Anti-inflammatory and anti-proliferative properties of chebulagic acid, a COX-2/5-LOX dual inhibitor isolated from *Terminalia chebula*

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Anti-inflammatory and anti-proliferative properties of chebulagic acid, a COX-2/5-LOX dual inhibitor isolated from *Terminalia chebula*"** 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
(Research Supervisor)

D. Bharat Kumar Reddy
(Research Scholar)

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CERTIFICATE

This is to certify that **Mr. D. Bharat Kumar Reddy** 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 entitled **"Anti-inflammatory and anti-proliferative properties of chebulagic acid, a COX-2/5-LOX dual inhibitor isolated from *Terminalia chebula*"** for submission for the degree of **Doctor of Philosophy** of this University.

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Bharat

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ABRIVIATIONS

°C	:	Degree centigrade/degree Celsius
µM	:	Micro molar
AA	:	Arachidonic acid
ABTS	:	2, 2'-azino-bis (3-ethylbenzthiazoline-6-Sulphonic acid)
CA	:	Chebulagic acid
COX	:	Cyclooxygenase
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DPPH	:	2, 2'-diphenyl-1-picrylhydrazyl
DTT	:	Dithiothreitol
EDTA	:	Ethylenediaminetetraacetic acid
ERK	:	Extracellular signal regulated kinase
FACS	:	Fluorescence activated cell sorter
FBS	:	Fetal bovine serum
FDA	:	Food and Drug Administration
g	:	Gram
GI	:	Growth inhibitory
GPx	:	Glutathione peroxidase
GSH	:	Glutathione
h	:	Hour(s)

HE	:	Haematoxylin and eosin
HETE	:	Hydroxyeicosatetraenoic acid
iNOS	:	Inducible nitric oxide synthase
JNK	:	c-Jun N-terminal kinase
kDa	:	Kilodalton
l	:	Litre
LA	:	Linoleic acid
LOX	:	Lipoxygenase
LT	:	Leukotriene
MAPK	:	Mitogen activated protein kinase
mg	:	Milligram
min	:	Minute(s)
ml	:	Millilitre
mM	:	Milli molar
MTT	:	[3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide]
NDGA	:	Nordihydroguaretic acid
NF-κB	:	Nuclear factor-kappa B
nm	:	Nanometers
NO	:	Nitric oxide
NSAIDs	:	Non-steroidal anti-inflammatory drugs
PAGE	:	Polyacrylamide gel electrophoresis
PARP	:	Poly (ADP) ribose polymerase

PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PG	:	Prostaglandin
PI	:	Propidium iodide
PMSF	:	Phenylmethanesulphonylfluoride
ROS	:	Reactive oxygen species
RP-HPLC	:	Reverse phase-HPLC
rpm	:	Revolutions per minute
RT-PCR	:	Reverse transcriptase-polymerase chain reaction
SCC	:	Squamous cell carcinoma
SDS	:	Sodium dodecyl sulfate
TEMED	:	N,N,N',N'-tetramethylene diamine
Tris	:	Tris-(Hydroxymethyl) aminoethane
UV	:	Ultraviolet

General introduction

Ⅰ. Inflammation

Inflammation is the body's immediate response to damage to its tissues and cells by pathogens, noxious stimuli such as chemicals, or physical injury (Haworth and Levy, 2007). It is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue. Excessive inflammation may lead to tissue injury and if severe can cause physiological decomposition, organ dysfunction and death. Inflammation is a complex stereotypical reaction of the body expressing the response to damage of its cells and vascularized tissues. The discovery of the detailed processes of inflammation has revealed a close relationship between inflammation and the immune response (Fig. 1). The five basic symptoms of inflammation - redness (rubor), swelling (tumour), heat (calor), pain (dolor) and deranged function (functio laesa) have been known since the ancient Greek and Roman era.

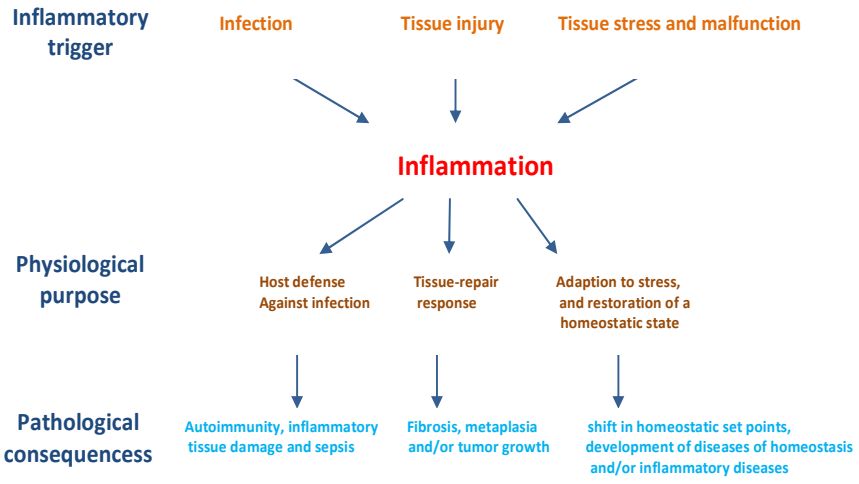

Fig. 1. Causes and physiological and pathological outcomes of inflammation

Hallmarks of inflammation are vasodilation, oedema and leukocyte infiltration. Vasodilatation is characterized by redness and warmth at the site of injury. The purpose of the vasodilatory response is facilitating the local delivery of soluble mediators and inflammatory cells. The white blood cells or leucocytes take an important role in inflammation; they extravasate from the capillaries into tissue, and carry on as phagocytes picking up bacteria and cellular debris. They may also aid by walling off an infection and preventing its spread. The main purpose of inflammation, this immensely complex response seems to be to bring fluid, proteins, and cells from the blood into the damaged tissues (Fig. 2).

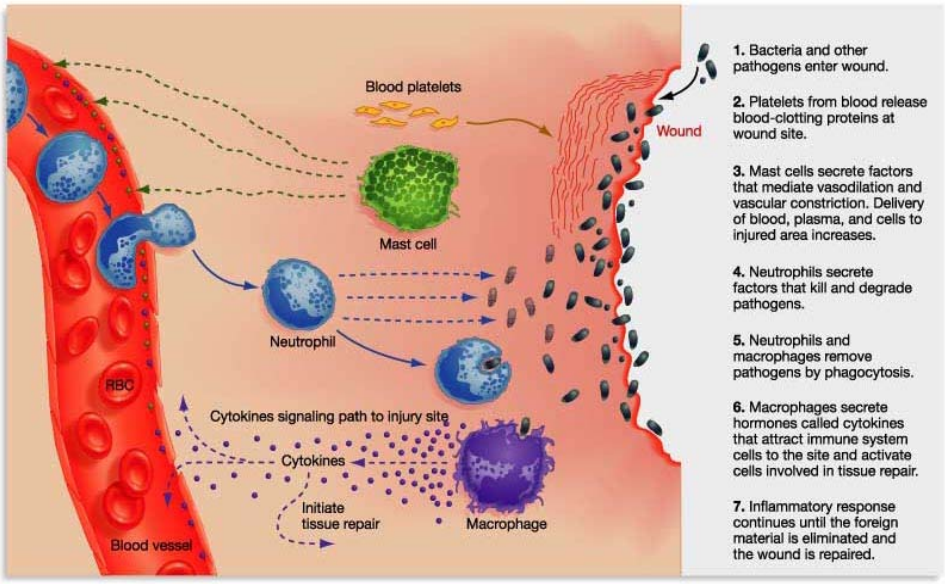

Fig. 2. Process of inflammation *(Source: www.uic.edu)*

The main features of the inflammatory response are, therefore: vasodilation, i.e. widening of the blood vessels to increase the blood flow to the infected area; increased vascular permeability, which allows diffusible

components to enter the site; cellular infiltration by chemotaxis, or the directed movement of inflammatory cells through the walls of blood vessels into the site of injury; changes in biosynthetic, metabolic, and catabolic profiles of many organs; and activation of cells of the immune system as well as of complex enzymatic systems of blood plasma. Of course, the degree to which these occur is normally proportional to the severity of the injury and the extent of infection.

Based on timing and pathological features, inflammation can be divided into two major categories-acute and chronic. Acute inflammation is a short-term response that usually results in healing: leukocytes infiltrate the damaged region, removing the stimulus and repairing the tissue. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Chronic inflammation, by contrast, is a prolonged, dysregulated and maladaptive response that involves active inflammation, tissue destruction and attempts at tissue repair. Such persistent inflammation is associated with many chronic human conditions and diseases, including allergy, atherosclerosis, cancer, arthritis and autoimmune diseases. These chronic inflammatory states do not seem to be caused by the classic instigators of inflammation: infection and injury. Instead, they seem to be associated with the malfunction of tissue: that is, with the homeostatic imbalance of one of several physiological systems that are not directly functionally related to host defense or tissue repair.

Ⅱ. Inducers of inflammation

Inducers of inflammation can be exogenous or endogenous.

Ⅱa. Exogenous inducers

Exogenous inducers can be classified into two groups: microbial and non-microbial. There are, in turn, two classes of microbial inducers: pathogen-associated molecular patterns (PAMPs) and virulence factors. The first class of microbial inducer, PAMPs, is a limited and defined set of conserved molecular patterns that is carried by all microorganisms of a given class (whether pathogenic or commensal) (Medzhitov and Janeway, 1997). The second class of microbial inducer comprises a variety of virulence factors and is therefore restricted to pathogens. In contrast to PAMPs, they are not sensed directly by dedicated receptors. Instead, the effects of their activity, particularly their adverse effects on host tissues, are responsible for triggering the inflammatory response.

Exogenous inducers of inflammation that are of non-microbial origin include allergens, irritants, foreign bodies and toxic compounds (Majno and Joris, 2004). Certain allergens are detected because they mimic the virulence activity of parasites; others can act as irritants on the mucosal epithelia. The inflammatory response induced by both types of allergen is largely similar because defense against parasites and environmental irritants relies on expulsion and clearance mediated by the mucosal epithelia.

Ⅱb. Endogenous inducers of inflammation

Endogenous inducers of inflammation are signals produced by stressed, damaged or otherwise malfunctioning tissues. The identity and characteristics of these signals are poorly defined. But they probably belong to various functional classes according to the nature and the degree of tissue anomalies on which they report. In addition to the inducers associated with infection and tissue damage, there is probably another, currently unidentified, class of inducers that trigger the inflammatory response in tissues that are malfunctioning or are under stress.

Ⅲ. Mediators and effectors of inflammation

Inducers of inflammation trigger the production of numerous inflammatory mediators, which in turn alter the functionality of many tissues and organs — the downstream effectors of the inflammatory pathway. Many of these inflammatory mediators have effects in common on the vasculature and on the recruitment of leukocytes. These mediators can be derived from plasma proteins or secreted by cells (Kumar *et al.,* 2003). The cellular mediators can be produced by specialized leukocytes (particularly tissue-resident macrophages and mast cells) or by cells present in local tissues. Some mediators (such as histamine and serotonin) are preformed and stored in the granules of mast cells, basophils and platelets. Others are preformed and circulate as inactive precursors in the plasma. The plasma concentration of these mediators can increase markedly as a result of increased secretion of the precursors by hepatocytes during the acute-phase response. Other mediators are produced

directly in response to appropriate stimulation by inducers of inflammation. Inflammatory mediators can be classified into seven groups according to their biochemical properties (Fig. 3).

- First, vasoactive amines -histamine and serotonin are released when mast cells and platelets degranulate. They have complex effects on the vasculature, causing increased vascular permeability and vasodilation, or vasoconstriction, depending on the context (Strassheim *et al.,* 2002). The immediate consequences of their release by mast cells can be highly detrimental in sensitized organisms, resulting in vascular and respiratory collapse during anaphylactic shock.

- Second, vasoactive peptides can be stored in an active form in secretory vesicles (for example, substance P) or generated by proteolytic processing of inactive precursors in the extracellular fluid (for example, kinins, fibrinopeptide A, fibrinopeptide B and fibrin degradation products). Substance P is released by sensory neurons and can itself cause mast-cell degranulation. Other vasoactive peptides are generated through proteolysis by the Hageman factor, thrombin or plasmin and cause vasodilation and increased vascular permeability either directly or by inducing the release of histamine from mast cells. The Hageman factor activates the kallikrein–kinin cascade, and the main product of this cascade, bradykinin, affects the vasculature, as well as having a potent pro-algesic (pain-stimulating) effect (Banik *et al.,* 2001).

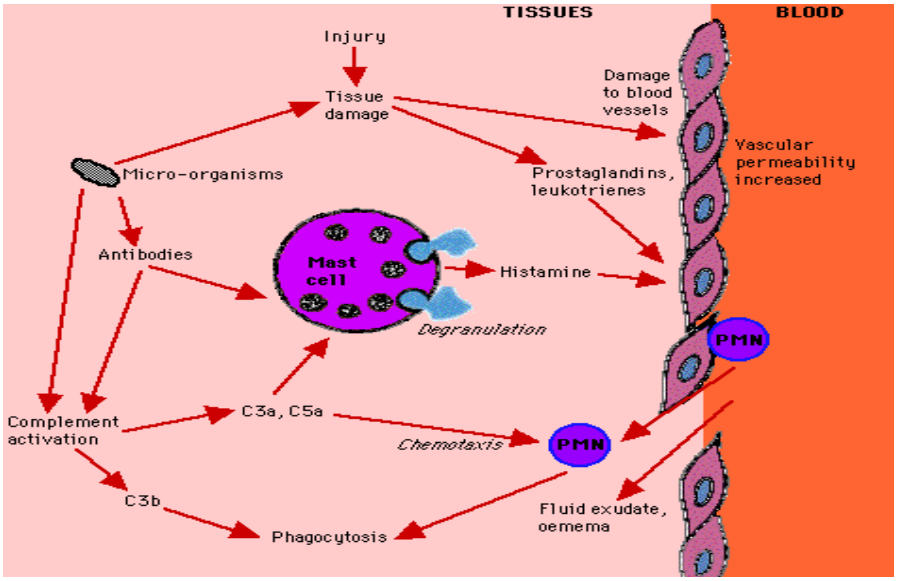

Fig. 3. Mediation of inflammation *(Source: www.microbiologybytes.com)*

- Third, the complement fragments C3a, C4a and C5a, also known as anaphylatoxins are produced by several pathways of complement activation. C5a and to a lesser extent C3a and C4a promote granulocyte and monocyte recruitment and induce mast-cell degranulation, thereby affecting the vasculature.

- Fourth, lipid mediators (eicosanoids and platelet-activating factors) are derived from phospholipids, such as phosphatidylcholine, that are present in the inner leaflet of cellular membranes. After activation by intracellular Ca^{2+} ions, cytosolic phospholipase A2 generates arachidonic acid and lysophosphatidic acid, the precursors of the two classes of lipid mediator listed above, from phosphatidylcholine. Arachidonic acid is

metabolized to form eicosanoids either by cyclooxygenases (COX-1 and COX-2), which generate prostaglandins and thromboxanes, or by lipoxygenases, which generate leukotrienes and lipoxins (Kumar *et al.,* 2003). The prostaglandins PGE_2 and PGI_2, in turn, cause vasodilation, and PGE_2 is also hyperalgesic and a potent inducer of fever (Higgs *et al.,* 1984). Lipoxins (and dietary ω3-fatty-acid-derived resolvins and protectins) inhibit inflammation and promote resolution of inflammation, and tissue repair (Serhan, 2007). The second classes of lipid mediator, platelet-activating factors, are generated by the acetylation of lysophosphatidic acid and activate several processes that occur during the inflammatory response, including recruitment of leukocytes, vasodilation and vasoconstriction, increased vascular permeability and platelet activation.

- Fifth, inflammatory cytokines tumour-necrosis factor-α (TNF-α), IL-1, IL-6 and many others are produced by many cell types, most importantly by macrophages and mast cells. They have several roles in the inflammatory response, including activation of the endothelium and leukocytes and induction of the acute-phase response.

- Sixth, chemokines are produced by many cell types in response to inducers of inflammation. They control leukocyte extravasation and chemotaxis towards the affected tissues.

- Seventh, several proteolytic enzymes (including elastin, cathepsins and matrix metalloproteinases) have diverse roles in inflammation, in part through degrading extracellular matrix and basement-membrane proteins. These proteases have important roles in many processes, including host defence, tissue remodelling and leukocyte migration.

Ⅳ. Inflammatory disorders

Abnormalities associated with inflammation comprise a large, unrelated group of disorders which underly a variety of human diseases. The immune system is often associated with inflammatory disorders, demonstrated in both allergic reactions and some myopathies, with many immune system disorders resulting in abnormal inflammation. Non-immune diseases with aetiological origins in inflammatory processes are thought to include cancer, atherosclerosis, and ischaemic heart disease. A large variety of proteins are involved in inflammation, and any one of them is open to a genetic mutation which impairs or otherwise dysregulates the normal function and expression of that protein. Examples of disorders associated with inflammation are represented in Table 1.

Table 1. Disorders of inflammation.

Asthma	Inflammatory bowel diseases
Autoimmune diseases	Pelvic inflammatory disease
Chronic inflammation	Reperfusion injury
Chronic prostatitis	Rheumatoid arthritis
Glomerulonephritis	Transplant rejection
Hypersensitivities	Vasculitis
Cancer	Diabetes II
Alzheimer's	Cardiovascular diseases

Ⅴ. Cancer

Cancer is a class of diseases in which a group of cells display uncontrolled growth, invasion and sometimes metastasis (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, do not invade or metastasize. Cancer occurs when cell division gets out of control (Fig. 4). Usually, the timing of cell division is under strict constraint, involving a network of signals that work together to say when a cell can divide, how often it should happen and how errors can be fixed. Mutations in one or more of the nodes in this network can trigger cancer, be it through exposure to some environmental factor (e.g. tobacco smoke) or because of a genetic predisposition, or both. The predominant mechanisms for the cancers featured here are (i) impairment of a

DNA repair pathway (ii) the transformation of a normal gene into an oncogene and (iii) the malfunction of a tumor supressor gene.

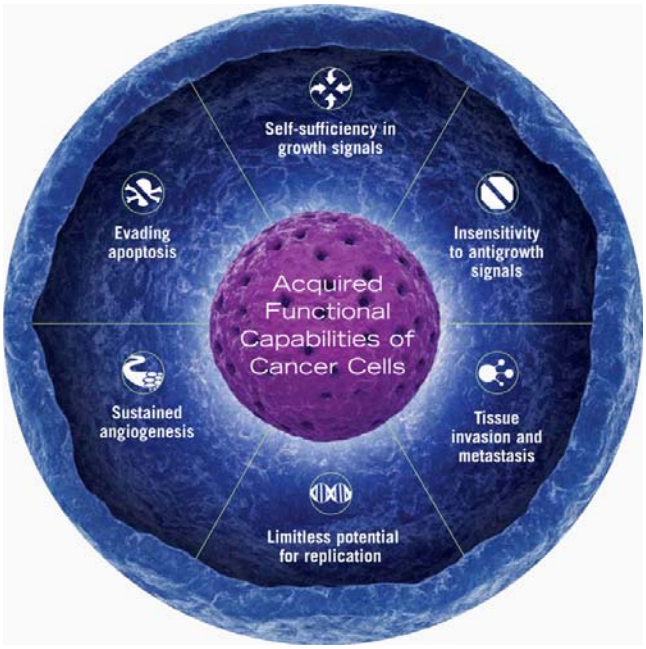

Fig. 4. Hall marks of cancer *(Source: Hanahan and Weinberg, 2000)*

Normal cell growth and division are largely under the control of a network of chemical and molecular signals. Disruption of the signaling process results in abnormal growth and division of cells. Aberrant expression of many proteins like MAP kinases, Bcl-2, Rac, AKT, NF-κB, AP-1, p53, β-catenin, COX-2, 5-LOX etc is required for the cancer cell proliferation. This condition of abnormal growth and uncontrolled division of cells is called cancer, which is one of the major causes of death worldwide, including India.

Cancer is a leading cause of death worldwide. The disease accounted for 7.9 million deaths (or around 13% of all deaths worldwide) in 2007*(WHO report,*

2007). The main types of cancer leading to overall cancer mortality each year are:

- Lung (1.4 million deaths/year)
- Stomach (866,000 deaths)
- Liver (653,000 deaths)
- Colon (677,000 deaths)
- Breast (548,000 deaths).

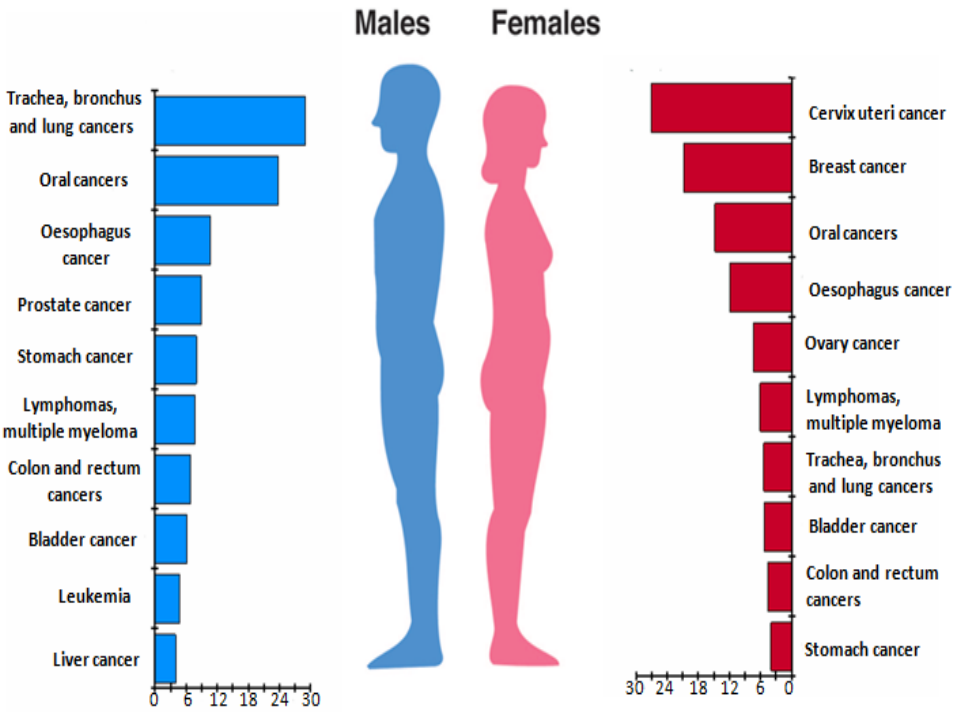

Fig. 5. Estimates of new cancer cases in India *(Source: www.who.int)*

About 72% of all cancer deaths in 2007 occurred in low- and middle-income countries, where resources available for prevention, diagnosis and treatment of cancer are limited or nonexistent. Based on projections, cancer deaths will continue to rise with an estimated 9 million people dying from cancer in 2015, and 12 million dying in 2030. The most frequent types of cancer worldwide (in order of the number of global deaths) are among men - lung, stomach, liver, colorectal, oesophagus and prostate and among women - breast, lung, stomach, colorectal and cervical.

Every year about 9,00,000 new cancer cases are diagnosed in India resulting in about 6,00,000 cancer related death every year. India has the highest number of the oral and throat cancer cases in the world. Every third oral cancer patient in the world is from India. In males - oral, lung and stomach cancers are the three most common causes of cancer incidence and death. In females - cervical, breast and oral cancers are the three main causes of cancer related illnesses and death.

Ⅵ. Cancer types

Cancers are classified by the type of cell that resembles the tumor and, therefore, the tissue presumed to be the origin of the tumor. Cancers are grouped into five major categories: carcinoma, sarcoma, myeloma, leukemia, and lymphoma. In addition, there are also some cancers of mixed types.

- Carcinoma refers cancer of epithelial origin or of the internal or external lining of the body. Carcinomas account for 80 to 90 percent of all cancer cases.

- Sarcoma originates in supportive and connective tissues such as bone, tendon, cartilage, muscle, and fat. Sarcoma tumors usually resemble the tissue in which they grow.
- Myeloma is cancer that originates in the plasma cells of bone marrow.
- Leukemias ("liquid cancers" or "blood cancers") are cancers of the bone marrow (the site of blood cell production).
- Lymphomas develop in the glands or nodes of the lymphatic system, a network of vessels, nodes, and organs (specifically the spleen, tonsils, and thymus) that purify bodily fluids and produce infection-fighting white blood cells, or lymphocytes. Unlike the leukemias which are sometimes called "liquid cancers," lymphomas are "solid cancers." Lymphomas may also occur in specific organs such as the stomach, breast or brain.

Ⅶ. Eicosanoids

In mammalian cells, eicosanoid biosynthesis is usually initiated by the activation of phospholipase A2 and the release of arachidonic acid (AA) from membrane phospholipids. The AA is subsequently transformed by cyclooxygenase (COX) and lipoxygenase (LO) pathways to prostaglandins, thromboxane and leukotrienes. Eicosanoid production is considerably increased during inflammation. Eicosanoids, the oxygenated metabolites of eicosapolyenoic fatty acids such as arachidonic acid (AA), have been the most actively studied of all the physiological components contributing to inflammation (Williams and Higgs, 1988; Wymann and Schneiter, 2008). Arachidonic acid generated from cellular membrane phospholipids gets oxygenated by either the cyclooxygenase (COX) pathway that generates

prostaglandins (PGs) or the lipoxygenase (LOX) pathway that forms hydroperoxy derivatives. In addition to COX and LOX pathways, AA is also oxygenated by the epoxygenase pathway involving cytochrome P450s to generate epoxyeicosatrienoic acids (EETs).

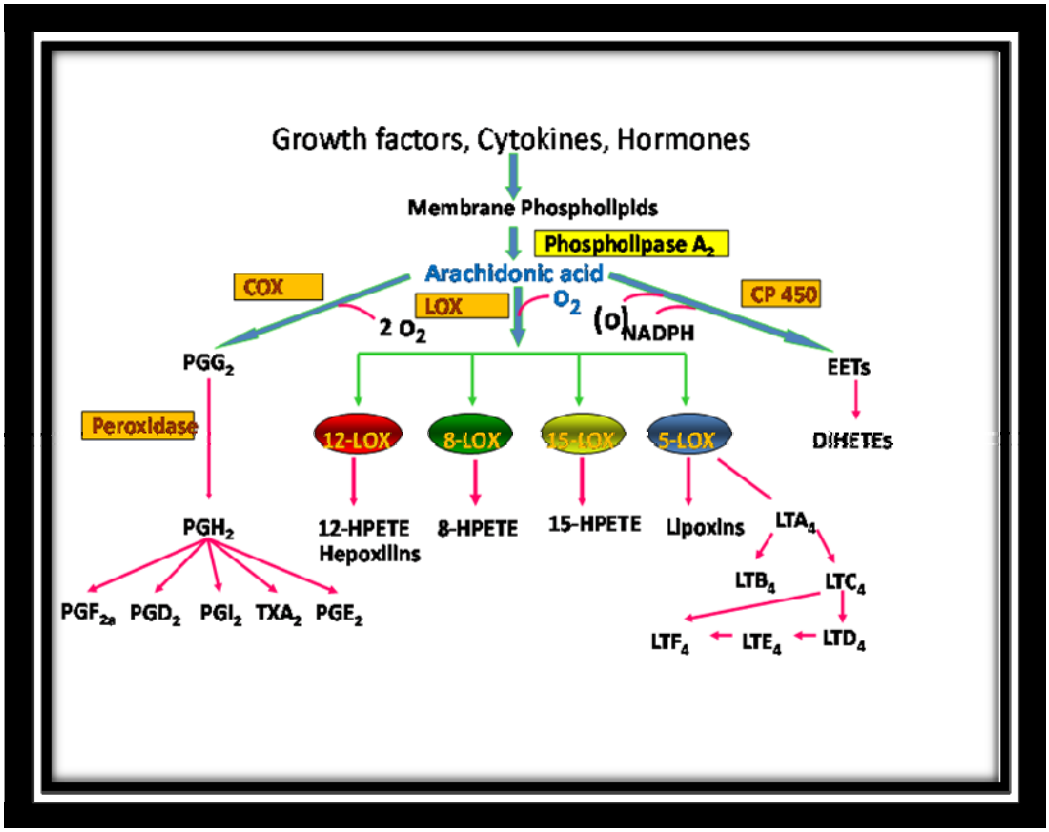

Fig. 6. Arachidonic metabolism

Ⅷ. Cyclooxygenases

Cyclooxygenase (COX), also known as Prostaglandin H synthase (PGHS) (EC 1.14.99.1), is the rate limiting enzyme in the biosynthesis of prostanoids (DeWitt *et al.,* 1990). It converts arachidonic acid to prostaglandin H_2 that gets

further metabolized tissue specifically to various prostaglandins, prostacyclin and thromboxanes together called as prostanoids. These prostanoids are extremely potent biologically active compounds with bewildering variety of actions. The enzyme has two distinct activities: a cyclooxygenase activity, which catalyzes the formation of PGG_2 from arachidonate, and a peroxidase activity, which reduces the hydroperoxide group of PGG_2 to form PGH_2 (Miyamoto *et al.,* 1976; Pagels *et al.,* 1983). COX exists in at least two isoforms. COX-1 is typically constitutive enzyme whereas COX-2 is expressed in most tissues and cells at very low levels unless induced by mitogenic or hormonal stimuli (Kujubu *et al.,* 1991). COX-1 is expressed constitutively in nearly all mammalian tissues and is the source of prostaglandins central to "housekeeping" functions such as renal water reabsorption, vascular homeostasis, and gastric protection (Smith *et al.,* 1996). COX-2 is absent from most cells but can be rapidly and dramatically induced in many cell types upon treatment with inflammatory cytokines, growth factors, v-*src*, and tumor promoters (Foegh *et al.,* 1989; Jones *et al.,* 1993; Hulkower *et al.,* 1994).

