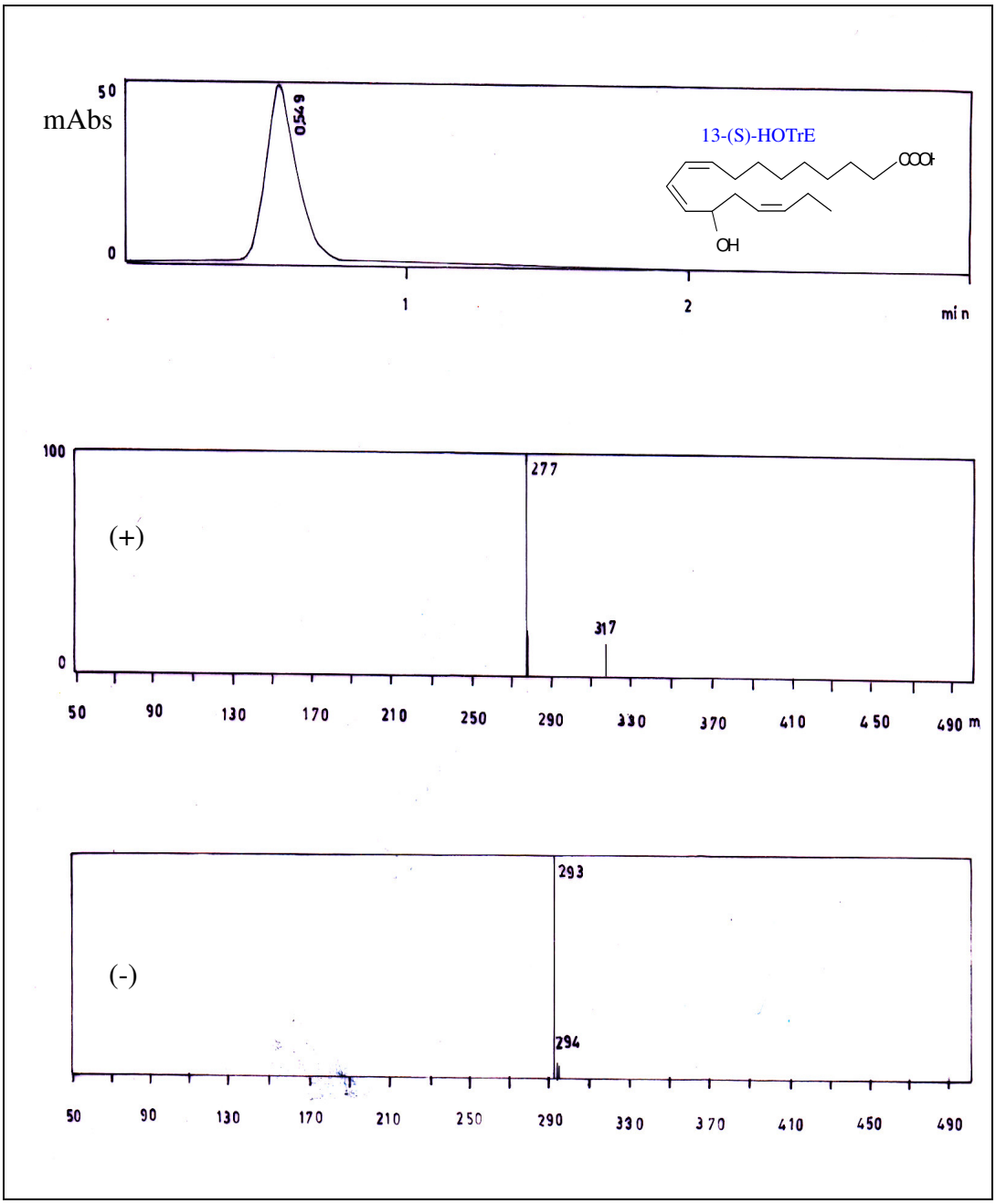


Fig. 26: LC-MS analysis of 13-(S)-HOTrE. LC-MS analysis of 13-(S)-HOTrE showed characteristic 277, 317 fragments in positive mode and 293, 294 fragments in negative mode.

Chapter 2

Antiproliferative effects of PUFAs and 15-LOX metabolites on acute lymphoblastic T-cell leukemia cell line (Jurkat)

4.3. Antiproliferative effects of PUFAs on acute lymphoblastic T-cell leukemia cell line (Jurkat)

Polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA), linoleic acid (LA) and α-linolenic acid (ALA) serve as substrates for 15-LOX. In the present study the effect of the above PUFAs was tested on the growth of on Jurkat cells was determined by MTT assay.

Jurkat cells were cultured in RPMI medium containing 1% FBS in presence or absence of 1-100 µM AA, LA and ALA for 3, 6, 12 and 24 h and MTT assay was carried out as described in methodology. All the three PUFAs showed no significant inhibition on cell proliferation upto 12 h at 10 µM and showed 15-25% inhibition after incubation at 100 µM for 24 h (Fig 27, 28 & 29) suggesting that PUFAs are not by themselves cytotoxic to leukemic cells at the concentrations tested in the present study.

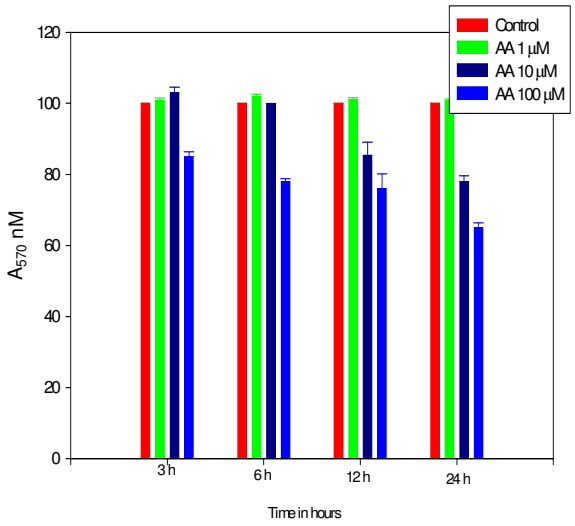


Fig. 27: Effect of Arachidonic acid on the growth of human acute lymphoblastic T-cell leukemia cell line-Jurkat. Cells (5 X10^3) were treated with various concentrations (as indicated in the figure) of arachidonic acid and the cell viability was measured by MTT assay at 3, 6, 12 and 24 h post treatment. The values represent the mean ± S.E from three independent experiments.

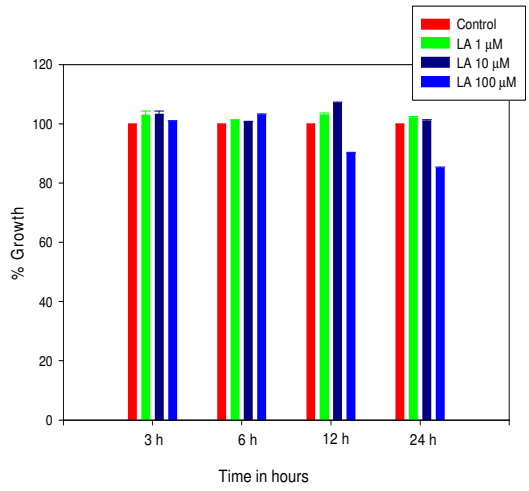


Fig. 28: Effect of Linoleic acid on the growth of human acute lymphoblastic T-cell leukemia cell line -Jurkat. Cells (5 X10^3) were treated with various concentrations (as indicated in the figure) of linoleic acid and the cell viability was measured by MTT assay at 3, 6, 12 and 24 h post treatment. The values represent the mean ± S.E from three independent experiments.

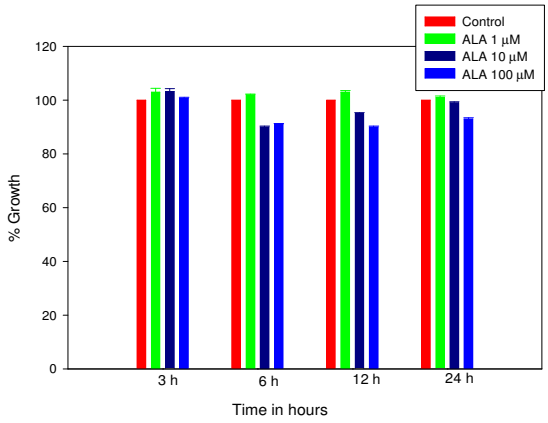


Fig. 29: Effect of α-Linolenic acid on the growth of human acute lymphoblastic T-cell leukemia cell line-Jurkat. Cells (5 X10^3) were treated with various concentrations (as indicated in the figure) of α-linolenic acid and the cell viability was measured by MTT assay at 3, 6, 12 and 24 post treatment. The values represent the mean ± S.E from three independent experiments.

4.4. Antiproliferative effects of 15-lipoxygenase metabolites of AA, LA and ALA on the Jurkat cell line

4.4.1. Antiproliferative effects of 15-(S)-HPETE and 15-(S)-HETE

Effect of 15-LOX metabolites on the survival and proliferation of Jurkat cells was evaluated by MTT assay. Jurkat cells were cultured in RPMI 1640 + 1% FBS medium containing 1–20 µM 15-(S)-HPETE, or 1–80 µM 15-(S)-HETE for 3, 6, 12 and 24 h and the cytotoxicity and cell proliferation were evaluated by the MTT assay. Under these experimental conditions, 15-(S)-HPETE inhibited the growth of Jurkat cells rapidly with IC_{50} value of 10 µM by 3 h (Fig. 30 a). 15-(S)-HETE, on the other hand inhibited the growth only at higher concentrations, with an IC_{50} of 80 µM by 3 h and IC_{50} of 40 µM by 6 h (Fig. 30 b). Further studies to elucidate the mechanism of cell death were carried out with 10 µM 15-(S)-HPETE and 40 µM 15-(S)-HETE at time periods of 3 h and 6 h respectively (Fig. 30). The above mentioned doses and time periods were used to study the mechanism behind 15-LOX metabolite mediated cell death. The IC_{50} values and time periods were used for further studies to elucidate the mechanism of cell death.

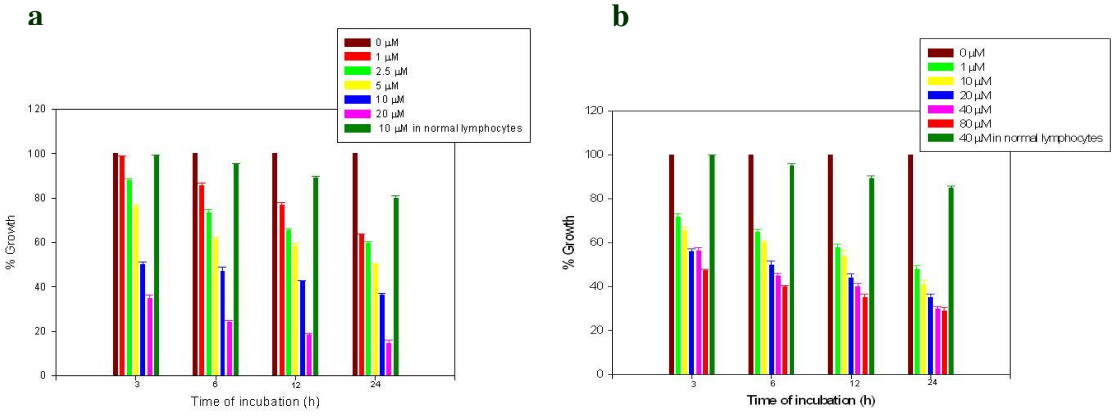


Fig. 30: Effect of 15-(S)-HPETE and 15-(S)-HETE on the growth of human acute lymphoblastic T-cell leukemia cell line-Jurkat. Cells (5 X10^3) were treated with various concentrations (as indicated in the figure) of **a.** 15-(*S*)-HPETE **b.** 15-(*S*)-HETE, and proliferation was determined by MTT assay at 3, 6, 12 and 24 h post treatment. The values represent the mean ± S.E from three independent experiments

In order to test the effect of these metabolites on normal cells, primary lymphocytes in culture were incubated with 15-(S)-HPETE (10 µM) and 15-(S)-HETE (40 µM) respectively for varying time periods. These studies showed negligible effect on primary lymphocytes (Fig. 30 a & b). The cells were showing only 20% growth inhibition even after exposure for 24 hours.

4.4.2. Antiproliferative effects of 13-(S)-HPODE and 13-(S)-HODE

Effect of linoleic acid metabolites [13-(S)-HPODE and 13-(S)-HODE] was also determined by MTT assay at concentrations 1-20 µM for 13-(S)-HPODE and 1-160 µM of 13-(S)-HODE. 13-(S)-HPODE inhibited the growth of Jurkat cells with an IC_{50} value of 20 µM by 3 h (Fig. 31 a). 13-(S)-HODE, on the other hand, showed IC_{50} value of 140 µM by 6 h (Fig. 31 b)

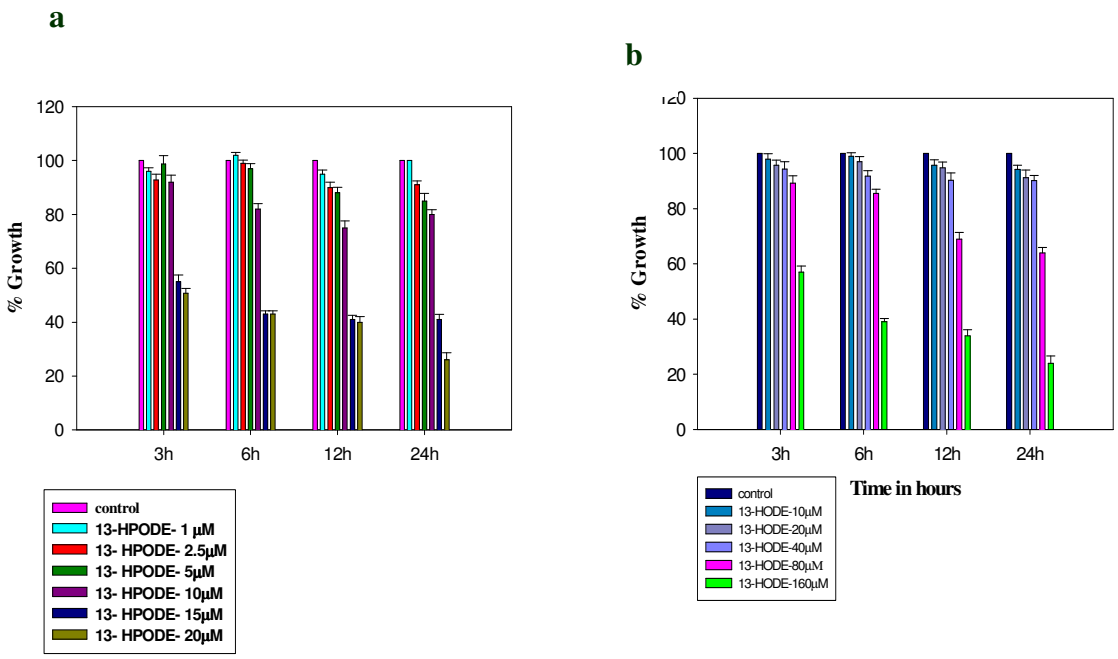


Fig. 31: Effect of 13-(S)-HPODE and 13-(S)-HODE on the growth of human acute lymphoblastic T-cell leukemia cell line-Jurkat. Cells (5 $X10^3$) were treated with various concentrations (as indicated in the figure) of **a.** 13-(S)-HPODE **b.** 13-(S)-HODE, and the cell viability was measured by MTT assay at 3, 6, 12 and 24 h post treatment. The values represent the mean ± S.E from three independent experiments.

