Molecular mechanisms involved in the anti-inflammatory and anti-proliferative properties of chebulagic acid, a COX-2 / 5-LOX dual inhibitor

Thesis Submitted for the degree of

DOCTOR OF PHILOSOPHY

BY

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Dedicated

to

my beloved parents

UNIVERSITY OF HYDERABAD
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DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Molecular mechanisms involved in the anti-inflammatory and anti-proliferative properties of chebulagic acid, a COX-2/5-LOX dual inhibitor"** 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.

In keeping with the general practice of reporting scientific investigations, due acknowledgements have been made wherever the work described is based on the findings of other investigators. Any omission or error that might have crept in is regretted.

Prof. P. Reddanna
(Research Supervisor)

Chandrani Achari
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CERTIFICATE

This is to certify that **Ms. Chandrani Achari** 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 her thesis **"Molecular mechanisms involved in the anti-inflammatory and anti-proliferative properties of chebulagic acid, a COX-2/5-LOX dual inhibitor"** for submission for the degree of Doctor of Philosophy of this University.

Prof. P. Reddanna
(Research Supervisor)

Head
(Department of Animal Sciences)

Dean
(School of Life Sciences)

PREFACE

The present thesis entitled **"Molecular mechanisms involved in the anti-inflammatory and anti-proliferative properties of chebulagic acid, a COX-2/5-LOX dual inhibitor"** has been divided into five chapters. ***Chapter 1*** provides a brief introduction on Cancer and inflammation. It also describes the role of eicosanoids in inflammation and cancer as well as the usefulness of NSAID's and COXIB's in overcoming cancer and inflammation. Furthermore it highlights the importance of a natural COX-2/5-LOX dual inhibitor chebulagic acid as a potential candidate for the treatment of inflammation disorders. ***Chapter 2*** demonstrates the potent anti-inflammatory effects of chebulagic acid in LPS induced acute lung injury model in mice. These effects were mediated through inhibition of NF-κB activation and MAP kinase phosphorylation and through activation of Nrf-2. In ***Chapter 3*** the anti-proliferative properties of chebulagic acid in human hepatocellular carcinoma cell lines, HepG2 and Hep3B have been illustrated. Further studies showed the involvement of Wnt/β-catenin signaling in chebulagic acid mediated effects. ***Chapter 4*** describes the efficacy of chebulagic acid to overcome MDR-1 mediated drug resistance in HepG2 cells through COX-2 dependant modulation of MDR-1. Studies on the signaling mechanisms revealed the inhibition of NF-κB activity and suppression of phosphorylation of MAP kinases and AKT to be involved in these effects. ***Chapter 5*** summarizes the findings of the present investigation.

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At this juncture, I remember with gratitude my beloved parents for their whole hearted support and immense blessings to achieve this goal in my life. This dissertation is cordially devoted to them.

What I am today is by the Grace of God Almighty. I stoop before Him for having strengthened me to complete this endeavour.

.

Chandrani Achari…

Abbreviations

AA	Arachidonic acid
ATP	Adenosine triphosphate
°C	Degree centigrade
CA	Chebulagic Acid
COX	Cyclooxygenase
DMSO	Dimethyl sulphoxide
DNA	Deoxy ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
g	Gram
h	Hour(s)
kDa	Kilodalton
l	Litre
LOX	Lipoxygenase
LT	Leukotriene
mg	Milligram
min	Minutes

mL	Milliliter
mM	Millimolar
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor-kappa B
nm	Nanometer
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylene diamine
Tris	Tris-(Hydroxymethyl) aminoethane
UV	Ultraviolet