COX isozymes share 60% primary sequence identity and X-ray crystal structures of the proteins are virtually superimposable (Xie and Herschman, 1995). Kinetic profiles suggest similar if not identical reaction mechanism. There are, however, significant differences between the two isozymes with respect to their pharmacological profiles and each isozyme plays an independent role in cell physiology (Picot *et al.,* 1994; Xie and Herschman, 1995). COX-1 and COX-2 share all critical amino acids required for prostaglandin H_2 synthesis from arachidonate. In their purified forms, the COX

isoforms show nearly identical catalytic properties toward arachidonate. Both enzymes are glycoproteins (Kurumbail *et al.,* 1996), associated with membranes of endoplasmic reticulum and nuclei (Langenbach *et al.,* 1995).

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin ameliorate many aspects of arthritis, and many diseases, but their deleterious side effects (e.g., gastric ulceration and renal insufficiency) are significant. Selective inhibitors of COX-2 provide the benefits of NSAIDs with minimized gastric side effects.

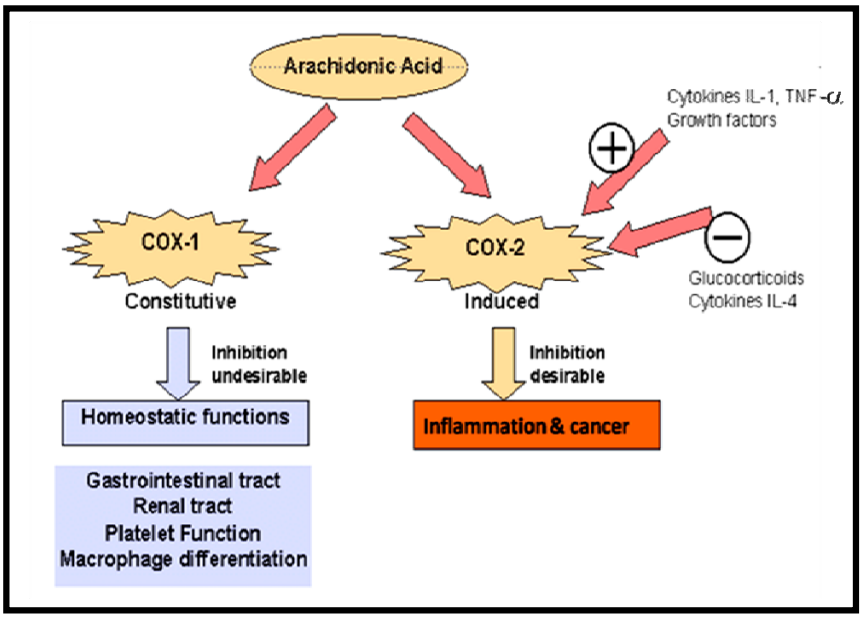

Fig. 7. COX path way *(Source: www.arthritis.co.za)*

Ⅸ. Lipoxygenases

Lipoxygenases comprise a family of non-heme, iron containing dioxygenases which incorporate molecular oxygen into poly unsaturated fatty acids with 1-cis, 4-cis-pentadiene structures such as arachidonic acid and linoleic acid, to give rise to 1-hydroperoxy-2, 4-trans, cis-pentadiene products. Lipoxygenases catalyse conversion of arachidonic acid to hydroperoxy-eicosatetraenoic acids (HPETEs), leukotrienes (LTs) and lipoxins. Lipoxygenation involves the formation of radical intermediates, which usually remain enzyme bound. Thus, the LOX reaction itself may not be considered an efficient source of free radicals. There are five active LOXs found in humans: 5-LOX, 12*S*-LOX, 12*R*-LOX, 15-LOX-1 and 15-LOX-2. Lipoxygenase products have been implicated in the pathogenesis of hypersensitivity, asthma, psoriasis, atherosclerosis and cancer. Leukotrienes (LTs) play a major part in the inflammatory process (Morham *et al.,* 1995). They are synthesized via the 5-LOX pathway (Fig. 9).

Ⅹ. 5-Lipoxygensae

5-LOX is the key enzyme in leukotriene biosynthesis (Ford-Hutchinson *et al.,* 1994) and is a current target for pharmaceutical intervention in a number of diseases. It translocates to the nuclear membrane upon stimulation, where it co-localizes with 5-LOX activating protein (FLAP) and cytosolic phospholipase A_2 ($cPLA_2$). This event converts arachidonic acid to leukotrienes.

The final and biologically active metabolites of the 5-LOX cascade are LTB_4 and the so-called cysteinyl LTs (LTC_4, LTD_4, and LTE_4), formerly known as slow

reacting substance (SRS) related to anaphylaxis, which are derived from the unstable intermediate LTA_4 (Futaki *et al.,* 1994). Leukotrienes are potent mediators of inflammation.

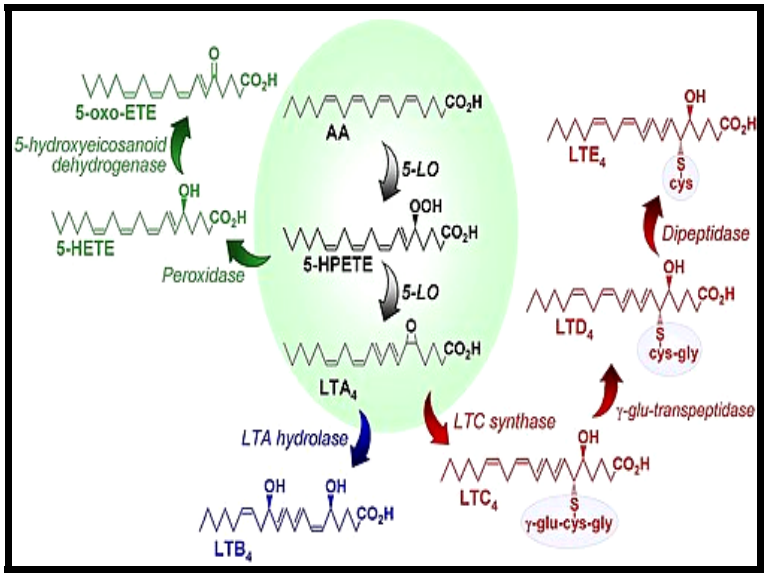

Fig. 8. 5-Lipoxygenase pathway showing the formation of leukotrienes.

(Source: Menard et al., 1996)

The target for the biological effects of LTB_4 has been found to be primarily inflammatory cells. LTB_4 is a potent stimulator of leukocyte activation, and adhesion of these cells to vascular endothelium, elicits chemokinetic and chemotactic responses (Mao *et al.,* 2004). During brief exposure to LTB_4, polymorpho nuclear leukocytes (PMN) are predominantly recruited, whereas during prolonged exposure, as probably occurs when LTB_4 is formed *in vivo*, other granulocytes, including neutrophils and eosinophils, are found in tissues and exudates(Sala *et al.,* 1998; Penrose and Austen, 1999). Furthermore, LTB_4 has been shown to be involved in the pathogenesis of a variety of inflammatory diseases (Bray *et al.,* 1981). It has been observed that LTB_4 stimulates the

production and release of pro inflammatory cytokines from macrophages and lymphocytes, and, recently, from synovial membranes (Smith *et al.,* 1980).

XI. Role of COX and 5-LOX in inflammation

Eicosanoids of COX and LOX pathways have been the most actively studied of all the physiological components contributing to inflammation (Wymann and Schneiter, 2008) (Fig. 9). These eicosanoids, formed tissue specifically, mediate various inflammatory responses in a receptor coupled mechanism. For example, thromboxane A2 (TXA2) from platelets, aggregates platelets, constricts blood vessels (Huang *et al.,* 2004). Prostacyclin (PGI_2) from the vessel wall, prevents platelet aggregation, and dilates vessels. Prostaglandin E_2 (PGE_2), also a potent vasodilator, greatly potentiates the ability of bradykinin to cause pain, and seems to be the local mediator of fever production from the hypothalamus. Both PGE_2 and prostacyclin potentiate permeability. Other prostaglandins exert a host of effects. Together, eicosanoids contribute to the inflammatory reaction at all steps (Rocca and Fitzgerald, 2002). All of the inflammatory cells except lymphocytes produce leukotrienes, products of the LOX pathway of arachidonic acid metabolism. It was reported to play a role in atherosclerosis, pulmonary fibrosis and cancer (Jala and Haribabu, 2004; Flamand *et al.,* 2007). Leukotrienes C_4 (LTC_4) and its products D_4, and E_4 increase vascular permeability and constrict smooth muscle. LTC_4 gets converted into a least potent LTF_4 by the action of carboxy peptidase (Reddanna *et al.,* 2003). Leukotriene B_4 is a potent chemotactic agent.

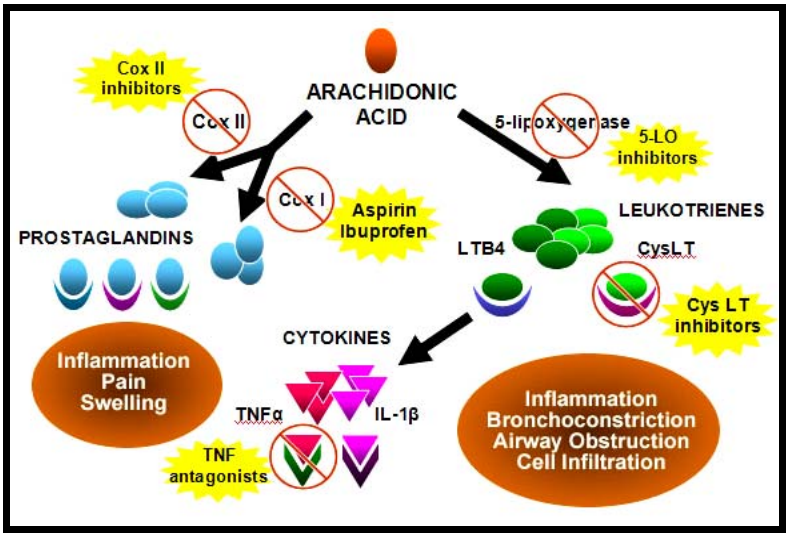

Fig. 9. Role of COX-2 and 5-LOX in inflammation

(Source: www.mydietaryfats.org)

XII. COX and LOX: Role in cancer

Overexpression of COX-2 appears to be a consequence of both increased transcription and enhanced mRNA stability. Importance of COX-2 in apoptosis was recognized when it was realized that NSAIDs induce apoptosis in cancer cell lines, and dysregulation of apoptosis is an important mechanism of cancer progression contributed by COX-2. Selective inhibitors of COX-2 have been demonstrated to induce apoptosis in variety of cancer cell lines including colon, stomach, prostate, and breast (Subhashini *et al.,* 2005; Sarkar *et al.,* 2007). COX-2 expression in colon cancer cells has been found to promote angiogenesis of co-cultured endothelial cells by stimulating the production of angiogenic factors (Tsujii *et al.,* 1998). Human gastric and breast tumors express higher levels of COX-2 than the surrounding normal tissue (Brown and Lippman, 2000). The importance of PGs in tumorigenesis is evidenced by the data demonstrating the

ability of NSAIDs to inhibit growth and metastasis of tumors *in vivo* (Snyderman *et al.,* 1995) and mostly of colon cancer (Dial *et al.,* 2006; Antonakopoulos and Karamanolis, 2007). High levels of prostaglandins, possibly reflecting the over expression of the COX-2 enzyme and playing important roles in proliferation and differentiation of various cancer cell lines, have been detected in different tumor types (Sheng *et al.,* 2001).

5-LOX is involved in the biosynthesis of leukotrienes, pro-inflammatory mediators participating in various forms of acute and sub acute inflammation. 5-HETE, product of 5-LOX was shown to be a potential survival factor for prostate cancer cells and inhibition of 5-LOX triggered massive apoptosis (Ghosh and Myeres, 1998). It has been established that lipoxygenases and their products are required for cancer growth.

Accumulating evidence indicates that 5-LOX may play a relevant role in development and progression of numerous cancers. Increased 5-LOX expression and induction of apoptosis by its inhibitors were shown in renal (Matsuyama *et al.,* 2005), oesophageal (Chen *et al.,* 2004; Hoque *et al.,* 2005) and breast (Kim *et al.,* 2005) cancers. In this last case, blockade of 5-LOX pathway induces apoptosis through the cytochrome c release and caspase-9 activation, with changes in the levels of Bcl-2 family proteins. Moreover, in human melanoma cells, lipoxygenase inhibitors may inhibit the expression of intracellular adhesion molecule-1 (ICAM-1) and might be valuable for treatment of melanoma metastasis (Wang *et al.,* 2004).

A number of studies reported the role of 5-LOX and leukotrienes in colon, prostate and pancreas cancers. 5-Lipoxygenase and leukotriene receptor $CysLT_1$ are up regulated in colon cancer (Nielsen *et al.,* 2003). So, via an increase in PI3K phosphorylation, LTD_4 cannot only increases proliferation and survival of intestinal epithelial cells but also triggers a motile response in these cells (Paruchuri *et al.,* 2005). Moreover, inhibition of 5-LOX decreases expression of VEGF, MMP-2 and MMP- 9 (Ye *et al.,* 2005).

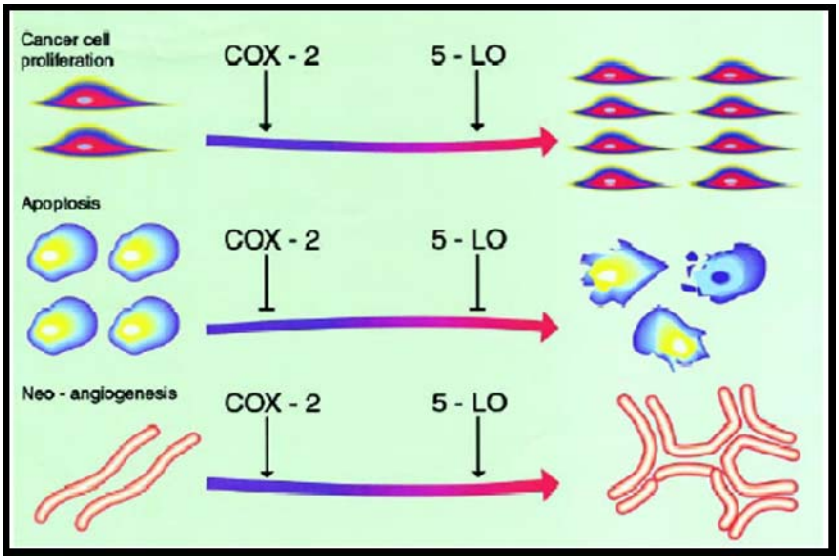

Fig. 10. Role of COX-2 and 5-LOX in cancer

Arachidonic cascade seems to show an important role in the genesis of pancreatic cancer and 5-LOX is suggested as marker for early pancreatic intraepithelial neoplastic lesions in order to detect this fourth leading cause of cancer death in an non-invasive stage (Hennig *et al.,* 2005). A double-blinded, placebo controlled phase II clinical trial with LY-293111, an orally stable LTB_4

receptor antagonist is currently underway (Ding *et al.,* 2005). This inhibitor could prevent ROS generation via 5-LOX and downstream NADPH oxidase, which mediates the prosurvival effect of extracellular matrix (ECM) in pancreatic cancer cells (Edderkaoui *et al.,* 2005). Finally, many authors have reported increase in the 5-LOX mRNA and protein expression in malignant tissue as compared with benign tissue of human prostate samples (Matsuyama *et al.,* 2005; Moretti *et al.,* 2004). One critical survival factor could be the dehydrogenated derivative of 5(S)-HETE, 5-oxoeicosatetraenoic acid (5-oxoETE) (Sundaram and Ghosh, 2006).

XIII. NSAIDs

Historically, anti-inflammatory drugs had their origins in the serendipitous discovery of certain plants and their extracts being applied for the relief of pain, fever and inflammation. When salicylates were discovered in the mid-19th century to be the active components of Willow Spp., this enabled these compounds to be synthesized and from this, acetyl-salicylic acid or Aspirin was developed. Likewise, the chemical advances of the 19th-20th centuries lead to development of the non-steroidal anti-inflammatory drugs (NSAIDs), most of which were initially organic acids, but later non-acidic compounds were discovered. NSAIDs, gained immense interest not just because they are the major targets of cyclooxygenases, but also because they are involved in a range of pathologies that for COX-1 include thrombosis and for COX-2 include inflammation, pain and fever, various cancers, and also Alzheimer's disease (Kawai *et al.,* 2002; Marnett, 2002). Specific inhibition of COX-2 might have therapeutic actions similar to those of NSAIDs, but without causing the

unwanted side effects, was the rationale for the development of specific inhibitors of the COX-2 enzyme as a new class of anti-inflammatory and analgesic agents with improved gastrointestinal tolerability (Vane, 1998).

ⅩⅣ. NSAIDs as chemopreventives

NSAIDs reduce pain by interfering with the production of prostaglandins from arachidonic acid. Prostaglandins, produced at a site of inflammation, sensitize pain receptors in the area. NSAIDs block prostaglandin production from arachidonic acid through the inhibition of the enzymes cyclo-oxygenase 1 and 2 (COX-1 and COX-2). NSAIDs are commonly used to treat headache, muscle soreness from strains or other injuries, arthritis, menstrual pain and cramping, and mild fever. More severe pain and inflammation may require the use of steroidal anti-inflammatory drugs, or opiate pain relievers. Epidemiological studies have shown that long-term intake of aspirin is associated with a reduction in the incidence of colorectal cancer (Thun *et al.,* 1991; Giovannucci *et al.,* 1995) and breast cancer (Garcia and Gonzalea, 2004). In a clinical trial, celecoxib (Steinbach *et al.,* 2000) was found to reduce the number and size of polyps in patients with familial adenomatous polyposis (FAP). The effects of several non-steroidal anti infflammatory drugs (NSAIDs) on tumor growth have also been demonstrated in animal models of FAP (Oshima *et al.,* 1996) and chemical colon carcinogenesis (de Jong *et al.,* 2000; Brown *et al.,* 2001) and prostate cancer. These observations suggest that NSAIDs have a potent chemopreventive effect.

Ⅹ Ⅴ. Mechanism of action of NSAIDs

NSAIDs inhibit the cyclooxygenase (COX) enzymes, COX-1 and COX-2, which catalyse the conversion of arachidonic acid to prostaglandins. COX-1 is expressed constitutively and is required for physiological processes such as maintenance of gastrointestinal mucosa and platelet aggregation, whereas COX-2 is induced by cytokines, growth factors, and mitogens.

NSAIDs vary in their abilities to inhibit COX-1 and COX-2 (Thun *et al.,* 2002). Classic NSAIDs not only inhibit COX-2, but also inhibit COX-1, resulting in the common side effect of gastric mucosal damage. To reduce the gastrointestinal side effects of NSAIDs, selective COX-2 inhibitors were developed (Cannon and Breedveld, 2001; Aisen, 2002). Selective inhibitors of COX-2 have been demonstrated to induce apoptosis in a variety of cancer cells, including those of colon (Takahashi *et al.,* 2006), stomach (Lazebnik *et al.,* 2005), prostate and breast (Liu *et al.,* 1998). These observations are consistent with the cancer chemopreventive effects of NSAIDs. Tumour inhibition by NSAIDs may be mediated by distinct cellular processes. These processes involve the ability of NSAIDs to restore apoptosis, induce cell-cycle arrest, and inhibit angiogenesis (Chan, 2002; Subhashini *et al.,* 2004). One of the main ways by which NSAIDs exert their effects is modulation of apoptosis, although there is considerable debate about how these effects are mediated.

Compounds that are structurally similar to NSAIDs, but do not inhibit COX, also have chemopreventive and pro-apoptotic properties. The precise mechanisms by which, inflammation stimulates cancer development are not

fully understood and are likely to be complex and multifactorial (Coussens and Werb, 2002). Indeed, NSAIDs have been shown to inhibit other pathways that contribute to inflammation. For example, NSAIDs inhibit activation of NF-kB, which controls the transcription of a variety of proinflammatory cytokines, independently of COX inhibition (Tegeder *et al.,* 2001; Subhashini *et al.,* 2004). It is therefore quite possible that NSAIDs inhibit inflammation stimulated carcinogenesis by affecting the function of these different pathways in addition to those of COX-1 or COX-2. However, because these agents are potent inhibitors of COX catalytic activity, it might be difficult to judge the relative contribution of these COX and non-COX mechanisms.

Chapter-1

Isolation and characterization of COX-LOX dual inhibitor from *Terminalia chebula*

1.1. Introduction

NSAIDs work by inhibiting the cyclooxygenase (COX) enzymes, COX-1 and COX-2, so preventing the formation of inflammatory prostaglandins from metabolism of arachidonic acid. However, by inhibiting COX-1 they can also cause serious gastrointestinal (GI) side effects and adversely affect platelet function. Increasingly, conventional NSAIDs are being replaced by COX-2 specific inhibitors, such as Celecoxib, for symptomatic relief of pain and inflammation. At therapeutic concentrations these drugs inhibit COX-2, which is associated with tissue injury, but spare COX-1 and so cause less GI toxicity. Recent studies suggest that NSAIDs induced GI toxicity may involve shunting arachidonic acid metabolism to the 5-LOX pathway, so increasing the production of gastrotoxic leukotrienes (de Gaetano *et al.,* 2003; Ziboh *et al.,* 2004). 5-lipoxygenase (5-LOX) is an enzyme associated with the production of pro-inflammatory and gastrotoxic leukotrienes (Sala and Folco, 2001). Inhibition of 5-LOX may therefore offer a new approach to reducing the GI toxicity associated with NSAID use, while retaining the analgesic and anti-inflammatory properties of NSAIDs and COX-2 specific inhibitors (Fiorucci *et al.,* 2001). Interestingly, 5-LOX has been implicated in the deterioration of joints in Osteoarthritis (OA) (Hinz and Brune, 2004). Inhibition of 5-LOX may therefore help protect arthrodial cartilage and slow disease progression. Leukotrienes are pro-inflammatory, they recruit pro-inflammatory immune cells, increase vascular permeability, acts as powerful bronchoconstrictor agents, and are damaging to the gastrointestinal tract (Bias *et al.,* 2004). The selective COX-2 inhibitors in the market, also were shown to have cardiac side effects on the

long-term use. This has lead to the withdrawl of refecoxib, another selective inhibitior of COX-2, from the market. These gastro-intestinal and cardiac side effects of conventional and selective NSAIDs respectively may be due to the shunt of AA metabolism towards 5-LOX pathway (Rainsford, 1993). Such a shunt has been shown *in vivo* and an enhanced synthesis of LTB4 was demonstrated in patients with rheumatoid arthritis or osteoarthritis taking NSAIDs for more than 3 months (Hudson *et al.,* 1993). Similarly, COX-2 inhibition by Celecoxib in cancer cell lines was shown to increase the formation of 5-HETEs, which is having tumor cell proliferative property (Ye *et al.,* 2005).

Both 5-LOX and COX-2 are co-expressed and up-regulated in inflammation and in many forms of human cancers, including colon, prostate, breast and lung cancers and form targets for development of anti-inflammatory and anti-cancer drugs (Pommery *et al.,* 2004). Hence there is need for the development of potent COX-LOX dual inhibitors without side effects. In this connection natural products isolated from medicinal plants form a very good alternative. We have earlier reported that c-Phycocyanin and betanin are potent selective inhibitors of COX-2 and 5-LOX respectively (Subhashini *et al.,* 2004; Sreekanth *et al.,* 2007). But since there is a need for a dual COX-LOX inhibitor, the present study is designed to screen various natural herbal sources for the identification of potent 5-LOX and COX-2 inhibitors. In our attempt to isolate a natural product with of COX-LOX dual inhibition, we identified *Terminalia chebula* as a potential source.

1.1.1. *Terminalia chebula*

Terminalia chebula Retz. (Combretaceae) has an esteemed origin according to Indian mythology and is well known for its medicinal properties. *Terminalia chebula* is called the "king of medicines" and is always listed first in the Ayurvedic meteria medica because of its extraordinary powers of healing. In Ayurveda it is considered to destroy all diseases and eliminate all waste from the body. At the same time, it is known to promote tissue growth and health. The fruits of *Terminalia chebula* are very important and its uses have been described by Charaka, the most eminent Ayurvedic physician, in the first chapter in his text *"Charaka Samhita"*, the best yet known text for Ayurvedic medicines and formulations. Its fruits have been described as Mridu Virechaka (mild laxative), Medhakara (enhances memory), Rasayana (rejuvenating, prevents aging and disease), Netra Hithakara (good for eyes), Laghu Paki (digests easily), Ayurvardhaka (increases life span) and Brimhana (nourishes body tissues).*Terminalia chebula* is used in India to treat many diseases such as digestive diseases, urinary diseases, diabetes, skin diseases, parasitic infections, heart diseases, irregular fevers, flatulence, constipation, ulcers, vomiting, colic pain and hemorrhoids (Barthakur and Arnold, 1991). Its fruit powder is one of the constituents of *Triphala,* a well known Ayurvedic medicine used for treatment of many chronic diseases such as ageing, heart ailments, diabetes, kidney diseases and gastro intestinal disorders. Its aqeous extract reported to have free radical scavenging activities and radioprotector properties (Naik *et al.,* 2004). A number of biological activities in water or alcoholic extracts of *Terminalia chebula* have been reported.

Fig. 11. *Terminalia chebula*. The insets show the fully ripen (right) and dried (left) fruits.

Lee *et al*. (2005) reported that methanolic extract of *Terminalia chebula* has anti-tumor activity. Saleem *et al*. (2001) reported higher phenolic contents and stronger *in vitro* lipid peroxidation inhibition capability for *Terminalia chebula*. The anti-oxidants are known to play a key role in reducing cancer cell proliferation and tannins are stronger lipid peroxidation inhibitors (Chinery *et al.,* 1998). Fourteen hydrolyzable tannins and related compounds have been isolated and identified from *Terminalia chebula* (Okuda *et al.,* 1983) which can be categorized into four groups: (i) phenolcarboxylic acids: gallic acid (1),

ellagic acid (11), and chebulic acid (2); (ii) gallotannins: 1,6-di-O-galloyl-b-D-glucose (3), 3,4,6-tri-O-galloyl-b-D-glucose (5), and 1,2,3,4,6-penta-O-galloyl-b-D-glucose (14); (iii) ellagitannins: punicalagin (4), casuarinin (6), corilagin (8), and terchebulin (10); (iv) others: chebulanin (7), neochebulinic acid (9), chebulagic acid (12), and chebulinic acid (13) (Fig. 12).

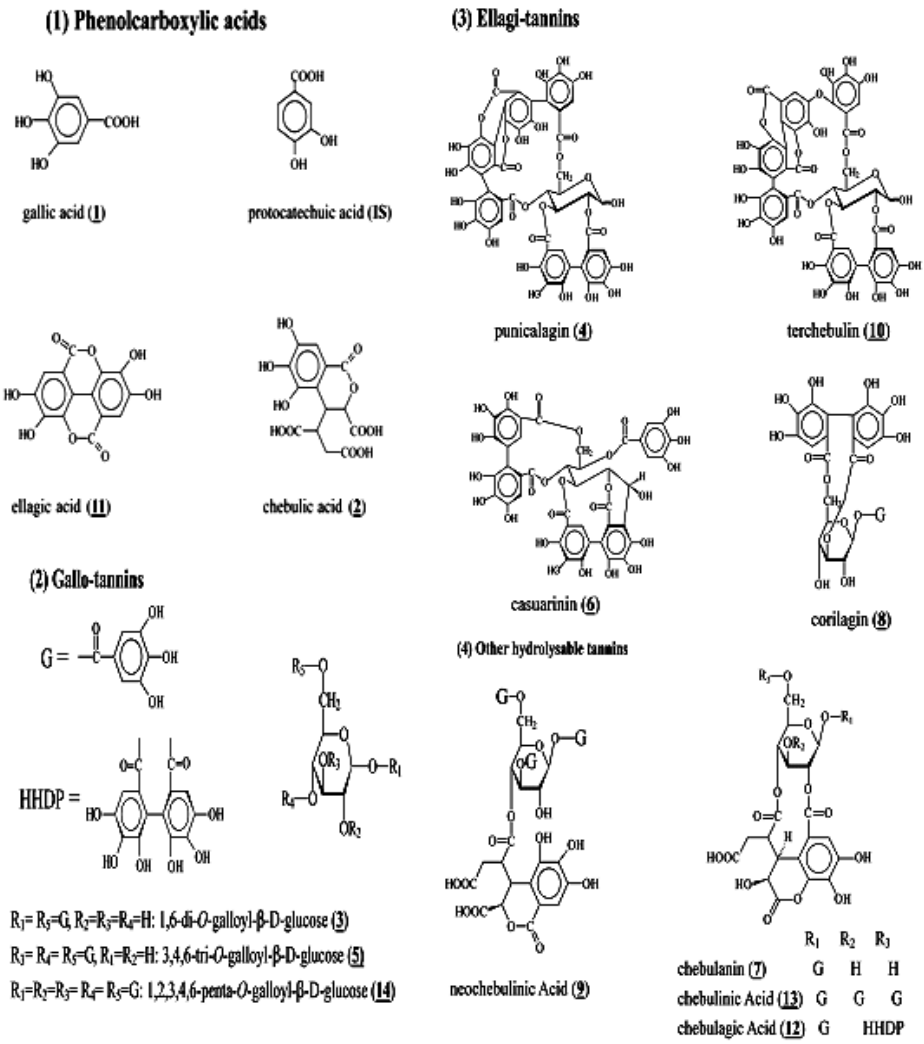

Fig. 12. Structures of the fourteen hydrolyzable tannin constituents in *Terminalia chebula.*

As pointed above both cyclooxygenases and lipoxygenases form targets for development of anti-inflammatory and anti-cancer drugs. The conventional as well as selective COX-2 inhibitors in the market, however, are reported to have many side effects due to the activation of 5-LOX pathway. Hence, there is need for the development of potent COX-LOX dual inhibitors without side effects. In this connection natural products isolated from medicinal plants form a very good alternative. The present study, therefore, is designed to screen *Terminalia chebula* fruit for the identification of potent 5-LOX and COX-2 inhibitors.