4.4.3. Antiproliferative effects of 13-(S)-HPOTrE and 13-(S)-HOTrE

Similarly the effect of ALA metabolites [13-(S)-HPOTrE and 13-(S)-HOTrE] on Jurkat cells was studied at concentrations 1-20 µM of 13-(S)-HPOTrE and 1-160 µM of 13-(S)-HOTrE. 13-(S)-HPOTrE showed an IC_{50} value around 20 µM at 3 hours (Fig. 32 a) and the value was 160 µM at 6 hours for 13-(S)-HOTrE (Fig. 32 b).

a **b**

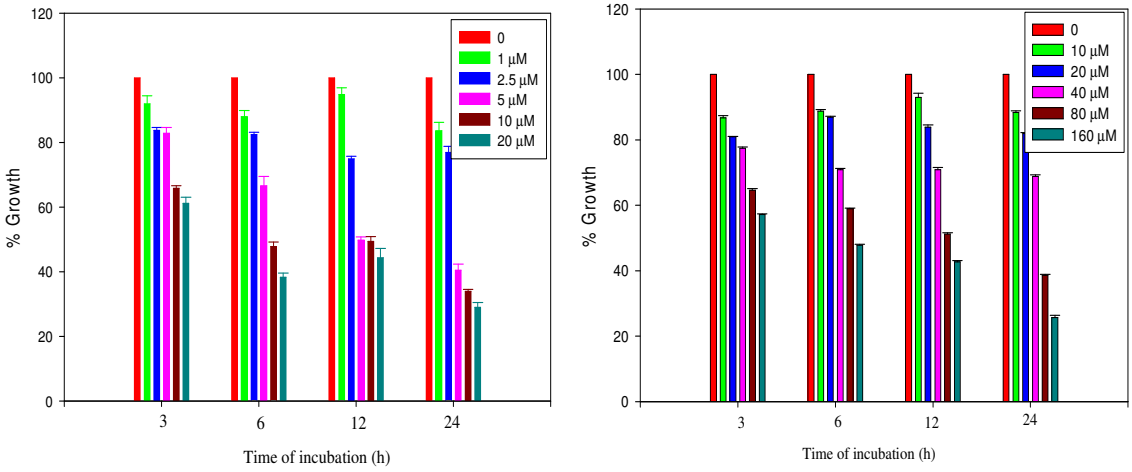


Fig. 32: Effect of 13-(S)-HPOTrE and 13-(S)-HOTrEon the growth of human acute lymphoblastic T-cell leukemia cell line-Jurkat. Cells (5 X10^3) were treated with various concentrations (as indicated in the figure) of **a.** 13-(S)-HPOTrE **b.** 13-(S)-HOTrE, and the cell viability was measured by MTT assay at 3, 6, 12 and 24 h post treatment. The values represent the mean ± S.E from three independent experiments.

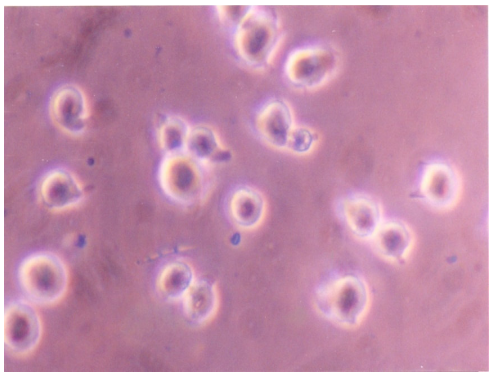
Of all these metabolites of 15-LOX tested, 15-(S)-HPETE and 15-(S)-HETE, the metabolites of arachidonic acid, showed more potent anti-proliferative effects when compared to the metabolites of LA and ALA tested. Hence further studies on the molecular mechanisms were carried only with 15-(S)-HPETE and 15-(S)-HETE.

Chapter 3

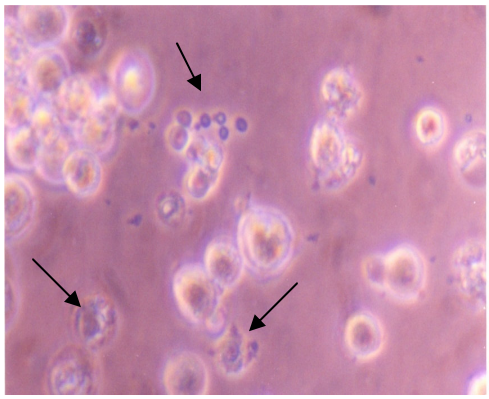
4.5. Morphological changes in Jurkat cells induced by 15-(S)-HPETE and 15-(S)-HETE

Jurkat cells treated with 10 µM 15-(S)-HPETE or 40 µM 15-(S)-HETE for 3 h and 6 h respectively were viewed under phase contrast microscope. The treated cells showed formation of apoptotic bodies (indicated by arrows) unlike the untreated cells (Fig. 33).

a Control Jurkat Cells (400X)



b 15-(S)-HPETE (10 µM) (400X)



c 15-(S)-HETE (40 µM) (400X)

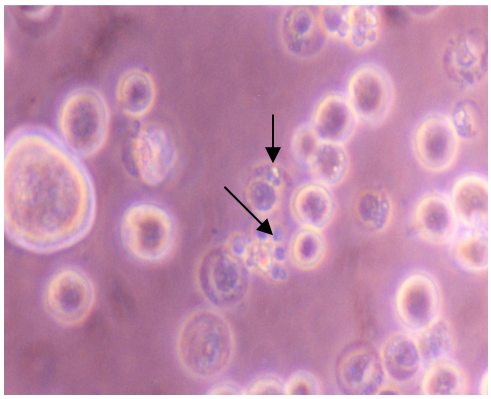


Fig. 33: Phase contrast microscopic analysis of 15-(S)-HPETE and 15(S)-HETE treated Jurkat cells. Jurkat cells treated with 15-(S)-HPETE for 3 h and 15-(S)-HETE for 6 h were photographed under phase contrast microscope**. a.** Control Jurkat cells **b.** Jurkat cells treated with 10 µM 15-(S)-HPETE **c.** Jurkat cells treated with 40 µM 15-(S)-HETE.

Apoptotic bodies were clearly evident in all the treatments. In the control, the cells were clear with many bunches of cells which is the characteristic feature of Jurkat cell line (Fig. 33 a).

4.6. DNA fragmentation induced by 15-(S)-HPETE and 15-(S)-HETE in Jurkat cells

Induction of apoptosis in Jurkat cells treated with 15-LOX metabolites was further evaluated by DNA fragmentation which is considered as a hallmark of apoptosis. Jurkat cells treated with 10 µM 15-(S)-HPETE (lane 2) and 40 µM 15-(S)-HETE (lane 3) showed DNA fragmentation showing a ladder of 180-200 base pairs (Fig. 34), which corresponds to internucleosomal cleavage, a characteristic feature of apoptotic cells. Untreated Jurkat cells (lane 1) did not show any such fragmentation pattern.

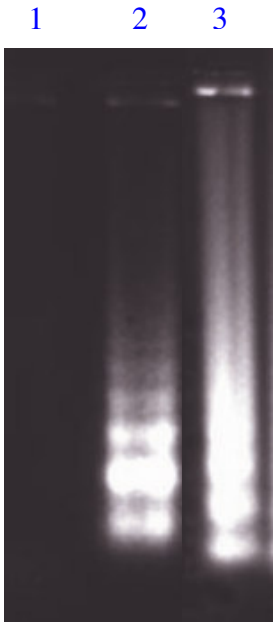


Fig. 34: Analysis of DNA fragmentation in Jurkat cells treated with 15-(S)-HPETE and 15-(S)-HETE. Jurkat cells were treated with 15-(S)-HPETE (10 µM) and 15-(S)-HETE (40 µM) for 3 h and 6 h respectively. DNA was isolated from the treated cells and separated on 1.5% agarose gels. DNA was stained and visualized under UV light. Lane. 1: Vehicle treated control; lane. 2. Cells treated with 15-(S)-HPETE; lane. 3. Cells treated with 15-(S)-HETE.

4.7. Effect of 15-(S)-HPETE and 15-(S)-HETE on cell cycle profile of Jurkat cells: Flow cytometric analysis.

The induction of apoptosis in cells treated with 15-(S)-HPETE and 15-(S)-HETE was further evaluated by flow cytometric analysis of DNA content. Jurkat cells treated with 15-(S)-HPETE (10 µM) (Fig. 35 b) or 15-(S)-HETE (40 µM) (Fig. 35 c) along with untreated cells (Fig. 35 a) were stained with propidium iodide and subjected to FACS analysis. Fig. 35 illustrates the DNA content histograms obtained after PI staining of permeabilized cells that were treated with 15-lipoxygenase metabolites.

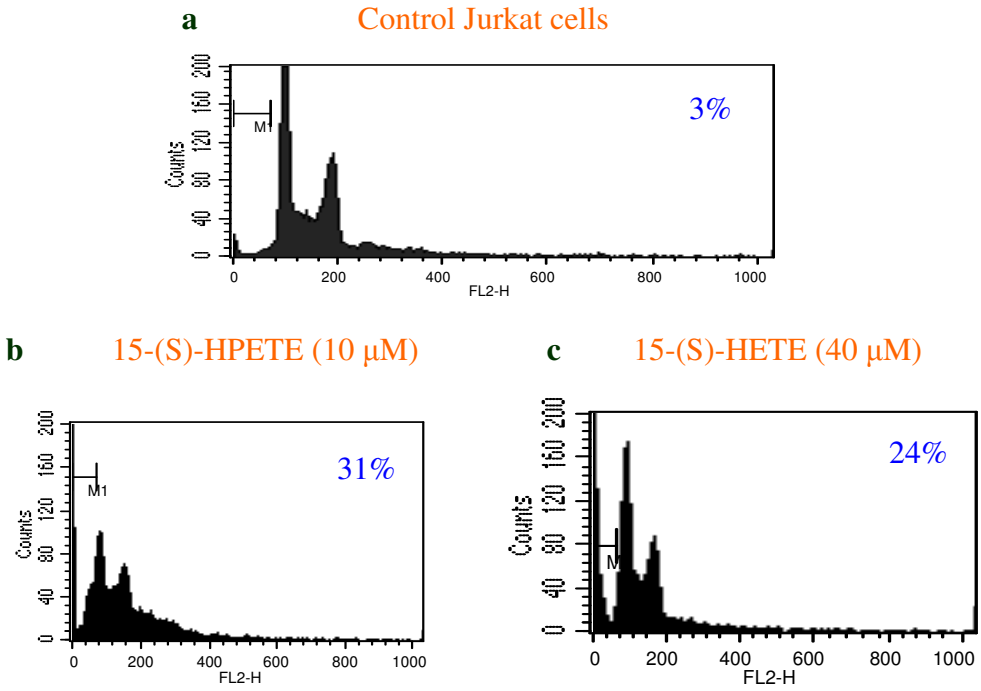


Fig. 35: 15-(S)-HPETE and 15-(S)-HETE induce apoptosis in Jurkat cells- Flow cytometric analysis. Jurkat cells (1.3 x 10^6) were treated with 10 µM 15-(S)-HPETE for 3 h and 40 µM 15-(S)-HETE for 6 h and were fixed in 1ml of 70% ethanol and suspended in PBS. The cells were then stained with PI solution for 1 h and analyzed for DNA content by flow cytometer. Data represent the result from one of three similar experiments. **a.** Control; **b.** 15-(S)-HPETE (10 µM) **c.** 15-(S)-HETE (40 µM).

The FACS analysis of control cells, showed prominent G1, followed by S and G2/M phases. Only around 3.8% of these cells showed hypodiploid DNA

(subG0/G1 peak). The percentage of hypodiploid DNA increased to 31% in case of cells treated with 10 µM 15-(S)-HPETE (Fig. 35 b) and to 24 % with 15-(S)-HETE at 40 µM conc (Fig. 35 c).

4.8. Effect of 15-(S)-HPETE and 15-(S)-HETE on FAS ligand and FAS receptor

Jurkat cells treated with 10 µM 15-(S)-HPETE (0.5 h, 1h, 2 h, and 3 h) and 40 µM 15-(S)-HETE (1h, 2 h, 4 h, 6 h) showed increased expression of Fas ligand (Fig. 36 a & b.) where as the receptor levels remained unchanged (Fig. 37 a & b).

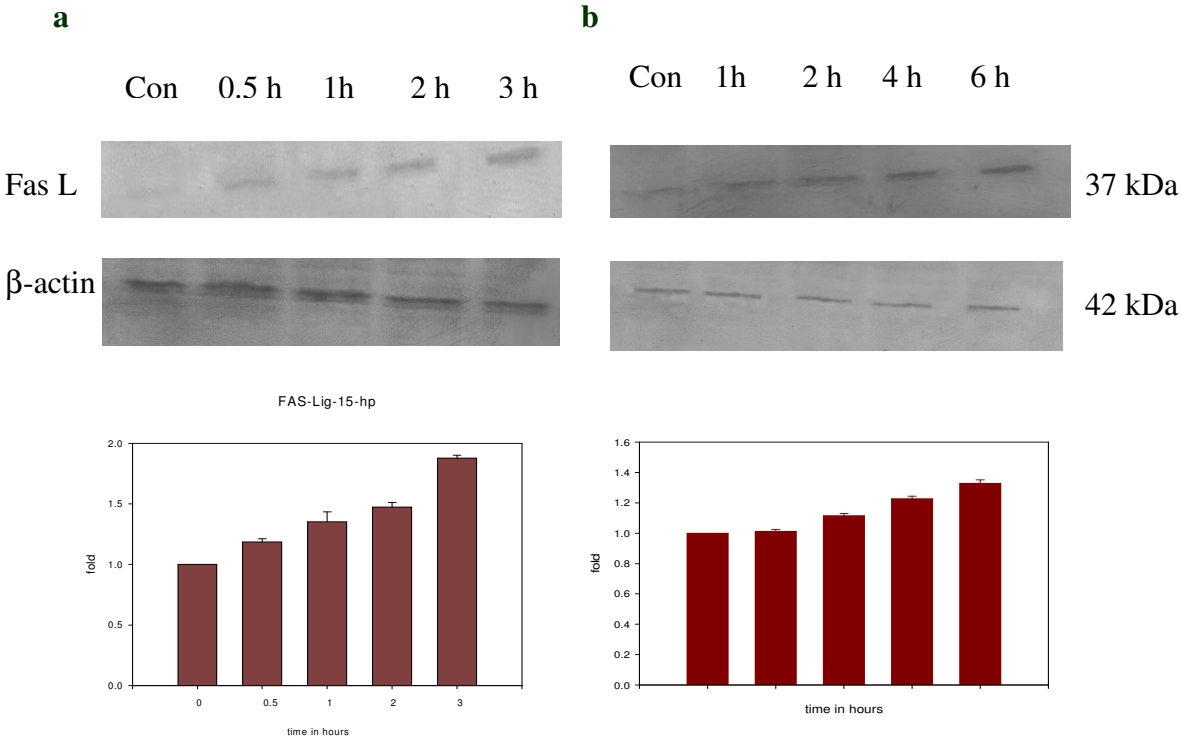


Fig. 36: Western blot analysis of Fas ligand expression during 15-S)-HPETE and 15-(S)-HETE induced apoptosis in Jurkat cells. After the treatment of Jurkat cells for various time periods with **a.** 15-(S)-HPETE (10 µM) and **b.** 15-(S)-HETE (40 µM) as indicated.The cell extracts were resolved on 12% SDS-PAGE and probed against Fas ligand antibodies. β-actin was probed to confirm equal loading. The bar diagrams show the Scion image analysis of the bands normalized with β-actin bands.