CONTENTS

Chapter 1

General Introduction

1.1. What is Cancer?

A major feature of all higher eukaryotes is the defined life span of the organism, a property that extends to the individual somatic cell, whose growth and division are highly regulated. A notable exception is provided by cancer cells, which arise as variants that have lost their usual growth control. These cancer cells form a mass of tissue called tumor. The cells in malignant (cancerous) tumors are abnormal and divide without control or order. They can invade and damage nearby tissues. Also, cancer cells can break away from a malignant tumor and spread to other parts of the body by a process called metastasis. Genetic alterations in two types of genes can contribute to the cancer process. Proto-oncogenes are normal genes that are involved in cell growth and division. Change in regulation of these genes lead to the development of oncogenes, which can promote excessive cell growth and division. Tumor suppressor genes are involved in controlling cell division. When tumor suppressor genes dysfunction, cells grow and divide abnormally, which leads to tumor growth. De regulation of genes or genetic changes that are not corrected by the cell can lead to the production of abnormal proteins. Damaged proteins may not respond to normal signals, may over-respond to normal signals, or otherwise fail to carry out their normal functions. These malfunctions of proteins lead to disruption of normal crosstalk between the signaling components of cell division machinery. 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.

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. There were previously six recognized hallmarks of cancer - unlimited replicative potential, self-sufficiency in growth signals, insensitivity to growth inhibitors, evasion of programmed cell death, ability to develop blood vessels, and tissue invasion and metastasis [1] , cancer related inflammation now emerges as number seven (Figure 1.1).

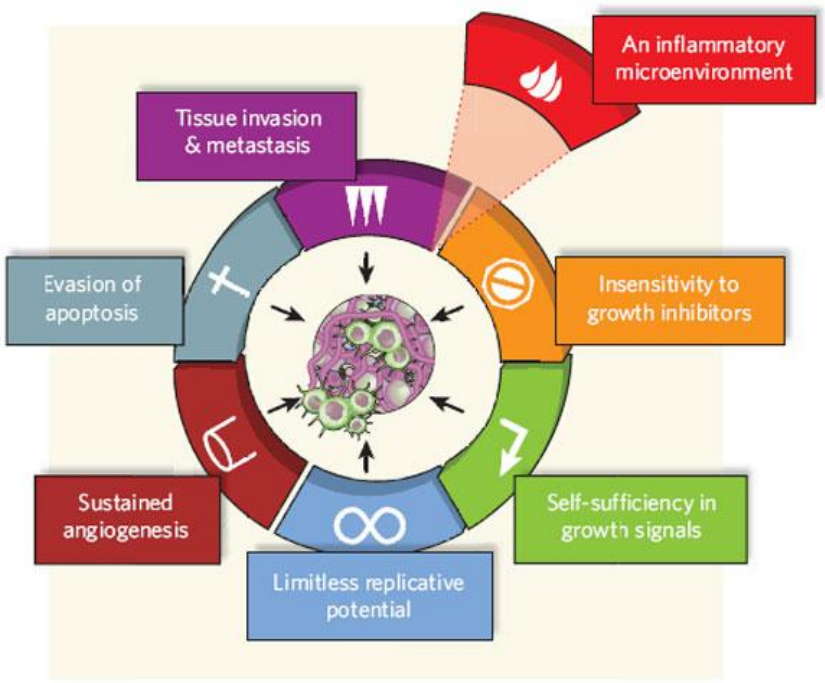


Figure 1.1. *Hall marks of cancer (Source: Alberto Mantovani, 2009).*

1.2. Cancer statistics

The World Health Organization (WHO) recently projected that in 2010, cancer would overtake ischemic heart disease as the leading cause of death in the world.

Between 2005 – when some 7.6 million people died from cancer, accounting for 13% of global deaths – and 2015, it is anticipated that 84 million people will die of cancer. The main types of cancer leading to overall cancer mortality in the world each year are:

- Lung (1.4 million deaths/year)
- Stomach (740,000 deaths)
- Liver (700,000 deaths)
- Colon (610,000 deaths)
- Breast (460,000 deaths).

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. The most frequent types of cancer worldwide (in order of the number of global deaths) among men are 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 (Figure 1.2). 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.