1.1.2. Objectives

The specific objectives of present study are:

- To screen *Terminalia chebula* fruit extracts for COX/5-LOX dual Inhibition.
- To isolate and characterize the active principle(s) involved in the COX/5-LOX dual inhibition.

1.2. Materials and Methods

1.2.1. Plant material collection

Dried fruits of *Terminalia chebula* (locally known as *Karakkaya*) were procured from the local vendors of traditional herbs and nuts of Adilabad, Andhra Pradesh, India. The identity of the fruits was cross checked with Prof. Appa Rao, of University College of Pharmaceutical Sciences, Kakatiya University, Warangal, who worked on its chemistry earlier (Reddy *et al.,* 1994).

1.2.2. Chemicals

TMPD (N, N, N', N'-tetramethyl p-phenylenediamine) and hematin were purchased from Sigma Chemical Company (St.Louis, USA). Arachidonic acid purchased from Nu-check Prep, Inc (MN, USA). All chemicals for chromatography were of HPLC grade. All other chemicals and solvents used were of analytical grade.

1.2.3. Preparation of ethanolic extract of *Terminalia chebula* fruit

Dried fruits were broken to remove the seeds and fruit coat was finely powdered using mixer. Absolute alcohol was added to the powder and thoroughly mixed by vortexing intermittently for 1 h and centrifuged at 5000g for 5 minutes. The extract was then lyophilized, redissolved in absolute alcohol and checked for inhibitory effects against cyclooxygenases (COX) and lipoxygenases (LOX).

1.2.4. RP-HPLC of the ethanolic extract

The lyophilized ethanolic extract of *Terminalia chebula* fruits, obtained above, was redissolved at 20 mg/ml in absolute alcohol and subjected to reverse phase HPLC (RP-HPLC) by employing C18 column (Shim-pack column with dimensions 250X4.6 mm and particle size 5 μm) with 1 ml/min flow rate and the eluants were monitored at 280 nm. The mobile phase consists of a complex gradient of solvent A (water: acetic acid-1000:1), and solvent B (acetonitrile: acetic acid- 1000:1). The elution was carried out according to the following gradient profile: 0-5 min, 95% of A, 5% of B; 5-10 min, 95-80% of A, 5-20% of B; 10-25 min, 80-70% of A, 20-30% of B; 25-45 min, 70-65% of A, 30-35% of B; 45-55 min, 65-50% of A, 35-50% of B; 55-65 min, 50-5% of A, 50-95% of B; 65-70 min, 5-95% of A, 95-5% of B. The flow rate was maintained constant at 1 ml/min and the peaks were monitored at 280 nm. Isolated peaks were tested for the COX-LOX inhibitory effect. The large scale purification of individual peaks with COX/LOX dual inhibition was carried on preparative RP-HPLC column (Shim-pack PREP-ODS column with dimensions 500x46 mm and particle size 5 μm) and the fractions were eluted by employing complex gradient described above, with a flow rate of 8 ml/min.

1.2.5. Isolation, purification and assay of COX-1 and COX-2

A. Cyclooxygenase-1

COX-1 was isolated from the microsomes obtained from ram seminal vesicles. The solubilized microsomal fraction was passed through DE-52 column

and the active fractions pooled were used for the inhibitory assays (Hemlar and Land, 1976).

B. Cyclooxygenase-2 (COX-2)

Human recombinant COX-2 expressed in *Spodoptera frugiperda* (Sf9) cells by baculovirus expression system was employed for the inhibitory assay as described earlier (Reddy *et al.,* 2000).

C. Cyclooxygenase Assay

Enzymatic activities of COX-1 and COX-2 were measured according to the method of Copeland et al. (1994), with slight modifications using a chromogenic assay based on the oxidation of N,N,N,N,-tetra methyl-p-phenylene diamine (TMPD) during the reduction of PGG_2 to PGH_2 (Egan *et al.,* 1976; Pagels *et al.,* 1983). Briefly, the assay mixture contained Tris-HCl buffer (100 mM, pH 8.0), hematin (15 μM), EDTA (3 μM), enzyme (100 μg COX-1 or COX-2) and the test compound. The mixture was pre-incubated at 25 °C for 15 min and then the reaction was initiated by the addition of arachidonic acid and TMPD, in total volume of 1 ml. The enzyme activity was determined by estimating the velocity of TMPD oxidation for the first 25 seconds of the reaction by following the increase in absorbance at 603 nm. A low rate of non-enzymatic oxidation observed in the absence of COX-1 and COX-2 was subtracted from the experimental value while calculating the percent inhibition.

1.2.6. Purification and assay of 5-Lipoxygenase

5-Lipoxygenase from potato tubers was purified and assayed as per the method described by Reddanna et al. (1990). Enzyme activity was measured using polarigraphic method with a Clark's oxygen electrode on Strathkelvin Instruments, model 782, RC-300. Typical reaction mixture contained 50-100 µl of enzyme and 10 µl of substrate and final volume made to 3 ml with100 mM phosphate buffer pH 6.3. The substrate solution contained 133 µM (final concentration) arachidonic acid in the reaction mixture. Since lipoxygenases are oxygen-consuming enzymes the concentration of oxygen decrease in the reaction mixture, the rate of decrease in oxygen was taken as a measure of enzyme activity. Reaction was allowed to proceed at 25 °C and the maximum slope generated was taken for calculating enzyme activity. The activity was expressed as units/mg protein, where one unit is defined as one µmole of oxygen consumed per minute.

1.2.7. LC-MS, IR and NMR analyses

LC-MS was performed according to method described by Han et al. (2006). Negative ion mass spectra was recorded setting the capillary temperature to 300 °C and maintaining the ESI spray voltage at 4 KV on Bruker micrOTOF-Q instrument. NMR spectra were recorded with a Bruker- Avance-400 instrument (^{1}H, 400 MHz). IR (KBr) spectra were recorded on JASCO FT-IR spectrophotometer Model 5300.

1.2.8. DPPH radical scavenging activity

DPPH scavenging activity was carried out as described by Kirby and Schmidt (1997). The assay is based on the decolorization of DPPH solution and decreased absorbance at 517 nm, under the influence of antioxidant substance. In brief, the reaction mixture contained ethanolic solution of DPPH (1 mM), equal volumes of test compounds at different concentrations in a final volume of 1 ml ethanol. The mixture is then incubated in dark for 20 min. The inhibition of absorbance at 517 nm is plotted as a function of concentration of antioxidants.

1.2.9. ABTS assay

The ABTS assay was employed to measure the antioxidant activity of isolated compound from ethanolic extract of *Terminalia chebula* fruit powder as described by Re et al. (1999). ABTS was dissolved in deionised water to 7 mM concentration, and potassium persulphate added to a concentration of 2.45 mM. The reaction mixture was left to stand at room temperature overnight (12 to 16 h) in the dark, before usage. The resultant intensely-coloured ABTS•+ radical cation was diluted with 0.01 M PBS (phosphate buffered saline), pH 7.4, to give an absorbance value of ~0.70 at 734 nm. The test compounds were diluted 100x with the ABTS•+ solution to a total volume of 1 ml. Absorbance was measured spectrophotometrically at time intervals of 1, 2, 5, 10, and 15 min after addition for a range of four to eight concentrations. The assay was performed at least in triplicates. Controls without ABTS•+ were used to allow for any absorbance of the extracts themselves, and 990 µl of PBS was added to these control samples instead.

1.3. Results

1.3.1. Inhibitory studies on COX and LOX with ethanolic extract of *Terminalia chebula* fruit

The effect of ethanolic extract of fruit coat powder of *Terminalia chebula* was studied on COX-1 and COX-2 *in vitro* as measured by TMPD assay. The IC_{50} values obtained for COX-1 and COX-2 enzymes were 90 and 3.75 μg/ml respectively. From this data it is clearly evident that ethanolic fraction of fruit coat powder of *Terminalia chebula* inhibits COX-2 and COX-1, with 25 fold preference towards COX-2. The ethanolic extract also showed potent inhibition of 5-LOX activity, as estimated polarigraphically by measuring decrease in the oxygen concentration, with an IC_{50} value of 20 μg/ml.

1.3.2. HPLC analysis of ethanolic extract of *Terminalia chebula* fruit powder

As shown in Fig. 13, ethanolic extract of *Terminalia chebula* fruits, resolved into 8-major Peaks (TC-I to TC-VIII). These major peaks were collected and checked for COX and LOX inhibitory effects. Among the fractions, TC-V alone showed inhibition of both COX and LOX (Table 2). TC-II and TC-III showed only COX inhibition, while TC-VI showed inhibition of LOX activity only. TC-V was purified in large quantities on preparatory RP-HPLC and lyophilized. Fine lyophilized powder of TC-V was dissolved in water, checked for inhibitory activities of COX-1, COX-2 and 5-LOX and IC_{50} values were determined.

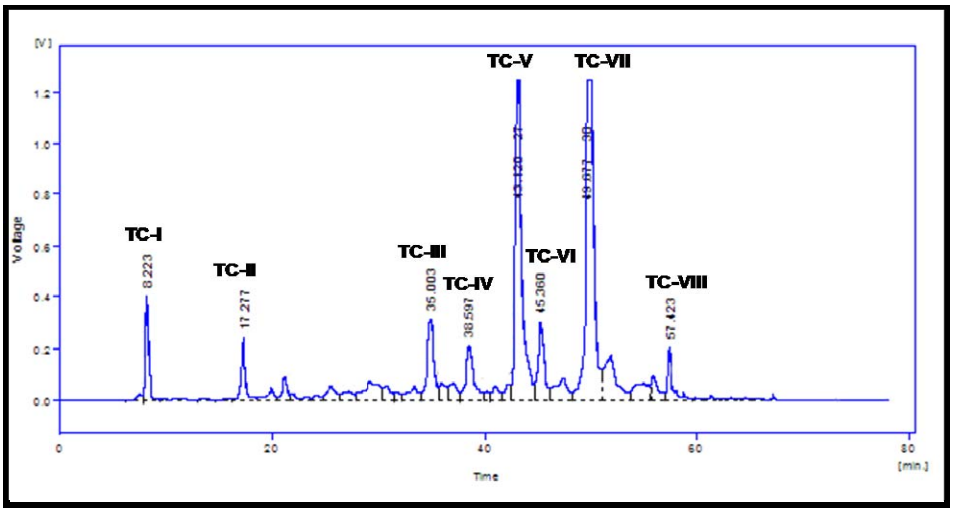

Fig. 13. RP-HPLC analysis of ethanolic extract of *Terminalia chebula* fruit

1.3.3. The LC-MS, NMR, IR analyses of TC - V

In view of dual inhibitory action of the TC-V fraction, it was subjected to electron spray ionization LC-MS analysis (Fig. 14) to identify the compound. Strong molecular ion peak $[M-H]^-$ at 953 amu (*m/z*), corresponds exactly to chebulagic acid with the molecular formula of $C_{41}H_{29}O_{27}$ reported in the literature (Gao et al., 2007). ^1H NMR δ (acetone-d6) ppm (*J* in Hz): 2.18 (1H, d, *J*=7.6 Hz), 3.7 (1H, m), 4.64 (2H,m), 4.82 (1H, d, *J*= 7.2) 4.98 (1H, d), 5.09 (1H, s), 5.37 (1H, s), 5.81 (1H, s), 6.37 (1H, s), 6.52 (1H, s), 6.95 (1H, s), 7.06 (2H, s), 7.39 (1H, s) (Fig. 17). The chemical nature of the compound was further confirmed based on IR analysis (Fig. 16).

Table 2. Enzymatic inhibitory activity of isolated peaks by RP-HPLC on COX-1, COX-2 and 5-LOX.

Peak	Retention time (minutes)	COX-1 inhibition	COX-2 inhibition	5-LOX inhibition
TC-I	8.223	-	-	-
TC-II	17.277	+	+	-
TC-III	35.003	+	+	-
TC-IV	38.597	-	-	-
TC-V	43.120	+	+	+
TC-VI	45.560	-	-	+
TC-VII	49.577	-	-	-
TC-VIII	57.423	-	-	-

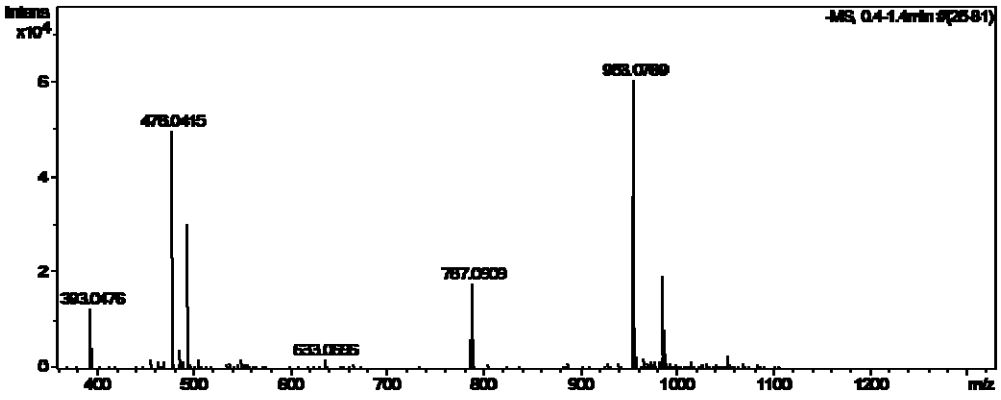

Fig. 14. LC-MS analysis of TC-V. HPLC separated peak TC-V from EtOH extract of *Terminalia chebula* fruit powder was subjected to electron spray ionization LCMS. Strong molecular ion [M-H] - at 953 amu represents the chebulagic acid.

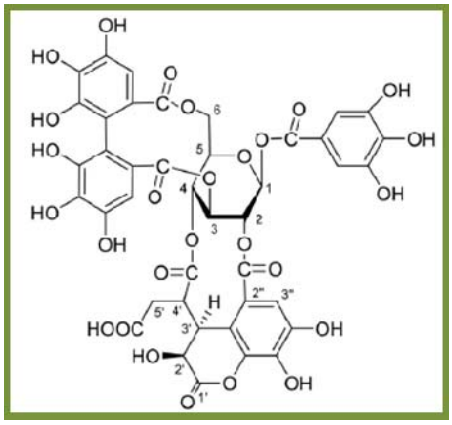

Fig. 15. Chebulagic acid. Chemical structure of chebulagic acid, with chemical formula $C_{41}H_{29}O_{27}$ (Mol.wt = 953) isolated from ethanolic extract (EtOH extract) of *Terminalia chebula* fruit powder.

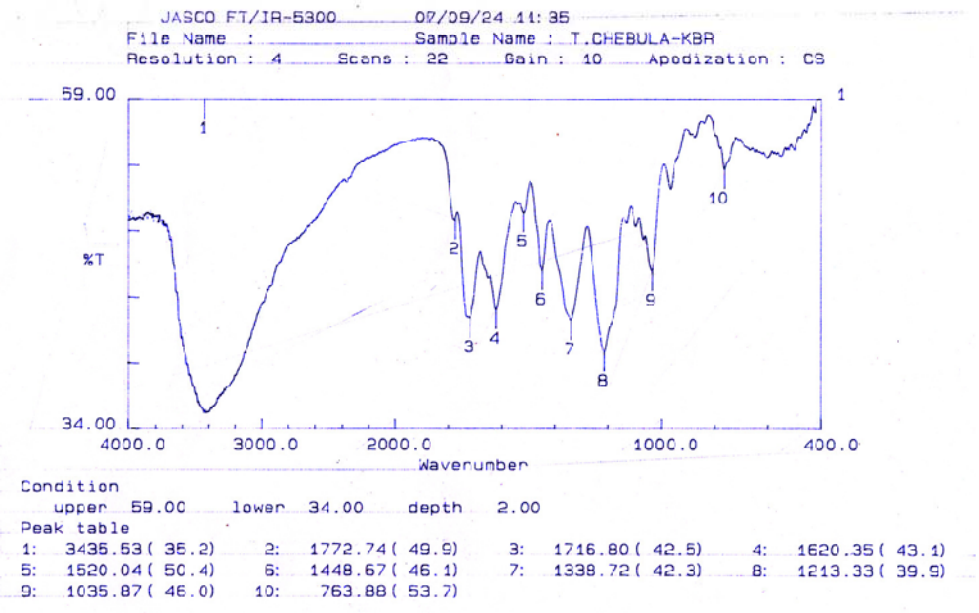

Fig. 16. IR Spectra of TC-V. IR (KBr) ν_{max} : 3435 (-OH), 1772 (>C=O), 1716 (>C=O), 1620 (>C=O),1520, 1448, 1338, 1213, 1035, 763 cm-.

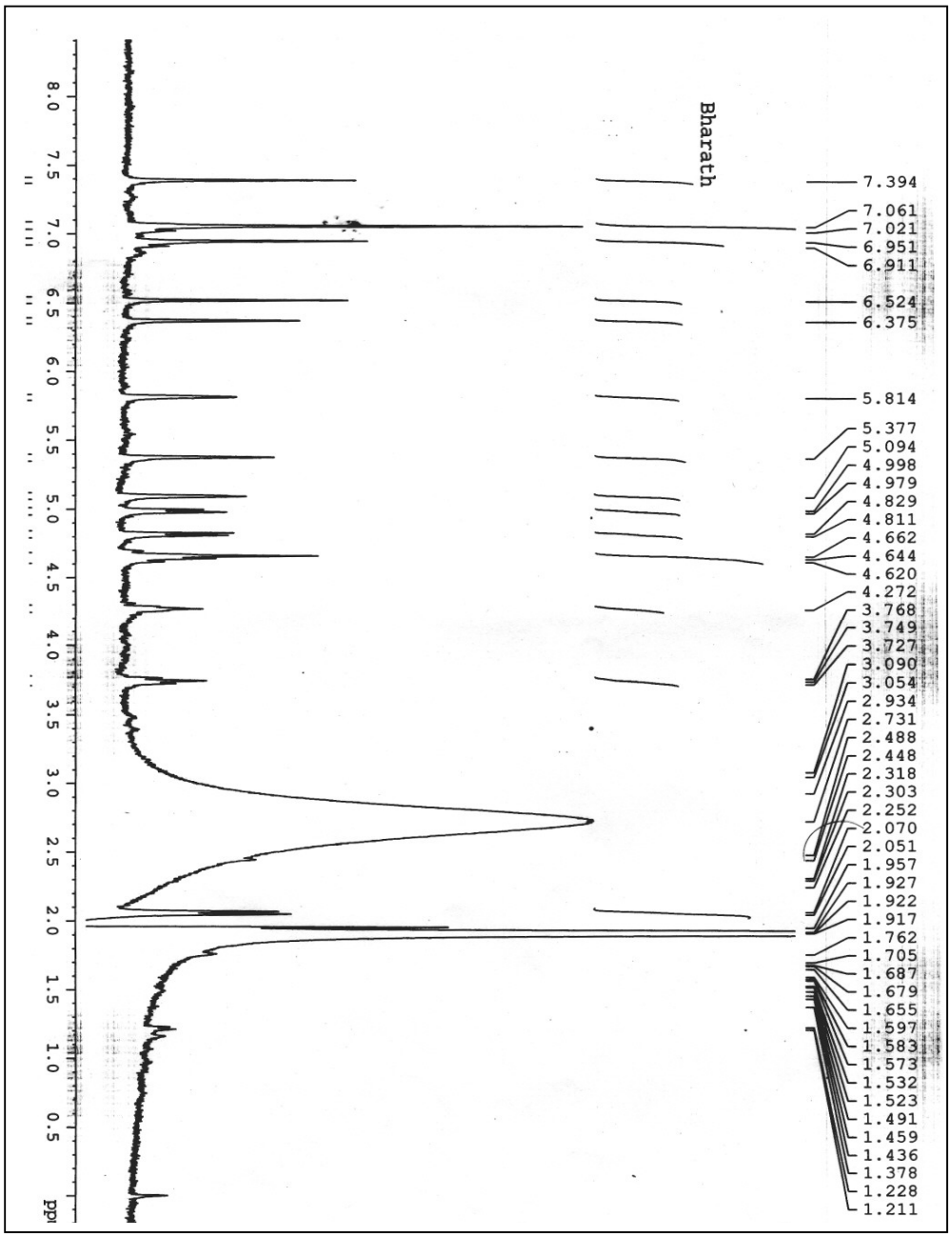

Fig. 17. ^{1}H NMR of TC-V.

^{1}H NMR δ (acetone-d6) ppm (*J* in Hz): 2.18 (1H, d, *J*=7.6 Hz), 3.7 (1H, m), 4.64 (2H,m), 4.82 (1H, d, *J*= 7.2) 4.98 (1H, d), 5.09 (1H, s), 5.37 (1H, s), 5.81 (1H, s), 6.37 (1H, s), 6.52 (1H, s), 6.95 (1H, s), 7.06 (2H, s), 7.39 (1H, s).

1.3.4. Inhibitory studies on COX and LOX with TC-V (chebulagic acid)

HPLC purified TC-V peak was tested against COX-1 and COX-2 by TMPD assay. TC-V showed dose dependent inhibition of both the enzymes with IC_{50} values of 15 ± 0.288 μM for COX-1 and 0.92 ± 0.011 μM for COX-2 (Fig. 18). These results indicate that TC-V has more specificity towards COX-2 than COX-1, with ~16 fold preference towards COX-2. In addition, TC-V showed potent inhibition of 5-LOX (Fig. 18) with an IC_{50} value of 2.1 ± 0.057 μM.

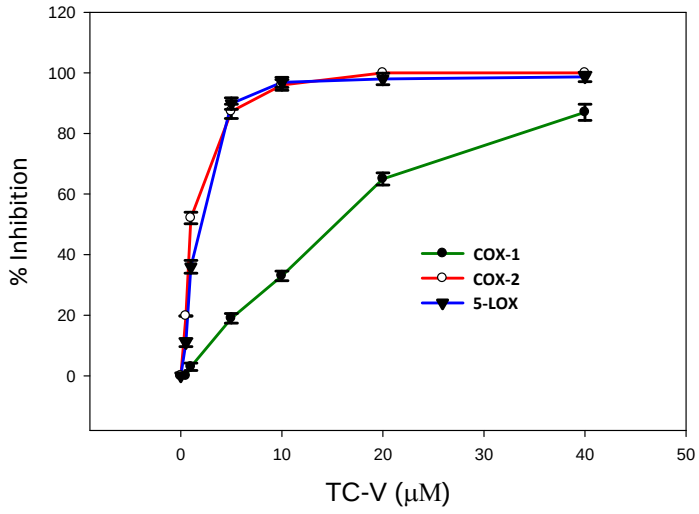

Fig. 18. Effect of TC-V (chebulagic acid) on COX-1, COX-2 and 5-LOX activities. TC-V preferentially inhibited COX-2 than COX-1 with ~16 fold preference. The IC_{50} values for COX-1, COX-2 and 5-LOX are 15±0.288, 0.92±0.011 and 2.1±0.057 μM respectively. Data are mean ± SEM of three independent experiments (N = 3).

1.3.5. Anti oxidant studies

Chebulagic acid (TC-V) showed potent DPPH radical scavenging activity in a concentration dependent manner with an IC_{50} value of 1.4 ± 0.0173 μM. At a concentration of ~4 μM, the chebulagic acid, almost completely inhibited DPPH absorption (Fig. 19). Similar strong inhibition of ABTS radical was recorded by chebulagic acid with an IC_{50} value of 1.7 ± 0.023 μM (Fig. 19).

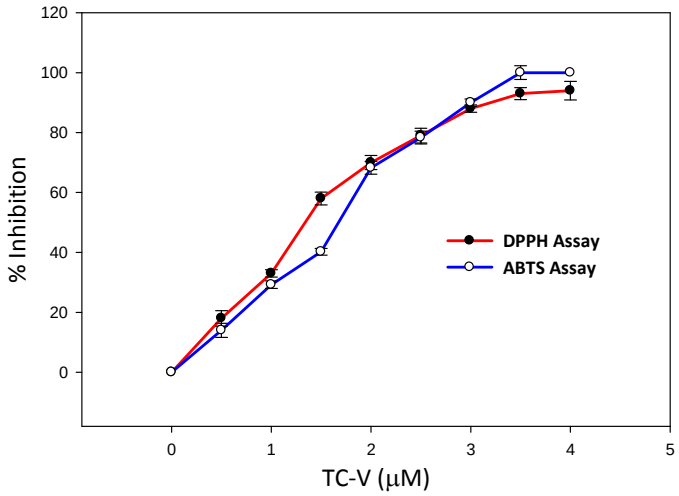

Fig. 19. Free radical scavenging effects of chebulagic acid (TC-V). Chebulagic acid showed potent anti-oxidant properties as measured by DPPH and ABTS decolourising assays with an IC_{50} values 1.4±0.0173 μM and 1.7±0.023 μM respectively. Data are mean ±SEM of three independent experiments (N = 3).

1.4. Discussion

Eicosanoids are potent biologically active arachidonic acid derived lipid mediators that are intimately involved in inflammation and cancer. Cyclooxygenases (COX) and 5-Lipoxygensase (5-LOX) are the key enzymes in the arachidonic acid metabolism. COX enzyme exists in two distinct isoforms, COX-1 and COX-2. COX-1 is expressed constitutively in nearly all mammalian tissues and is the source of prostaglandins central to "housekeeping" functions such as renal water reabsorption, vascular homeostasis, and gastric protection (Smith *et al.,* 1996). COX-2, an inducible enzyme, is mainly expressed under pathological conditions such as inflammation and carcinogenesis. Hence, COX-2 inhibitors were developed as novel non-steroidal anti-inflammatory drugs (NSAIDs) without gastric side effects that are associated with the conventional NSAIDs.

In addition to their role in inflammation, selective inhibitors of COX-2 have been demonstrated to induce apoptosis in variety of cancer cell lines including colon, stomach, prostate, and breast (Elder *et al.,* 1997). 5-LOX is involved in the biosynthesis of leukotrienes, pro-inflammatory mediators participating in various forms of acute and sub acute inflammation. 5-HETE, product of 5-LOX was shown to be a potential survival factor for prostate cancer cells and inhibition of 5-LOX triggered massive apoptosis (Ghosh *et al.,* 1998). Both 5-LOX and COX-2 are co-expressed and up-regulated in inflammation and in many forms of human cancers, including colon, prostate, breast and lung cancers and form targets for development of anti-inflammatory and anti-cancer drugs (Pommery *et al.,* 2004). Moreover, COX-2 inhibition by Celecoxib in cancer cell

lines was shown to increase the formation of 5-HETEs, which is having tumor cell proliferative property (Ye *et al.,* 2005). Hence there is need for the development of potent COX-LOX dual inhibitors without side effects.

In our attempt to isolate a natural product with COX-LOX dual inhibition, we identified *Terminalia chebula* as a potential source. The initial studies with the ethanolic extract of *Terminalia chebula* showed potent inhibition of COX-1, COX-2 and 5-LOX, with more preference towards COX-2 and 5-LOX. Further HPLC fractionation of the ethanolic extract resulted in the identification of peak TC-V as a potent COX-LOX dual inhibitor. TC-V was further subjected to LC-MS, NMR and IR and identified it as chebulagic acid. Chebulagic acid showed potent COX/5-LOX dual inhibition with IC_{50} values of 15 ± 0.288, 0.92 ± 0.011 and 2.1 ± 0.057 μM for COX-1, COX-2 and 5-LOX respectively. These studies suggest that chebulagic acid showed ~16 fold preference towards COX-2 and with a COX-2/COX-1 ratio of IC_{50} values of 0.0613. This is comparable to that of celecoxib, a selective inhibitor of COX-2, which was approved by U.S.FDA (United States Food and Drug Administration) for the treatment of rheumatoid arthritis patients and colon and rectum polyps in patients with familial adenomatous polyposis (FAP).

Though selective COX-2 inhibitors were shown to have less or no gastric side effects, recent studies indicate their increased reports of cardiac problems. This infact has lead to the withdrawal of rofecoxib (vioxx), from the market by Merck & Co Inc. These cardiac side effects of COX-2 inhibitors, however, were shown to vary from one to the other and depend on the degree of selectivity towards COX-1 and COX-2 (Bombardier *et al.,* 2000). Rofecoxib with a high

degree of COX-2 selectivity (COX-2/COX-1 IC_{50} ratio of 0.0056) was shown to have more cardiac side effects than celecoxib (COX-2/COX-1 IC_{50} ratio of 0.0263). The degree of selectivity of chebulagic acid (COX-2/COX-1 ratio IC_{50} of 0.0613), isolated in the present study is more towards celecoxib suggesting its possible lesser cardiac side effects. Further, chebulagic acid is a potent antioxidant and COX-LOX dual inhibitor unlike the synthetic celecoxib. In view of the above, chebulagic acid could form a better candidate for development of anti-inflammatory drugs. Further studies, however, are required to test its efficacy and toxicity in animal models and clinical trials.

Chapter-2

Anti-inflammatory and anti-allergic properties of chebulagic acid: *In vitro* studies

2.1. Introduction

2.1.1. Inflammation and macrophages

Inflammation is a manifestation of the body's response to tissue damage and infection. The discovery of the detailed processes of inflammation has revealed a close relationship between inflammation and the immune response. Inflammation is a complex stereotypical reaction involving a number of cellular and molecular components and important changes in the physiological systems as well. Inflammation is the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. Abnormalities associated with inflammation comprise a large, unrelated group of disorders which underly a variety of human diseases. Some of the inflammatory disorders include arthritis, asthma, hypersensitivity etc.