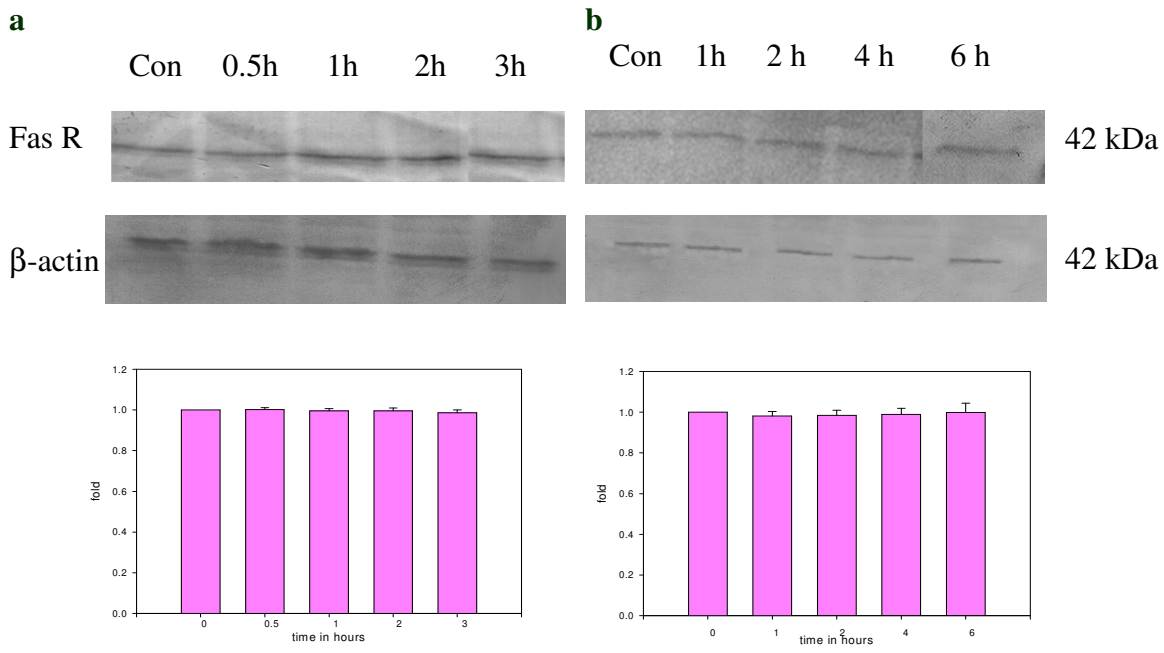


Fig. 37: Immunoblot analysis of Fas receptor expression in 15-(S)-HPETE and 15-(S)-HETE treated Jurkat cells. The cell extracts of Jurkat cells treated with **a.** 15-(S)-HPETE (10 µM) and **b.**15-(S)-HETE (40 µM) for various time periods were probed against Fas receptor antibodies. Equal loading was confirmed by probing with β-actin antibodies. The bar diagrams show the Scion image analysis of the bands normalized with β-actin bands.

4.9. Effect of 15-(S)-HPETE and 15-(S)-HETE on FADD

Binding of FasL to Fas causes a higher order aggregation of receptor molecules and recruitment of adoptor molecule-Fas-associated death domain (FADD) via death domain (DD-DD) interactions. FADD levels increased significantly when the cells were treated with 15-LOX metabolites. (Fig. 38 a & b).

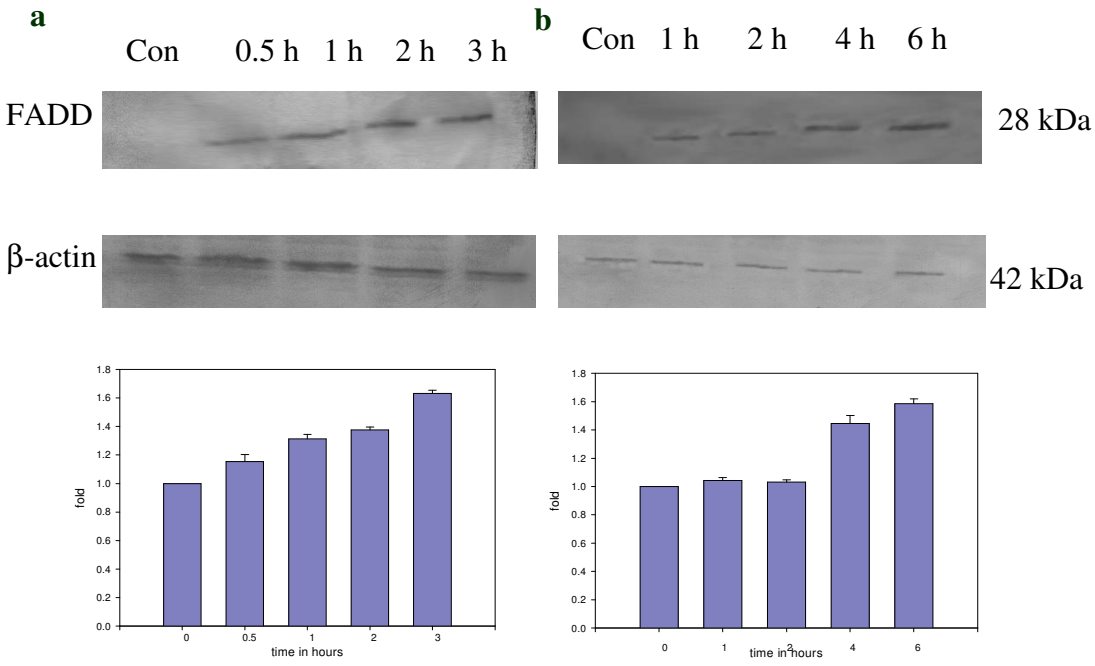


Fig. 38: Western blot analysis of FADD expression in Jurkat cells treated with 15-(S)-HPETE and 15-(S)-HETE. Jurkat cells were treated with **a.** 15-(S)-HPETE (10 μM) and **b.** 15-(S)-HETE (40 μM) metabolites for various time points as indicated. The cell extracts were resolved on 12% SDS-PAGE and probed against FADD antibodies. Equal loading was confirmed by β-actin. The bar diagrams show the Scion image analysis of the bands normalized with β-actin bands.

The same cell extract was used for the western blot analysis of Fas ligand, Fas receptor and FADD. So, the same β-actin blots were shown for the three proteins.

4.10. Caspase-8 activation in response to 15-(S)-HPETE and 15-(S)-HETE treatments.

FADD also has another domain called the death effector domain, which in turn recruits pro-caspase-8 (FLICE) and /or pro-caspase-10 to the receptor. The resulting multimeric protein complex is called death-inducing signaling complex

(DISC), and forms within seconds of receptor engagement. At the DISC, pro-caspase-8 (and/or-10) is activated.

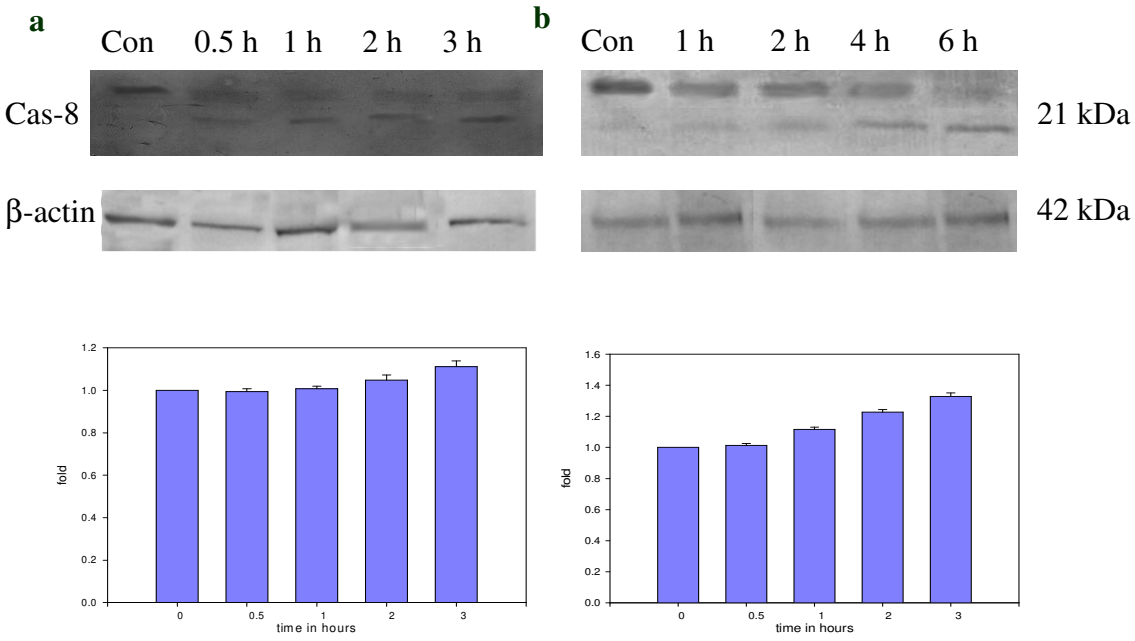


Fig. 39: Caspase-8 activation in 15-(S)-HPETE and 15-(S)-HETE treated Jurkat cells. Cells were treated with either **a.** 15-(S)-HPETE (10 μM) or **b.** 15-(S)-HETE (40 μM) for indicated time periods. After the treatments cell extracts were separated on 12% SDS PAGE and probed against caspase-8 antibodies that detect cleaved caspase-8 fragments. β-actin was probed to confirm equal loading. The bar diagrams show the Scion image analysis of the bands normalized with β-actin bands.

Western blot analysis of 15-LOX metabolite treated Jurkat cells showed increased expression of caspase-8 levels compared to control cells. Active caspase-8 expression levels were increased when the cells were treated with 10 μM 15-(S)-HPETE or 40 μM 15-(S)-HETE in a time dependent manner (Fig. 39 a & b). In order to quantify caspase-8 activity, a fluorometric assay was carried with caspase-8 substrate, Ac-IETD-AMC. As shown in Fig. 40 a & b, 15-(S)-HPETE and 15-(S)-HETE treated cells showed increase in caspase-8 activity in time dependent manner reaching maximum at 3 h and 6 h respectively. Z-IETD-FMK, a caspase-8 inhibitor, inhibited caspase-8 activity completely in both the treatments.

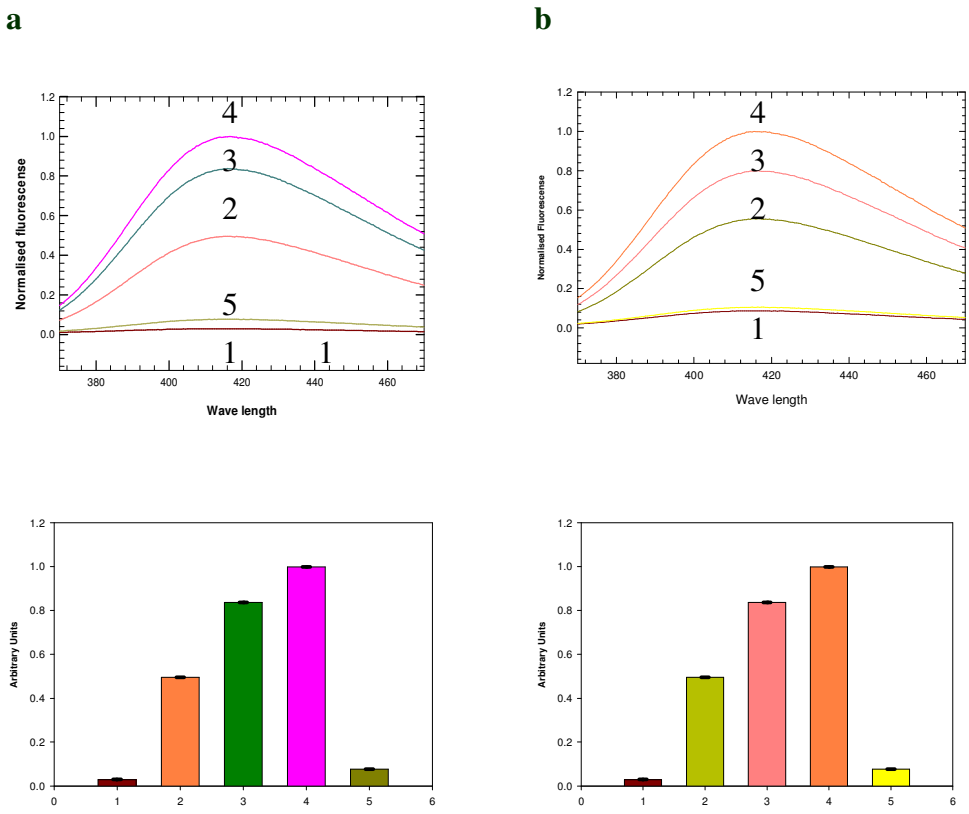


Fig. 40: Fluorometric analysis of 15-(S)-HPETE and 15-(S)-HETE induced caspase-8 activation. Jurkat cells after treatment with 10 µM 15-(S)-HPETE (for 1, 2 and 3 h) and 40 µM 15-(S)-HETE (for 2, 4 and 6 h) were lysed and assayed for caspase-8 activity with a fluorescence caspase-8 substrate- Ac-IETD-AMC. Z-IETD-FMK, a specific inhibitor for caspase-8 was used as an assay control. **a.** 15-(S)-HPETE (10 µM) treatments. 1. Control; 2. 10 µM 15-(S)-HPETE -1 h; 3. 10 µM 15-(S)-HPETE -2 h; 4. 10 µM 15-(S)-HPETE-3 h; 5. 10 µM 15-(S)-HPETE- 3 h + Z-IETD-FMK (1µg/ml) **b.** 15-(S)-HETE (40 µM) treatments. 1. Control; 2. 40 µM 15-(S)-HETE -2 h; 3. 40 µM 15-(S)-HETE -4 h; 4. 40 µM 15-(S)-HETE-6 h; 5. 40 µM 15-(S)-HETE-6 h + Z-IETD-FMK (1µg/ml). The changes in the activity levels were also shown by bar diagrams

Caspase-8 mediates cleavage of the Bcl-2 family member Bid. Truncated Bid translocates to the mitochondria where it can induce both the oliogomerization of pro-apoptotic Bax and /or Bak in the membrane and the release of pro-apoptotic molecules, including cytochrome c, from the mitochondrial intermembrane space. Treatment of Jurkat cells with 15-LOX metabolites showed decreased expression of Bid levels time dependently (Fig.41).