Estimated New Cases*

Males				Females		
Prostate	217,730	28%		Breast	207,090	28%
Lung & bronchus	116,750	15%		Lung & bronchus	105,770	14%
Colon & rectum	72,090	9%		Colon & rectum	70,480	10%
Urinary bladder	52,760	7%		Uterine corpus	43,470	6%
Melanoma of the skin	38,870	5%		Thyroid	33,930	5%
Non-Hodgkin lymphoma	35,380	4%		Non-Hodgkin lymphoma	30,160	4%
Kidney & renal pelvis	35,370	4%		Melanoma of the skin	29,260	4%
Oral cavity & pharynx	25,420	3%		Kidney & renal pelvis	22,870	3%
Leukemia	24,690	3%		Ovary	21,880	3%
Pancreas	21,370	3%		Pancreas	21,770	3%
All Sites	**789,620**	**100%**		**All Sites**	**739,940**	**100%**

Estimated Deaths

Males				Females		
Lung & bronchus	86,220	29%		Lung & bronchus	71,080	26%
Prostate	32,050	11%		Breast	39,840	15%
Colon & rectum	26,580	9%		Colon & rectum	24,790	9%
Pancreas	18,770	6%		Pancreas	18,030	7%
Liver & intrahepatic bile duct	12,720	4%		Ovary	13,850	5%
Leukemia	12,660	4%		Non-Hodgkin lymphoma	9,500	4%
Esophagus	11,650	4%		Leukemia	9,180	3%
Non-Hodgkin lymphoma	10,710	4%		Uterine Corpus	7,950	3%
Urinary bladder	10,410	3%		Liver & intrahepatic bile duct	6,190	2%
Kidney & renal pelvis	8,210	3%		Brain & other nervous system	5,720	2%
All Sites	**299,200**	**100%**		**All Sites**	**270,290**	**100%**

Figure 1.2. *Ten Leading Cancer Types for the Estimated New Cancer Cases and Deaths by Sex in US in 2010.*

1.3. 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.

1.4. Inflammation: Gearing the journey to cancer

An association between the development of cancer and inflammation has long been appreciated [2,3]. The inflammatory response orchestrates host defenses to microbial infection and mediates tissue repair and regeneration, which may occur due to infectious or non-infectious tissue damage. Epidemiological evidence points to a connection between inflammation and a predisposition for the development of cancer, i.e. long-term inflammation leads to the development of dysplasia. Epidemiologic

studies estimate that nearly 15 percent of the worldwide cancer incidence is associated with microbial infection [4].

Chronic inflammation represents a major pathological basis for tumour development. Although inflammation acts as host defence mechanism against infection or injury and is primarily a self limiting process, inadequate resolution of inflammatory responses lead to various chronic disorders associated with cancers. In 1863, Rudolf Virchow proposed that chronic inflammation supports cancerogenesis. Since then, accumulating studies support this hypothesis and it is estimated that 20% of all cancer deaths are associated with chronic infection and inflammation. Microbial infections (e.g. Helicobacter pylori is associated with gastric cancer and gastric mucosal lymphoma), viral infections (e.g. hepatitis B or C virus are associated with hepatocellular carcinoma), autoimmune disease (e.g. inflammatory bowel disease is associated with colon cancer) and inflammatory conditions of unknown origin (e.g. prostatitis is associated with prostate cancer) are recognized as triggers of chronic inflammation associated with cancer development [1]. Recent evidence has indeed demonstrated that the expression of the inflammation-related programs is driven by the activation of different classes of oncogenes. Borrello and colleagues observed that in freshly isolated human thyrocytes, activation of the oncogene RET promotes the same inflammatory transcriptional program found in patients affected by papillary thyroid carcinoma [5]. In analogy, other oncogenes, (e.g. RAS and MYC) and tumour suppressor genes, (e.g. von Hippel-Lindau tumour suppressor (VHL), transforming growth factor-β (TGFβ) and phosphatase and tensin homologue (PTEN)), activate