Macrophages are key players in the immune response to foreign invaders such as infectious microorganisms. Macrophages help destroy bacteria, protozoa, and tumor cells. Macrophages are activated by IFN-γ, pro-inflammatory cytokine (Berenbaum, 2000), heat-killed gram-positive bacteria, bacterial lipopolysacharride (LPS) (Zhang and Ghosh, 2000), yeast glucans, GM-CSF and phorbol esters. Activated macrophages play an important role in inflammatory diseases by the production of cytokines, interleukin-1 beta (IL-1β), IL-6, tumor necrosis factor-alpha (TNF-α) and other inflammatory mediators such as nitric oxide (NO), and prostaglandins (PGE_2) (Paul *et al.,* 1999; Fujiwara and Kobayashi, 2005). Overproduction of the inflammatory mediators involves many diseases, such as rheumatoid arthritis (Manzi and Wasko, 2000), atherosclerosis (Libby *et al.,* 2000), asthma (Tak and Firestein,

2001) and pulmonary fibrosis (Coker and Laurent, 1998). Thus, inhibition of the production of these inflammatory mediators in activated macrophages may prevent or suppress a variety of inflammatory diseases, including atherosclerosis, sepsis, and endotoxemia.

2.1.2. Role of NF-κB and MAP kinases in inflammation

Nuclear transcription factor kappa-B (NF-κB) is one of the most important transcription factors, and regulates various cellular genes involved in immune and acute phase inflammatory responses, and in cell survival (Li and Verma, 2002). NF-κB is located in the cytoplasm of nonstimulated cells by interaction with inhibitory proteins, like IκBs (Bowie *et al.,* 1997). NF-κB activation in response to proinflammatory stimuli, involves the rapid phosphorylation of IκBs by IKK signalosome complex (Karin and Delhase, 2000) and degradation by 26S proteosome (Sanchez-perez *et al.,* 2002). The resulted free NF-κB then translocates into the nucleus where it binds to κB binding sites in the promoter region of target genes, and induces the transcription of proinflammatory mediators, such as, iNOS, COX-2, TNF-α, IL-1β, IL-6 and IL-8 (Baeuerle and Baltimore, 1996). The activation of the NF-κB, although a series of other pathways are also triggered, includes interferon regulatory factor 3 (IRF-3), extracellular signal related kinase (ERK1/2), p38 mitogen-activated kinase (p38) and c-Jun N-terminal kinase (JNK) pathways (Triantafilou *et al.,* 2004).

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that react to extracellular stimuli (mitogens) and control various cellular activities like gene expression, mitosis,

differentiation, and cell survival/apoptosis (Pearson *et al.,* 2001). They transmitt extracellular signals to nucleus by inducing transcription of various genes by phosphorylation and activation of transcription factors. These classical MAPKs, identified in vertebrates include extra cellular signal regulated kinase (ERK), p38 MAPK, and c-Jun NH_2-terminal kinase (JNK). p38 and JNK regulate inflammatory proteins as well as immune responses and expression of various cytokines like, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) (Hommes *et al.,* 2003) and their signaling pathways are involved in LPS-induced COX-2 and iNOS expression in macrophages (Uto *et al.,* 2005). ERK is activated by growth factors, cytokines, virus infection and carcinogens and activation of ERK is thought to be involved in LPS induced macrophage responses, such as, in the increased production of pro-inflammatory cytokines and iNOS (Ajizian etal., 1999).

2.1.3. ROS (reactive oxygen species) and inflammation

The generation of reactive oxygen species (ROS) by phagocytic leukocytes (neutrophils, monocytes, macrophages, and eosinophils) is one of the most important hallmarks in the inflammatory process. The overproduction of reactive oxygen species (ROS) by macrophages causes oxidative damage to membrane lipids, DNA, proteins and lipoproteins (Ciz *et al.,* 2008). ROS are involved in a variety of cellular stress mechanisms. Therefore, inhibition of ROS production is a popular target for the attenuation of many inflammatory diseases (Shen *et al.,* 2002). Many reports indicate that the ROS participates in modulating NF-κB activation (D'Acquisto *et al.,* 2002) and the antioxidants block NF-κB activation (Singh and Aggarwal, 1995; Chen *et al.,* 2007).

2.1.4. Basophils and allergy

Basophils are nonphagocytic granulocytes that play a part in the allergic response as they have IgE on their surface, and release chemical mediators causing allergic symptoms when the IgE binds to its specific allergen. The rat basophilic leukaemia (RBL) cell line has been widely used as a convenient model system to study regulated secretion in mast cells. Activated mast cells and basophils undergo degranulation, during which they release histamine and other inflammatory chemical mediators (cytokines, interleukins, leukotrienes, and prostaglandins) from their granules into the surrounding tissue (Schleimer et al, 1985). The release of histamine and other inflammatory mediators from mast cell or basophils is the primary event in several allergic responses such as food allergies, hay fever, asthma and drug-induced allergies, allergic asthma, atopic dermatitis, allergic rhinitis and several ocular allergic diseases. Many natural products reported to suppress the degranualtion of activated RBL-2H3 (rat blastocyte leukemia) cells (Itoh *et al.,* 2008).

2.1.5. Chebulagic acid

The dried fruit powder of *Terminalia chebula* are traditionally used to treat many allergic and inflammatory diseases and to treat many diseases such as digestive, urinary, skin, and heart diseases, parasitic infections, diabetes, colic pain and hemorrhoids (Barthakur and Arnold, 1991). In the prevous chapter we have isolated chebulagic acid, from the ethanolic extract of *Terminalia chebula,* a potent anti-oxidant that exhibits COX/5-LOX dual inhibition activities. Hamada et al. (1997) reported the immunosuppressive effects of chebulagic acid on

cytotoxic T lymphocyte mediated cytotoxicity. Chebulagic acid also inhibited the mammalian α-glucosidase activity (Gao *et al.,* 2007). Murakami et al. (2006) reported the suppressive effect of chebulagic acid on NO production in RAW 264.7 cells stimulated with LPS. However, the molecular mechanism of chebulagic acid mediated immune modulation has not been fully understood.

In the light of the above pharmacological properties shown by chebulagic acid, the present study is undertaken to evaluate its anti-inflammatory and anti-allergic properties. Anti-inflammatory studies were taken up in mouse macrophage cell line (RAW 264.7) treated with LPS and anti-allergic properties were studied in A23187-sensitized rat basophilic leukemia (RBL-2H3) cells.

2.1.6. Objectives

The objectives of the present study are:

- To study the anti-inflammatory properties of chebulagic acid in LPS-induced RAW 264.7 cells and understand the signaling mechanisms involved.
- To study anti-allergic effects of chebulagic acid in A23187 induced RBL-2H3 cells.

2.2. Materials and methods

2.2.1. Chemicals

Phosphate buffered saline (PBS), RPMI 1640, Fetal Bovine Serum (FBS), Penicillin and Streptomycin were purchased from Gibco BRL (California, USA). *Escherichia coli* lipopolysaccharide (LPS), MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), Trypsin-EDTA, Protease Inhibitor Cocktail and β-actin antibodies were purchased from Sigma Chemical Company (St.Louis, USA). iNOS, COX-1, COX-2, 5-LOX, p65, p50, p-IκB-α, IκB-α, p-ERK, ERK, p-p38, p38, p-JNK, JNK and PARP monoclonal antibodies and the alkaline phosphatase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (California, U.S.A). The enzyme immuno assay kits for the measurement of PGE_2 and NF-κB were purchased from Cayman Chemical (Ann Arbor, MI, USA) and Invitrogen Inc., (California, U.S.A) respectively.

2.2.2. Cell culture

RAW 264.7 (murine macrophages) and RBL-2H3 (rat basophilic leukemia) cell lines were obtained from NCCS, Pune and American Type Culture Collection (Rockville, MD) respectively. The cell lines were maintained in a humidified atmosphere with 5% CO_2 at 37^0C. Medium for all the cell lines was RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2mM L-glutamine.

2.2.3. Nitrite measurement

RAW 264.7 cells were plated at 5×10^5 cells/well in 24-well plates and then incubated with or without LPS (1 μg/ml) in the absence or presence of various concentrations (5,10 and 20 μM) of chebulagic acid for 16 h. Nitrite levels in culture media were determined using the Griess reaction. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl), and incubated at room temperature for 10 min. Absorbance was then measured at 540 nm using microtiter plate reader, Quant Bio-Tek Instruments, VT. Fresh culture media were used as blanks in all experiments. Nitrite levels in samples were determined using a standard sodium nitrite curve.

2.2.4. MTT assay for cell viability

To determine the appropriate concentration, not toxic to cells, cytotoxicity studies were performed 16 h after treating cells with various concentrations of chebulagic acid in RAW 264.7 cells. Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to purple formazan. After treatment with the chebulagic acid for 16 h, cells were incubated with MTT (0.5%) for 4 h at 37 °C. The medium was removed by aspiration, and formazan crystals were dissolved in DMSO. The extent of the reduction of MTT was quantified by measurement of absorbance at 570 nm using microtiter plate reader, Quant Bio-Tek Instruments, VT.

2.2.5. Determination of PGE_2, NF-κB and IL-6 levels

PGE_2, NF-κB and IL-6 concentrations in the supernatant of the culture medium were determined by using the respective EIA kits according to the manufacturer's instructions.

2.2.6. Preparation of cytoplasmic and nuclear extracts

RAW cells were cultured in 6-well plates (4 x 10^6 cells/well) with/without LPS (1μg/ml) and in the presence or absence of chebulagic acid (0-25 μM). The cytoplasmic and nuclear protein extracts were prepared for measuring the protein levels by Western blotting and enzyme immuno assay (EIA). Briefly, after culture the cells were collected and washed twice with cold PBS, lysed in 400 μL of cold buffer A (HEPES 10 mmol/L pH 7.9, KCl 10 mmol/L, 1mM EDTA, phenylmethanesulphonylfluoride (PMSF) 1 mmol/L, 1mM EGTA, dithiothreitol (DTT) 1 mmol/L, aprotinin 1 mg/L, leupeptin 1 mg/L, and pepstatin A 1 mg/L). After 15-min incubation on ice, 0.1 % NP-40 was added to the homogenates and the tubes were vigorously rocked for 1 min. Then the homogenates were centrifuged (20,800×*g*, 5 min) in a microcentrifuge at 4 °C. The supernatant fluid (cytoplasmic extracts) was collected and stored in aliquots at -70 °C. The nuclear pellets were washed once with cold buffer A, then suspended in 50 μL of cold buffer B (HEPES 20 mmol/L, pH 7.9, NaCl 420 mmol/L, edetic acid 0.1 mmol/L, egtazic acid 0.1 mmol/L, PMSF 1 mmol/L, DTT 1 mmol/L, aprotinin 1 mg/L, leupeptin 1 mg/L, and pepstatin A 1 mg/L) and vigorously rocked at maximum speed at 4 °C for 30 min. The solution was clarified by centrifugation at 20,800x*g* for 5 min, and the supernatant fluid (nuclear extract) was stored in

aliquots at -70 °C. The protein concentration was determined according to the Bradford method (Bradford, 1976).

2.2.7. SDS-PAGE and Western blotting

An equal quantity of cytosolic/nuclear proteins from each treatment (70 μg of total protein/lane) was resolved on 8–12% SDS-PAGE gels and then transferred on to nitrocellulose membranes. Membranes were stained with 0.5% ponceau in 1% acetic acid to confirm equal loading. The membranes were blocked with 5% w/v non-fat dry milk and then incubated with the primary antibodies in 10 ml of antibody-diluted buffer (Tris-buffered saline and 0.05% Tween-20 with 5% milk) with gentle shaking at 4^{o}C for 8-12 h and then incubated with respective conjugated secondary antibodies. Signals were detected using Western blot detection reagents.

2.2.8. RNA preparation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from both the controlled and treated cells in a 6-well plate by using TRI REAGENT™ according to the manufacturer's recommendations (Sigma Chemical Co., St. Louis, MO). From each sample, 1 μg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM dNTP, and oligo ($dT_{12–18}$) 0.5 μg/μl. Then PCR analyses were performed on the aliquots of the cDNA preparations to detect iNOS, COX-2, TNF-α, IL-6 and β-actin (as an internal standard). The reactions were carried out in a volume of 25 μl containing (final concentration) 1 U of Taq DNA polymerase, 0.2 mM dNTP, 10x reaction buffer and 100 pmol of 5′ and 3′ primers. After initial denaturation

for 2 min at 95 °C, thirty amplification cycles were performed for iNOS (1 min of 95 °C denaturation, 1 min of 60 °C annealing, and 1.5 min 72 °C extension), COX-1 (1 min of denaturation at 94 °C, 1 min of 60 °C annealing, 2 min 68 °C extension), COX-2 (1 min of 94 °C denaturation, 1 min of 60 °C annealing, and 1 min 72 °C extension), 5-LOX (1 min of 94 °C denaturation, 1 min of 56 °C annealing, and 1 min 72 °C extension), TNF-α (1 min of 95 °C denaturation, 1 min of 55 °C annealing, and 1 min 72 °C extension) and IL-6 (1 min of 94 °C denaturation, 1 min of 56 °C annealing, and 1 min 72 °C extension). PCR primers used in this study are listed below and were purchased from MWG Inc., (Bangalore, India): sense strand iNOS, 5′-AAT GGC AAC ATC AGG TCG GCC ATC ACT-3′, anti-sense strand iNOS, 5′-GCT GTG TGT CAC AGA AGT CTC GAA CTC-3′; sense strand COX-1, 5'-ACT GGC TCT GGG AAT TTG TG-3', anti-sense strand COX-1, 5'-AGA GCC GCA GGT GAT ACT GT-3'; sense strand COX-2, 5′-GGA GAG ACT ATC AAG ATA GT-3′, anti-sense strand COX-2, 5′-ATG-GTC AGT AGA CTT TTA CA-3′; sense strand 5-LOX, 5'-GGC ACC GAC GAC TAC ATC TAC-3′, anti-sense strand 5-LOX, 5'-CAA TTT TGC ACG TCC ATC CC-3'; sense strand TNF-α, 5′-ATG AGC ACA GAA AGC-ATG ATC-3′, anti-sense strand TNF-α, 5′-TAC AGG CTT GTC ACT CGA ATT-3′; sense strand IL-6, 5′-GAG GAT ACC ACT CCC AAC AGA CC-3′, anti-sense strand IL-6, 5′-AAG TGC-ATC ATC GTT GTT CATACA-3′; sense strand β-actin, 5′- TCATGA AGT GTG ACG-TTG ACATCCGT-3′, anti-sense strand β-actin, 5′- CCTAGA AGC ATT TGC GGT-GCA CGATG-3′. After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

2.2.9. Measurement of reactive oxygen species (ROS) production

ROS production in control, LPS and/or chebulagic acid treated RAW 264.7 cells was measured using the dye 2, 7-dichloro dihydro fluorescein diacetate (DCFH-DA). DCFH-DA, a non-fluorescent cell-permeable compound becomes the fluorescent compound, 2, 7-dichlorofluorescein (DCF), upon oxidation by ROS. Cells seeded at a density of 2 x 10^6 in 60 mm culture dishes were first pre-incubated with chebulagic acid (10 and 20 μM) and NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI) (10 μM) for 1 h and then LPS (1 μg/ml) was added to the culture dishes except to the control cells. Cells were harvested 16 h after the LPS treatment and incubated with DCFH-DA (10 μM) for 30 min. Then cells were washed and harvested in PBS and ROS measurement was carried out on FACS Calibur flow cytometer. Data were collected using the data acquisition program CELL Quest (Becton Dickinson, San Jose, CA). DCF was measured with the following excitation and emission wavelengths: λ_{exc} = 488 nm and λ_{emi} = 525 nm. 10,000 cells were analyzed per sample.

2.2.10. Histamine assay

RBL-2H3 cells in culture dishes were first treated with/without different concentrations of chebulagic acid for 30 min before sensitizing them with A23187 (2 μM) for 30 min. A23187, a calcium-ionophore, used to degranulate RBL-2H3 cells to release histamine into the medium. The medium was collected from culture dishes and the histamine was extracted according to method of Siraganian (1974). The histamine content released into the medium was

measured using the spectroflourimeter at the following excitation and emission wavelengths: λ_{exc} = 350 nm and λ_{emi} = 450 nm.

2.2.11. Statistical analysis

Data were presented as mean ± SEM and the *P* values were determined using the unpaired Student's t-test. *P* value of less than 0.05 was considered as significant.

2.3. Results

2.3.1. Inhibition of LPS-induced NO and PGE_2 secretion in RAW 264.7 macrophages

To examine the potential anti-inflammatory properties of chebulagic acid on LPS-induced NO and PGE_2 production in RAW 264.7 cells, cells were treated with/without chebulagic acid (5, 10, 15, 20 and 25 μM) for 1 h and then treated with LPS (1 μg/ml) for 16 h. NO and PGE_2 concentrations were measured in the culture supernatants by Griess reaction and ELISA methods respectively. LPS (1μg/ml) significantly increased the concentrations of NO and PGE_2 as compared to the basal level without LPS. As shown in Fig. 20, Chebulagic acid significantly inhibited LPS-induced NO (Fig. 20A) and PGE_2 (Fig. 20B) production in a concentration-dependent manner.

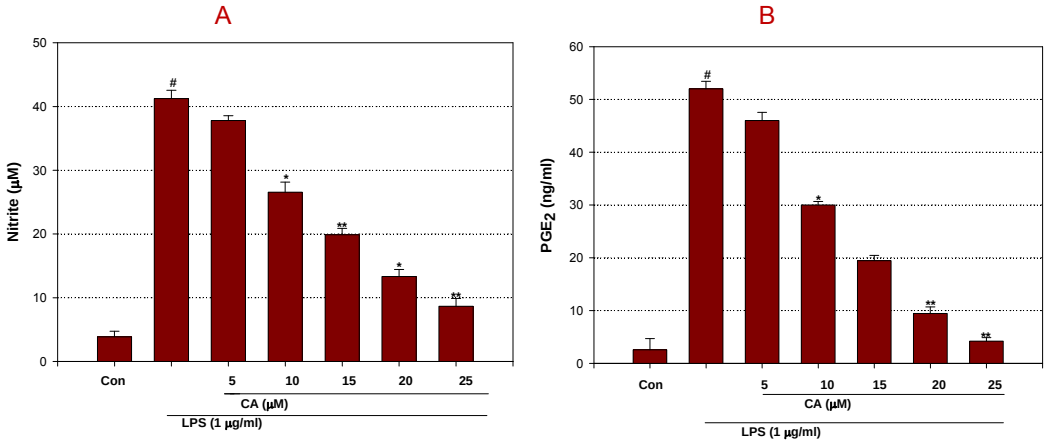

Fig. 20. Effect of chebulagic acid on LPS-induced nitrite (A) and PGE_2 (B) production in RAW 264.7 cells. Cells were pretreated with indicated concentrations of chebulagic acid for 1 h, LPS (1μg/ml) was then added, and cells were incubated for 16 h. The culture supernatants were subsequently isolated and analyzed for nitrite (A) and PGE_2 (B) levels. Control (Con) values were obtained in the absence of LPS and of chebulagic acid. Data are mean ± SEM of three independent experiments (N = 3). $^{\#}P<0.05$ compared with control group (Con), $^{*}P<0.05$ and $^{**}P<0.01$ when compared with the LPS (1 μg/ml) only treated group.

2.3.2. Effect of chebulagic acid on RAW 264.7 cell viability

To examine whether chebulagic acid is cytotoxic to RAW 264.7 cells, cells were incubated with 10–50 μM chebulagic acid along with LPS (1 μg/ml) for 16 h. Within tested concentrations (10-50 μM), no cytotoxic effect of chebulagic caid was observed in these cell lines (Fig. 21). Hence, the inhibitory effects of chebulagic acid on NO and PGE_2 levels were not attributable to cytotoxic effects.

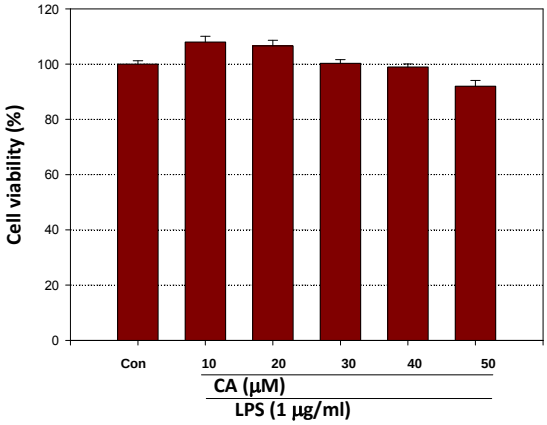

Fig. 21. Effect of chebulagic acid on the cell viability. RAW 264.7 cells were incubated with or without chebulagic acid (10-50 μM) and LPS (1μg/ml). Data are mean ±SEM of three independent experiments (N = 3). $^{*}P<0.05$ compared to the none treated control group.

2.3.3. Effects of chebulagic acid on LPS-induced expressions of iNOS, COX and 5-LOX mRNAs and proteins

Since chebulagic acid was found to inhibit NO and PGE_2 production, we used Western blotting and RT-PCR to determine whether these inhibitory effects were related to iNOS and COX-2 levels. These studies revealed marked up-regulation in the expression of iNOS, COX-2 and 5-LOX protein levels in response to LPS (Fig. 22B), and chebulagic acid strongly inhibited the expression of these proteins in a concentration-dependent manner. RT-PCR analysis on the expression of iNOS, COX-2 and 5-LOX mRNAs correlated well with their protein levels (Fig. 22A). Chebulagic acid on the other hand, did not affect the expression of COX-1, a housekeeping gene required for maintaining normal physiological functions.

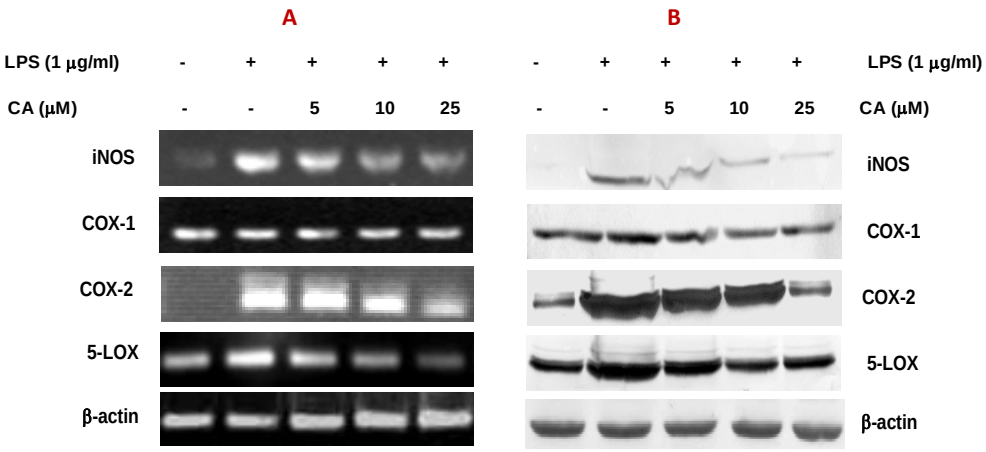

Fig. 22. Effect of chebulagic acid on LPS-induced expression of iNOS, COX and 5-LOX mRNA (A) and protein (B) in RAW 264.7 cells. Cells were treated with different concentrations (5,10, 25 μM) of chebulagic acid for 1 h, LPS (1 μg/ml) was then added and cells were incubated for 16 h. Total cellular proteins (70 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected with specific antibodies, as described in Materials and Methods (B). Total RNA was prepared for RT-PCR analysis iNOS and COX and 5-LOX gene expression in RAW 264.7 macrophages (A).

2.3.4. Effect of chebulagic cid on LPS-induced IL-6 release and mRNA expression of TNF-α and IL-6

We have studied the effect of chebulagic acid on LPS-induced IL-6 release and on the expression of pro-inflammatory cytokines TNF-α and IL-6 using RT-PCR. Pretreatment with chebulagic acid (5-25 μM) significantly reduced IL-6 release into the medium (Fig. 23A). Chebulagic acid also attenuated the TNF-α and IL-6 mRNA expressions in a dose-dependent manner in RAW 264.7 cells stimulated with LPS (Fig. 23B).

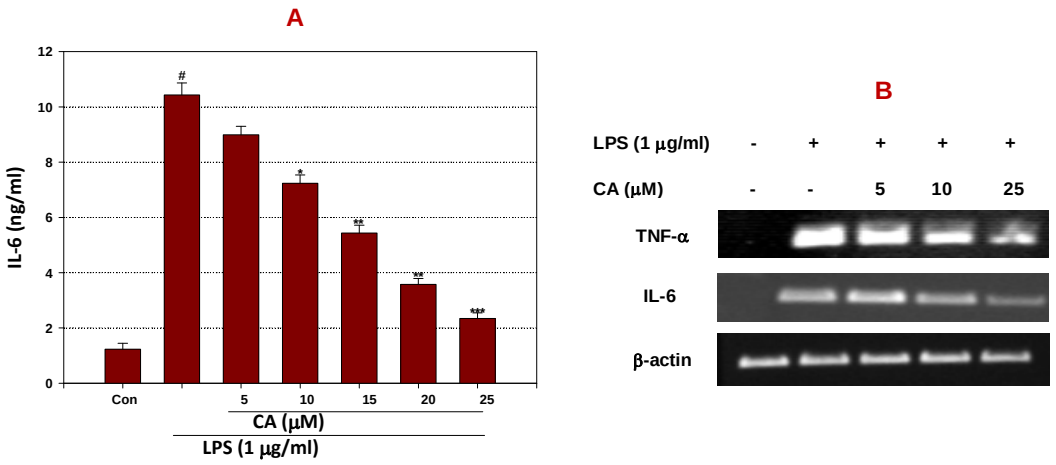

Fig. 23. Effect of chebulagic acid on LPS-induced IL-6 release (A) and mRNA expression of IL-6, TNF-α (B) in RAW 264.7 cells. (A) Cells were pretreated with different concentrations (5, 10, 15, 20, 25 μM) of chebulagic acid for 1 h, LPS (1μg/ml) was then added, and cells were incubated for 16 h. The culture supernatants were subsequently isolated and analyzed for IL-6 release (A) by EIA kit as described in Materials and Methods. The experiment was repeated three times and similar results were obtained. Data are mean ± SEM of three independent experiments (N = 3). $^{\#}P<0.05$ compared with control group (Con), $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ when compared with the LPS (1 μg/ml) only treated group. (B) Total RNA was prepared, and RT-PCR was then performed to compare the mRNA levels of TNF-α and IL-6. β-Actin was used as a PCR control. The experiment was repeated three times and similar results were obtained.

2.3.5. Effect of chebulagic acid on ROS (Reactive Oxygen Species) production

ROS have important role in NF-κB activation and pro-inflammatory cytokines production in LPS-induced macrophages. Macrophages produce reactive oxygen species (ROS) during stimulation with a wide variety of agents through activation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase that is assembled at the plasma membrane from resident

plasma membrane and cytosolic protein components (Forman and Torres, 2002). It is reported that antioxidants inhibit NF-κB activation and subsequently suppress NF-κB-dependent gene expression (D'Acquisto *et al.,* 2002). In view of anti-oxidant nature of chebulagic acid we have investigated whether chebulagic acid inhibits LPS-induced ROS generation in RAW 264.7 cells by FACS analysis. As shown in Fig. 24, ROS production was markedly increased in LPS-treated cells and chebulagic acid attenuated the LPS-induced ROS production in a concentration dependent manner. DPI, a NADPH oxidase inhibitor, reversed the LPS-induced ROS production in RAW 264.7 cells.

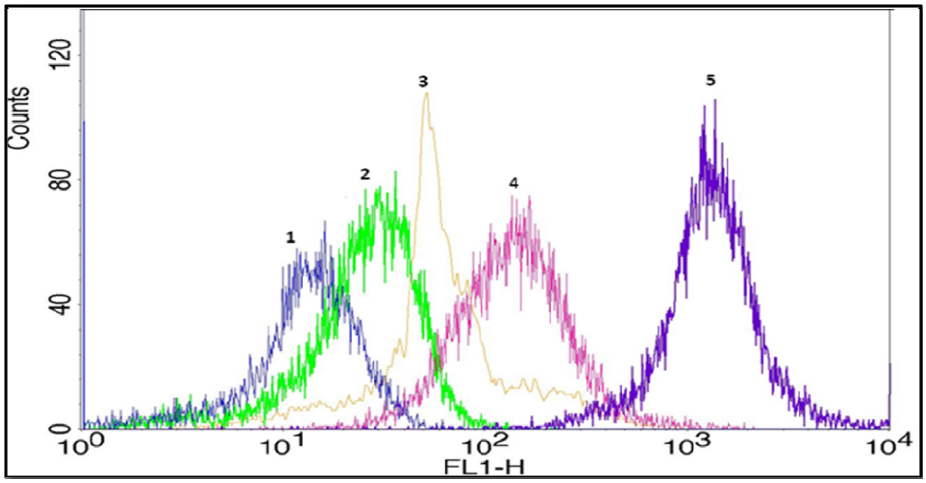

Fig. 24. Effect of chebulagic acid on LPS-induced ROS generation. RAW 264.7 macrophages were pretreated with H_2DCFDA (5 μM) for 30 min in serum free medium and then exposed to LPS (1 μg/ml) at 37 °C for another 30 min. Cells were collected and fluorescence was measured with a flow cytometer using Cell Quest software. (1) Control RAW 264.7 cells, (2) LPS + CA (25 μM), (3) LPS + DPI (10 μM) (4) LPS + CA (10 μM) and (5) RAW 264.7 cells treated with LPS (1 μg/ml) only. The figure was obtained from three independent experiments with similar patterns. DPI, a NADPH oxidase inhibitor, was used as a positive control at 10 μM.

2.3.6. Effect of chebulagic acid on LPS-induced NF-κB activation

Since the activation of NF-κB is critically required for the activation of iNOS, COX-2, TNF-α and IL-6 by LPS, ELISA was performed using NF-κB EIA kit, in order to examine whether chebulagic acid suppresses the LPS-induced NF-κB activation.