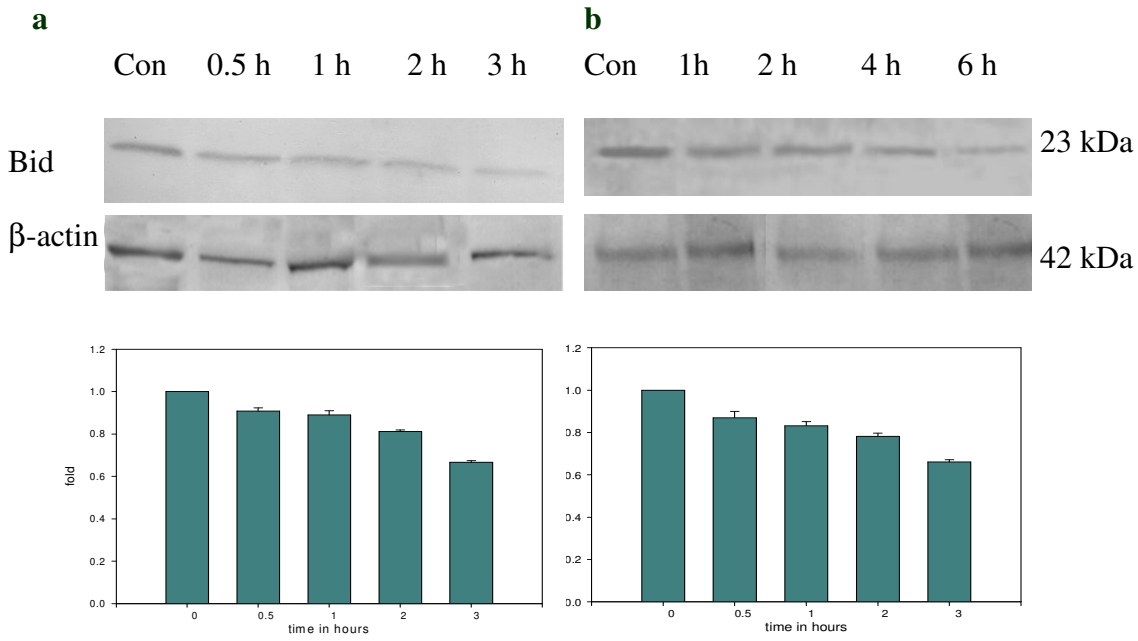


Fig. 41: Western blot analysis of Bid expression during 15-(S)-HPETE and 15-(S)-HETE induced apoptosis in Jurkat cells. After the treatment of Jurkat cells with **a.** 15-(S)-HPETE (10 μM) and **b.** 15-(S)-HETE (40 μM) for various time periods as indicated. The cell extracts were resolved on 12% SDS-PAGE and probed against Bid antibodies. β-actin was probed to confirm equal loading. The bar diagrams show the Scion image analysis of the bands normalized with β-actin bands.

The same cell extract was used for the western blot analysis of caspase-8, Bid and caspase-3. So, the same β-actin blots were shown for the three proteins.

4.11. Effect of 15-(S)-HPETE and 15-(S)-HETE on Akt and pAkt

Possible involvement of Akt in the 15-LOX metabolite induced apoptosis was analyzed. The Western blot analysis of Akt, p-Akt in Jurkat cells treated with 15-(S)-HPETE (10 μM) and 15-(S)-HETE (40 μM) is presented in Fig. 42 and Fig. 43 respectively. The level of pAkt decreased in the cells treated with 15-LOX metabolites (Fig. 43). The levels of Akt, on the other hand, increased in all the treatments compared to the control (Fig. 42). Taken together, these results indicate that 15-LOX metabolites induce apoptosis in Jurkat cells through Akt/p-Akt signaling pathway.

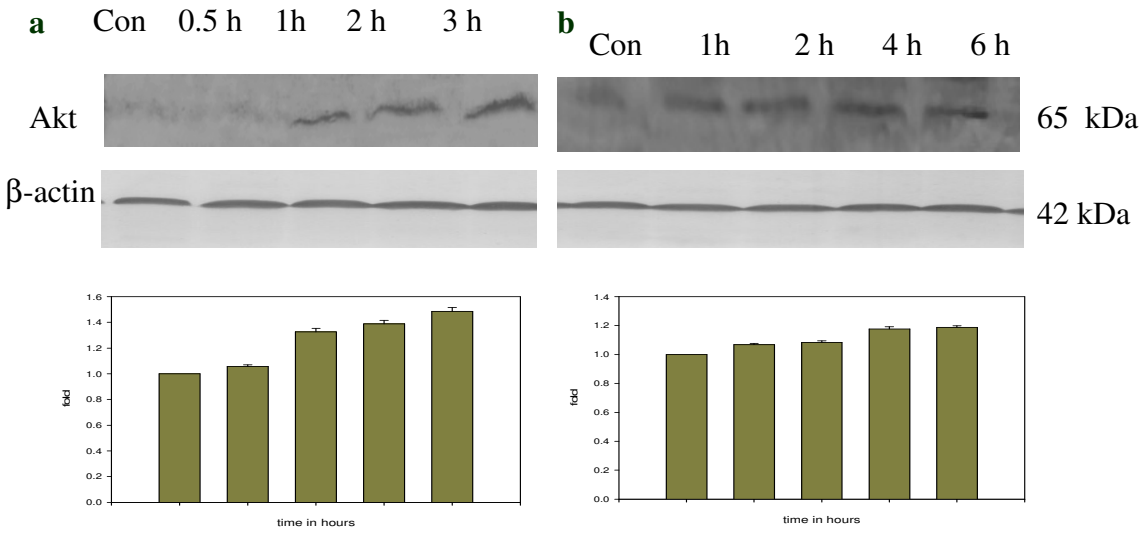


Fig. 42: Western blot analysis of Akt expression in Jurkat cells treated with 15-(S)-HPETE and 15-(S)-HETE. Jurkat cells were treated with **a.** 15-(S)-HPETE (10 µM) and **b.** 15-(S)-HETE (40 µM) for various time points as indicated. The cell extracts were resolved on 12% SDS-PAGE and probed against Akt antibodies. Equal loading was confirmed by β-actin. The bar diagrams show the Scion image analysis of the bands normalized with β-actin bands.

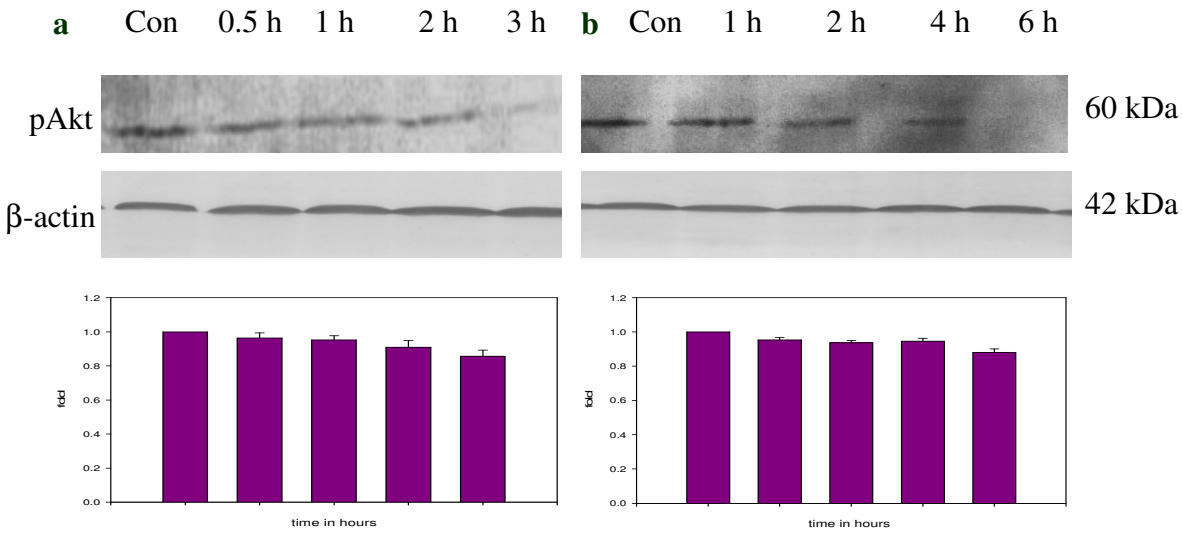


Fig. 43: Western blot analysis of pAkt expression in Jurkat cells treated with 15-(S)-HPETE and 15-(S)-HETE. Jurkat cells were treated with **a.** 15-(S)-HPETE (10 µM) and **b.** 15-(S)-HETE (40 µM) for various time points as indicated. The cell extracts were resolved on 12% SDS-PAGE and probed against pAkt antibodies. Equal loading was confirmed by β-actin. The bar diagrams show the Scion image analysis of the bands normalized with β-actin bands.

The same cell extract was used for the western blot analysis of Akt and pAkt. So, the same β-actin blots were shown for the two proteins.

4.12. Study of mitochondrial membrane potential (Flow cytometric analysis)

In cell death, the outer membrane permeability often increases, allowing for the release of soluble proteins that usually are retained within mitochondria, in the intermembrane space (IMS). The death associated outer membrane permeabilization is not only an accidental process but also a tightly regulated phenomenon, with major consequences for health and disease. The decrease in mitochondrial membrane potential ($\Delta\Psi m$) is associated with mitochondrial dysfunction (Seuduto and Grotyohann, 1999). In the present study changes in the membrane potential of Jurkat cells exposed to 10 μM 15-(S)-HPETE and 40 μM 15-(S)-HETE were investigated. The cells were harvested and the mitochondrial membrane potentials were measured by the uptake of lipophilic cationic dye-Rhodamine 123 into mitochondria (Kroemer and Reed, 2000). Untreated control cells were used to determine the normal uptake of rhodamine and the percentage of treated cells with low membrane potentials was calculated. Fig. 44 a & b shows a decrease of 70% in the fluorescence intensity in the cells treated with 10 μM 15-(S)-HPETE and 60% with 40 μM 15-(S)-HETE, compared to that of control. These results indicate impaired mitochondrial membrane potentials in the cells treated with 15-LOX metabolites and thus leading to the leakage of mitochondrial components.

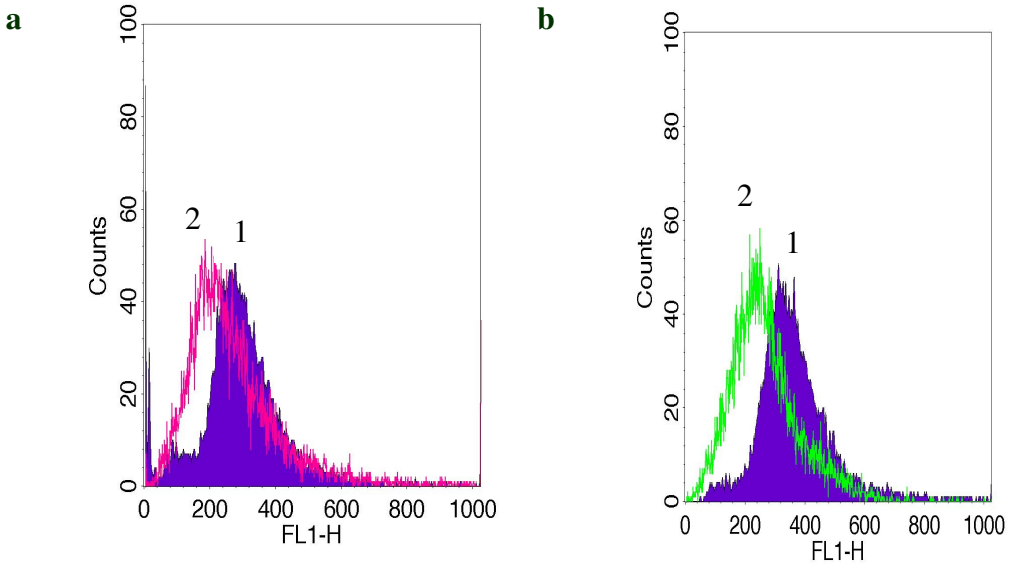


Fig. 44: Measurement of mitochondrial membrane potentials upon treatment of Jurkat cells with 15-(S)-HPETE and 15-(S)-HETE. Induction of mitochondrial dysfunction and relative mitochondrial membrane potential (Δψm) was measured by fluorescent emission from rhodamine 123 uptake by mitochondria. Jurkat cells were treated with 10 µM 15-(S)-HPETE for 3 h **a.**. Histogram-1 represents fluorescence intensity from control Jurkat cells, histogram-2 represents fluorescence intensity from Jurkat cells treated with 15-(S)-HPETE (10 µM). and **b.** 40 µM HETE for 6 h and stained with rhodamine 123 and subjected to FACS analysis. Histogram-1 represents fluorescence intensity from control Jurkat cells histogram-2 represents fluorescence intensity from Jurkat cells treated with 15-(S)-HETE (40 µM).

4.13. Effect of 15-(S)-HPETE and 15-(S)-HETE on cytochrome *c* release, Bcl-2 and Bax proteins in Jurkat cells.

The process of apoptosis is associated with the disruption of mitochondrial membrane potentials, which results from the opening of permeability transition pores in the mitochondrial membrane, leading to the release of cytochrome *c*. To determine whether there is any release of cytochrome *c* from the mitochondria into the cytosol, cytosolic fractions from the cells treated with 10 µM 15-(S)-HPETE and 40 µM 15-(S)-HETE for indicated times, were subjected to Western blot analysis. A time dependent elevation in the levels of cytochrome *c* with maximum increase at 3 and 6 h after 15-(S)-HPETE and 15-(S)-HETE treatments respectively was observed (Fig. 45 a & b).

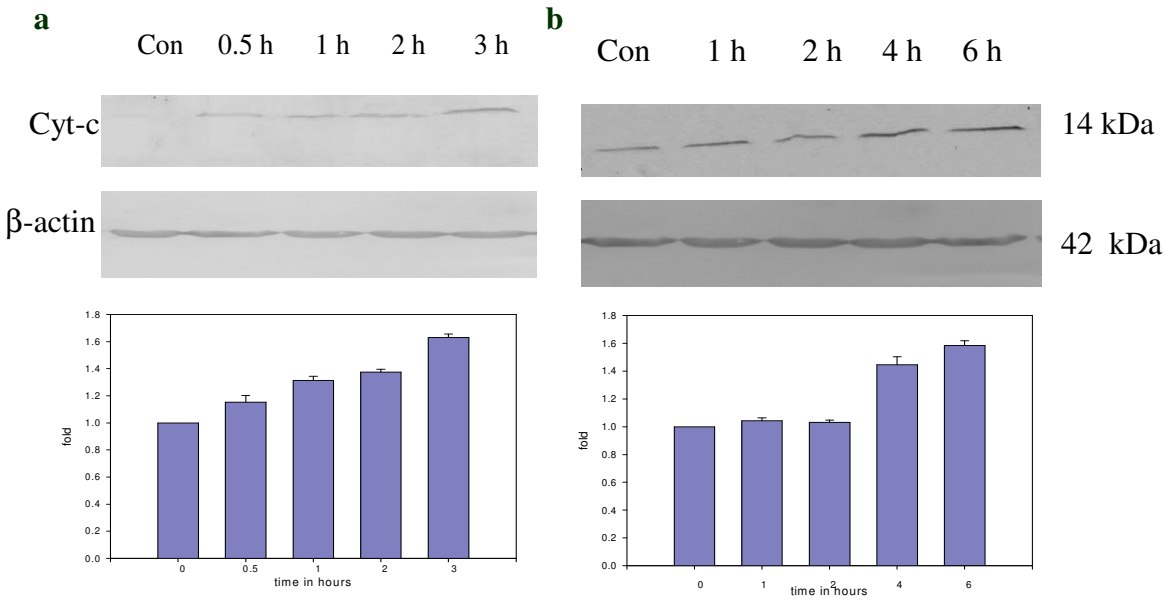


Fig. 45: Effect of 15-(S)-HPETE and 15-(S)-HETE on cytochrome *c* release. Jurkat cells were treated with **a.** 15-(S)-HPETE (10 μM) and **b.** 15-(S)-HETE (40 μM) for indicated times. Equal quantities of protein (30 μg) were analyzed by 15% SDS-PAGE and immunoblotted with anti-cytochrome *c* antibody. β-actin was used as control for equal loading of protein. The bar diagrams show the Scion image analysis of the bands normalized with actin bands.