signalling pathways involved in inflammation [6-13]. Both extrinsic and intrinsic pathways of cancer- related inflammation activate transcription factors (mainly NF-kB, HIF-1a, STAT3), which are the key inducers of inflammatory mediators (e.g. cytokines, chemokines, prostaglandins and nitric oxide) [1]. The switch to ‘‘smouldering’’ inflammation contributes to tumour development through different mechanisms, including induction of genomic instability, alteration in epigenetic events and subsequent inappropriate gene expression, enhanced proliferation and resistance to apoptosis of initiated cells, induction of tumour angiogenesis and tissue remodelling with consequent promotion of tumour cells invasion and metastasis [1] (Figure. 1.3). Genomic instability has been identified as yet another hallmark of cancer [14]. Despite this evidence, genetic studies of mouse models have demonstrated that the inflammatory response supported by innate immune cells is crucial for the activation of an adaptive immune response capable to eliminate nascent tumours [14]. It is generally accepted that immune cells continuously recognize and destroy nascent tumour cells but, due to the genetic instability that characterize neoplastic cells, the arising of new variants able to evade the immune surveillance results in tumour establishment and progression (‘‘immunoediting’’process) [15]. In this regard several studies aim to elucidate the mechanisms driving immune escape. They emphasise that the smouldering inflammation associated with established tumours tunes the adaptive immune response. Indeed, tumour-associated dendritic cells mainly show an immature phenotype [16] and myelomonocytic cells recruited in

tumours express an alternative M2 functional phenotype, mainly oriented towards the suppression of the adaptive immune response [1, 17].