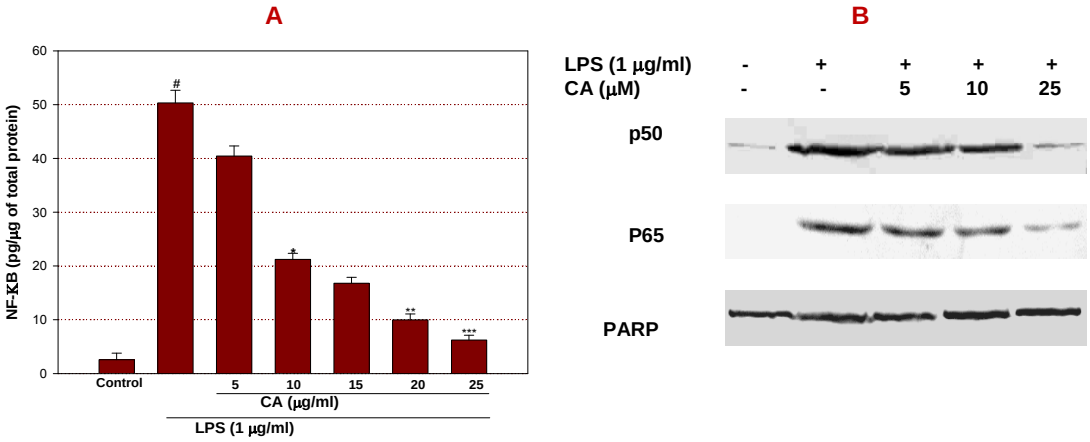

Fig. 25. Effect of chebulagic acid on activation of NF-κB (p65) in LPS-stimulated RAW 264.7 macrophages (A) and nuclear translocation of NF-κB (B). (A) Nuclear extracts were prepared from controls or pretreated with different concentrations (5,10,15,20,25 µM) of chebulagic acid for 1 h and then with LPS (1 µg/ml) for 1 h and analyzed for NF-κB activation by EIA kit as described in Materials and Methods. Data are mean ±SEM of three independent experiments (N = 3). $^{\#}P<0.05$ compared with control group (Con), $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ when compared with the LPS (1 µg/ml) only treated group. (B) Nuclear extracts (50 µg/ml) were prepared for the western blot analysis of p65 and p50 of NF-κB using specific anti-p65 and anti-p50 NF-κB monoclonal antibodies. Experiments were repeated two times and similar results were obtained.

Treating the RAW 264.7 cells with LPS (1 μg/ml) was found to increase the translocation of NF-κB into the nucleus and treating these cells with chebulagic acid prior to induction with LPS, reduced the NF-κB translocation into the nucleus in a concentration-dependent manner as evidenced by quantification using EIA kit.

2.3.7. Effect of chebulagic acid on the LPS-induced phosphorylation of IκB-α and on the nuclear translocation of NF-κB

Since, p50 and p65 are major components of NF-κB, which are activated by LPS in macrophages, we examined the translocation of p50 and p65 from cytosol to nucleus by Western blotting. RAW 264.7 cells were incubated with LPS in the presence or absence of chebulagic acid for 1 h and the nuclear proteins were probed with respective antibodies. Negligible levels of p50 or p65 proteins were detected in control cell nuclei, but treatment with LPS alone for 1 h caused marked increase of their levels in the nucleus. Pretreatment with chebulagic acid inhibited LPS-induced nuclear translocation of p50 and p65 sub units of NF-κB in a concentration dependent manner (Fig. 25B). PARP was used as internal control.

Translocation of NF-κB to the nucleus requires IκB-α protein phosphorylation, ubiquitination and degradation, since nuclear translocation of NF-κB is directly linked to IκB-α degradation and phosphorylation (Moynagh, 2005). So we have investigated whether chebulagic acid prevents the phosphorylation of IκB-α. It was found that pretreatment with chebulagic acid prior to induction with LPS, attenuated the phosphorylation of IκB-α in a

concentration dependent manner as revealed by Western blotting (Fig. 26A). Taken together these results suggest that chebulagic acid inhibits LPS-induced translocation of NF-κB by attenuating the phosphorylation of IκB-α.

2.3.8. Effects of chebulagic acid on LPS-induced MAP kinase phosphorylation

MAP kinases play critical role in the regulation of cell growth and differentiation, and control cellular responses to cytokines and stresses (Vandenberghe *et al.,* 1998). In addition they have critical role in the modulation of NF-κB activity (Surh *et al.,* 2001). To investigate the molecular mechanism of NF-κB inhibition by chebulagic acid in LPS-stimulated RAW 264.7 cells, we monitored the effect of CA on the activation of p38, ERK1/2, and JNK.

Maximal MAPK (ERK1/2, p38, and JNK) phosphoprotein expression levels are known to occur 10–30 min after LPS treatment in human and murine monocytes/ macrophages (Bian *et al.,* 1995). Therefore, we have carried out Western blotting for MAPK, 30 min after treating with LPS (1 μg/ml). RAW 264.7 cells were pretreated with chebulagic acid in the indicated concentrations for 30 min and then stimulated with 1 μg/ml LPS for 30 min. Total cell lysates were then probed with phosphospecific antibodies for p38, ERK1/2, and JNK. The phosphorylation of p38, ERK1/2, and JNK were increased in cells treated with LPS alone. However, chebulagic acid inhibited phosphorylation of p38, ERK1/2, and JNK levels in RAW 264.7 cells stimulated with LPS in a concentration dependent manner (Fig. 26B). No changes in non-phosphorylated ERK, JNK and p38 kinase expressions were observed in cells treated with LPS or

LPS plus chebulagic acid. These results suggest that the phosphorylation of MAP kinases may be involved in the inhibitory effect of chebulagic acid on LPS-stimulated NF-κB activation in RAW 264.7 cells.

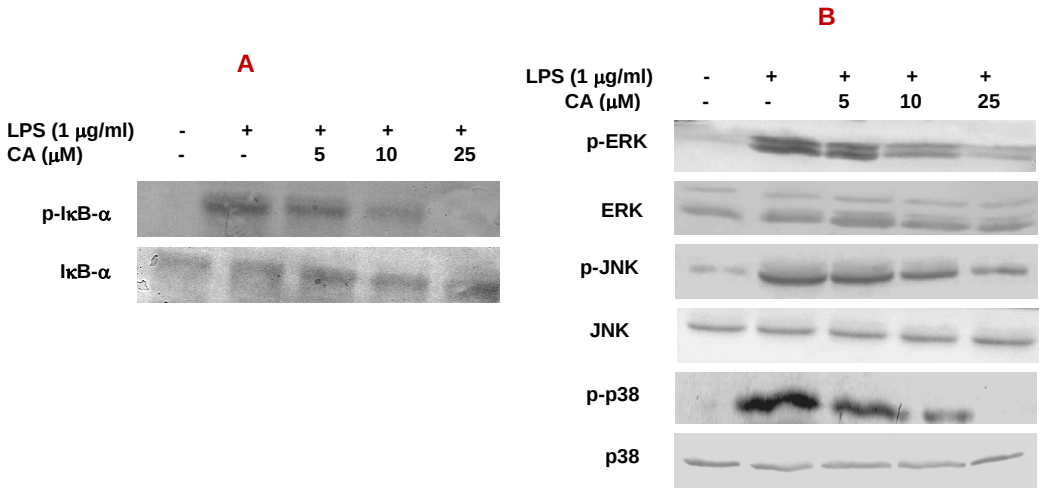

Fig. 26. The Effects of chebulagic acid on the LPS-induced Phosphorylation of IκB-α (A) and MAP Kinases (B). (A) RAW 264.7 macrophage cells were pretreated or not with the indicated concentrations of of chebulagic acid for 1 h and then the cells were incubated with LPS (1 μg/ml) for 15 min. Total cellular proteins were prepared and Western blotted for IκB-α and p-IκB-α using specific IκB-α and p-IκB-α antibodies. (B) The conditions of sample treatment were identical to those described for Fig. 26A. Western blot analysis using specific p-ERK, ERK, p-p38, p38, p-JNK and JNK antibodies.

2.3.9. Anti-allergic effect of chebulagic acid on A23187 induced degranulation in RBL-2H3 cells

Anti-allergic potential of chebulagic acid was studied in A23187 induced RBL-2H3 cells. Treatment with chebulagic acid potently inhibited the release of histamine into the medium in A23187 induced RBL-2H3 cells at concentrations of 5-25 μM (Fig. 27) in a dose dependent manner. NDGA, a LOX inhibitor (10 μM) was used as a positive histamine release inhibitor. The concentration of chebulagic acid treatment used in this study had no significant effect on the viability of RBL-2H3 cells (data not shown).

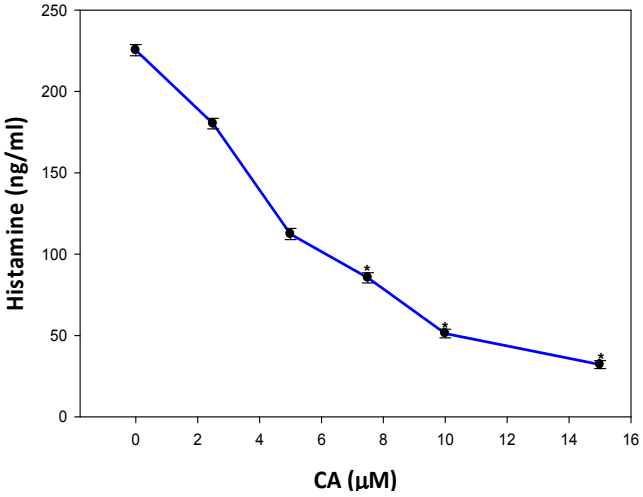

Fig. 27. Effect of chebulagic acid on histamine release from A23187 sensitized rat basophilic leukemia RBL-2H3 cells. As a marker of degranulation, we examined the release of histamine, which was measured as follows: RBL-2H3 cells were pretreated with or without different concentrations of chebulagic acid (0-15 μM) for 30 min and then sensitized with A23187 (2 μM) for another 30 min. The histamine content released into the medium was quantified by measuring the fluorescence as described in Materials and Methods. Data are mean ±SEM of three independent experiments (N = 3). $^{*}P<0.05$ compared with the A23187 (2 μM) only treated group.

2.4. Discussion

Fruits of *Terminalia chebula* are being used in various Ayurvedic preparations for the treatment of variety of disorders. Their fruit powder is one of the main constituents of *Triphala,* a well known Ayurvedic medicine used to treat allergies and common health disorders and chebulagic acid is one of the main bio active constituents of *Terminalia chebula* fruit powder. In the first chapter we have demonstrated that chebulagic acid is COX-2/5-LOX dual inhibitor. However, the molecular mechanism of chebulagic acid mediated immuno modulation has not been fully understood.

Inflammation is a complex process regulated by a cascade of cytokines, growth factors, NO and prostaglandins produced by activated macrophages. Hence inhibition of inflammatory cytokines, iNOS, COX-2 and 5-LOX serves as a key mechanism in the control of inflammation, and agents that suppress the expression of these inflammation-associated genes have therapeutic potential for treatment of inflammatory diseases.

In the present study we found that chebulagic acid potently inhibits the production of NO and prostaglandin E_2 (PGE_2) in a dose dependent manner. This may partly be due to inhibition of COX-2 by chebulagic acid in LPS stimulated mouse macrophage cell line, RAW 264.7. To further explore the possible mechanism underlying these inhibitions by chebulagic acid, we examined the expression levels of iNOS, COX and 5-LOX proteins and iNOS, COX, 5-LOX, TNF-α and IL-6 mRNA. The inhibition in the expression of iNOS and COX-2 genes was evidenced by reductions in their protein and mRNA levels with increasing

concentration of chebulagic acid. This observed decrease in the production of NO and PGE_2 may also be attributed to the down regulation in the expression of iNOS and COX-2 at mRNA and protein levels. The inhibition in the LPS-stimulated expression of iNOS and COX-2 in RAW 264.7 cells by chebulagic acid was not due to cytotoxic effect of chebulagic acid as assessed by MTT assay and the expression of the housekeeping gene β-actin. Furthermore, chebulagic acid didn't change the expression level of COX-1, which is constitutively expressed in most tissues and seems to be responsible for housekeeping and normal physiological functions including maintenance of the integrity of the gastric mucosa and regulation of renal blood flow.

TNF-α, mainly produced by macrophages and known to have a key role in inflammatory processes (Nathan, 1987), is mainly involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. Bacterial lipopolysaccharide (LPS) stimulates the large production of macrophage TNF-α, which in turn is responsible for induction of other pro-inflammatory mediators (Chandel *et al.,* 2000) and activation of NF-κB and MAP Kinase pathways. Interleukin-6 (IL-6) mainly produced by macrophages and T cells, acts as both a pro-inflammatory and anti-inflammatory cytokine. However, IL-6 production is rapidly increased in acute inflammatory responses associated with fever, infection, injury, trauma and other stress conditions. In the present study chebulagic acid suppressed the LPS-stimulated expression of TNF-α and IL-6 mRNAs in a concentration dependent manner.

NF-κB, a mammalian transcription factor which is activated by LPS, is known to control the expression of cell survival genes, and coordinates the expression of proinflammatory genes, such as iNOS, COX-2, TNF-α, and IL-6. NF-κB is a heterodimer composed of p50 and p65 (Barnes and Karin, 1997). In unstimulated cells, NF-κB is present in the cytoplasm through interactions with the inhibitory protein, IκB (Baeuerle and Baltimore, 1996). NF-κB is activated by IκB-α degradation following phosphorylation of IκB-α. This causes NF-κB to translocate into the nucleus and interact with the NF-κB binding motif in the promoters of target genes (Baeuerle and Henkel, 1994). In the present study we found that chebulagic acid inhibits the LPS-induced translocation of NF-κB/p65 and the nuclear translocations of p65 and p50 proteins as a consequence of the inhibition of IκB-α phosphorylation in a concentration dependent manner as evidenced by ELISA and Western blotting analyses. These results suggest that the inhibition of LPS induced expression of iNOS and COX-2 genes is mediated by blocking the activation of NF-κB.

The activation of NF-κB is regulated by cellular kinases, including MAPKs (Guha and Mackman, 2001). MAPKs are a highly conserved family of serine/threonine protein kinases and include the p38, ERK1/2, and JNK subgroups (Ruland and Mak, 2003). p38 is activated by LPS stimulation and has been postulated to play an important role in the control of iNOS and TNF-α gene expression (Bhat *et al.,* 1998). In addition, the activation of ERK is thought to be involved in LPS-induced macrophage responses, such as, in the increased production of pro-inflammatory cytokines and iNOS (Ajizian *et al.,* 1999). It was found that JNK may associate with the c-Rel subunit of NF-κB and directly

enhances NF-κB activation in the yeast two-hybrid system (Meyer *et al.,* 1996). In the present study, we investigated the effects of chebulagic acid on the LPS-induced phosphorylation of MAP kinases in RAW 264.7 cells. Treatment with chebulagic acid was found to significantly inhibit LPS-induced JNK, ERK and p38 phosphorylation, suggesting that JNK, ERK and p38 are involved in the inhibition of LPS-stimulated NF-κB activation by chebulagic acid in RAW 264.7 cells.

Mast cells and basophils play a central role in inflammatory and immediate allergic reactions. Histamine is important mediator for inflammation and/or allergy. Most of the histamine in the body is stored in mast cells or basophils. Rat basophilic leukemia (RBL-2H3) cells display properties of mucosal-type mast cells. Upon induction by IgE anti body or calcium ionophore histamine will be released into the medium. To explore the anti-allergic properties of chebulagic acid we have estimated the histamine concentration in chebulagic acid pretreated RBL-2H3 cells induced with A23187, a calcium ionophore. Treating the RBL-2H3 cells with chebulagic acid 30 min prior to induction with A23187, significantly reduced the release of histamine into the medium. These studies suggest that chebulagic acid could be a potential candidate for treating allergic diseases. Further studies on animal models, however, are required to evaluate the anti-allergic potential of chebulagic acid.

In conclusion, the present study reveals that chebulagic acid, a COX-2/5-LOX dual inhibitor, acts as a potent anti-inflammatory agent by down regulating the expression of pro-inflammatory genes iNOS and COX-2, leading to decreased production of NO and PGE_2 in LPS-stimulated mouse macrophage cell line, RAW

264.7. These effects of chebulagic acid appear to be mediated through the inhibition of NF-κB activation by blocking the phosphorylation of IκB-α and MAPK. Furthermore, chebulagic acid successfully blocked the release of histamine in A23187 stimulated RBL cells suggesting that it could be a potential candidate for treating allergic diseases. However, further *in vivo* studies are required to qualify the mechanism and therapeutic potential in the inflammatory and allergic disorders.

Chapter-3

Anti-inflammatory properties of chebulagic acid: *In vivo* studies on rat air pouch model of inflammation

3.1.1. Introduction

The rat air pouch is a convenient model to study acute inflammation (Sedgwick and Lees, 1986). It is formed by the subcutaneous injection of air over several days and is composed of a lining of cells that consists primarily of macrophages and fibroblasts. Injection of carrageenan into the fully formed air pouch produces an inflammatory granulomatous reaction characterized by the production of chemical mediators in the fluid exudates, including prostaglandins and leukotrienes, as well as a significant influx of polymorphonuclear leukocytes (PMNLs) and macrophages. The degree of non-immunologically-mediated, carrageenan-induced acute inflammation was characterized by the analysis of the contents of the air pouch. This was manifested by the volume of fluid exudation, number of infiltrated cells, especially neutrophils and the content of the inflammatory mediators. Potential anti-inflammatory drugs can be tested by measuring the reduction in carrageenan-induced inflammation (Masferrer *et al.,* 1994).

Carrageenan is a mucopolysaccharide derived from Irish Sea moss, which provokes a local antigenic inflammatory response primarily attributed to neutrophil mediated injury and is highly reproducible. Its involvement during the inflammatory response may be related to its ability to increase vascular permeability and edema through changes in local blood flow. Carrageenan-induced inflammation in the rat air pouch model is believed to mimic the pathological process occurring in joint diseases such as arthritis. This is because the connective tissues formed along the air pouch are similar to those found in chronic joint diseases (Sedgwick and Lees, 1986).

Carrageenan-induced inflammation and chronic joint diseases share other features, including markedly elevated PGE_2, neutrophil infiltration, cytokine formation, and tissue damage. Histamine, serotonin, and bradykinin are involved in the initial phase of inflammation (0-1 h), whereas the late phase (1-6 h) is mainly sustained by prostaglandins (Di Rosa *et al.,* 1971; Vinegar *et al.,* 1979), attributed to the induction of cyclooxygenase (COX-2) in the inflamed tissue (Nantel *et al.,* 1999). Studies have established that in this model, COX-2, which is quickly induced in the lining macrophages and fibroblasts, is the primary enzyme responsible for the elevation of PGE_2 (Masferrer *et al.,* 1994). Thus, various COX-2 inhibitors have been shown to inhibit the formation of PGE_2 in the pouch (Masferrer *et al.,* 1994).

Another key mediator in acute inflammation is nitric oxide (NO), a potent vasodilator. Among the three distinct isoforms of NO synthase (NOS), it has been suggested that inducible NOS (iNOS) mainly contributes to the development and maintenance of acute inflammation. This is further supported by the observation that its protein expression is up-regulated during the late stage of inflammation in air pouch after injection of carrageenan.

3.1.1. Objectives

In the previous chapter we have studied the anti-inflammatory properties of chebulagic acid in LPS-induced mouse macrophage cell lines (RAW 264.7 cells), an *in vitro* system. To check the anti-inflammatory potential of chebulagic acid *in vivo*, the present study is undertaken to evaluate its anti-inflammatory activity in carrageenan induced rat air pouch model of inflammation. The objectives of the present study are:

- ❖ To check the anti-inflammatory activity of chebulagic acid in carrageenan induced rat air pouch model of inflammation.
- ❖ To study the signaling mechanisms involved in anti-inflammatory activity of chebulagic acid.

3.2. Materials and methods

3.2.1. Chemicals

Carrageenan and β-actin antibodies were purchased from Sigma-Aldrich (St.Louis, USA). Monoclonal antibodies to iNOS, COX-1, COX-2 and TNF-α were purchased from Santa Cruz Biotechnology (California, U.S.A). All other chemicals and solvents were of analytical grade.

3.2.2. Experimental animals

Adult Wistar strain male rats (150 to 200 gm) were used for all the experiments in the present study. They were fed a standard chow pellet diet, had free access to water, and were maintained on a 12:12-h light-dark cycle. All

procedures in this study were approved by the Animal ethical committee of the University of Hyderabad.

3.2.3. Air pouch model of inflammation

Carrageenan treated rat air pouch model of inflammation was developed as described previously (Sedgwick *et al.,* 1985). Air cavities were produced by subcutaneous injections of 20 ml of sterile air into the intracapsular area on the dorsal side of the animal. An additional 10 ml of air was injected into the cavity every three days. Seven days after the initial air injection, 2 ml of 1.5% (w/v) solution of carrageenan dissolved in saline was injected directly into the pouch to produce an inflammatory response. Control animals received 2 ml of saline only. For the time course studies, animals were sacrificed by cervical dislocation at various time points after the injection. Pouch tissue was carefully dissected and cut open to aspirate the inflammatory exudates into graduated tubes. The pouch lining was separated from the muscle and dissected out, and rinsed in saline before processing further.

3.2.4. Administering of chebulagic acid

All the treatments were given along with carrageenan directly into the pouch cavity. Chebulagic acid and celecoxib were administered into the rat air pouch. The stock solutions of chebulagic acid (10 mM) and celecoxib (10 mM) were prepared in absolute ethanol and dimethyl sulphoxide respectively and further dilutions were made at the time of experiment. Animals were divided into 4 different groups. They are:

1. Saline treated

2. Carrageenan (2ml of 1.5% (w/v) carrageenan in saline) treated

3. Carrageenan + Celecoxib (20mg /Kg body weight) treated

4. Carrageenan + Chebulagic acid (10 mg/Kg body weight) treated.

3.2.5. Histology of rat air pouch tissue

Rat air pouch tissues from control and experimental animals were rinsed in PBS and fixed in Bouin's fixative (70% saturated picric acid, 25 % formaldehyde and 5% glacial acetic acid) overnight followed by thorough washing with distilled water. Tissues were then dehydrated sequentially in 70%, 80%, 90% alcohol and finally in absolute alcohol for 10 minutes each. After dehydration the tissue was processed in alcohol and benzene (3:1 for 10 min, 1:1 for 10 min, benzene and paraffin (1:1) for 10 min) to embed in paraffin wax. The tissue was placed in molten paraffin for 2-3 h to allow infiltration of paraffin into the tissue and then allowed to harden. Thin sections (10 μm) were taken on Leitz microtome and mounted on polylysine-coated slides. Sections were deparaffinised by incubating in xylene for 10 minutes, rehydrated by sequential incubations in 90, 80, and 70 % alcohol for 10 minutes each. The tissue sections were observed under light microscope at 400x magnification and photographs were taken.

3.2.6. SDS-PAGE and Western Blotting

Pouch tissue homogenate was prepared by homogenizing the pouch lining tissue in 100 mM Tris-HCl (pH 8.0) buffer containing 0.3 M mannitol, 1 mM EGTA, 1 mM EDTA, 4 mM K_2HPO_4, 1 mM DTT, 1 mM Sodium orthovanadate,

0.1% SDS, 2 mM PMSF and 40 µl/ml of complete protease inhibitor solution. The homogenate was centrifuged for 30 min at 10,000 rpm, 4 °C and the resultant supernatant was used for SDS-PAGE and Western blot analysis. Protein content in the supernatant was measured by Lowry method (Lowry *et al.,* 1951). SDS-PAGE and Western blot analyses for the detection of COX-1, COX-2, iNOS and TNF-α and β-actin in the air pouch tissue homogenate were performed by the procedure as mentioned earlier (Chapter-2, Section 2.2.7).

3.2.7. Biochemical parameters

3.2.7.1. Reduced glutathione (GSH) estimation

250 mg of saline, carrageenan and/or chebulagic acid treated rat air pouch tissues were homogenized in 3.5 ml of 100 mM phosphate buffer pH 7.0 with 1 ml of 25% phosphoric acid. Homogenate was centrifuged at 10,000 rpm at 4 °C for 30 min. 0.5 ml of supernatant was diluted with 4.5 ml of 100 mM phosphate buffer pH 7.0, and this diluted sample was used as the source for GSH estimation. The reaction mixture for each sample consisted of 1800 µl of 100 mM phosphate buffer pH 7.0, 100 µl of tissue homogenate and 100 ml of Ophthalaldehyde (OPT) (1% solution). This reaction mixture was incubated at room temperature for 15 min and the emission of fluorescence was monitored at 420 nm with excitation at 350 nm on Hitachi spectrofluorometer.

3.2.7.2. Glutathione peroxidase (GPx) activity

Perfused tissues were homogenized (10% w/v) in 50 mM phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM PMSF and 250 mM sucrose. The cytosolic fraction was used as the enzyme source to estimate the peroxidase

activity. The reaction mixture in a final volume of I ml contained 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.24 U/ml yeast glutathione reductase, 0.3 mM glutathione (reduced), 0.2 mM NADPH, 1.5 mM cumene hydroperoxide and cytosolic fraction. Reaction was initiated by adding NADPH and its oxidation was monitored at 340 nm by observing the decrease in OD for 1 min.

$$ROOH + 2GSH \longrightarrow ROH + H_2O + G\text{-}S\text{-}S\text{-}G$$

Activity of enzyme was calculated according to the following equation:

$$\frac{\text{Difference in absorbance for 1min X volume of the reaction mixture in ml}}{\xi \text{ NADPH (6.2) X volume of the enzyme in ml}}$$

Specific activity was expressed as units per mg protein, where one unit was defined as one nano mole of NADPH oxidised per minute.

3.2.8. Statistical analysis

Each experiment was performed in triplicates. Data were expressed as mean ± standard error. Correlations between the various parameters were analyzed using regression analysis. *P* value was determined by the Student's T-test. *P* value of less than 0.05 was considered as a significant difference.

3.3. Results

3.3.1. Inflammatory reaction

The classical symptoms of acute inflammation - redness and swelling were clearly observed in the air pouch lining of carrageenan treated animal. The inflammatory reaction gradually progressed with time and reached a peak at 24 h after carrageenan treatment. In the chebulagic acid treated animals the inflammatory reaction was relatively less when compared to animals treated with carrageenan alone (Fig. 28D).

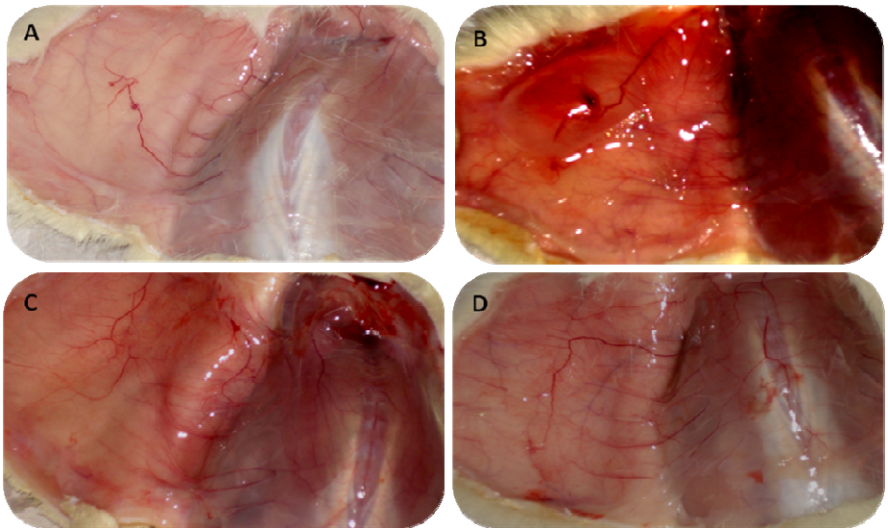

Fig. 28. Photographs of the exposed air pouch tissue 24 h after the administration of saline(A), carrageenan (B), carrageenan + celecoxib (C) and carrageenan + chebulagic acid (D). The saline treated animal was given 2 ml of saline only while the carrageenan treated animal was given 2ml of 1.5 % carrageenan dissolved in saline. The test compounds celecoxib (20 mg/Kg body wt) and chebulagic acid (10 mg/Kg body wt) were given to the animals along with carrageenan.

3.3.2. Histopathological observations of granulomatous tissue

The air pouch tissue is a freshly formed granulomatous tissue that is created by the loose association of macrophages, neutrophils, fibroblasts and plasma cells. The section of the pouch lining showed a large number of air cavities. Carrageenan treated pouch tissue showed heavy infiltration of blood cells at various sites in the tissue (Fig. 29B). These observations clearly demonstrate the induction of inflammation in the air pouch of the carrageenan treated animals. In chebulagic acid treated animals, however, reduced inflammatory reaction was seen as indicated by less degree of cellular infiltration.

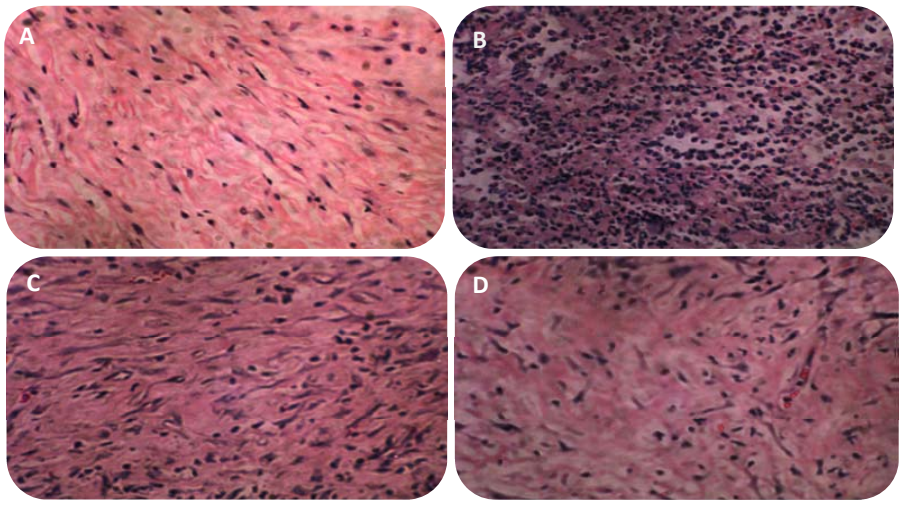

Fig. 29. Photomicrographs (400x) showing the histological sections of the pouch tissue 24 h after saline (A), carrageenan (B), carrageenan + celecoxib (C) and carrageenan + chebulagic acid (D) treatments. The air pouch tissue was fixed in buffered formalin, sectioned and stained with hematoxylin and eosin.