Furthermore, the expression levels of Bcl-2 and Bax proteins are associated with mitochondrial membrane integrity and play a crucial role in the regulation of apoptosis. Time dependent decrease of Bcl-2 protein levels was observed after treatment (Fig. 46 a & b), but no appreciable change in the levels of Bax protein was observed at all the time periods (Fig. 47 a & b). These results suggest an altered Bcl-2/Bax ratio in the cells treated with 15-(S)-HPETE and 15-(S)-HETE.

The same cell extract was used for the western blot analysis of Bcl-2 and Bax. So, the same β-actin blots were shown for the two proteins.

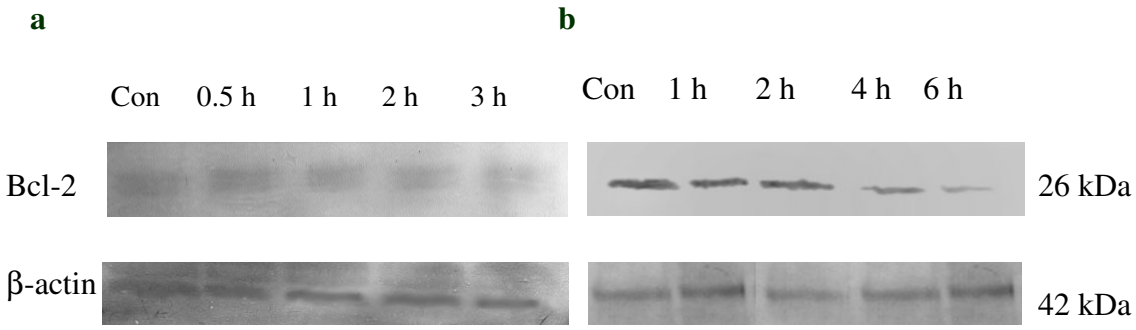


Fig. 46: Immunoblot analysis of Bcl-2 expression in 15-(S)-HPETE and 15-(S)-HETE treated Jurkat cells. The cell extracts of Jurkat cells treated with **a.** 15-(S)-HPETE (10 µM) and **b.** 15-(S)-HETE (40 µM) metabolites were resolved on 15% SDS-PAGE and probed against Bcl-2 antibodies. β-actin was probed to confirm equal loading. The bar diagrams show the Scion image analysis of the bands normalized with β-actin bands.

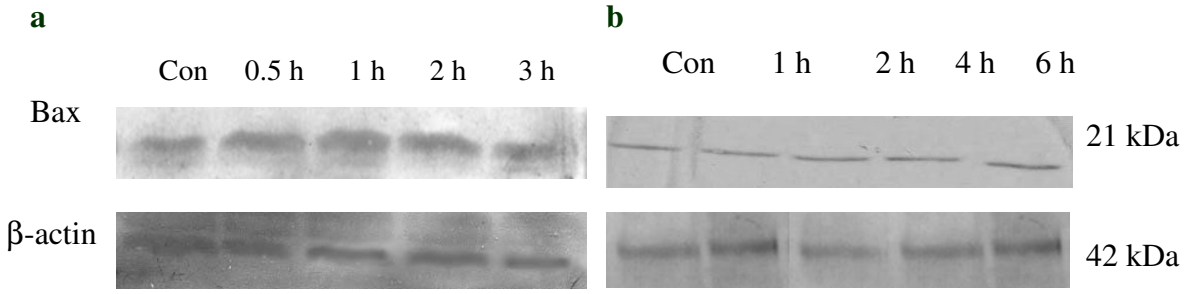
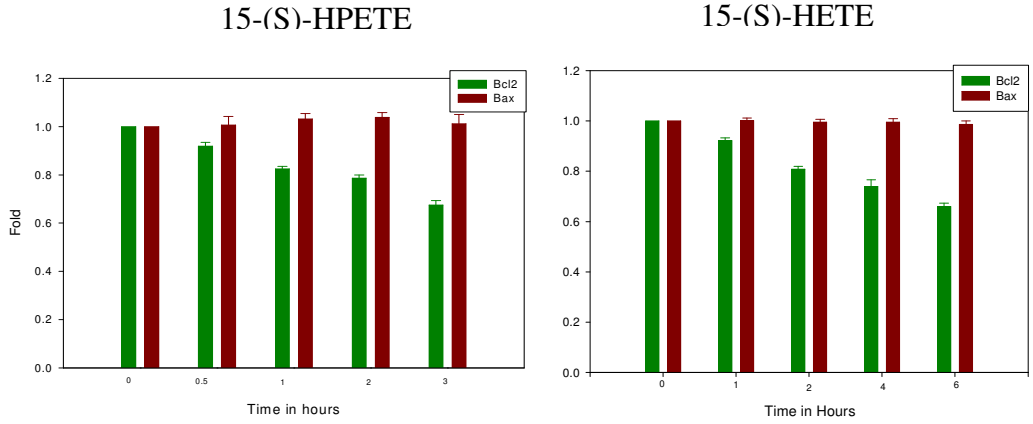


Fig. 47: Western blot analysis of Bax expression in 15-(S)-HPETE and 15(S)-HETE treated Jurkat cells. The cell extracts of Jurkat cells treated with **a.** 15-(S)-HPETE (10 µM) and **b.** 15-(S)-HETE (40 µM) metabolites were resolved on 15% SDS-PAGE and probed against Bax antibodies. β-actin was probed to confirm equal loading. The bar diagrams show the Scion image analysis of the bands normalized with β-actin bands.



4.14. Caspase-3 activation in response to 15-(S)-HPETE and 15-(S)-HETE treatments.

Cytochrome *c* leakage into the cytosol results in the activation of caspase-3 which cleaves the cellular proteins and eventually steer a cell to undergo apoptosis. As 15-LOX metabolite treatment of Jurkat cells resulted in the leakage of cytochrome *c*, we examined for the activation of caspase-3. Indeed caspase-3 was activated during 15-LOX metabolite-induced apoptosis as shown by Western blot analysis and caspase-3 activity assay. Western blot analysis carried out with antibodies specific to cleaved caspase-3 showed time dependent increase in the levels of the caspase-3 protein in cells treated with both 10 µM 15-(S)-HPETE and 40 µM 15-(S)-HETE (Fig. 48 a & b).

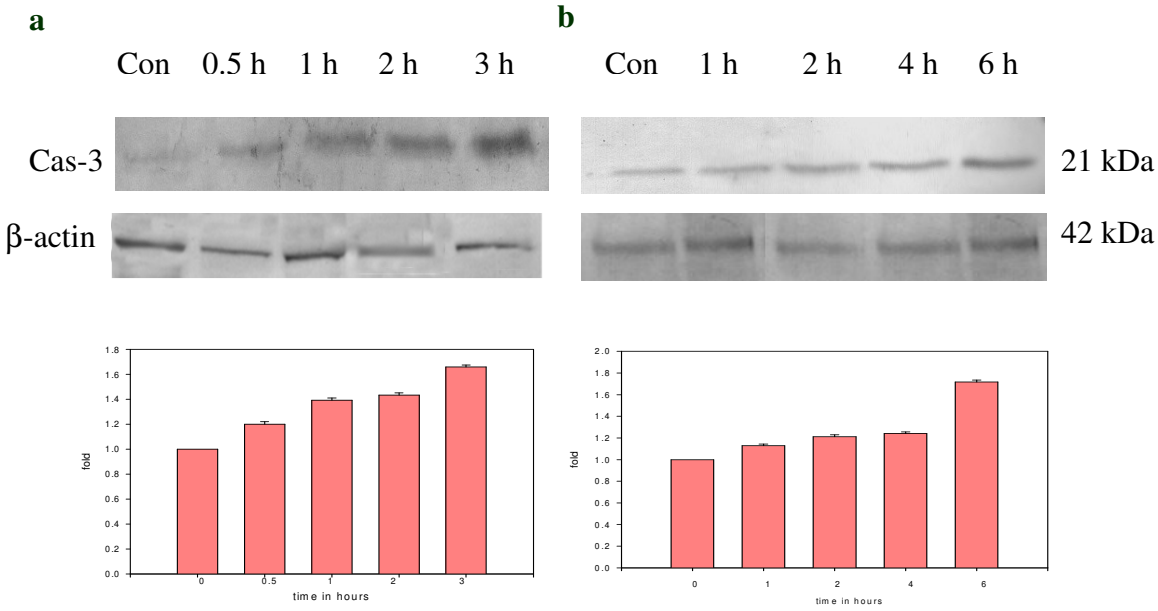


Fig. 48: Caspase-3 activation during 15-(S)-HPETE and 15-(S)-HETE induced apoptosis in Jurkat cells. Cells were treated with either **a.** 15-(S)-HPETE (10 µM) and **b.**15-(S)-HETE (40 µM) for indicated time periods. After the treatments cell extracts were separated on 12% SDS PAGE and probed against caspase-3 antibodies that detect cleaved caspase-8 fragments. β-actin was probed to confirm equal loading. The bar diagrams show the Sion image analysis of the bands normalized with β-actin bands.

In order to quantify the caspase-3 activity, a fluorometric assay was carried with caspase-3 substrate, Ac-DEVD-AFC. As shown in Fig. 49 a & b, 10 µM 15-(S)-HPETE treatment for 3 h increased caspase-3 activity compared to control. Cells treated with 40 µM 15-(S)-HETE also showed increase in caspase-3 activity by 6 h. Ac-DEVD-CHO (1 µg/ml), a caspase-3 specific inhibitor, when added to the assay mixtures, complete inhibition of the caspase-3 activity was observed in both the treatments.

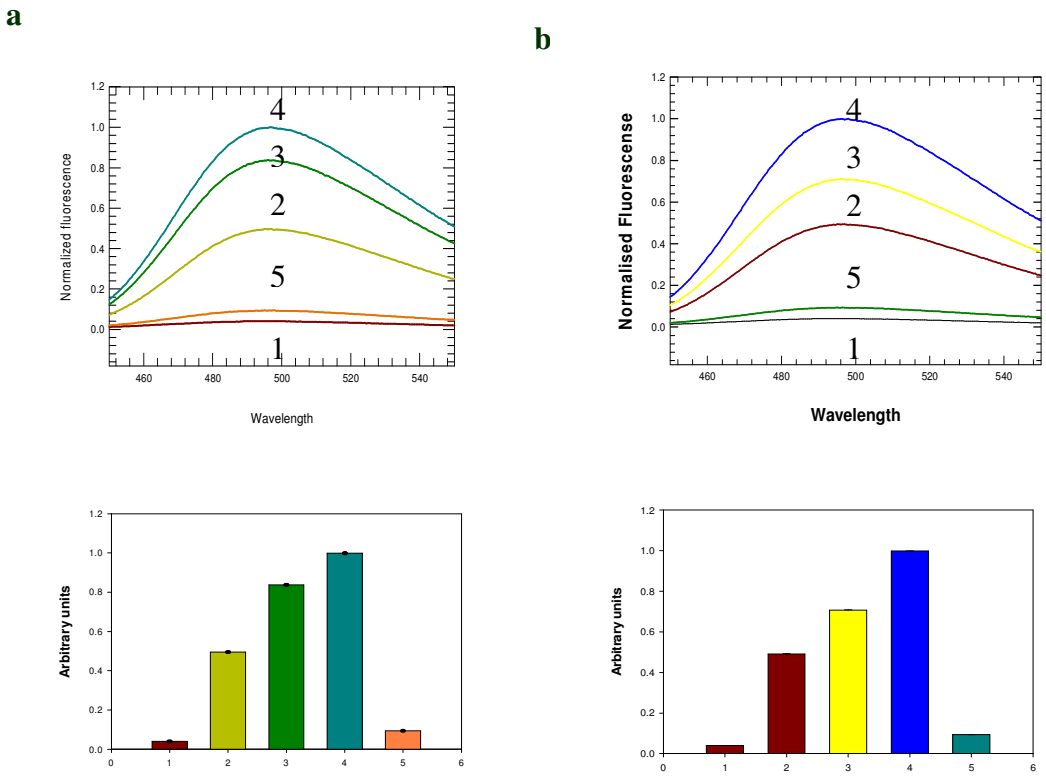


Fig. 49: Fluorometric analysis of 15-(S)-HPETE and 15-(S)-HETE induced caspase-3 activation. Jurkat cells after treatment with 10 µM 15-(S)-HPETE (for 1, 2 and 3 h) and 40 µM 15-(S)-HETE (for 2, 4 and 6 h) were lysed and assayed for caspase-3 activity with a fluorescence caspase-3 substrate- Ac-DEVD-AFC. AC -DEVD-CHO, a specific inhibitor for caspase-3 was used as an assay control. **a.** 15-(S)-HPETE (10 µM) treatments. 1. Control; 2. 10 µM 15-(S)-HPETE -1 h; 3. 10 µM 15-(S)-HPETE -2 h; 4. 10 µM 15-(S)-HPETE-3 h; 5. 10 µM 15-(S)-HPETE- 3 h + AC -DEVD-CHO (1µg/ml) **b.** 15-(S)-HETE (40 µM) treatments. 1. Control; 2. 40 µM 15-(S)-HETE -2 h; 3. 40 µM 15-(S)-HETE -4 h; 4. 40 µM 15-(S)-HETE-6 h; 5. 40 µM 15-(S)-HETE-6 h + AC -DEVD-CHO (1 µg/ml). The changes in the activity levels were also shown by bar diagrams.

4.15. PARP cleavage in response to 15-(S)-HPETE and 15-(S)-HETE treatments

PARP, poly (ADP ribose) polymerase, is a nuclear enzyme implicated in many cellular processes including apoptosis and DNA repair. During apoptosis PARP (116 kDa) is cleaved to yield two fragments of 85 and 23 kDa. To determine whether PARP is cleaved in 15-LOX metabolite-induced cell death, Jurkat cells were treated with 10 µM 15-(S)-HPETE and 40 µM 15-(S)-HETE for indicated times and PARP cleavage was monitored by employing PARP antibodies that recognize the 85 kDa fragment of cleaved PARP and uncleaved 116 kDa full length PARP. The data presented in Fig. 50 a & b show the gradual increase in the 85 kDa fragment of PARP with simultaneous decrease of 116 kDa uncleaved PARP at all the indicated times.