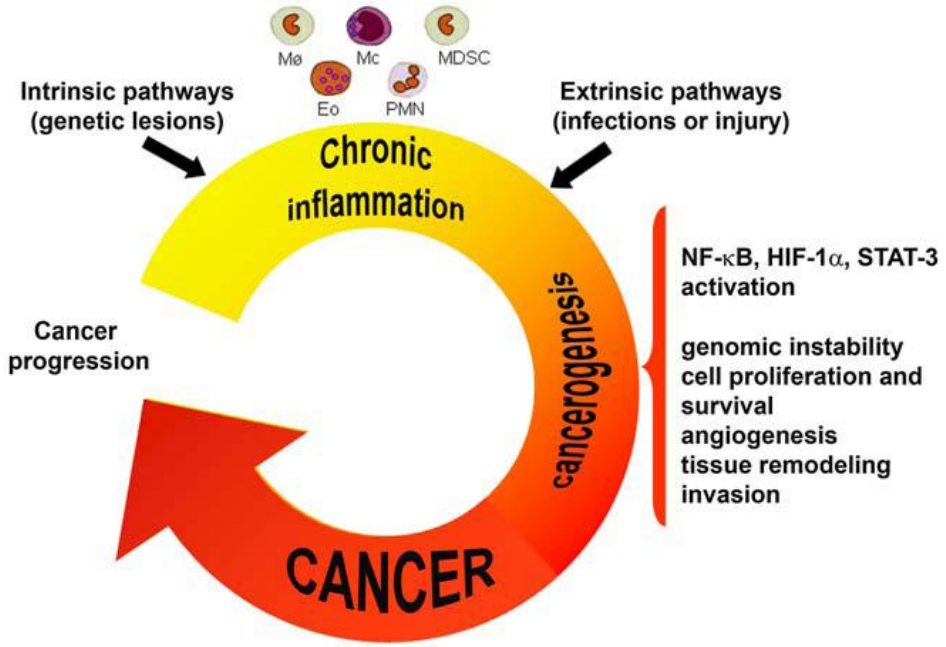


Figure 1.3. *Inflammation and cancer connection. Irrespective of the trigger for the development both intrinsic (driven by genetic alteration) and extrinsic (driven by inflammatory cells and mediators) pathways result in inflammation and neoplasia. Both neoplastic cells and leukocytes, mainly belonging to the myelomonocytes lineage, contribute to the ''smouldering'' inflammation associated with tumour initiation and progression. The transcription factors NF-kB, HIF-1a and STAT-3 are key modulators of the inflammatory response that promotes cancer development through different mechanisms including induction of genomic instability, alteration in epigenetic events and subsequent inappropriate gene expression, enhanced proliferation and resistance to apoptosis of initiated cells, induction of tumour angiogenesis and tissue remodelling with consequent promotion of tumour cells invasion and metastasis. (Mø, macrophages; Mc, mastcells; MDSC, myeloid derived suppressor cells; Eo, eosinophil; PMN, polymorphonuclear cells). (Source: Porta et al 2009, Immunobiology).*

1.5. Eicosanoids

Eicosanoids (from the Greek *eikosi* for "twenty") are a family of oxygenated metabolites of the eicosapolyenoic fatty acids such as arachidonic acid (AA) formed via the enzymatic and non-enzymatic pathways. Although originally recognized for their capacities to elicit biological responses such as smooth muscle contraction, edema, and platelet aggregation, eicosanoids are now appreciated to influence processes ranging from inflammation and immune responses to chronic tissue remodeling and cancer [18]. 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 (LOx) pathways to prostaglandins, thromboxane and leukotrienes (Figure 1.4).

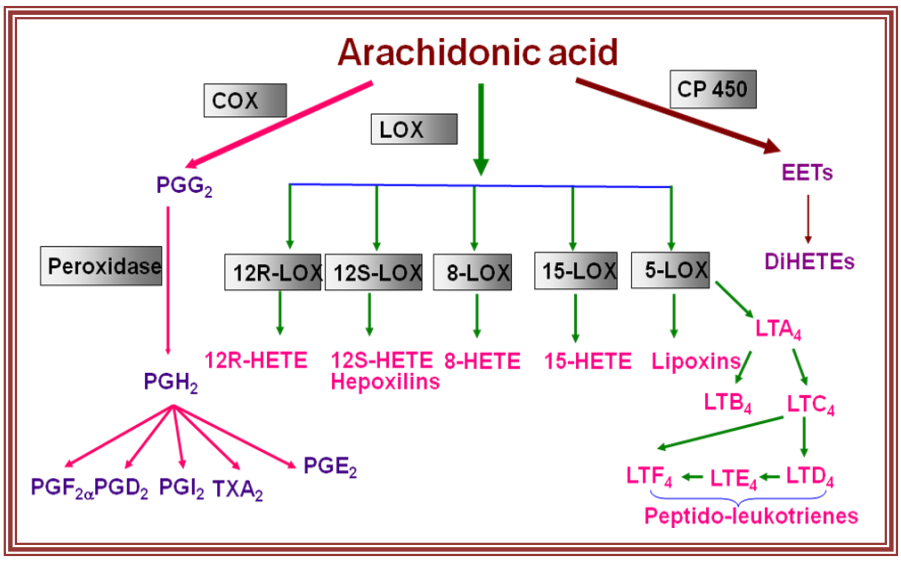


Figure 1.4. *Arachidonic metabolism.*

Eicosanoids have been the most actively studied of all the physiological components contributing to inflammation [19, 20]. In addition to COX and LOX pathways, AA is also oxygenated by the epoxygenase pathway involving cytochrome P450s to generate epoxyeicosatrienoic acids (EETs).

1.5.1. 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 [21]. It converts arachidonic acid to prostaglandin H_2 that gets further metabolized tissue specifically to various prostaglandins, prostacyclin and thromboxanes, together called as prostanoids (Figure 1.5). 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 [22, 23]. 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 [24]. 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 [25]. COX-2 is absent in 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 [26, 27].

COX isozymes share 60% primary sequence identity and X-ray crystal structures of the proteins are virtually super imposable [28]. 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 [28, 29].