3.3.3. Exudate volume in the Pouch

The air pouch was carefully dissected by keeping the pouch intact and the fluid was aspirated, and the volume measured. As shown in the Fig. 30, pouch fluid was seen with in 6 h after carrageenan treatment with gradual increase at later periods and reaching maximum at 24 h. In chebulagic acid treated animals, fluid volume was found to be significantly lower i.e. 1.5 ml compared to that of 4.5 ml collected in carrageenan treated animals, at 24 h time period.

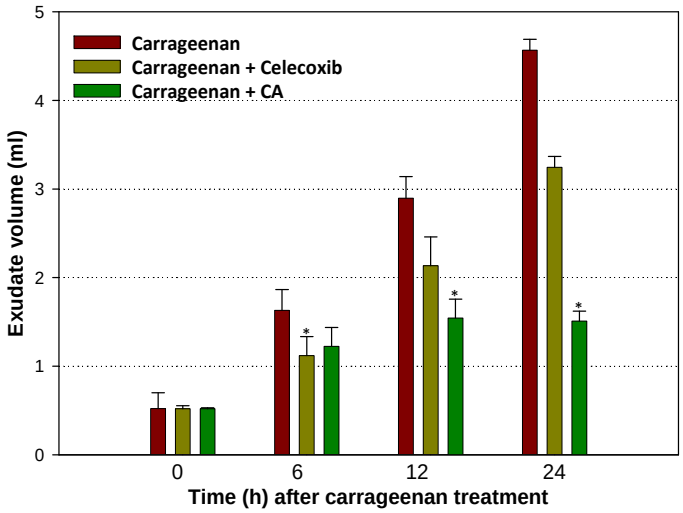

Fig. 30. Effect of chebulagic acid on accumulated fluid volume in the air pouch of either carrageenan or carrageenan+ chebulagic acid treated rats.

Rats were sacrificed at various time points after treatments. The values were the mean ± SE of data obtained from 3 different animals. *$p < 0.05$ compared to carrageenan treated rats.

3.3.4. Infiltration of leukocytes into the pouch fluid

Cell population in the pouch cavity was measured by gavaging about 20 ml of saline into the pouch repeatedly. This procedure ensures the complete recovery of cells from the pouch. For cell counting the collected fluid was centrifuged and the cell pellet was washed in RPMI medium twice to remove the debris and dissolved in saline, then counted on hemocytometer. As shown in the Fig. 31, cells started infiltrating into the pouch as early as 6 h and increased continuously to about 100 million cells by 24 h in carrageenan treated rats. The cell population mainly consisted of neutrophils, monocytes and macrophages. Infiltration of cells into the air pouch was significantly lower in chebulagic acid treated animals (28 million) at 24 h indicating the arrest of inflammatory reaction.

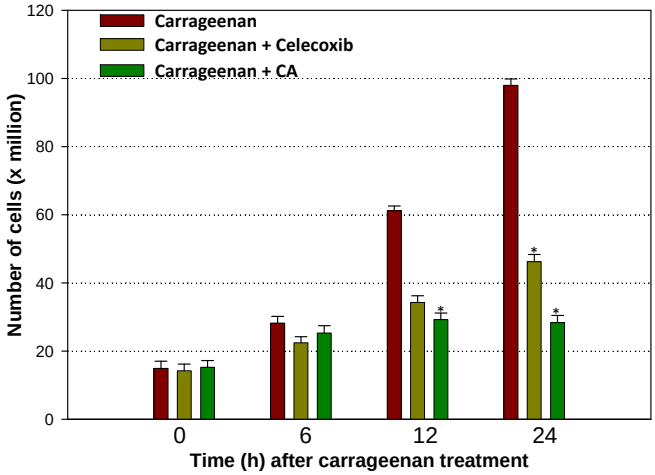

Fig. 31. Effect of chebulagic acid on number of cells infiltrated into the air pouch of either carrageenan or carrageenan + chebulagic acid (CA) treated rats. Animals were sacrificed at various time points after treatments. The values were the mean ± SE of data obtained from 3 different animals. *$P < 0.05$ compared to carrageenan treated animals.

3.3.5. Effect of chebulagic acid on the expression of COX, iNOS and TNF-α proteins

Since chebulagic acid reduced the inflammatory conditions in rat air pouch, further, Western blot was performed to check whether chebulagic acid has any effect on the expression of pro-inflammatory proteins, iNOS, COX-2 and TNF-α. iNOS, COX-2 and TNF-α protein levels were markedly up-regulated in response to carrageenan and chebulagic acid potently inhibited their expression in a concentration-dependent manner (Fig. 32). The expression of COX-1, however, was not affected by the treatment of either carrageenan or chebulagic acid.

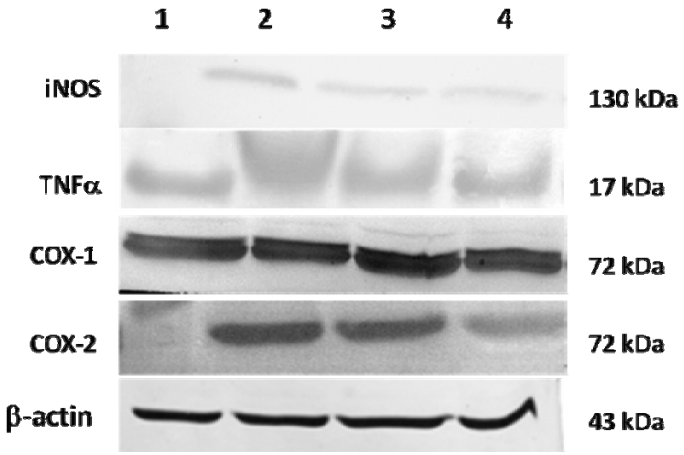

Fig. 32. Effect of chebulagic acid on the expression of COX-1, COX-2, iNOS and TNF-α proteins in rat air pouch tissue. Western blot analysis showing the expression of COX- 1, COX-2, iNOS, and TNF-α proteins in air pouch tissues obtained from rats treated with saline (lane 1), carrageenan alone (lane 2), carrageenan + chebulagic acid (10 mg/Kg body weight) (lane 3) and carrageenan + chebulagic acid (20 mg/Kg body weight) for 24 h. β-actin used as an internal control for equal loading of the proteins.

The above results indicate that chebulagic acid does not have any effect on COX-1, a house keeping gene required for maintaining normal physiological functions, but inhibits the progressive increase in the intensity of the expression of COX-2, iNOS, and TNF-α. This down regulation in the expression of pro-inflammatory proteins, in addition to its direct inhibitory effect on COX-2 activity may account for the strong anti-inflammatory effects of chebulagic acid observed in the present study.

3.3.6. Antioxidant properties of chebulagic acid

Oxidative stress is another important component of pathophysiology of inflammation. ROS are now recognized to be both mediators and modulators of inflammation. Oxidative stress in any system can be assessed by the levels of reactive oxygen species and the antioxidant potential of the system. ROS are the major cause of tissue damage during inflammation. Hence, reducing oxidative stress should be a part of any anti-inflammatory therapy. In the present study, antioxidant enzymes like glutathione peroxidase and reduced glutathione levels were measured in the inflammatory tissue to assess the antioxidant potential of chebulagic acid in the rat air pouch tissue.

3.3.6.1. Effect of chebulagic acid on reduced glutathione levels

Glutathione is a tripeptide and exists either in reduced form (GSH) or in the oxidized form (GSSG) within cells. while GSH is a potent anti-oxidant, GSSG is an indicator of the degree of oxidative stress in the system. Air pouch tissues from the animals were homogenized in phosphate buffer containing phosphoric acid in order to precipitate the proteins as quickly as possible and thus avoid

conversion of GSSG to GSH during homogenization. As shown in Fig. 33 levels of GSH increased gradually with time reaching to a maximum at 24 h in both the treatments. There was a significant increase in the levels of GSH in chebulagic acid treated rat air pouch tissue when compared to carrageenan alone treated pouch tissue.

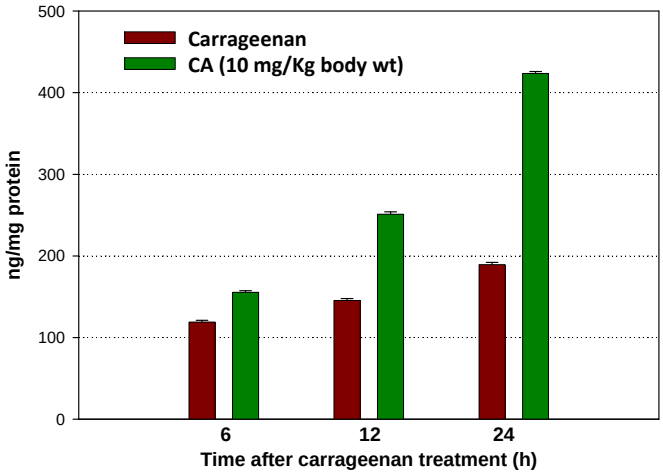

Fig. 33. Effect of chebulagic acid on reduced glutathione levels in the air pouch tissue at various time points after carrageenan and carrageenan + chebulagic acid (CA) treatments. GSH levels increased gradually reaching to a maximum by 24 h in both the treatments. However, in chebulagic acid treated rats GSH levels were significantly higher when compared to carrageenan alone treated rats, implying the protective role of chebulagic acid against oxidative stress.

3.3.6.2. Effect of chebulagic acid on glutathione peroxidase (GPx) activity

The levels of GPx were measured in carrageenan and carrageenan + chebulagic acid treated rat air pouch tissues and the results were shown in Fig. 34. As shown in Fig. 34, the levels of GPx increased gradually with time reaching

to a maximum at 24 h in both the treatments. There was a significant increase in the levels of GPx in carrageenan + chebulagic acid treated rat air pouch tissue when compared to carrageenan alone treated pouch tissue implying that chebulagic acid confers a protective sheath to the air pouch tissue against oxidative stress.

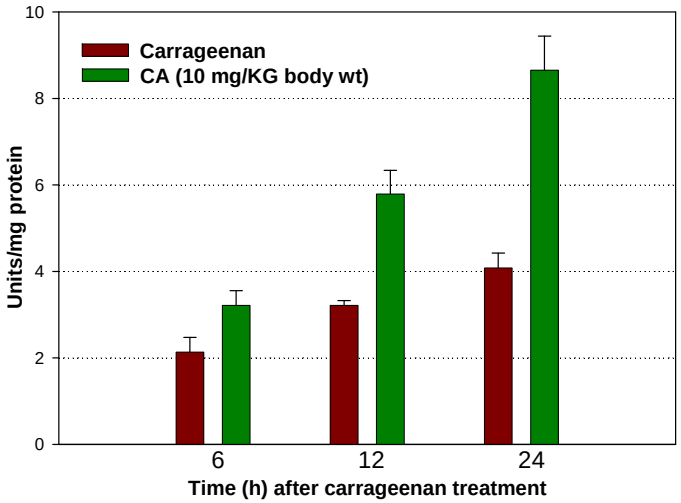

Fig 34. Effect of chebulagic acid on levels of GPx in the pouch tissues of carrageenan and carrageenan + chebulagic acid (CA) treated rats at various time intervals. Supernatants from pouch tissues treated with carrageenan alone or carrageenan + chebulagic acid were assayed for GPx as mentioned in methodology. All the values represent mean ± SE of data obtained from 3 different animals. *$p<0.05$ compared to carrageenan treated animals.

3.4. Discussion

Inflammation, which was initially recognized as a simple allergic reaction for centuries is now understood to be extremely important in pathogenesis of a large number of diseases. The process of inflammatory reaction, initiated by carrageenan, begins by utilizing the locally released population of mediators. The stimulus and the low level of released mediators specifically LTB_4 lead to chemotactic attraction of leukocytes into the area of stimulus. This in turn leads to induction of COX-2, and other cytokines. PGE_2 and LTB_4 regulate the synthesis of many catabolic factors (He *et al.,* 2002; Martel-Pelletier *et al.,* 2003; Boileau *et al.,* 2005; Marcouiller *et al.,* 2005). In fact, drugs which inhibit these eicosanoids resulted in significant reduction of arthritis (Nickerson-Nutter and Medvedeff, 1996). In particular, dual inhibitors of cyclooxygenase-2 and 5-lipoxygenase have been selected as anti-arthritic drug candidates (Inagaki *et al.,* 2000) and some of them, including darbufelone and licofelone, are currently in clinical development for the treatment of osteoarthritis (Bannwarth, 2003; Alvaro-Gracia, 2004).

Animal experiments have been extremely useful in understanding the entire inflammatory reaction since they demonstrate a complete window of events from the time when the stimulus is given till the reaction naturally resolves (Dawson *et al.,* 1991; Martin *et al.,* 1994; Serhan and Chiang, 2004). In the present study, air pouch animal model was chosen to study the progress of inflammatory reaction. Repeated injections of air into the subcutaneous tissue on the rodent's back, results in the formation of a cavity with many features similar to the synovial cavity of arthritic joints (Hambleton and Miller, 1989).

Air pouch model is a popular animal model of inflammation since it is easy, relatively quick and gives reproducible results. Air pouch model is considered very similar to the arthritic condition in humans since the synovial fluid and the air pouch fluid were found to be very similar (Sedgwick and Lees, 1986).

The histological staining of pouch lining shows infiltration of blood cells, in response to carrageenan treatment and thus establishing the role of pouch lining in the initiation and maintenance of inflammatory reaction (Sedgewick *et al.,* 1985). The pouch lining cells such as polymorphonuclear leukocytes (PMNLs), fibroblasts, monocytes and macrophages initiate and elaborate an array of proinflammatory mediators, which lead to expression and release of cytokines, specifically LTB_4 (Schumann *et al.,* 1994). These molecules act as chemoattractants and bring about changes in the endothelial layer to accommodate movement of infiltrating cells (Yang *et al.,* 2003). This process is simultaneously enhanced by induction of different pro-inflammatory proteins such as COX-2 (Kirsching *et al.,* 1997). Thus the initiation of an inflammatory reaction is complex and requires the concerted efforts of cells of the pouch lining, endothelial cells and peripheral blood cells. Once initiated the inflammatory reaction progresses and reaches a maximum at 24 h after carrageenan treatment and resolves spontaneously by removal of carrageenan either by ingestion by macrophages or by diffusion from the site of injection.

In the present study, the inflammatory response was defined acute since the pouch showed large number of cells mainly PMNLs migrating into the pouch with increasing time of exposure to inflammatory stimulus. PMNLs and macrophages appeared in increasing numbers with increasing time after

carrageenan treatment into the pouch. These cells are known to work together with the cells of the pouch lining to bring about the inflammatory reaction. The inflammatory reaction can be quantified by measuring the exudates volume and number of infiltering cells into the pouch - the two parameters that are of significance in assessing the efficacy of anti-inflammatory agents. In the present study, a decrease in these two parameters was observed in response to chebulagic acid treatment suggesting its anti-inflammatory property.

In the previous chapter (chapter-1) we have shown that chebulagic acid inhibits both COX-2 and 5-LOX activities. This will result in the decreased formation of PGE_2 and LTB_4 respectively, the pro-inflammatory mediators, and thus chebulagic acid may act as a potent anti-inflammatory agent. Hence in the present study, the air pouch model of inflammation was used to further evaluate the efficacy of chebulagic acid, as anti-inflammatory agent, and to understand the molecular mechanisms involved in its action. This study was carried out by analyzing the expression of COX-1, COX-2, iNOS and TNF-α and different parameters of oxidative stress.

Arachdonic acid which is accumulated in the membrane lipid, can be selectively released from the phospholipid pool by chemical or mechanical stimulation, and is subsequently converted to prostaglandins (PGs) by two enzymes, COX-1 and COX-2. COX-2 is primarily responsible for PGs produced in inflammation and COX-1 for PGs involved in normal homeostasis. In this regard, COX-2 is up-regulated in the air pouch and catalyzes the production of large amounts of PGE_2 (Masferrer *et al.,* 1994). The up-regulation of COX-2 associated with the increase of PGE_2 may be a major event in carrageenan-induced

inflammation. Moreover, non-steroidal anti-inflammatory drugs that inhibit COX-2 isoforms can suppress the inflammation of the rat air pouch (Masferrer *et al.,* 1994). In the present study chebulagic acid effectively down regulated the expression of COX-2 in the pouch tissue. These studies indicate that chebulagic acid acts as anti-inflammatory agent by both the mechanisms – inhibition of COX-2 and 5-LOX and down regulation of expression of COX-2. However, it is not clear how chebulagic acid is affecting the regulation of expression of COX-2. One assumption is that chebulagic acid could inhibit the carrageenan induced release of cytokines such as IL-1 and TNF-α, which are known inducers of COX-2 (Morikawa, 2003). Several *in vivo* results demonstrated that iNOS is involved in inflammatory reaction. The production of iNOS is region specific and induced with time, thus correlating with the development of inflammatory disorders. In the present study carrageenan induced iNOS expression was effectively down regulated by chebulagic acid in dose-dependent manner, further supporting the anti-inflammatory role of chebulagic acid.

Oxidative stress is another important component of pathophysiology of inflammation. ROS are now recognized to be both mediators and modulators of inflammation. Oxidative stress in any system can be assessed by the levels of reactive oxygen species and the antioxidant potential of the system. Hence reducing oxidative stress should be a part of any anti-inflammatory therapy in curing inflammatory disorders. If the cell is insufficiently protected by enzymatic and non-enzymatic antioxidants, free radicals can react with bio molecules and thus damage cellular structure. Antioxidants may prevent genetic changes by preventing DNA damage directly induced by free radical attack.

Thiol homeostasis is regulated to guarantee basic function and defense mechanism against xenobiotics. In the previous study we have shown that chebulagic acid is a potent anti-oxidant (chapter-1). This evidence indicates that chebulagic acid could prevent inflammation induced oxidative stress. Hence the activities of enzymes like glutathione peroxidase and reduced glutathione levels were measured in the inflammatory tissue to assess the antioxidant potential of chebulagic acid in the rat air pouch tissue. Chebulagic acid significantly elevated GSH levels suggesting its role in potentiating antioxidant defenses. Glutathione peroxidase (GPx) protects the cells from oxidative damage by reducing the levels of highly reactive organic as well as inorganic peroxides. Enhanced glutathione peroxidase (GPx) levels in chebulagic acid treated tissue further strengthens its anti oxidant potential.

The studies on air pouch model of inflammation, thus, further substantiate the anti-inflammatory potential of chebulagic acid under *in vivo* conditions also. In addition to its anti-ionflammatory effects, chebulagic acid offered protection from inflammation induced oxidative stress. These studies suggest that chebulagic acid could become an ideal anti-inflammatory drug candidate. Further studies, however, are required on its toxic side effects and clinical trials to substantiate these findings.

Chapter-4

Anti-proliferative properties of chebulagic acid on COLO-205 cells

4.1. Introduction

In a way to overcome the side effects of selective COX-2 inhibitors, COX-LOX dual inhibitors are emerging as potential anti-inflammatory drug candidates with least side effects (Kirchner *et al.,* 1997; Claria and Romano, 2005). A number of COX-LOX dual inhibitors are undergoing preclinical and clinical development for inflammatory disorders (Celotti and Laufer, 2001; Bannwarth, 2003; Charlier and Michaux, 2003). The chebulagic acid isolated from *Terminalia chebula* falls into that category of COX-2/5-LOX dual inhibitors.

It is now being clear that COX-2 and 5-LOX have converging functions not only in inflammation but also in cell proliferation and neo-angiogenesis. This is based on the reports on the co-expression and up-regulation of COX-2 and 5-LOX not only in inflammatory disorders but also in various neoplastic tissues. Also the COX-2 or 5-LOX inhibitors were shown to have potential anti-cancer effects (Avis *et al.,* 2001; Rao *et al.,* 2004). In the light of these findings it is reasonable to propose that COX-2, 5-LOX dual inhibitors would be a valid therapeutic approach for various cancers (Celotti *et al.,* 2003). Though a number of studies have proposed COX-2/5-LOX dual inhibitors as anti-inflammatory agents, very little is known about the potential of such dual acting molecules as anti-tumor agents.

In the previous chapters we have isolated and characterized COX-2, 5-LOX dual inhibitor and demonstrated its effectiveness as anti-inflammatory agent. This chapter presents data on the anti-proliferative effects of the COX-2/5-LOX

dual inhibitor, chebulagic acid against different cancer cell lines. Further, in depth apoptotic studies were undertaken on COLO-205 (colon cancer) cell lines.

4.1.1. Objectives

In view of COX/5-LOX dual inhibition shown by chebulagic acid the present study was taken up to evaluate the anti-proliferative properties of chebulagic acid in different cancer cell lines. The specific objectives of the present study are:

- To evaluate the anti-proliferative properties of chebulagic acid on cancer cell lines.
- To understand the molecular mechanisms of cell death induced by chebulagic acid.

4.2. Materials and methods

Cancer cell lines- MDA-MB-231 (breast carcinoma), HCT-15, COLO-205 (colon cancer), DU-145 (prostate cancer) and K-562 (chronic myeloid leukemia) were obtained from National Centre for Cell Science (NCCS), Pune, India. Phosphate buffered saline (PBS), RPMI 1640, Fetal Bovine Serum (FBS), Penicillin and Streptomycin were purchased from Gibco BRL (California, USA). MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), Trypsin and β-actin antibodies were purchased from Sigma Chemical Company (St.Louis, USA). PARP (Poly ADP-ribose polymerase), Bcl-2, Bax and Cytochrome c antibodies were purchased from Santa Cruz Biotechnology (California, USA). All other chemicals and solvents were of analytical grade.

4.2.1. Cell culture and treatment

All the cell lines used in this study except K-562 were maintained in monolayer in tissue culture Petri dishes. Medium for all the cell lines was RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37° C. The cultured cells were passaged twice a week, seeding at a density of 5 x 10^3 cells per well in 96 well plate before the day of experiment. Before the treatment with test compound cells were washed with PBS and fresh medium was added.

4.2.2. Cell proliferation assay

Cell proliferation was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) staining as described by Mosmann *et al.* (1983). The MTT assay is based on the reduction of the tetrazolium salt, MTT, by viable cells. The NADH or NADPH generated in the living cells, convert the yellow form of the MTT salt to insoluble, purple formazan crystals. The absorbance of the formazan solution can be measured spectrophotometrically after dissolving the crystals in an organic solvent (DMSO). Cells (5 × 10^3 cells per well) were incubated in 96-well plates in the presence or absence of chebulagic acid (0-50 μM) for 24 h in a final volume of 100 μl. At the end of the treatment, 20 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for an additional 4 h at 37 °C. The purple-blue MTT formazan precipitate was dissolved in 100 μl of DMSO. The activity of the mitochondria, reflecting cellular growth and viability, was evaluated by measuring the optical density at 570 nm

on μ Quant Bio-tek Instruments, Inc. micro titer plate reader. Each concentration was tested in three different experiments run in four replicates.

4.2.3. DNA fragmentation assay

The Cells were incubated with vehicle or test compound (chebulagic acid) for 24 h. 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 any genomic DNA contamination (Hermann *et al.,* 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 with Proteinase K (0.5 mg/ml) and 1% SDS for 1 hour at 50 °C. DNA was extracted with buffered phenol and precipitated with 140 mM NaCl and 2 volumes of ethanol at -20 °C overnight. DNA precipitates were washed in 70% ethanol, dissolved in TE, and treated for 1 hour at 37 °C with RNase A. 15 μl of DNA was mixed with 3 μl DNA sample buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol), and DNA was resolved in 1 % agarose gel in TBE (44.6 mM Tris, 44.5 mM boric acid and 1 mM EDTA) using 100 bp ladder as DNA standard. DNA fragmentation was visualized upon staining the gel with ethidium bromide (0.5 mg/ml) and exposing to UV light. The presence of apoptosis was indicated by the appearance of a ladder of oligonucleosomal DNA fragments that are approximately 180–200 bp multiples.

4.2.4. Quantification of apoptosis by flow cytometry

To quantitate apoptosis, flow cytometric analysis was performed using propidium iodide as described previously (Reddy *et al.,* 2003). Cells, which were less intensively stained than G1 cells (sub-G0/G1 cells) in flow cytometric histograms, were considered as apoptotic cells. Briefly, 3.5 x 10^6 cells (COLO-205) were plated in 6 well culture plates, cultured in 10% FBS with or without test compound for 24 h. After treatment, cells were harvested and washed in PBS, and the viability was determined by tryphan blue exclusion method. For analyzing DNA content 106 cells were fixed in 70% ethanol, washed in PBS, incubated with 0.1 mg/ml RNase A and stained with Propidium iodide (final concentration 50 μg/ml). The red fluorescence at excitation wave length- 530 nm and emission wave length-615 nm of individual cells was measured on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). A minimum of 10,000 events were analyzed per sample.

4.2.5. Preparation of the cell extracts and Western transfer

Cells were exposed to 25 μg of the purified compound for various time points (0, 6, 12, 24h) and whole cell extracts were prepared based on the method of (Pardhasaradhi *et al.,* 2003). 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% Triton X-100, 0.5% deoxycholic acid, 1 mM glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mg/ml leupeptin, 20 mg/ml aprotinin). After 30 min of shaking at 4 °C, the mixtures were centrifuged (10000xg) 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 and then transferred on to nitrocellulose membranes. Membranes were stained with 0.5% ponceau in 1% acetic acid to confirm equal loading. The membranes were blocked with 5% w/v non-fat dry milk and then incubated with the primary antibodies (PARP, Cytochrome c, Bcl-2, Bax and β-Actin) in 10 ml of antibody-diluted buffer (Tris-buffered saline and 0.05% Tween-20 with 5% milk) with gentle shaking at 4 ^{o}C for 8–12 h and then incubated with respective conjugated secondary antibodies. Signals were detected using Western blot detection reagents.

4.2.6. Statistical Analysis

Data are presented as mean ± SEM and the *P* values were determined using the unpaired Student's t-test. *P* value of less than 0.05 was considered as significant.

4.3. Results

4.3.1. *In vitro* anti cancer studies

To test the anti-cancer effect of the isolated compound, chebulagic acid (TC-V), cancer cell lines- MDA-MB-231 (breast carcinoma), COLO-205, HCT-15 (colon cancer), DU-145 (prostate cancer) and K-562 (chronic myeloid leukemia) were used. The cells were incubated with different concentrations of compound for 24 hr and the cell viability was examined by MTT assay. Cells were cultured in RPMI-1640 medium containing 10% FBS, with or without compound and viability was evaluated (Fig. 35). The GI_{50} values for different cancer cell lines

obtained were shown in Table 2. As can be seen from the Table 2, chebulagic acid showed broad spectrum anti-cancer activities with GI_{50} ranging from 18 to 31 μM. Further mechanistic studies, however, were undertaken only with COLO-205 cells.

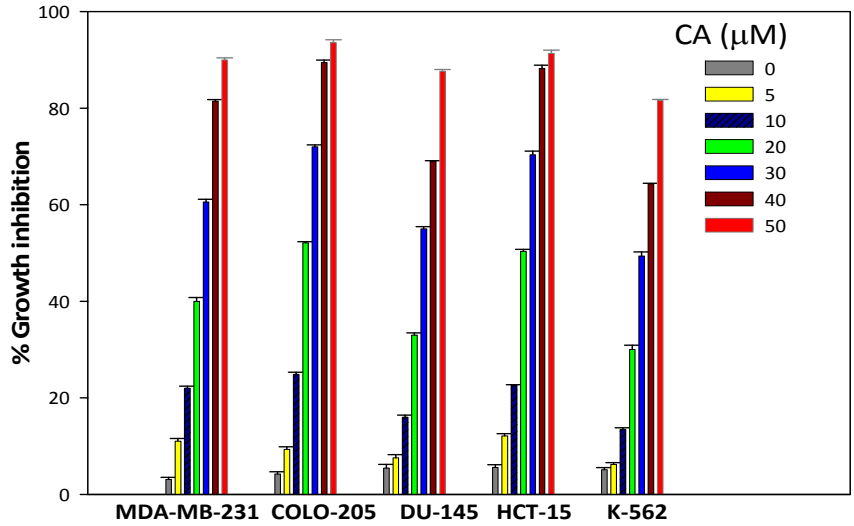

Fig. 35. Effect of chebulagic acid on the proliferation of cancer cell lines. The cells were incubated with different concentrations (as shown in graph) of chebulagic acid for 24 h and the cell viability was examined by MTT assay. Dose dependent growth inhibition was observed in all the tested cell lines. Data were mean ± SEM of three independent experiments (N = 3). [a]$P<0.01$, [b]$P<0.05$ compared with control.

Table 3. Cytotoxicity of chebulagic acid on cancer cell lines.

Cell line	IC_{50} (μM)
MDA-MB-231	26.2±0.472
COLO-205	18.1±0.2186
DU-145	28.54±0.389
HCT-15	20.3±0.23
K-562	30.66±0.36

Growth inhibition–50 (GI_{50}) was calculated by plotting, % inhibition vs. concentration. Cells were treated with different concentrations of chebulagic acid for 24 h. Data were mean ± SEM of three independent experiments (N = 3).

4.3.2. DNA fragmentation assay

As illustrated in Fig. 36, agarose gel electrophoresis of DNA extracted from COLO 205 cells treated with Chebulagic acid at concentrations of 25 and 50 μM for 24 h revealed a progressive increase in the non-random fragmentation into a ladder of 180–200 bp (lanes 2 and 3). The degree of nuclear DNA fragmentation was directly proportional to the concentration of compound. Such a pattern corresponds to internucleosomal cleavage, reflecting the endonuclease activity characteristic of apoptosis. Control cells did not show any internucleosomal DNA fragmentation (lane 1).