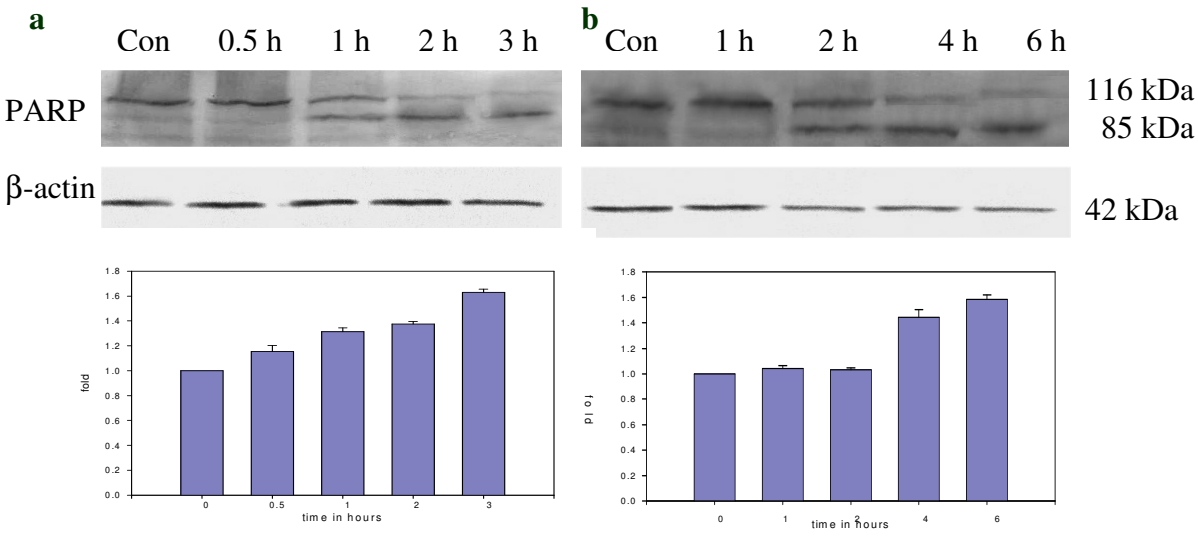


Fig. 50: Detection of PARP cleavage by western blot analysis in 15-(S)-HPETE and 15-(S)-HETE treated Jurkat cells. Jurkat cells with 15-(S)-HPETE (10 µM) **a.** and 15-(S)-HETE (40 µM) **b.** for indicated time periods. 100 µg of total protein extract was separated on 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane. Intact PARP (116 kDa) and the cleavage product of PARP (85 kDa) were detected using anti-PARP antibody. β-actin was probed to confirm equal loading. The bar diagrams show the Scion image analysis of the bands normalized with actin bands (for 85 kDa) protein.

4.16. Generation of Reactive Oxygen Species (ROS) upon treatment with 15-(S)-HPETE and 15-(S)-HETE

DCFH-DA analysis was done to see the ROS generation in Jurkat cells upon treatment with 10 µM 15-(S)-HPETE and 40 µM 15-(S)-HETE. Significant generation of ROS was observed within minutes as evidenced by the shift in DCF fluorescence when the cells were treated with 10 µM 15-(S)-HPETE and 40 µM 15-(S)-HETE (Fig. 51 a & b). The magnitude of ROS generation was more in case of cells treated with 15-(S)-HPETE when compared to 15-(S)-HETE.

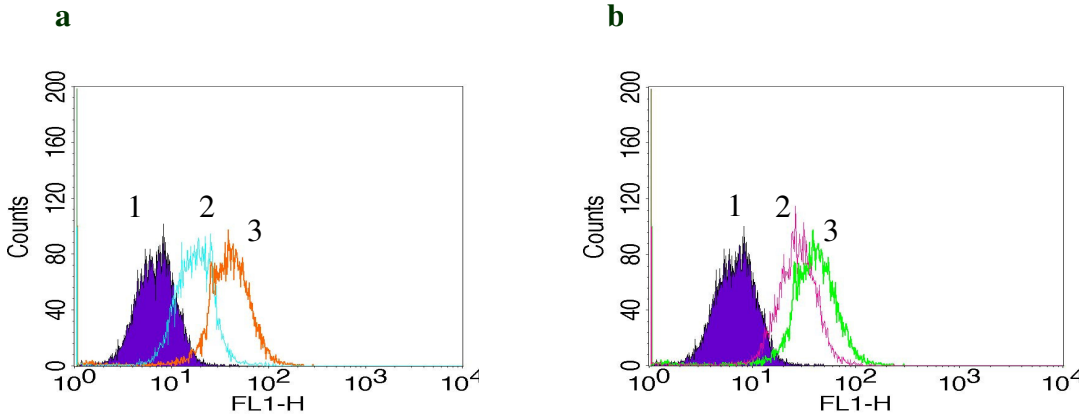


Fig. 51: DCFH-DA analysis of 15-(S)-HPETE and 15-(S)-HETE induced ROS generation in Jurkat cells. Jurkat cells were incubated with 10 µM DCFH-DA for 15 min and then washed and incubated in PBS with either 10 µM 15-(S)-HPETE or 40 µM 15-(S)-HETE for indicated time periods. DCF fluorescence was detected by flow cytometry after stipulated treatments using 530 nm emission filter. **a.** 1. Vehicle treated control; 2. 10 µM 15-(S)-HPETE –15 min; 3. 10 µM 15-(S)-HPETE – 30 min; **b.** 1. Vehicle treated control; 2. 40 µM 15-(S) - HETE –30 min; 3. 40 µM 15-(S)-HETE – 45 min.

4.17. Activation of NADPH Oxidase by 15-(S)-HPETE and 15-(S)-HETE

Pretreatment of Jurkat cells with 10 µM DPI, NADPH Oxidase inhibitor, for 45 min followed by treatments with 10 µM 15-(S)-HPETE for 1 h or 40 µM 15-(S)-HETE for 1.5 h, resulted in reduced intracellular ROS levels (Fig. 52 a & b). Pretreatment of cells with 10 µM DPI inhibited 85% of ROS production induced by 15-(S)-HPETE and 76% of ROS production induced by 15-(S)-HETE. These results demonstrate that NADPH oxidase activation is responsible for ROS production in case of both 15- (S)-HPETE and 15-(S)-HETE treatments.

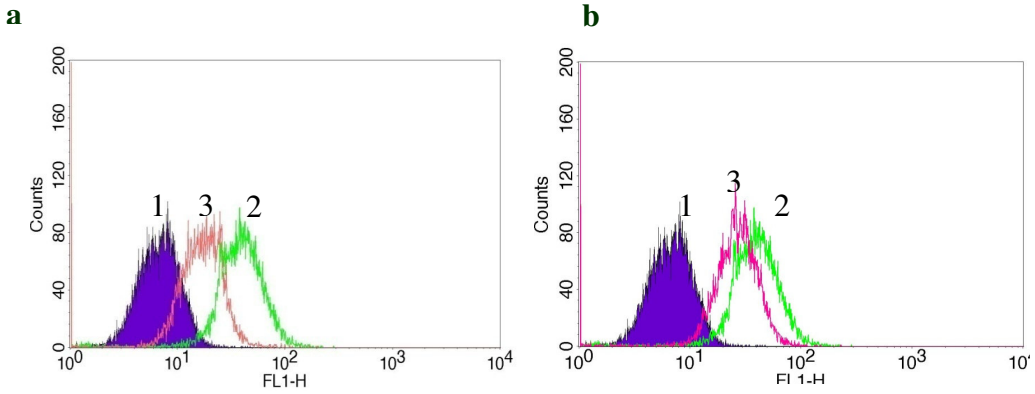


Fig. 52: Inhibition of ROS production in 15-(S)-HPETE and 15-(S)-HETE treated Jurkat cells by DPI, an NADPH oxidase inhibitor. Jurkat cells were pretreated with 10 µM DPI, an NADPH Oxidase inhibitor for 1 h and followed by the treatment with 15-lipoxygenase metabolites and analyzed for ROS production. **a.** 1. Vehicle treated control; 2. 10 µM 15-(S)-HPETE – 30 min; 3. 10 µM 15-(S)-HPETE + 10 µM DPI. and **b.** 1. Control, 2. 40 µM 15-(S)-HETE – 45 min 3. 40 µM 15-(S)-HETE – 45 min + 10 µM DPI. Data represent one of the three independent experiments.

To determine the role of ROS generation and glutathione depletion associated with it in 15-LOX metabolite-induced apoptosis, Jurkat cells were pretreated with 50 µM NAC (N-acetyl cysteine, a glutathione precursor and an antioxidant) for 3 h followed by exposure to 10 µM 15-(S)-HPETE or 40 µM 15-(S)-HETE and analyzed for ROS production and inhibition of apoptosis. NAC

pretreatment resulted in reduction of cellular ROS levels by 52 % incase of 15-(S)-HPETE (Fig. 53 a & b) and by 63 % incase of 15-(S)-HETE.

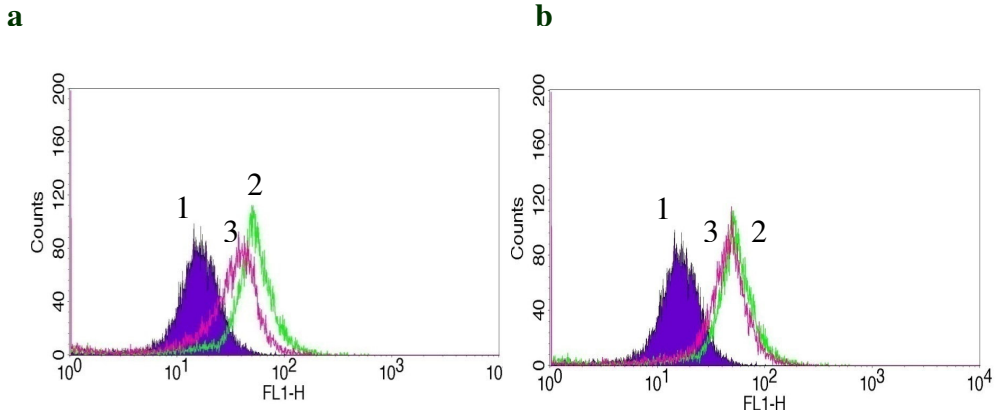


Fig. 53: DCFH-DA analysis of ROS production in Jurkat cells pretreated with N-Acetyl cysteine (NAC– glutathione precursor). Jurkat cells were pretreated with 50 µM NAC for 3 h followed by treatment with 15-LOX metabolites. **a.** 1. Vehicle treated control; 2. 10 µM 15-(S)-HPETE – 60 min; 3. 10 µM 15-(S)-HPETE – 60 min + 50 µM NAC; **b.** 1. Vehicle treated control; 2. 40 µM 15-(S)-HETE – 90 min; 3. 40 µM 15-(S)-HETE – 90 m in + 50 µM NAC. Data represent one of the three independent experiments.

Discussion

5. Discussion

Omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFAs) are essential fatty acids necessary for human health. Cardiovascular disease, cancer, obesity, and diabetes collectively are responsible for more than 80% of the disease-related mortality (Berquin et al., 2008). Lipids play critical roles in all of these diseases, and the relative amounts and the types of dietary lipids consumed are believed to be of critical importance. Total fat intake and the ratio of ω-6 to ω-3 PUFAs in the diet have increased significantly since the industrial revolution (Simopoulos, 2002, 1999,).

Linoleic acid and α-linolenic acid are essential polyunsaturated fatty acids, the former a ω-6 and the latter a ω-3 fatty acid (Fischer, 1989; Yamamoto et al., 1987). In mammalian cells, linoleic acid is converted to arachidonic acid by chain elongation and desaturation (δ-6 desaturase); α-linolenic acid is also converted to eicosapentaenoic acid (EPA) and then to docosahexaenoic acid (DHA) by chain elongation and desaturation.

Many investigators have demonstrated that ω-6 and ω-3 PUFAs- including linoleic acid (LA), gamma-linoleic acid (GLA), dihommo-γ-linolenic acid (DGLA), arachidonic acid (AA), alpha- linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)-inhibit growth and are cytotoxic to cancer cells *in-vitro* (Shirota et al., 2005; Menendz et al., 2001; Albino et al., 2000; Hawkins et al., 1998; Lai et al., 1996; Das, 1991; Chow et al., 1989; Begin et al., 1988, 1985; Fujiwara et al., 1986;), that the effects are associated with the production of lipid peroxides and aldehydes (Shirota et al., 2005; Hawkins et al., 1998; Das, 1991; Chow et al., 1989; Fujiwara et al., 1986) and that the cytotoxicity of the added PUFAs is reduced by the addition of antioxidants (Shirota et al., 2005; Hawkins et al., 1998; Das, 1991; Chow et al., 1989; Fujiwara et al., 1986). Studies on laboratory animals have also demonstrated that feeding a diet containing peroxidation products of fish oil (Hardman et al., 2001) reduces tumor growth, and that the effect is

reduced by administering antioxidants (Hardman et al., 2001; Gonzalez et al., 1993). However, these effects *in-vitro* are observed at PUFA concentrations (30 μM and above in most studies) exceeding normal plasma free fatty acid (FFA) levels.

Epidemiological literature on the association of ω-3 PUFAs and cancer, including corelational and migrational studies, suggest a protective effect of ω-3 PUFAs and a promoting effect of ω-6 PUFAs on cancer (Berquin et al., 2008). A number of biological effects that could contribute to cancer promotion by ω-6 PUFAs and cancer suppression by ω-3 PUFAs have been suggested (Chapkin et al., 2007 and Larsson et al., 2004). However, the results of such studies are mixed, and failed to demonstrate a statistically significant association between n-3 PUFAs and reduced cancer risk (Chen at al., 2007; Chen et al., 2006; MacLean et al., 2006; Larsson et al., 2004; Terry et al., 2003; Simopoulos, 2002).

A number of studies have shown the protection offered by ω-3 fatty acids in rodent models of breast cancer. These include dietary supplementation of mouse transplantable tumors (Gabor et al., 1985) and human cell xenograft models (Rose and Connolly, 1995, 1993) as well as chemically induced tumors in rats (Braden and Carroll, 1986; Jurkowski and Cave 1985; Reddy et al., 1980). Dietary ω-6 and ω-3 PUFAs can be metabolized to prostaglandins (PG), thromboxanes (TX), hydroxyeicosatetraenoic acids (HETEs), and leukotrienes (LT) by the enzymatic activity of COX and LOX. Generally speaking, eicosanoids derived from ω-6 PUFAs have pro-inflammatory effects whereas those derived from ω-3 precursors have anti-inflammatory effects (Calder et al., 2002). Likewise, eicosanoids derived from these two series have opposing effects in cancer cell growth (Rose and Connolly, 1990; Abou-el-Ela et al., 1989), invasion (Brown et al., 2006) and angiogenesis (Rose and Connolly, 2000; McCarty, 1996).

The cytotoxic effect of PUFAs on lymphocytes or leukemic cells has long been known. A growing body of evidence indicates that PUFAs might impair proliferation and exert proapoptotic and necrotic effects in leukemic cell lines (Finstad et al., 1998, 1994).

5.1. Polyunsaturated fatty acids (PUFAs) are less effective in inhibiting the growth of acute lymphoblastic T-cell leukemia cell line-Jurkat.