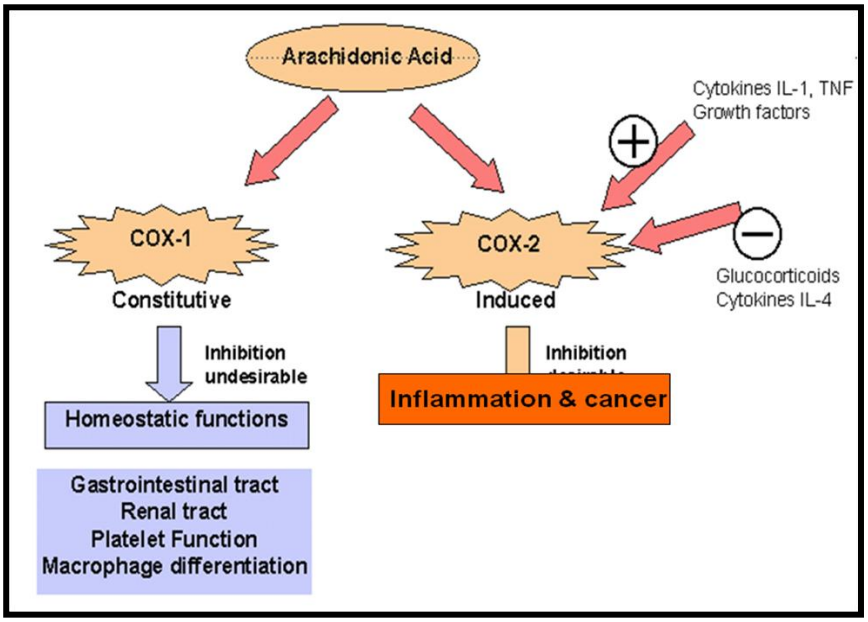


Figure 1.5. *COX pathway (Source: www.arthritis.co.za).*

Prostanoids exert their actions via G protein coupled membrane receptors on the surface of target cells [30]. There are ten types and subtypes of receptors for prostanoids that are conserved in mammals from mouse to human, the PGD receptors (DP1 and DP2), four subtypes of the PGE receptors (EP1, EP2, EP3, and EP4), the

PGF receptor (FP), the PGI receptor (IP), and the TXA receptors (TPα and TPβ) [31]. The prostanoids exert a variety of effects, sometime opposing effects. While PGE_2 is pro-inflammatory in nature, PGD_2 exerts anti-inflammatory effects. TXA_2 promotes platelet aggregation and is vasoconstrictor in nature but PGI_2 is anti-platelet aggregatory with vasodilator functions. PGE_2 relaxes smooth muscle but $PGF_{2\alpha}$ promotes smooth muscle contraction. Despite the varied effects of prostanoids, COX-2 coupled with PGE_2 has principally been associated with inflammation in a variety of cells and tissues.

1.5.2. 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 hydroperoxyeicosatetraenoic acids (HPETEs), leukotrienes (LTs) and lipoxins (Figure 1.6). Depending on the positional specificity of oxygenation, LOXs have been classified as arachidonate 5-, 8-, 12- and 15-lipoxygenases (5-LOX, 8-LOX, 12-LOX, and 15-LOX). The primary products are 5-, 8-, 12-, or 15 hydroperoxy eicosatetraenoic acids (5-, 8-, 12-, or 15-HPETE), which can be further reduced by peroxidases to the hydroxy forms (5-, 8-, 12- or 15-HETE) respectively. Among the lipoxygenases, the 5-LOX pathway has received much attention because of its involvement in pro-inflammatory leukotriene synthesis and its