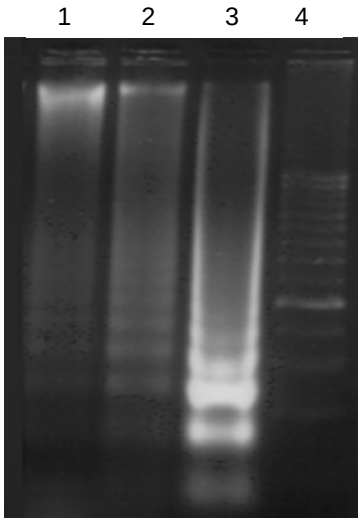

Fig. 36. Agarose gel electrophoresis showing internucleosomal DNA fragmentation of COLO-205 cells induced by chebulagic acid for 24 h. After treatment cells were lysed and total cellular DNA was extracted and electrophoresed on a 1% agarose gel containing 0.05 mg/ml ethidium bromide at 5 V/cm. The gels were then photographed under UV illumination. Lane 1: COLO-205 control cells, lane 2: COLO-205 cells treated with 25 μM chebulagic acid, lane 3: COLO-205 cells treated with 50 μM chebulagic acid and lane 4: 100 bp marker.

4.3.3. Effect of Chebulagic acid on cell cycle profile of COLO-205 cells: Flow cytometric analysis

The induction of apoptosis in COLO-205 cells treated with Chebulagic acid was further confirmed by flow cytometric analysis of DNA content. COLO-205 cells treated with Chebulagic acid (12.5, 25 and 50 μM) along with untreated cells were stained with propidium iodide and subjected to FACS analysis (Fig. 37). These studies showed 24.56% of cells treated with 12.5 μM (Fig. 37B), 42.44% of cells treated with 25 μM (Fig. 37C) and 62.65% of cells treated with 50 μM (Fig. 37D) of Chebulagic acid were at sub G0/G1 phase when compared to 3.46% in untreated cells (Fig. 37A).

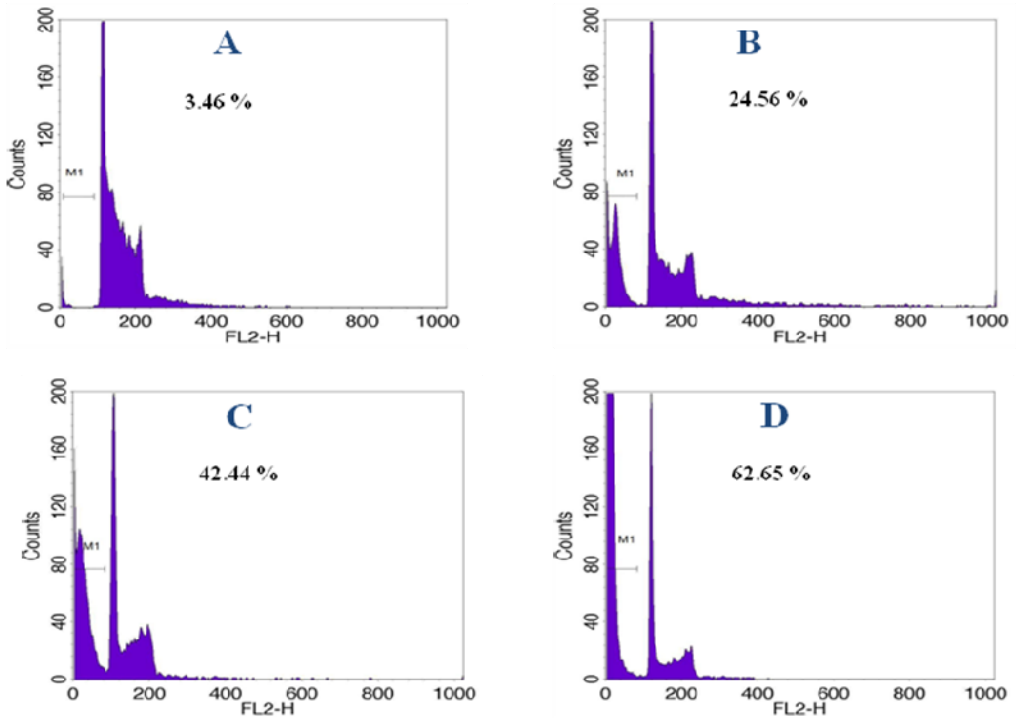

Fig. 37. Flow cytometric analysis of the control and chebulagic acid treated COLO-205 cells. Cells treated with or with out chebulagic acid for 24 h were fixed and stained with propidium iodide and the DNA contents were quantified by Flow cytometer. The number of hypo diploid (sub-G0/G1 phase) cells is expressed as a percentage of the total number of cells. (A) Control COLO-205 cells (3.46%), (B) COLO-205 cells treated with 12.5 μM of chebulagic acid (24.56%), (C) COLO-205 cells treated with 25 μM of chebulagic acid (42.44%) and (D) COLO-205 cells treated with 50 μM of chebulagic acid (62.65%).

4.3.4. Western blot analysis

4.3.4.1. Effects of Chebulagic acid on PARP cleavage and cytochrome c release

Preliminary apoptotic mechanisms were analyzed by Western blot analysis. 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. COLO 205 cells were treated with 25 μM chebulagic acid for 6, 12 and 24 h and PARP cleavage was monitored by employing PARP antibodies that recognize the 85 kDa fragment of cleaved PARP and uncleaved 116 kDa PARP. The data presented in Fig. 38, show the gradual increase in the appearance of 85 kDa signature fragment of PARP cleavage at all the indicated times. However, in control cells (lane-1) no 85kDa fragment of PARP was observed, except the uncleaved 116 kDa protein.

The process of apoptosis is associated with the disruption of mitochondrial membrane potentials, which result from the opening of permeability transition pores in the mitochondrial membrane, leading to the release of cytochrome c. A time dependent elevation in the cytosolic levels of cytochrome c with maximum increase at 12 and 24 h after treatment with chebulagic acid at 25 μM concentration was observed in the present study (Fig. 38).

4.3.4.2. Effects of Chebulagic acid on the expression of Bcl-2 and Bax proteins

The expression levels of Bcl-2 and Bax proteins are associated with mitochondrial membrane integrity and they play a crucial role in the regulation of apoptosis. In the present study, a time dependent decrease of Bcl-2 protein levels was observed after treatment with chebulagic acid, but no appreciable change in the levels of Bax protein was observed at all the time periods (Fig. 39). These results suggest a disturbed Bcl-2/Bax ratio in the cells treated with chebulagic acid.

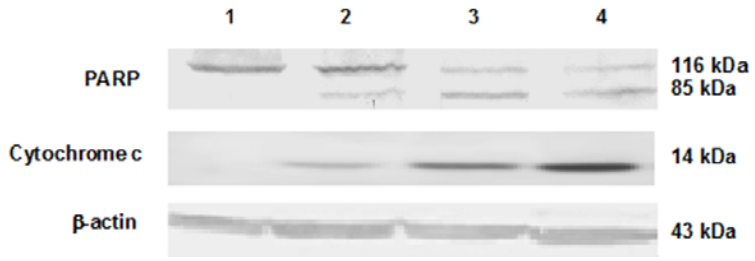

Fig. 38. Western blot analysis showing the effect of chebulagic acid on PARP cleavage and expression of Cytochrome c. Equal protein (75 μg) from the COLO-205 cells treated with 25 μM of chebulagic acid at indicated times (0, 6, 12 and 24 h) were analyzed by 12% SDS–PAGE, and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with PARP, Cytochrome-c and β-actin antibodies. β-actin was used as an internal control. Lane 1: 0 h; lane 2: 6 h; lane 3: 12 h and lane 4: 24h.

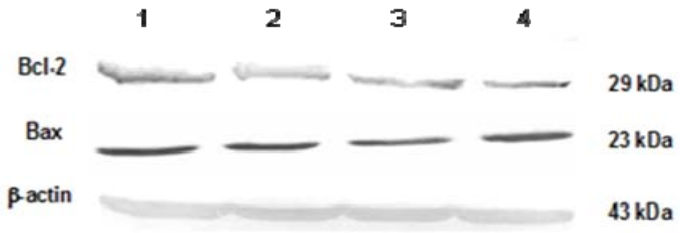

Fig. 39. Western blot analysis showing the effect of chebulagic acid on expression of Bcl-2, Bax proteins. Equal protein (75 μg) from the COLO-205 cells treated with 25 μM of chebulagic acid at indicated times (0, 6, 12 and 24 h) were analyzed by 12% SDS–PAGE, and after electrophoresis, proteins on the gel were transferred to nitrocellulose membrane and probed with Bcl-2, Bax and β-actin antibodies. β-actin was used as an internal control. Lane 1: 0 h; lane 2: 6 h; lane 3: 12 h and lane 4: 24 h.

4.4. Discussion

Natural products discovered from medicinal plants have played an important role in the treatment of cancer. A number of natural phytochemicals and antioxidants such as C-phycocyanin (Subhashini *et al.,* 2004; Pardhasaradhi *et al.,* 2003), Gallic acid (Inoue *et al.,* 1994), Capsaicin (Wolvetang *et al.,* 1996), Gingerol (Samaha *et al.,* 1997), - (-) epigallocatechin gallate (Suganuma *et al.,* 1999), Resveratrol (Clement *et al.,* 1998) etc., were shown to have anticancer properties. In the present study we have analyzed anti-proliferative properties of chebulagic acid, a COX-LOX dual inhibitor isolated from *Terminalia chebula* in five different cancer cell lines (MDA-MB-231 (breast carcinoma), HCT-15, COLO-205 (colon cancer), DU-145 (prostate cancer) and K-562 (chronic myeloid leukemia)). Chebulagic acid showed dose dependent broad spectrum anti-proliferative properties in all the above cancer cell lines with GI_{50} (growth inhibition-50) concentrations ranging from 18-30 μM.

Further in-depth studies were taken up to understand the molecular mechanisms involved in chebulagic acid-induced cell death in COLO-205 cells. Apoptosis is a process of cell death that is critically regulated based on the expression of intrinsic suicide machinery (Vaux and Korsmeyer, 1999), which further leads to the characteristic pattern of morphological, biochemical, and molecular changes. The present study showed typical apoptotic characteristics such as DNA fragmentation in the cells treated with chebulagic acid. The flow cytometric analysis of chebulagic acid treated cells showed an increase in the hypo diploid apoptotic DNA content with a decrease in the number of cells at the S and G2 phases of the cell cycle.

Accumulating scientific evidences indicate the pivotal role of mitochondria in the execution of apoptosis of the cells exposed to various stimuli (Green and Reed, 1998; Desagher and Martinou, 2000). The present study showed accumulation cytochrome c in the cytosolic fractions of cells exposed to chebulagic acid. Further decrease in the expression of Bcl-2 protein coupled with a decrease in the Bcl-2/Bax ratios strongly support mitochondrial membrane alterations in the cells treated with chebulagic acid. Caspase-3, an executioner caspase in apoptotic cascades is implicated in the cleavage of a number of proteins, including Poly (ADP-ribose) polymerase (PARP), which is a hallmark of apoptosis. In the present study a time dependent increase in the PARP cleavage in cells treated with chebulagic acid was observed, suggesting the activation of caspase-3 activity. The foregoing studies, thus, clearly demonstrate that chebulaguic acid induces apoptosis in COLO-205 cells by intrinsic death pathway.

Chapter-5

Chemopreventive effects of chebulagic acid on 1, 2-dimethylhydrazine induced tumors in rat colon

5.1. Introduction

5.1.1. Colon cancer

Cancer of the colon is the disease characterized by the development of malignant cells in the lining or epithelium of the first and longest portion of the large intestine. Malignant cells have lost normal control mechanisms governing growth. These cells may invade surrounding local tissue, or they may spread throughout the body and invade other organ systems. Colon cancer is the third most common disease in the United States, prevalent in both men and women. Globally, colon cancer is the fourth most common cancer in men and the third most common cancer in women. Most cases of colon cancer begin as small, noncancerous (benign) clumps of cells called adenomatous polyps. Over time some of these polyps become colon cancers and removal of these polyps can prevent colon cancer.

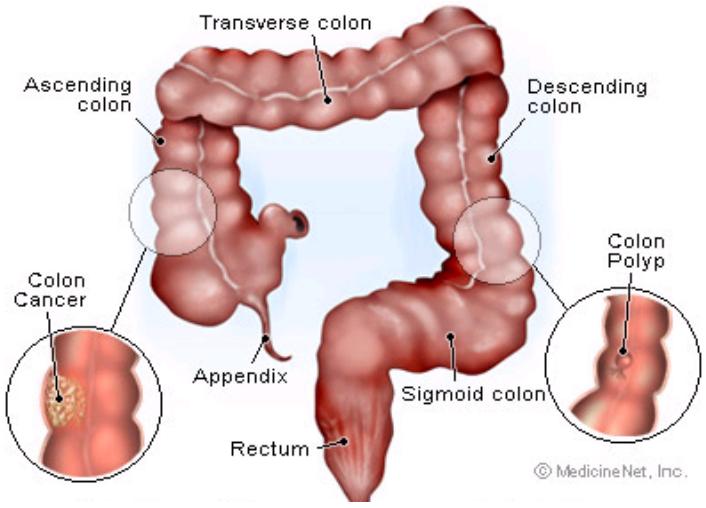

Fig. 40. Colon cancer and polyp (source: *cancer-colon.org*)

Colon cancer has no early symptoms and hence screening regularly is important. Surgery is a common treatment for colon cancer. The development of colon cancer results from the sequential accumulation of activating mutations in oncogenes, such as *ras*, and inactivating mutations, truncations, or deletions in the coding sequence of several tumor suppressor genes, including *p53* and *adenomatous polyposis coli* (*APC*), together with aberrant activity of molecules controlling genomic stability.

5.1.2. Molecular biomarkers in colon cancer

The most important studied/altered molecular markers/targets involved in signal transduction pathways, which can be considered under surrogate endpoints in colon carcinogenesis, are COX-2, iNOS, HMG-CoA, ER-β, β-catenin, 5-LOX, and STAT3. Effect of different agents on these molecular markers/targets will influence the endpoint markers in colon carcinogenesis.

Ⅰ. COX-2

COX-1 is constitutively expressed in the colon, but COX-2 is inducible and markedly up-regulated in all types of colon cancers (Sano *et al.,* 1995). Increased COX-2 gene expression has been shown in human colorectal adenocarcinomas and in carcinogen induced rat colonic tumors (Tsujii *et al.,* 1997). Notable over-expression of COX-2 protein in tumors located in the rectum was observed when compared with other locations in the colon (Dimberg *et al.,* 1999). Recent observations on COX-2 expression in colon tumor samples from patients explain a progressive over-expression of COX-2 during stepwise sequence from adenoma to carcinoma (Chan *et al.,* 2007). Based on

various evidences, suppression of COX-2 is now considered as a crucial target for control of tumors with chronic/persistent inflammation. Epidemiological (Kune *et al.,* 1988), human (Thun *et al.,* 1993; Giovannucci *et al.,* 1995), and animal studies (Charalambous *et al.,* 1996) indicate that the regular use of nonsteroidal antiinflammatory drugs (NSAIDs) potentially reduces the risk of colorectal cancer by 40–60%. Celecoxib, selective COX-2 inhibitor, has been approved for the treatment of FAP and is still in use. A number of clinical trials are at different phases with celecoxib individually and also in combination with other drugs (curcumin, docetaxel, prednisolone, zoledronate, eflornithine) on colon cancer patients.

Ⅱ. iNOS

NO is a highly reactive compound that is produced by three isoforms of Nitric Oxide Synthase (nNOS, eNOS and iNOS). Under normal physiological conditions, endogenous NO is produced by the constitutive NOS isoforms, eNOS and nNOS (neuronal NOS). These two are important for peristalsis (nNOS) and maintaining mucosal blood flow (eNOS). Inducible NOS (iNOS) is expressed in many cells, extravascular resident leucocytes (macrophages), intravascular and/or infiltrating leucocytes (neutrophils and monocytes), endothelium, and parenchymal cells, including intestinal epithelium after exposure to various inflammatory stimuli like lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β. iNOS produces large amounts of NO for a limited period of time and is an element of the innate immunity. Excessive and

prolonged NO production has been suggested to cause intestinal dysfunction in inflammatory bowel disease (IBD) and sepsis.

It is assumed that chronic inflammation and continuous exposure to NO produced by iNOS leads to neoplastic transformation, which is a key step in carcinogenesis. Studies on iNOS indicate that NO produced by iNOS can initiate and/or promote tumorigenesis (Hofseth *et al.,* 2003). Mice with mutations in both adenomatous polyposis coli (Apc) and iNOS showed fewer adenomatous polyps in the small and large intestines of mice compared with mice with the mutation in *Apc* alone (Ahn and Ohshima, 2001). These findings identify iNOS as a target for tumor chemoprevention in colon cancer. Indeed, iNOS inhibitors reduced azoxymethane-induced colon cancer and tumorigenesis in *Apc*-mutant mice and rats (Rao *et al.,* 2002). Preclinical studies with iNOS selective inhibitors in colon cancer models are encouraging in the prevention of tumorigenesis.

III. β-catenin

β-catenin is a subunit of the cadherin protein complex and is found at the plasma membrane in association with cadherins, the tumor suppressor promoter APC and microtubules, in the cytoplasm and in the nucleus. So, it cannot be presumed that it works only in the nucleus to "signal" a response. It is a multifunctional protein involved in cell adhesion, signaling and many more (Cadigan and Nusse, 1997; Huelsken *et al.,* 2001). There are a number of reports pertaining to the functional interactions between nuclear receptors and the canonical, Wnt/β-catenin signaling pathway cascade (Mulholland *et al.,* 2005).

The deregulation of β-catenin has been found to lead to various forms of cancer, particularly colorectal cancer (Powell *et al.,* 1992). Mutations in *APC* or β-catenin can result in the failure of β-catenin to be degraded and they are retained in the cytoplasm of cells which is often seen in colon cancer (Yang *et al.,* 2006). Subsequently, there will be an increase in β-catenin-TCF complex formation, causing alterations in gene transcription (myc, cyclinD1, c-jun, TCF-1, Lef-1, conductin/axin2 and MMP7), leading to carcinogenesis (Morin *et al.,* 1997). Somatic mutations in genes in the β-catenin pathway are found in >80% of colon cancers (FAP and sporadic disease), and aberrant beta catenin activity is known to play an early and causative role in colon cancer. This may occur by mutations in the APC protein, axin or in the β-catenin itself, which leads to dysregulation of β-catenin turnover and activation of genes involved in tumorigenesis (Behrens *et al.,* 1996). As these mutations are exclusively found in colorectal cancers, β-catenin is considered to be a potential molecular target in colon carcinogenesis (Korinek *et al.,* 1997). A number of compounds that inhibit the beta-catenin pathway and show selective toxicity toward cancer cells have been identified. The development of drugs that selectively target the nuclear entry or exit of regulatory proteins altered in cancer may be another option for treatment and prevention.

IV. 5-LOX

Lipoxygenase (LOX) is one of the two important enzyme classes that metabolizes polyunsaturated fatty acids and affect carcinogenesis. Cell membrane phospholipids are converted to arachidonic acid, which serves as a

substrate that gives rise, in turn, to two powerful and potentially damaging classes of inflammation mediators, known as eicosanoids: the prostaglandins and leukotrienes. Arachidonic acid release and production of eicosanoids are prerequisites for inflammation. The inflammatory prostaglandins and leukotrienes are formed by the action of cyclooxygenase (COX-2) and lipoxygenase (5-LOX) enzymes, respectively (Borgeat *et al.,* 1976). This forms the crux of dual inflammatory pathways: COX-2 and 5-LOX. Many reports documented the clear evidence of COX pathway generating inflammatory prostaglandins and its role in colon carcinogenesis. But medical research has largely ignored the potentially damaging effects of 5-LOX, the enzyme that forms the second branch of the dual Arachidonic acid inflammation pathways. Most emphasis was given to block COX-2 activity, ignoring the effects of 5-LOX, which may actually increase the 5-LOX levels, worsening the inflammation. This may be due to shifting of Arachidonic acid toward synthesis of leukotrienes through the 5-LOX pathway when COX-2 is blocked, which plays a vital role in inflammation. COX-2 inhibition alone was ineffective in slowing the progression of clinically diagnosed cancers. It is clearly evident from animal models and *in vitro* studies that expression of 5-LOX appears to be occasionally upregulated during neoplastic transformation (Ye *et al.,* 2005). Expression of 5-LOX has been characterized in early colon neoplasms and found up regulated in colon polyps and colon cancers (Soumaoro *et al.,* 2006). Recently, it has been shown that even these products may contribute to the development of colon and several other human tumors (Chen *et al.,* 2004). These observations provide evidence that 5-LOX plays a role in colon cancer development and may be an early target for chemoprevention of colon cancer.

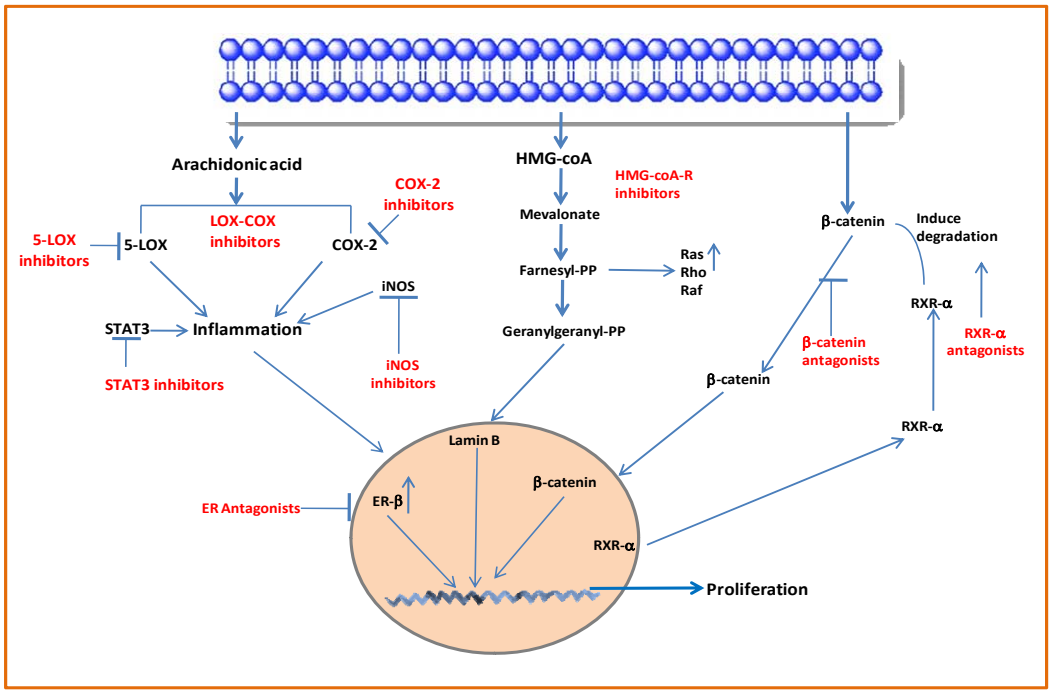

Fig. 41. Molecular markers and targets in colorectal cancer.

5.1.3. DMH model of colon cancer

The induction of colonic tumours in mice and rats by 1, 2-dimethylhydrazine (DMH) is widely used as an experimental model for studies on the role of dietary factors in colon carcinogenesis (Goldin, 1998). High or repetitive doses of DMH result in a spectrum of antecedent and neoplastic changes analogous to those seen in man with regard to type of lesion and response to chemotherapy (Haase *et al.,* 1973). Aberrant crypt foci (ACF), a preneoplastic change in the colonic mucosa, may represent a critical event in the stepwise progression of colon cancer. The study of premalignant

hyperproliferative lesions and of aberrant crypts is crucial for understanding the progression of early changes to malignancy in the pathogenesis of colon cancer (Bird, 1987).

Intestinal tumors in man, including metastasis, can be reproduced to a fair resemblance in rodents after a certain time of induction; frequency and localizations vary according to different combination of rodents and DMH administration schedules (Wiebecke *et al.,* 1973). DMH is known as a chemical carcinogen of choice for investigating tumor pathogenesis and various other factors affecting oncogenesis (Martin *et al.,* 1973; Garmaise *et al.,* 1975).

5.1.4. Objectives

In the previous chapter we have studied the anti-proliferative properties of chebulagic acid in COLO-205 cells, an *in vitro* system. To check the anti-tumor effect of chebulagic acid under *in vivo* conditions, the present study is undertaken to study its anti-tumor effect in DMH-induced ACF (Aberrant crypt foci) in rat colon. The objectives of the present study are:

- ❖ To check the anti-tumor effect of chebulagic acid on DMH-induced ACFs in rat colon.
- ❖ To check the signaling mechanisms involved in anti-tumor activity.

5.2. Materials and methods

5.2.1. Chemicals

1, 2-dimethylhydrazine (DMH) and β-actin antibody were purchased from Sigma Chemical Company (St.Louis, USA). β-catenin, cyclin D1, iNOS and c-Myc monoclonal antibodies and the alkaline phosphatase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (California, U.S.A).

5.2.2. Experimental animals

Adult Wistar strain male rats (150 to 200 gms) were used for all the experiments in the present study. They were fed a standard chow pellet diet, had free access to water, and were maintained on a 12:12-h light-dark cycle. All procedures in this study were approved by the Animal ethics committee of the University of Hyderabad.

5.2.3. Study Design

Animals were assorted into the following groups:

A. Control Group, Vehicle Treated: Animals were administered the vehicle (1mM EDTA-saline subcutaneously (s.c.) in weekly injection for 8 weeks.

B. DMH Group: Animals were administered with DMH weekly at a dose of 30 mg/Kg body weight, sub cutaneously (s.c.). DMH was freshly prepared in 1mM EDTA-saline, pH adjusted to 7.0 using dilute NaOH solution.

C. DMH + CA Group: Chebulagic acid was given daily per oral (p.o) to the animals along with the weekly administration of DMH.

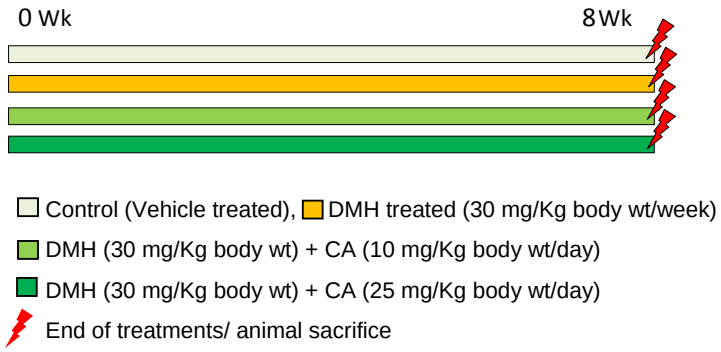

Fig. 42. Treatment design followed to evaluate the chemopreventive response by chebulagic acid on DMH induced colon tumorigenesis in 8 weeks treatment groups. Daily doses of chebulagic acid were administered per oral.

A minimum of 8 animals from each group were sacrificed to study various parameters determining the progression/ regression of tumors during the treatment. Animals were kept overnight fasting with drinking water *ad libitum* before sacrifice.

5.2.4. Gross anatomical observation and chemopreventive response

All the animals were sacrificed under an overdose of ether anesthesia. Colon was removed, flushed with chilled physiological saline, and opened longitudinally. These were thoroughly examined macroscopically for the presence of any tumors. Different regions of colon were carefully examined under a hand held lens for counting the tumors. Chemopreventive response was assessed on the basis of tumor incidence (Tumor incidence: percentage of animals having tumors).

5.2.5. Histopathological observations

The colonic segments were divided into proximal, middle and distal regions, fixed immediately in 10% formalin and processed routinely. Paraffin embedded sections were stained with Hematoxylin and Eosin (HE) for histopathological examinations. Presence of carcinoma, hyperplasia and dysplasia, aberrant crypt focci associated with aggregates of lymphoid tissues and mucosal inflammation were observed in the stained sections of colon under the light microscope.

For histological studies, small pieces of colonic segment were taken, washed with ice-cooled 0.9% saline and fixed in 10% formalin for 24 h. After fixation, the tissues were processed carefully in paraffin wax (58-60 $^{\circ}$C), according to the standard technique (Pearse, 1968). Paraffin sections were cut at 5-7 μ thickness in a microtome, stretched on an albumin coated slide and then subjected to the histological technique, where sections were stained with Delafield haematoxylin-eosin. Sections were dewaxed in xylene, downgraded (hydrated) in decreasing percentage of alcohols and brought to water, and stained with haematoxylin for 20 sec, washed in tap water, till the appearance of blue color, rinsed in ammonia water, again washed with water, treated with acid water (if over stained). The sections were upgraded (dehydrated) in alcohol till 70%, stained with 1% alcoholic eosin for 30 sec, differentiated in 90% alcohol, cleared in xylene and finally mounted in distyrene plasticizer xylene (DPX).

5.2.6. Homogenisation of colon tissue

Colon tissue was carefully dissected out and homogenized in 100 mM Tris-HCl (pH 8.0), 0.3 M mannitol, 1 mM EGTA, 1 mM EDTA, 4 mM K_2HPO_4, 1 mM DTT, 1 mM Sodium ortho vanadate, 0.1% (w/v) SDS, 2 mM PMSF and 40 µl/ml of complete protease inhibitor solution, centrifuged for 30 min at 10,000 rpm, 4 °C. Protein content in the supernatant was measured by Bradford method (Bradford, 1976) and this was used for further experiments.

5.2.7. SDS-PAGE and Western Blotting

SDS PAGE and Western blot analyses for the detection of β-catenin, cyclin D1, iNOS, and c-Myc and β-actin in the colon tissue homogenate were performed by the procedure as mentioned earlier (Chapter-2, Section 2.2.7).