In the present study the effects of PUFAs like AA, LA and ALA were analyzed on the growth of acute lymphoblastic T-cell leukemia cell line-Jurkat. All the three PUFAs which include ALA of n-3 series and LA, AA of n-6 series showed no significant inhibition on the growth of Jurkat cells even at a concentration of 100 µM exposed for a period of 24 hours. At 24 hours they showed only 15-25% growth inhibition. These studies indicate that PUFAs as such are not showing any anti-cancer effects, atleast at the concentrations employed in the present study.

The present study indicates that PUFAs, in general, have very less or negligible effect on Jurkat cells. The effects reported in the literature at very high concentrations may be mediated through their oxygenated metabolites.

As the PUFAs of both ω-3 series and ω-6 series have negligible effect, on the growth of Jurkat cells, further studies were undertaken to check the effect of the metabolites of PUFAs.

Several LOX metabolites within the arachidonic acid pathway, infact, appear to enhance tumorigenesis. These metabolites include 5-(S)-HETE and LTB4 formed via the 5-LOX pathway and other products like 8-(S)-HETE, 12-(S)-HETE and 12-(R)-HETE (Bortuzzo et al., 1996). 5-LOX overexpression and 5-(S)-HETE formation has recently been documented in prostate, lung, and other cancer cell lines (Ghosh and Myers, 1997). 5-LOX is known to regulate the growth of prostate cancer cells (Ghosh and Myers, 1997). The expression of platelet-type 12-(S)-LOX mRNA and protein have been detected in prostate, melanoma (Timar et al., 2000; Gao et al., 1995), and 12-(S)-LOX overexpression was identified with tumor metastatic potential, skin tumorigenesis, and angiogenesis in human prostate cancers and enhanced angiogenic response in normal endothelial cells (Nie et al., 1998; Liu et al., 1995; Timar et al., 1992; Hohn et al., 1992; Chopra et al., 1991).

Arachidonate 15-lipoxygenase (15-LOX) can be sub classified according to specificity of tissue distribution and enzymatic characteristics into 15-LOX-1 and 15-LOX-2. 15-LOX-1 is expressed in reticulocytes, eosinophils, macrophages, tracheo brachial epithelial cells, skin and colon (Kamitani et al., 1998; Funk, 1996;) and converts LA to 13-(S)-HPODE (Brash, 1997). 15-LOX-2, on the other hand, is expressed in prostate, lung, skin and cornea (Shappel et al., 1997) and converts AA to 15-(S)-HETE but metabolizes LA poorly (Brash, 1997).

In general, many reports on 15-LOX-1 are controversial. Contrasting results were reported on the expression of 15-LOX-1 in colorectal tissues. Ikawa et al. (1999) reported that 15-LOX-1 is expressed at higher levels in human colorectal carcinoma than in normal adjacent tissues. However, Shureiqi et al. (2000) reported the opposite, higher expression of 15-LOX-1 in normal tissues compared to the tumors. 15-LOX-1 is expressed in bronchial epithelial cells, and increased expression has been noted in lung cancer (Moody et al., 1998; Shankaranarayanan and Nigam, 1998). Increased expression has also been reported in prostate cancer (; Kelavkar et al., 2002; Shureiqi and Lippman, 2001).

In prostate, for example, 15-LOX-2 expression is reduced in carcinoma (Shappel et al., 2001; Jack et al., 2000; Shappel et al., 1999) and 15-LOX-2 and 15-HETE have been shown to negatively regulate cell cycle progression in epithelial cells (Tang et al., 2002; Shappel et al., 1999), further suggesting a possible contribution of reduced expression of 15-LOX-2 to altered proliferation and differentiation in carcinoma. 15-(S)-HETE was shown to inhibit the proliferation of PC3 prostate carcinoma cells, possibly through activation of PPAR-γ (Shappell et al., 2001). Recently LOX products have also been shown to induce apoptosis in human T-cells, neutrophils, PC12h cells and Jurkat cells (Maccarrone et al., 2001). The above discussion gives an over all picture on the effects of various LOXs and in particular 15-LOXs. Studies from this laboratory have earlier shown that 15-LOX metabolites, 15-(S)-HPETE and 15-(S)-HETE, exert differential effects on BHK-21 cell proliferation (Kiran Kumar et al., 1993) and induce apoptosis in K-562 (chronic

myeloid leukemia) cell line by ROS generation, involving intrinsic pathway (Mahipal et al., 2007). The foregoing studies clearly indicate the role of LOX metabolites in the regulation of the growth in a variety of cancer cells. In the light of no effects of PUFAs observed in Jurkat cells, it would be interesting to study the effects of LOX metabolites on the regulation of growth in Jurkat cells.

5.2. Hydroperoxy metabolites of 15-LOX are more effective than the hydroxy metabolites in inhibiting the growth of acute lymphoblastic T-cell leukemia cell line- Jurkat

As the products of 15-LOX alone are proved to be anti-proliferative by various studies the effects of these metabolites alone are studied.

In the present study we analyzed the effects of metabolites of AA [15-(S)-HPETE and 15-(S)-HETE], LA [13-(S)-HPODE and 13-(S)-HODE] and ALA [13-(S)-HPOTrE and 13-(S)-HOTrE] on the growth of acute lymphoblastic T-cell leukemia cell line -Jurkat. Of all the metabolites tested the hydroperoxy forms in all the cases showed profound effects than the corresponding hydroxy forms. The hydroperoxy metabolite of AA (15-(S)-HPETE), LA (13-(S)-HPODE) and ALA (13-(S)-HPOTrE) showed potent inhibition in the growth of Jurkat cells with an IC_{50} of 10 µM, 20 µM and 20 µM respectively. The hydroxy forms, on the other hand, showed higher IC_{50} values with 40 µM by 15-(S)-HETE, 160 µM in the case of 13-(S)-HODE and above 160 µM in the case of 13-(S)-HOTrE. These results are in agreement with several other earlier reports wherein 50 µM of 13-(S)-HODE did not inhibit the proliferation of Caco-2 or DLD-1 colorectal carcinoma cells (Nixon et al., 2004) and LNCaP, PC3 and DU145 prostate cancer cell lines (Tang et al., 2002). Similar differential effects of hydroperoxy and hydroxy metabolites were reported on the growth of BHK 21 cell line (Kiran kumar et al., 1993).

5.3. 15-LOX metabolites of AA are more potent than those of LA and ALA

Among all the metabolites of AA, LA and ALA tested the metabolites of AA (15-(S)-HPETE and 15-(S)-HETE) are more potent than the metabolites of LA (13-(S)-HPODE and 13-(S)-HODE) and ALA (13-(S)-HPOTrE and 13-(S)-HOTrE). Though required in higher concentrations and exposure for prolonged periods, 15-(S)-HETE also inhibited the growth of Jurkat cells very similar to that of 15-(S)-HPETE. 15-(S)-HETE which has got doubly allelic methylene groups can undergo further oxygenation once entered into the cell (Chavis et al., 1996). These delayed effects of 15-(S)-HETE may be due to the delay in the formation of further oxygenated metabolites like 5-(OOH), 15-OH-di H(P)ETE or 8-(OOH), 15-(OH) di H(P)ETE metabolites depending on the type of LOX present in the cell as explained in the earlier studies (Mahipal et al., 2007). 13-(S)-HODE and 13-(S)-HOTrE, however, lack the divinyl methyl groups to undergo further oxygenations and induce apoptosis. Hence they are least potent in inhibiting the growth of Jurkat cells. The foregoing studies clearly demonstrate that PUFA metabolites are more potent in inhibiting the proliferation of Jurkat cells. Further, the hydroperoxy metabolites are more potent than the hydroxy metabolites of PUFAs tested, with 15-LOX metabolites of AA (15-(S)-HPETE and 15-(S)-HETE) being the most potent. In the light of the above, it would be interesting to study the mechanism of regulation of cell growth by these metabolites of AA.

5.4. Jurkat cells undergo apoptosis upon treatment with 15-(S)-HPETE and 15-(S)-HETE

Jurkat cells treated with 15-LOX metabolites showed the clear formation of apoptotic bodies when observed under phase contrast microscope. Where as the untreated cells showed bunches of healthy cells the characteristic feature of Jurkat cells. The cells showed fragmented DNA of 180-200 base pairs upon treatment with

15-(S)-HPETE (10 μM) and 15-(S)-HETE (40 μM). This further substantiates the induction of apoptosis by 15-LOX metabolites. This is in accordance with the earlier findings with 15-LOX metabolites on chronic myeloid leukemia cells-k562 (Mahipal et al., 2007). These findings suggest that 15-LOX metabolites exert very similar effects on leukemia cells of myeloid and lymphoid origin.

Flow cyometric analysis of cells showed a prominent sub G0/G1 peak in both 15-(S)-HPETE and 15-(S)-HETE treated cells. However, 15-(S)-HPETE and 15-(S)-HETE showed differential effects, 15-(S)-HPETE at 10 μM concentration by 3 hours induced 31% of cell death, whereas 15-(S)-HETE at 40 μM induced only 24% of cell death by 6 hours. This indicates that hydroxy metabolites need a threshold concentration and higher incubation periods to initiate the induction of apoptosis in Jurkat cells. Similar requirement of threshold concentration of PUFAs to induce apoptosis in various cancer cell lines was reported (Jiang, 1998). The foregoing studies indicate that 15-LOX metabolites of AA induce apoptosis in Jurkat cells. Of these metabolites, 15-(S)-HPETE was more potent than 15-(S)-HETE. It would be interesting to understand the molecular mechanisms involved in the induction of apoptosis by 15-LOX metabolites.

5.5. 15-(S)-HPETE and 15-(S)-HETE induce apoptosis in Jurkat cells by extrinsic and intrinsic death path ways.

As the Jurkat cells treated with 15-LOX metabolites showed apoptotic features as confirmed by phase contrast microscopic pictures, DNA fragmentation and flow cytometric analysis, further studies were undertaken to find the pathway by which apoptosis is taking place in these cells.

Cell death with apoptotic morphology can be triggered by involving several stimuli, intracellular stress and receptor mediated signaling (Guimaraes and Linden, 2004). These signals feed into an evolutionarily conserved intracellular machinery of execution (Hengartner, 2000; Green, 2000), the mechanisms of which have mainly

been traced to the activity of the caspase family of cysteine proteases (Cryns and Yuan, 1998; Zhivotovsky et al., 1997; Yuan et al., 1993).

FasL is a transmembrane protein belonging to the tumor necrosis factor (TNF) super family that can trigger apoptotic cell death following ligation to its receptor, Fas (CD95/APO-1) (Houstan and Connell, 2004). Upon interaction with its agonistic antibody or its natural Fas ligand, Fas induces apoptosis in a number of cell lines including lymphocytes (Nagata and Golstein, 1995; Nagata and Suda, 1995; Daniel and Krammer, 1994). In the present study Jurkat cells treated with 15-LOX-2 metabolites showed increased expression of Fas ligand whereas receptor levels remained constant. Binding of FasL to Fas causes a higher order aggregation of receptor molecules (Holler et al., 2003) and recruitment of adopter molecule, Fas activated death domain (FADD) via DD-DD interactions. FADD levels increased in Jurkat cells undergoing apoptosis. In the present study caspase-8 levels increased upon treatment with 15-LOX metabolites. The cells treated with Ac-IETD-AMC, a specific caspase-8 substrate showed, increase in the activity of caspase-8 in a time dependent manner. The cells preincubated with Z-IETD-FMK, a specific caspase-8 inhibitor, showed decrease in caspase-8 activity, suggesting the activation of extrinsic pathway in 15-LOX metabolite induced apoptosis. Activated caspase-8 further cleaves Bid to a truncated form, which translocates into mitochondria; releases cytochrome- *c* into the cytosol. Decreased expression of Bid levels in Jurkat cells treated with 15-LOX metabolites, observed in the present study, indicates the same. Cytochrome- *c* participates in a protein-protein interaction with Apaf-1, which leads to the sequential activation of procaspase-9 and procaspase-3 (Li et al., 1997). Increase in the levels of cytochrome- *c*, a14 kDa protein, was seen in the 15-LOX metabolite treated cells. Caspase-3 protein levels were increased in 15-(S)-HPETE and 15-(S)-HETE treated cells. Caspase-3 activity levels, when measured by treating the cells with a caspase-3 substrate, Ac-DEVD-AFC, were increased time dependently. The activity was decreased when the cells were preincubated with specific caspase-3 inhibitor i.e., Ac-DEVD-CHO. PARP cleavage products observed

in Jurkat cells exposed to 15-(S)-HPETE and 15-(S)-HETE provides further evidence for the activation of caspase-3. The decrease in pAkt despite increase in total Akt was consistently observed in the present study. The decrease in pAkt, in the presence of 15-LOX metabolites, might have triggered compensatory mechanisms to further enhance the levels of Akt by either increasing mRNA stability or enhancing the gene expression. These findings demonstrate that 15-LOX metabolites induce apoptosis by activating the extrinsic as well as intrinsic death pathways through a series of events involving Fas ligand expression, caspase-8 activation, Bid cleavage, cytochrome *c* release, caspase-3 activation, PARP cleavage and DNA fragmentation. However, the mechanisms involved in the induction of apoptosis by 15-LOX metabolites are not known. Hence further studies were undertaken to see whether 15-LOX metabolites induce ROS generation.

5.6. 15-(S)-HPETE and 15-(S)-HETE induced apoptosis is mediated by ROS generation in Jurkat cells.

ROS generation has been linked to rapid induction of apoptosis in many cases (Ling et al., 2003). Reactive oxygen species generation in 15-LOX metabolite mediated apoptosis was investigated through DCFH-DA analysis. The present study indicates that there is many fold induction of ROS within minutes after treatment with 15-LOX metabolites. 15-(S)-HPETE is more potent in generating ROS than 15-(S)-HETE. These results indicate a 6 to 8 fold increase in ROS generation in 10 μM 15-(S)-HPETE treated cells with in 45 min compared to 4 to 5 fold with 40 μM 15-(S)-HETE. This might be responsible for differential effects of hydroperoxy (15-(S)-HPETE) and hydroxy (15-(S)-HETE) metabolites observed in the study. Pretreatment of cells with N-acetyl cysteine (NAC), a glutathione precursor, has prevented the ROS production in 15-LOX metabolite treated cells suggesting that ROS generated by 15-LOX metabolites triggers apoptosis in Jurkat cells. In order to find out the sources of ROS generation, further studies were undertaken to check

whether 15-LOX metabolites activate NADPH oxidase, one of the $O_2^{\cdot -}$ radicals generators.

5.7. NADPH oxidase mediates ROS generation in Jurkat cells treated with 15-LOX metabolites.

NADPH oxidase is a vital component of host defense. This enzyme produces superoxide anion, ($O_2^{\cdot -}$), the precursor to a number of reactive oxygen species (ROS) that play essential role in killing many types of bacteria and other invaders (DeCoursey and Ligeti, 2005). Similar induction of ROS by NADPH oxidase dependent pathway was reported earlier (Portanova et al., 2008; Mahipal et al., 2007). Diphenylene Idonium (DPI) inhibits NADPH Oxidase and decreases the production of ROS (Mahipal et al., 2007; Hyseni et al., 2006).