potential as a therapeutic target. Leukotriene A_4 (LTA_4), the biologically most important LT intermediate, is derived from 5-(S)-HPETE. Epoxide opening coupled with tagging of GSH by glutathione S-transferase (LTC_4 synthase) leads to the formation of LTC_4, whereas enzymatic hydrolysis with a stereo specific rearrangement of the triene by LTA_4-hydrolase yields leukotriene B_4 (LTB_4), a potent chemotactic agent. γ-Glutamyl transpeptidases (GGTP) and dipeptidases convert LTC_4 to LTD_4 and then to LTE_4 and LTF_4. A new pathway of direct conversion of LTC_4 to LTF_4 by carboxypeptidases has been reported [32]. LTB_4, best known as a neutrophil chemo-attractant, is now recognized as a major player contributing to inflammatory and immune diseases. Many recent studies in animal models have shown the critical role for LTB_4 and its receptors in the development of inflammatory arthritis [20]. Cysteinyl leukotrienes, on the other hand, are functionally involved in the pathophysiology of asthma by causing bronchial constriction. Hence, cysteinyl leukotriene receptor antagonists (lukasts) are effective anti-asthmatic drugs [33]. Recent studies indicate their possible therapeutic role in alleviating the symptoms of allergic rhinitis also [34]. In addition, 5-LOX inhibitor (Zileuton) also has entered the market for the treatment of asthma. Accumulating evidence suggests that the 5-LOX pathway has profound influence on the development and progression of human cancers [35, 36]. The effects of LOXs on tumor cell proliferation, their role in cell cycle control and cell death induction, effects on angiogenesis, migration and the immune response have been recently reviewed [37]. The eicosanoids thus generated by a variety of LOXs in a tissue specific manner play a key role in inflammation and

cancer. The LOX inhibitors may therefore prove useful in the treatment of inflammation and cancer, either alone or in combination with conventional therapies.

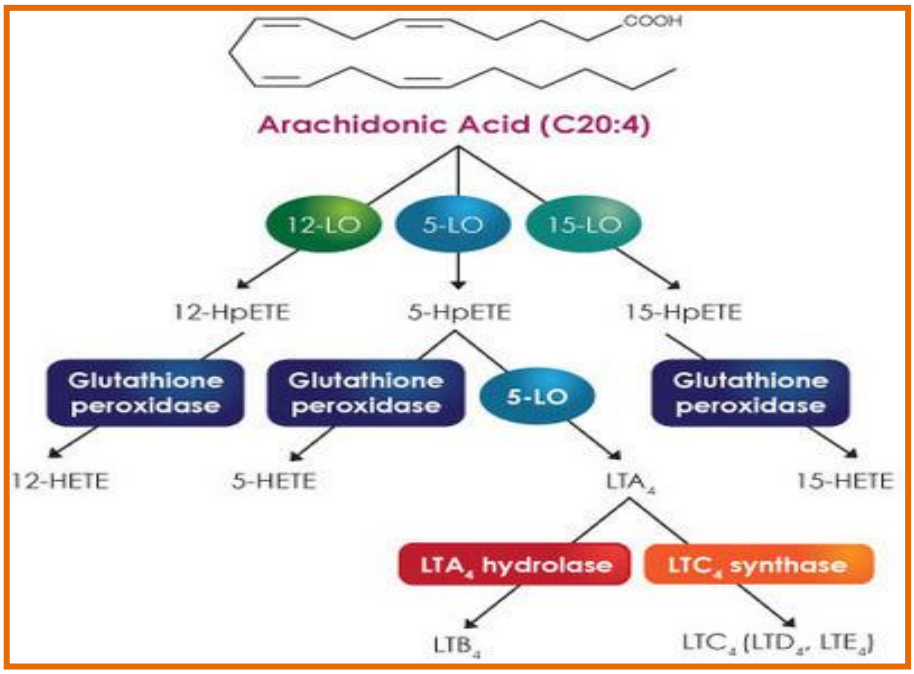


Figure 1.6. *Lipoxygenase pathway of Arachidonic acid metabolism (Source: www.caymanchem.com).*