5.3. Results

5.3.1. Gross anatomic observation and chemo preventive response

Colons of the animals treated with/without 1, 2-dimethylhydrazine (DMH) and chebulagic acid were dissected longitudinally to check the incidence of aberrant crypt foci (ACF). All the rats treated with DMH developed ACF and treatment with chebulagic acid reduced the tumor incidence in a dose dependent manner. The mean number of ACF developing in DMH treated animals over the 8 week treatment period was 3.33 (0.235). This number was significantly reduced with chebulagic acid in a dose dependent manner.

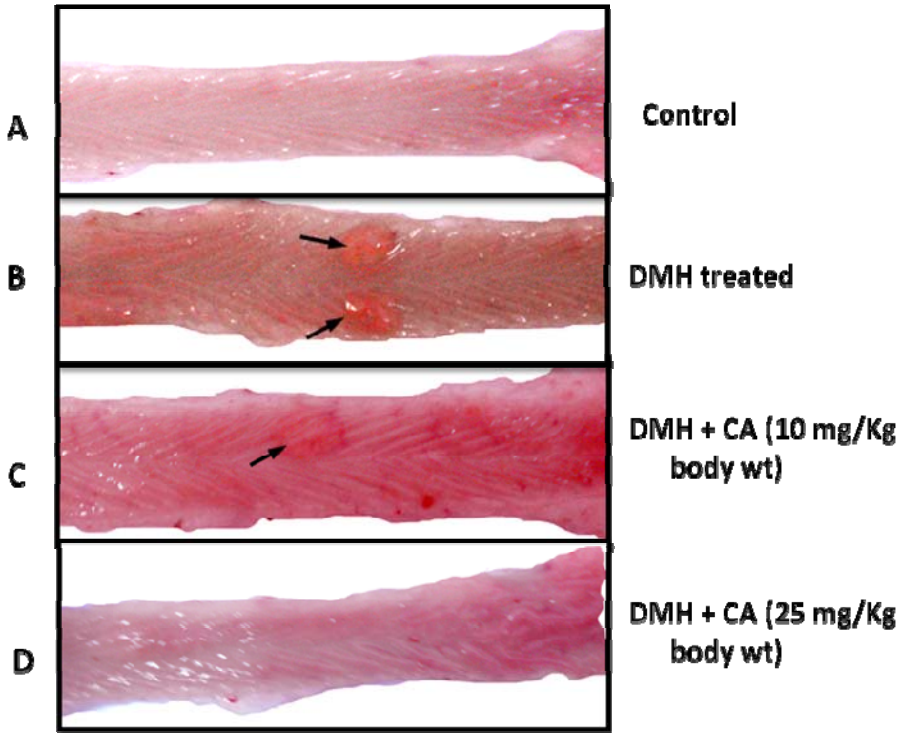

Fig. 43. Comparative gross anatomy of the colonic mucosal surface after 8 weeks of indicated treatments (locations shown by arrows).

Of animals treated with chebulagic acid at a concentration of 10 mg/Kg body wt, developed a mean of 1.33 (0.129) tumours, a 60% reduction ($p<0.01$), and in animals treated with chebulagic acid at a concentration of 25 mg/Kg body wt showed 0.11 (0.111) tumors (Table 4).

Table 4. Effect of 1, 2-dimethylhydrazine and chebulagic acid on the colonic tumor incidence.

Group	Number of rats	Number of tumour-bearing rats	Tumour Incidence (%)	Mean (SEM) number of tumours (ACF) developing per animal
Control (saline treated)	9	0	0	0
DMH	9	8	88.88	$3.33 \pm 0.235^{\#}$
DMH ± chebulagic acid (10 mg/Kg body wt)	9	4	44.44	1.33 ± 0.129*
DMH ± chebulagic acid (25 mg/Kg body wt)	9	1	11.11	0.11 ± 0.111**

Data represents mean ± SEM. $^{\#}P < 0.01$ in comparison with the control. $^{*}P < 0.01$, $^{**}P < 0.001$ in comparision with DMH treated group.

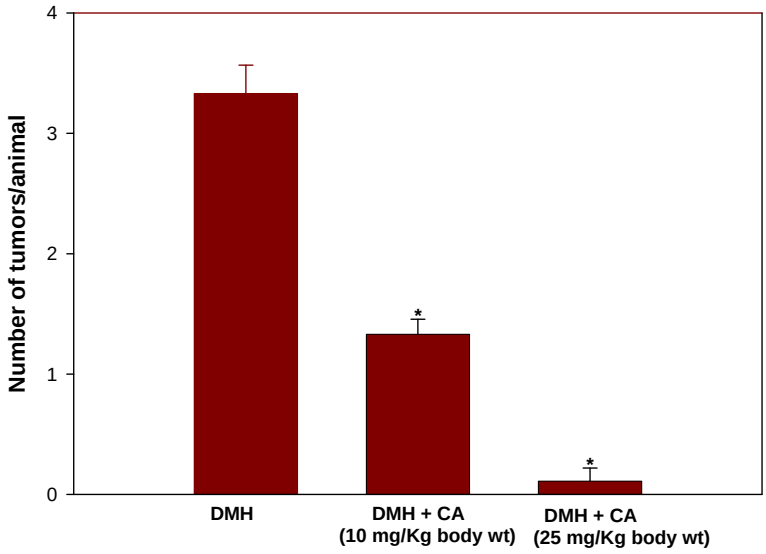

Fig. 44. Effect of chebulagic acid treatment on 1, 2-dimethylhydrazine (DMH)-induced tumors. Mean ± SEM number of tumors developing per animal over 8 weeks was significantly reduced compared with DMH alone treated animals in all treatment groups. *P < 0.01.

5.3.2. Histological observations in colon tissue

Histopathologic analysis in the colon of control rats revealed no signs of ACF and reported the normal epithelium (Fig. 45A) and DMH alone treated group reported the presence of hyper plastic ACF in colon (Fig. 45B). DMH + chebulagic acid (10 mg/Kg body wt) (Fig. 45C) reported 1.33 ± 0.129 ACF in colons of treated animals. In DMH + chebulagic acid (25 mg/Kg body wt) treated group (Fig. 45D) despite extensive sampling, there was no evidence of carcinoma *in situ* or frank malignancy animals.

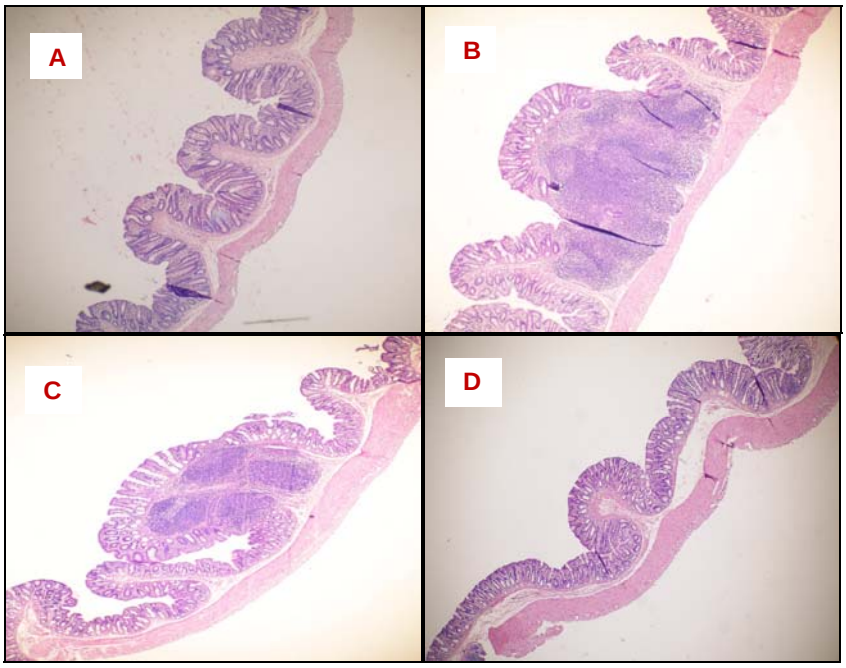

Fig. 45. Histological observations of mucosal epithelium of colon after the treatment with DMH and/or chebulagic acid. (A) Mucosal epithelium control rat (magnification ×40); (B) rat administered with DMH exhibiting various hyperplastic ACFs (magnification ×40); (C) and (D) mucosal surface of colon of rat administered DMH + chebulagic acid at 10 and 25 μg/ml respectively (magnification ×40).

5.3.3. Effect of chebulagic acid on the expression of β-catenin, iNOS, cyclin D1 and c-Myc proteins

To ascertain the anti-tumor effects of chebulagic acid Western blot was performed on the expression of β-catenin, iNOS, cyclin D1 and c-Myc protein levels in rat colons treated with/without DMH and chebulagic acid. As shown in Fig. 46, marked increase in the levels of β-catenin, iNOS, cyclin D1 and c-Myc proteins were observed in DMH alone treated group when compared with the

control group. Further, chebulagic acid reduced the expression of these proteins in a concentration dependent manner.

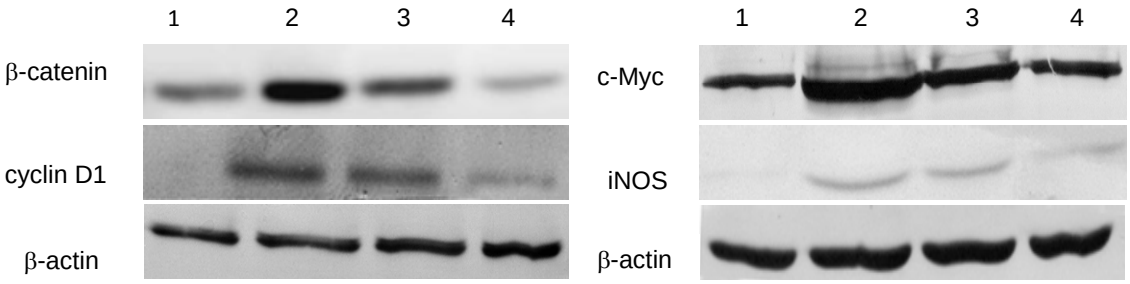

Fig. 46. Effect of chebulagic acid on the expression of β-catenin, iNOS, cyclin-D1 and c-Myc protein levels in rat colons. Western blot analysis showing effect of chebulagic acid on the expression of β-catenin, iNOS, cyclin D1 and c-Myc protein levels in different treatment groups. β-actin used as an internal control for equal loading of proteins. Lane 1: Control, lane 2: DMH treated group, lane 3: DMH + chebulagic acid (10 mg/Kg body wt) treated group and lane 4: DMH + chebulagic acid (25 mg/Kg body wt) treated group.

5.4. Discussion

Colorectal cancer is one of the most common malignancies and is a major cause of cancer deaths in the western world (Kune *et al.,* 1988). Chemoprevention of colorectal cancer by nonsteroidal anti-inflammatory drugs (NSAIDs) is arguably the most promising area for reducing the clinical problem. The NSAIDs have been shown in randomized controlled trials to induce inhibition of polyps in patients with familial adenomatous polyposis (FAP) (Nugent, 1993). A number of large case control and cohort studies have shown a reduction of 40–50% in colorectal cancer in subjects taking NSAIDs regularly, particularly aspirin (Thun *et al.,* 1993). In addition, experimental studies in the rat demonstrate inhibition of benign and malignant tumors by a range of NSAIDs.

DMH and its metabolite AOM cause colorectal cancer in rodents with a distribution and histology similar to those observed in human colorectal cancer (de Jong *et al.,* 2000) and are thus widely used as a model for human colorectal carcinogenesis. The rodent DMH colorectal cancer model thus provides an ideal whole animal system in which to study the effect of COX inhibitors (NSAIDs). Recent findings suggest that the prostaglandin E_2, the proinflammatory product of elevated cyclooxygenase-2 activity in colon cancer, stimulates cancer cell growth through a G protein–dependent signaling pathway coupling the prostaglandin E_2 receptor to β-catenin (Castellone *et al.,* 2006). Alterations in the *APC* or β-catenin gene are considered to play a gate keeper role in the development of colon cancers in both humans and preclinical models (Takahashi *et al.,* 1998). Numerous studies have focused on the significance of

ACF as early events in colon carcinogenesis and ACF are now regarded as putative preneoplastic lesions for colon cancers (Pretlow *et al.,* 1991). It has been widely accepted that the increase in cryptal size on the whole mount preparation is the most distinctive feature of ACF. ACF were considered to be biological precursors of colon cancer in rodents and man (Bird and Good, 2000).

In the previous chapter (Chapter-4) we reported the anti-proliferative properties of chebulagic acid, a COX-2 and 5-LOX dual inhibitor, in colon cancer cells. The present study was undertaken to study the anti-tumor effects of chebulagic acid in DMH-induced ACF in rat colon and to understand the molecular mechanisms involved in its action. This study was carried out by analyzing the efficacy of chebulagic acid in reducing the ACF, histology of rat colon and expression levels of β-catenin, iNOS, c-Myc, and cyclin D1 in DMH induced ACF in colons of rat treated with chebulagic acid.

In the present study, chebulagic acid treatment had a significant inverse influence on ACF formation in the colon. As shown in Table 4, chebulagic acid reduced the number of ACF in DMH induced colons of rats in a dose dependent manner. Compared to rats treated with DMH alone (3.33/rat), the number of ACF were reduced to 1.33/rat in rats treated with chebulagic acid at a concentration 10 mg/Kg body wt and where as in rats treated with chebulagic acid at concentration 25 mg/Kg body wt the number of ACF were further reduced to 0.11. Histopathologic analysis in control rats of the colon revealed no signs of ACF and reported the normal epithelium (Fig. 45A) and the mucosal crypts were well oriented. Colons of DMH treated rats showed irregular disposition of the crypts, reduction of the intercryptic spaces and lymphoid–

glandular complexes with severe inflammatory cell infiltrations (Fig. 45B). DMH + chebulagic acid (10 mg/Kg body wt) treated group (Fig. 45C) reported reduced cellular infiltration along with decreased number of ACF when compared with DMH alone treated group. Whereas in DMH + chebulagic acid (25 mg/Kg body wt) treated group reported further decrease in the occurrence of ACF and showed well oriented colonic epithelium as in control rat colon (Fig. 45D).

Dysregulation of catenin-related transcription (CRT) is a common early event in intestinal epithelial cells during human colorectal carcinogenesis (Wong and Pignatelli, 2002). Upregulation of β-catenin expression during colorectal carcinogenesis leads to accumulation of nuclear β-catenin, which is a prerequisite for increased CRT (Takahashi *et al.,* 1998; Herter *et al.,* 1999). This, in turn, leads to increased CRT via formation of a transcriptional complex with T-cell factor (TCF)/lymphoid enhancer factor transcription factors and subsequent trans-activation of several b-catenin/TCF target genes such as cyclin D1, peroxisome proliferator-activated receptor δ, iNOS and c-Myc, which are known to play a role in colorectal carcinogenesis (Wong and Pignatelli, 2002). Therefore, dysregulated CRT is a potential target for chemoprevention and treatment of colorectal carcinogenisis. In the present study treatment with DMH alone caused the over expression of β-catenin, and treatment with chebulagic acid reduced the expression of β-catenin protein in a dose dependent manner.

Cyclin D1 has central role in cell cycle control and is a downstream target for several signalling pathways and has a complex promoter with multiple

transcription factor binding elements (Lukas *et al.,* 1996; Wang *et al.,* 2001). Cyclin D1 is a known β-catenin/TCF target gene (Shtutman *et al.,* 1999). In the present study treatment with chebulagic acid inversed the over expression of cyclin D1 protein induced by DMH in a concentration dependent manner. Further chebulagic acid reduced the iNOS and c-Myc protein expressions which were induced by DMH treatment. Thus by reducing the expression levels of β-catenin, chebulagic acid reduced the transcription of target genes, resulting in the restoration of apoptosis and reduced cell proliferation.

In summary, the present study demonstrates that chebulagic acid, a COX-2, 5-LOX dual inhibitor isolated from *Terminalia chebula* prevents/reduces the number of ACF in the colons of DMH treated rats in dose dependent manner. These anti-tumor effects of chebulagic acid appear to be mediated by lowering the accumulation of β-catenin, which in turn inhibits the formation of a transcriptional complex with T-cell factor (TCF), and by further inhibiting the transactivation of β-catenin/TCF target genes such as cyclin D1, iNOS and c-Myc.

6. Summary and conclusions

Inflammation is the body's immediate response of tissues and cells to pathogen infection, exposure to noxious stimuli such as chemicals, or physical injury. It is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue. However, if triggered or directed inappropriately, the inflammatory response can itself become harmful, leading to cell, tissue and organ destruction. Abnormalities associated with inflammation comprise a large, unrelated group of disorders which underlay a variety of human diseases. Some of the inflammatory disorders include arthritis, asthma, hypersensitivity etc. Inflammation has been proven to be associated with many degenerative diseases, including heart diseases, cancer, stroke, respiratory diseases, and Diabetes mellitus, Alzheimer's disease, septicemia, liver and kidney diseases. Inflammation is a complex process regulated by a cascade of cytokines, growth factors, NO and prostaglandins produced by activated macrophages. Hence inhibition of inflammatory cytokines, iNOS, COX-2 and 5-LOX serves as a key mechanism in the control of inflammation, and agents that suppress the expression of these inflammation-associated genes have therapeutic potential for treatment of many inflammatory diseases including cancer.

Cancer is a class of disease in which a group of cells display uncontrolled growth, invasion and sometimes metastasis (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, do not invade or metastasize. Cancer occurs when cell division gets out of control. Usually, the

timing of cell division is under strict constraint, involving a network of signals that work together to say when a cell can divide, how often it should happen and how errors can be fixed. Mutations in one or more of the nodes in this network can trigger cancer, be it through exposure to some environmental factors (e.g. tobacco smoke) or because of a genetic predisposition, or both. The predominant mechanisms for the cancers featured here are (i) impairment of a DNA repair pathway (ii) the transformation of a normal gene into an oncogene and (iii) the malfunction of a tumor suppressor gene. Normal cell growth and division are largely under the control of a network of chemical and molecular signals. Disruption of the signaling process results in abnormal growth and division of cells. Aberrant expression of many proteins like MAP kinases, Bcl-2, Rac, AKT, NF-κB, AP-1, p53, β-catenin, COX-2, 5-LOX etc were required for the cancer cell proliferation. Among these, the Arachidonic acid metabolites formed via COX-2 and 5-LOX pathways, collectively called as eicosanoids, play an important role in promoting cell growth, invasion and metastasis which are the key components of malignant cancers.

Eicosanoids are potent biologically active Arachidonic acid derived lipid mediators that are intimately involved in inflammation and cancer. Cyclooxygenases (COX) and 5-Lipoxygensase (5-LOX) are the key enzymes in the Arachidonic acid metabolism. COX enzyme exists in two distinct isoforms, COX-1 and COX-2. COX-1 is expressed constitutively in nearly all mammalian tissues and is the source of prostaglandins central to "housekeeping" functions such as renal water re-absorption, vascular homeostasis, and gastric protection. COX-2, an inducible enzyme, is mainly expressed under pathological conditions

such as inflammation and carcinogenesis. Hence, COX-2 inhibitors were developed as novel non steroidal anti-inflammatory drugs (NSAIDs) without gastric side effects that are associated with the conventional NSAIDs.

In addition to their role in inflammation, selective inhibitors of COX-2 have been demonstrated to induce apoptosis in variety of cancer cell lines including colon, stomach, prostate, and breast. 5-LOX is involved in the biosynthesis of leukotrienes, pro-inflammatory mediators participating in various forms of acute and sub acute inflammation. 5-HETE, product of 5-LOX, was shown to be a potential survival factor for prostate cancer cells and inhibition of 5-LOX triggered massive apoptosis. Both 5-LOX and COX-2 are co-expressed and up-regulated in inflammation and in many forms of human cancers, including colon, prostate, breast and lung cancers and form targets for development of anti-inflammatory and anti-cancer drugs. The conventional as well as selective COX-2 inhibitors in the market, however, are reported to have many side effects, because the inhibition of COX may lead to a shunt of the Arachidonic acid metabolism towards the 5-LOX pathway. This was shown to be responsible for many of the side effects reported with conventional and COX-2 selective NSAIDs. Treatment with NSAIDs may, therefore, increase the formation of LTs, which can induce gastric damage and ulceration. Such a shunt has been shown *in vivo* and an enhanced synthesis of LTB_4 was demonstrated in patients with rheumatoid arthritis or osteoarthritis taking NSAIDs for more than 3 months. Moreover, COX-2 inhibition by celecoxib in cancer cell lines was shown to increase the formation of 5-HETEs, which is having tumor cell proliferative

property. Hence there is need for the development of potent COX-LOX dual inhibitors without side effects.

The isolates of natural products, viz., the medicinal plants are proving to be good alternatives to the synthetic chemicals, with little or no side effects. We have earlier reported that c-Phycocyanin and betanin are potent selective inhibitors of COX-2 and 5-LOX respectively (Subhashini *et al.,* 2004; Sreekanth *et al.,* 2007). But since there is a need for a dual COX-LOX inhibitor, the present study is designed to screen various natural herbal products for the identification of potent 5-LOX and COX-2 inhibitors. In our attempt to isolate a natural product with dual inhibition of COX-LOX, we identified *Terminalia chebula* as a potential source.

The initial studies with the ethanolic extract of *Terminalia chebula* showed potent inhibition of COX-1, COX-2 and 5-LOX, with more preference towards COX-2 and 5-LOX. Further HPLC fractionation of the ethanolic extract resulted in the identification of TC-V as a potent COX-LOX dual inhibitor with IC_{50} values of 15±0.288, 0.92±0.011 and 2.1±0.057 μM for COX-1, COX-2 and 5-LOX respectively. In view of dual inhibition, the TC-V fraction was subjected to electron spray ionization LC-MS, NMR and IR analyses and identified as chebulagic acid with molecular wt of 953 Daltons. Chebulagic acid showed potent anti-oxidant activity with IC_{50} values of 1.4±0.0173 μM and 1.7±0.023 μM respectively in DPPH radical scavenging and ABTS decolourization assays.

Anti-inflammatory properties of chebulagic acid were studied *in vitro*, in LPS-induced RAW 264.7 macrophages. In these cells, chebulagic acid potently

inhibited the production of NO and prostaglandin E_2 (PGE_2) in a concentration dependent manner. This may partly be due to inhibition of COX-2 by chebulagic acid in LPS stimulated mouse macrophage cell line RAW 264.7. To further explore the possible mechanism(s) underlying these inhibitions by chebulagic acid, we examined the expression levels of iNOS, COX and 5-LOX at gene and protein levels. The inhibition in the expression of iNOS and COX-2 was evidenced by reductions in their protein and mRNA levels in a dose dependent manner with increasing concentration of chebulagic acid. These studies thus reveal that the observed decrease in the production of NO and PGE_2 may also be attributed to the down regulation in the expression of iNOS and COX-2 at mRNA and protein levels, in response to chebulagic acid treatment.

In order to understand the signaling mechanisms involved in the anti-inflammatory properties shown by chebulagic acid in LPS-induced RAW 264.7 macrophages, further studies were undertaken on NF-κB that controls many pro-inflammatory genes. Treatment with chebulagic acid to the RAW 264.7 cell inhibited the NF-κB activation induced by LPS, and this was associated with the abrogation of IκB-α phosphorylation and with subsequent decreases in nuclear p65 and p50 protein levels. Moreover, the phosphorylations of p38, ERK1/2, and JNK in LPS-stimulated RAW 264.7 cells were suppressed by chebulagic acid in a dose-dependent manner. These results suggest that the anti-inflammatory properties of chebulagic acid might be mediated through the inhibition of iNOS, COX-2, 5-LOX, IL-6, and TNF-α expression through the down-regulation of NF-κB activation via suppression of IκB-α phosphorylation and MAP kinase (p38, ERK1/2, and JNK) phosphorylation in RAW 264.7 cells induced with LPS.

Encouraged with the potent anti-inflammatory properties of chebulagic acid *in vitro*, further studies were undertaken on carrageenan induced inflammation in rat air pouch. Air pouch model is a popular animal model of inflammation since it is easy, relatively quick and gives reproducible results. Air pouch model is considered very similar to the arthritic condition in humans since the synovial fluid and the air pouch fluid were found to be very similar. Inflammatory characteristics like exudate volume and number of infiltrated cells were reduced in chebulagic acid treated (10 mg/Kg body weight) animals compared to carrageenan alone treated animals. Histological sections of air pouch tissue of chebulagic acid treated animals also showed reduced inflammatory reaction and this was evidenced by the down regulated expression of COX-2 levels, in air-pouch tissues of chebulagic acid treated animals. Chebulagic acid also down regulated the expression of other pro-inflammatory mediators such as iNOS and TNF-α in carrageenan induced rat air-pouch tissue. As a result the levels of COX-2, iNOS and TNF-α were markedly lower in chebulagic acid treated air pouch tissue when compared to carrageenan alone treated animals. While down regulating the expression of pro-inflammatory genes, chebulagic acid increased the reduced glutathione (GSH) and glutathione peroxidase (GPx) activity levels in carrageenan treated rat air pouch tissues when compared to carrageenan alone treated animals. These studies suggest that chebulagic acid could form a potential candidate for the treatment of inflammatory disorders. Further, in depth preclinical and clinical trials, however, are required to evaluate its potential as the anti-inflammatory drug without side effects.

COX-2 and 5-LOX inhibitors have been shown to exhibit potent anti-cancer effects on cell lines and in animal models. In the present study also chebulagic acid, which is a COX-LOX dual inhibitor showed broad spectrum anti-cancer effects on HCT-15 (colon), COLO-205 (colon), MDA-MB-231 (breast), DU-145 (prostate) and K562 (chronic myeloid leukemia) cell lines, suggesting a broad spectrum anti-cancer effects of chebulagic acid. Further detailed studies on mechanism of action on COLO-205 (colon cancer) cell lines revealed that chebulagic acid induces apoptosis as evidenced by DNA fragmentation assay, PARP cleavage, cytochrome c release from mitochondria and alteration of Bcl2/Bax ratios.

Anti-cancer properties of chebulagic acid were also studied *in vivo* on DMH-induced ACFs (aberrant crypt foci) in rat colon. 1, 2 dimethylhydrazine (DMH) rat model has been proven to be a valuable animal model of colorectal cancer. Because of its morphological similarity to human colorectal cancer, DMH rat model was used to produce information on histology and biochemistry of tumors development as well as on factors that retard or enhance tumorigenesis. Epidemiological studies in humans suggest that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) especially aspirin significantly decreases the risk of developing colorectal cancer. In the present study chebulagic acid reduced the total number of tumors induced by DMH in the colons of rats. Histological observations also showed the occurrence of ACF (aberrant crypt foci) formation in colons of DMH treated rats and they were reduced by the treatment with chebulagic acid indicating that chebulagic acid prevents tumor formation induced by xenobiotics. In order to understand the mechanisms

underlying the anti-tumor property of chebulagic acid in DMH-induced rat colon carcinogenesis, further studies were taken up on signaling elements involved. Treatment with chebulagic acid inhibited the translocation of β-catenin from cytoplasm to nucleus and subsequent reduction in the expression levels of cyclin-D1, c-Myc and iNOS in DMH-induced rat colon carcinogenesis.

In conclusion, the present study demonstrates that chebulagic acid isolated from *Terminalia chebula* fruits is a potent of COX-2 and 5-LOX dual inhibitor and also a potent anti-oxidant. Through *in vitro* and *in vivo* studies it is demonstrated that chebulagic acid exhibits anti-inflammatory and anti-cancer properties. Further, the molecular mechanisms and signal transduction pathways involved in these anti-inflammatory and anti-cancer effects of chebulagic acid were elucidated.

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Research Publications:

- Chebulagic acid, a dual COX-LOX inhibitor from *Terminalia chebula* Retz. fruit induces apoptosis on COLO-205 cell line. **Bharat Reddy D,** C M Reddy T, Jyotsna Radhika G, Satish Sharan, Nalini Priya, Lakshmipathi V, Reddanna P. *Journal of Ethnopharmacology (In Review).*
- Chebulagic acid (CA) attenuates LPS-induced inflammation by suppressing redox based NF-κB and MAPK activation in RAW 264.7 macrophages. **D. Bharat Reddy** and Pallu Reddanna*. *Biochemical and Biophysical Research Communications*. *(In Review).*
- Synthesis of Methyl [6-(2-amino-1, 3-thiazol-4-yl)-3-oxo-1, 4-benzoxazin-2-yl]acetates as possible COX-2 / 5-LOX inhibitors. G. Jagath Reddy*, K. Srinivasa Rao, K N Jayaveera, S. Sailaja, P. Reddanna, and **D. Bharat Reddy**. Heterocyclic Communications, Vol 14, May 2008, 95-100.
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Posters and Papers presented:

- Presented the poster "Isolation, purification and characterization of dual COX-LOX inhibitor from *Terminalia chebula".* **Bharat Reddy D**, Jyotsna Radhika G, Satish Sharan, Nalini Priya, Lakshmipathi V, Reddanna P. Awarded as the **Best poster** at **SBC** (Society of Biological Chemists) Conference, Tirupathi, Nov 25-27, 2007.
- Presented the poster "Anti-oxidant, anti-inflammatory and anti-cancer properties of Clerodendrum serratum extracts" Jaipal reddy, sreekanth devalaraju, **Bharat Reddy D,** Anil kotha, Reddanna P. Awarded as the **Best poster** at **SFRR** (Society For Free Radical Research) Conference, Bangalore, Jan 10-12, 2005.
- Presented the Paper "Antioxidant and Antidiabetic Studies of Serendipitously Identified Proline- Ninhydrin Adduct" Prabhakar K.R, Veerapur V.P, Vipan Kumar P, Machendar Reddy Kandadi, **Bharat Kumar Reddy**, Reddanna. P, Atanu Barik, Priyadarsini K.I and Unnikrishnan M.K. SFRR conference, Mumbai, 2007.