In the present study the Jurkat cells were preincubated with 10 μM Diphenylene idonium (DPI) one hour before the treatment with 15-LOX metabolites. These cells when treated with either 10 μM 15-(S)-HPETE or 40 μM 15-(S)-HETE showed decrease in the generation of ROS as analyzed by flow cytometry. This decrease in ROS production by DPI, a specific inhibitor of NADPH oxidase, clearly indicates the involvement of NADPH oxidase in the production ROS, which in turn is responsible for activation of Fas mediated death pathway. These results are in accordance with earlier reports where ROS generation via NADPH oxidase activation was shown to be involved in Fas-mediated apoptosis (Suzuki et al., 1998). The present study thus reveals that PUFAs have limited effect on the growth of Jurkat cells. The 15-LOX metabolites of PUFAs, on the other hand, exhibit profound antiproliferative effects-the hydroperoxy metabolites being more potent than the hydroxy metabolites and 15-LOX metabolites of AA (15-(S)-HPETE and 15-(S)-HETE) being more effective than those of LA (13-(S)-HPODE and 13-(S)-HODE) and ALA (13-(S)-HPOTrE and 13-(S)-HOTrE). Further studies on the mode of cell death with 15-(S)-HPETE and 15-(S)-HETE showed the induction of apoptosis in

Jurkat cells, involving both extrinsic and intrinsic death pathways. These anti-proliferative effects were demonstrated to be mediated through the activation of NADPH oxidase, leading to the generation of ROS. These studies thus reveal the critical role played by 15-LOX metabolites of AA in the regulation of growth in acute lymphoblastic T-cell leukemia cell line, Jurkat.

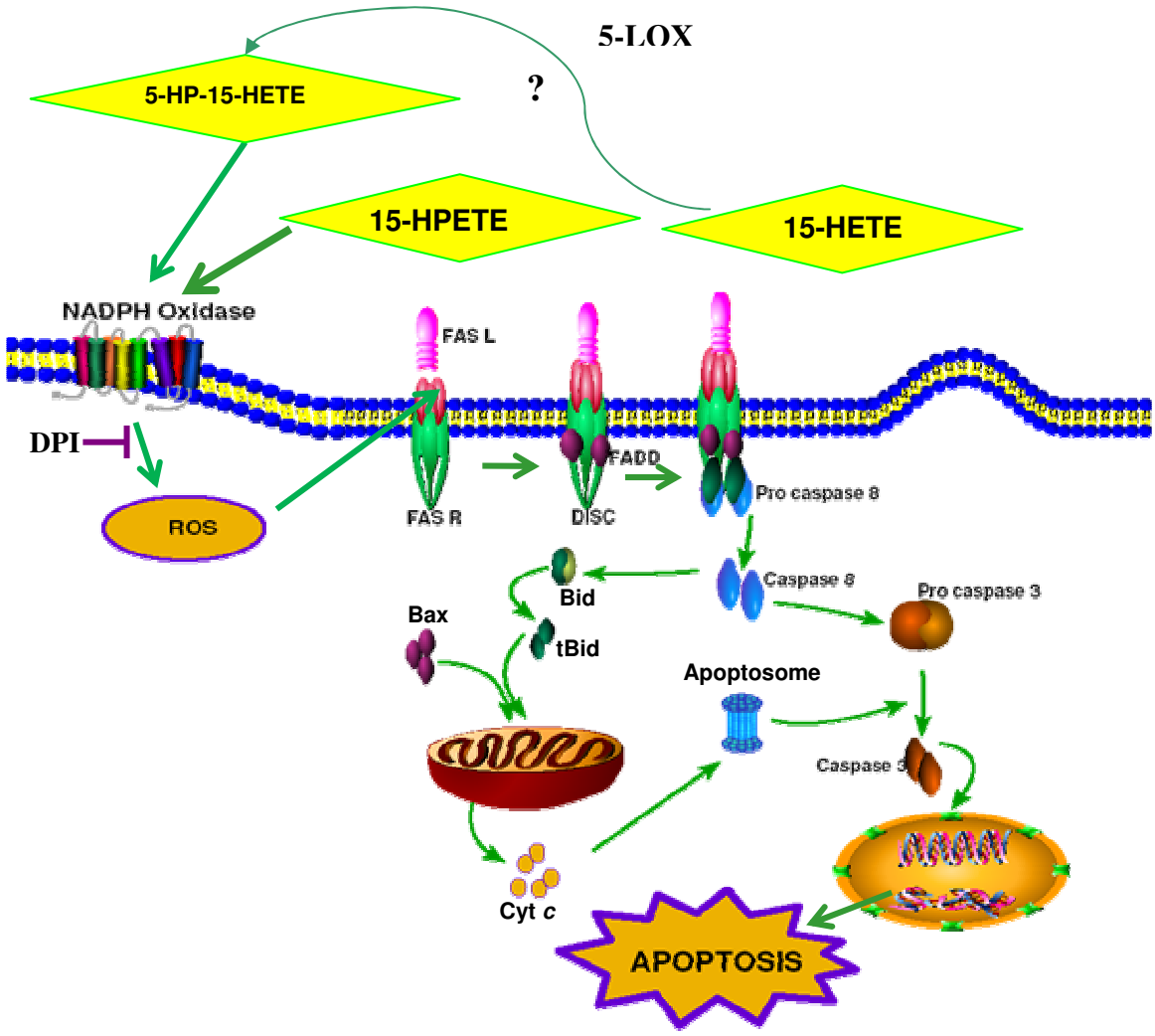


Fig. 54. Proposed mechanism of action of 15-(S)-HPETE and 15-(S)-HETE–induced apoptosis in acute lymphoblastic T-cell leukemia cells

Summary

Summary

Nutrition is an environmental factor which is of great importance in health related issues. Humans today live in a nutritional environment that differs greatly from that for which their genetic background prepared them several hundred years ago. There is evidence that human beings evolved on a diet with a (ω)-6 to (ω)-3 PUFA ratio of 1/1, where as in current western diets the ratio ranges from 15 to 20/1. Epidemiological literature on the association of ω-3 PUFAs and cancer including correlational studies and migrational studies, suggests a protective effect of ω-3 PUFAs and a promoting effect of ω-6 PUFAs on cancer.

Tumorigenesis studies have demonstrated that PUFAs must undergo oxidative metabolism to enhance tumorigenesis. The cyclooxygenases (COXs) and lipoxygenases (LOXs) mediate the oxidative metabolism of LA and AA forming an array of biologically active metabolites, such as protsglandins, HODEs and HETEs.

A wide variety of LOXs are found in nature, which are described based on the carbon atom that each enzyme peroxygenates on a particular substrate. The primary lipid targets of LOX activity are arachidonic acid and linoleic acid. Two forms of 15-LOXs occur in humans. 15-LOX-2, preferentially peroxidizes arachidonic acid on carbon 15 to generate 15-(S)-HPETE, which is hydrolyzed to 15-(S)-HETE. 15-LOX-1 preferentially peroxidizes linoleic acid on carbon 13 to form 13-(S)-HPODE which is hydrolyzed to 13-(S)-HODE. Even though the role of 15-LOX was well studied in relation with carcinogenesis, there are antithetical reports regarding the role of 15-LOX-1 in controlling carcinogenesis. On the other hand, 15-LOX-2 in several studies has been shown to negatively regulate cell proliferation. Even though the role of 15-LOX-2 in mediating anti-carcinogenic effects was very well established, the mechanisms behind these effects are still unclear. The apoptotic cascades mediated by 15-LOX metabolites are not well explored. The present study is therefore designed to understand the molecular mechanisms mediated by 15-LOX

metabolites induced apoptosis, on acute lymphoblastic T-cell leukemia cell line-Jurkat.

In order to generate the 15-LOX metabolites of PUFAs in large quantities, the cloning, expression and purification of 15-LOX was taken up as a part of the study. The gene for 15-LOX was isolated from green gram embryo. Green gram 15-lipoxygenase ORF was cloned into *E. coli* expression vector pETBlue-2. The recombinant protein was expressed and activity was assayed by analyzing the products formed on SP-HPLC. The major peak with the absorption maximum at 235 nm was isolated and co-chromatographed with standard 15-(S)-HPETE. The co-chromatographic spectrum of recombinant LOX product and standard 15-(S)-HPETE showed different retention times on reverse phase HPLC. To identify the LOX metabolite formed by the recombinant 15-LOX expressed in *E. coli*, LC-MS analysis of the peak was also performed. The fragments obtained were quite different from that of standard 15-(S)-HPETE. In the light of above results further studies on acute lymphoblastic T-cell leukemia cell line was carried out using products synthesized using commercially available soybean LOX only.

In the present study the effects of polyunsaturated fatty acids like arachidonic acid, linoleic acid and α-linolenic acid were analyzed on the growth of Jurkat cells. All the three PUFAs showed no significant inhibition on the growth of Jurkat cells even at a concentration of 100 μM exposed for a period of 24 hours. At 24 hours they showed only 15-25% growth inhibition. These studies indicate that PUFAs as such are not showing any anti-cancer effects, at the concentrations employed in the present study.

The LOX metabolites of LA [13-(S)-HPODE and 13-(S)-HODE], AA [15-(S)-HPETE, 15-(S)-HETE] and ALA [13-(S)-HPOTrE and 13-(S)-HOTrE] were prepared by employing commercially available soybean LOX, separated on SP-HPLC and identified based on LC-MS analysis. The effect of these metabolites on the growth of Jurkat cell line was analyzed. These studies revealed that hydroperoxy metabolites were more potent than the hydroxy metabolites of LA, ALA and AA.

Among all the metabolites tested 15-(S)-HPETE and 15-(S)-HETE were found to be more potent with IC_{50} values of 10 μM and 40 μM respectively. Hence further studies were undertaken by exposing Jurkat cells to 10 μM 15-(S)-HPETE for 3 h and 40 μM 15-(S)-HETE for 6 h.

The cells exposed to either 10 μM 15-(S)-HPETE for 3 h or 40 μM 15-(S)-HETE for 6 h showed typical apoptotic bodies, when observed under phase contrast microscope. Flow cytometric analysis of 15-LOX metabolites treated cells upon staining with propidium iodide (PI) has shown distinct sub G0/G1 peak characteristic feature of cells undergoing apoptosis. 31% of cells treated with 15-(S)-HPETE and 24% of cells treated with 15-(S)-HETE showed apoptotic features. The cells also showed fragmented DNA of 180-200 base pairs in the treatments of 10 μM 15-(S)-HPETE and 40 μM 15-(S)-HETE.

As the Jurkat cells treated with 15-LOX metabolites were undergoing apoptosis, the molecular mechanisms involved in this phenomenon were studied and the pathways responsible for apoptosis were elucidated. The cells treated with 10 μM 15-(S)-HPETE or 40 μM 15-(S)-HETE for different time periods showed increased expression of Fas ligand (FasL) and the receptor (FasR) levels remain unchanged. Fas activated death domain (FADD) levels were increased in all the treatments of 15-LOX metabolites. The active caspase-8 levels showed increased expression whereas the expression of Bcl-2 family member protein- Bid levels were decreased confirming the translocation of Bid into mitochondria which may responsible for observed release of cytochrome *c*. The Akt expression levels were increased with the decrease of pAkt levels indicating the involvement of Akt/pAkt signaling pathway. The decrease in pAkt despite increase in total Akt was consistently observed in the present study. The decrease in pAKT in the presence of 15-LOX metabolites might have triggered compensatory mechanisms to further enhance the levels of Akt by either increasing mRNA stability or enhancing the gene expression.

The cells treated with 15-LOX metabolites showed decreased expression of Bcl-2 protein whereas Bax levels remain unchanged. This suggests an altered Bcl-

2/Bax ratio. Impaired mitochondrial membrane potentials were observed in the cells treated with 15-(S)-HPETE and 15-(S)-HETE leading to the leakage of mitochondrial components. Cytochrome *c,* a 14 kDa protein was increased in all treated cells. Increased expression of caspase-3 protein was also observed in 15-LOX metabolites treated Jurkat cells. PARP, poly (ADP ribose) polymerase, is a nuclear enzyme implicated in many cellular processes including apoptosis and DNA repair. The increase in 85 kDa fragment of PARP with simultaneous decrease of 116 kDa un-cleaved PARP was observed in all treated cells. These findings clearly indicate that 15-LOX metabolite induce apoptosis in Jurkat cells by activation of both extrinsic and intrinsic death pathways, involving Fas ligand activation, Bid cleavage, caspase-8 activation, cytochrome *c* release, caspase-3 activation, PARP cleavage and DNA fragmentation.

The involvement of ROS in induction of apoptosis was shown in many studies. In the present study ROS generation was studied using DCHFDA on flow cytometer. The cells treated with 15-LOX metabolites showed ROS generation within minutes after treatments. When the cells were pre incubated with DPI (10 µM), a specific inhibitor of NADPH oxidase, ROS generation was reversed suggesting the involvement of NADPH oxidase in the generation of ROS. When cells were pre-incubated with N-acetyl cysteine (NAC) (50 µM) an anti-oxidant, ROS generation was reversed. The caspase-8 and caspase-3 levels were decreased when the cells were pre incubated both DPI and NAC. These studies conclusively demonstrate the involvement of ROS in the 15-LOX metabolites induced apoptosis in Jurkat cells.

In conclusion the present study demonstrates that PUFAs have limited effects on the growth of Jurkat cells. The 15-LOX metabolites of PUFAs, on the other hand, exhibit profound antiproliferative effects-the hydroperoxy metabolites being more potent than the hydroxy metabolites and 15-LOX metabolites of AA (15-(S)-HPETE and 15-(S)-HETE) being more effective than those of LA (13-(S)-HPODE and 13-(S)-HODE) and ALA (13-(S)-HPOTrE and 13-(S)-HOTrE). Further in-depth studies

with 15-(S)-HPETE and 15-(S)-HETE showed the induction of apoptosis in Jurkat cells, involving both extrinsic and intrinsic death pathways. These anti-proliferative effects were demonstrated to be mediated through the activation of NADPH oxidase, leading to the generation of ROS. These studies thus reveal the critical role played by 15-LOX metabolites of AA in the regulation of growth in acute lymphoblastic T-cell leukemia cell line, Jurkat.

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1: Biotechnol Appl Biochem. 2008 May 21. [Epub ahead of print]

Links

15-(S)-HPETE and 15-(S)-HETE effects on acute lymphoblasticleukemia cell line-Jurkat: Activation of Fas mediated death pathway.

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Anti-proliferative effects of 15-lipoxygenase (15-LOX) metabolites of arachidonic acid [15-(S)-HPETE and 15-(S)-HETE] and the mechanism(s) involved were studied in a human T-cell leukemia cell line, Jurkat. 15-(S)-HPETE, the hydroperoxy metabolite of 15-LOX inhibited the growth of Jurkat cells 3 h after exposure with an IC 50 value of 10 microM. The hydroxy metabolite of 15-LOX, 15-(S)-HETE, on the other hand, inhibited the growth of Jurkat cells after 6 h of exposure and with an IC 50 of 40 microM. The cells exposed to 10 microM 15-(S)-HPETE for 3 h or 40 microM 15-(S)-HETE for 6 h showed increased expression of Fas ligand and FADD, caspase-8 activation, Bid cleavage, decrease in mitochondrial membrane potential, cytochrome c release, caspase-3 activation, PARP-1 (poly (ADP) ribose polymerase-1) cleavage and DNA fragmentation, suggesting the involvement of both extrinsic and intrinsic death pathways. Further studies on Reactive Oxygen Species generation revealed the involvement of NADPH oxidase. In conclusion, this study indicates that NADPH oxidase induced ROS generation activates Fas mediated death pathway.

PMID: 18494609 [PubMed - as supplied by publisher