1.5.3. Role of COX-2 in Inflammation

Eicosanoids of COX pathways have been the most actively studied of all the physiological components contributing to inflammation [20]. These eicosanoids, formed tissue specifically, mediate various inflammatory responses in a receptor coupled mechanism. It is well known that PGs are primarily involved in vasodilatation in the inflammatory process and synergize with other mediators, such as histamine, to cause vascular permeability and edema. PGE_2 plays a key role in the development of the three cardinal signs of inflammation: “swelling-redness”, “pain”

and "fever" [38]. Many studies have shown that PGE_2 and PGI_2 are potent vasodilators that are present at high concentrations at inflammation sites that increase tissue blood flow, contributing to the appearance of the characteristic erythema ("redness") [39]. PGE_2 greatly potentiates the ability of bradykinin to cause pain, and seems to be the local mediator of fever production from the hypothalamus. PGD_2 has been shown to exert an important function in limiting neutrophil infiltration in the early phases of experimentally induced colitis in rat [40]. A specific function for PGD_2 in the resolution of inflammation has been indicated by a number of studies that have shown increased expression of the hematopoietic PGD synthase during resolution of heart inflammation after endotoxin treatment in mice [41]. Prostacyclin (PGI_2) acts on platelets and blood vessels to inhibit platelet aggregation and to cause vasodilatation and is thought to be important for vascular homeostasis. In inflammation, PGI_2 is an important mediator of the edema and pain that accompany acute inflammation. Thromboxane A_2 (TXA2) from platelets, aggregates platelets and constricts blood vessels [42]. Together, eicosanoids contribute to the inflammatory reaction at all steps [43].

1.5.4. Role of COX-2 in cancer

In vitro studies have revealed that cells over-expressing COX-2 undergo phenotypic changes such as exhibition of an increased adhesion to extra cellular matrix proteins and resistance to apoptosis, which could enhance their tumorogenic potential [44]. COX-2 over-expression in tumors is considered as a predictor of more advanced stage of disease and worse prognosis in a number of studies investigating

solid malignancies [45]. COX-2 has been found to be related to key events of tumor promotion such as cellular hyper-proliferation, inhibition of programmed cell death, and tumor angiogenesis [46]. Efforts to identify the molecular mechanisms by which COX-2 and PGE_2 promote tumor growth and metastasis demonstrate that the PGE_2 signaling cascade includes the epidermal growth factor receptor (EGFR) [47], nuclear receptor (PPARs) [48], and Ras-mitogen-activated protein kinase (MAPK pathways) [49]. The downstream targets of PGE_2 are angiogenic factors, anti- apoptotic factors, chemokines and their receptors, and immuno-suppressive mediators [47] (Figure 1.7). Further evidence comes from a large number of *in vivo* studies showing that selective COX-2-inhibiting agents are highly potent in preventing tumor development [50].

Treatment of cancer cells with COX inhibitors induces apoptosis by cytochrome C release from mitochondria, which in turn activates caspase-9 and then caspase-3. Induction of apoptosis seems to be directly related to COX inhibition since addition of PGE_2 can prevent NS398-induced cytochrome C release, caspase activation and PARP cleavage [51]. Over-expression of COX-2 increases Bcl-2 expression, which might be responsible for preventing cytochrome C release from mitochondria. A recent study showed that death receptors are also involved in COX-regulated cancer cell survival [52]. Forced COX-2 expression significantly attenuated TRAIL-induced apoptosis and was associated with transcriptional repression of death receptor-5 and up-regulation of Bcl-2 [53]. Over-expression of COX-2 also reduced caspase-8, caspase-3, and caspase-9 activation relative to corresponding parental cells. Several lines of evidences suggest that NF-κB and PI3 kinase are involved in NSAID-

induced cancer cell apoptosis. NSAIDs inhibit NF-κB and PI3 kinase activity, while restoration of NF-κB or PI3 kinase activity blocks NSAID-induced apoptosis [54].

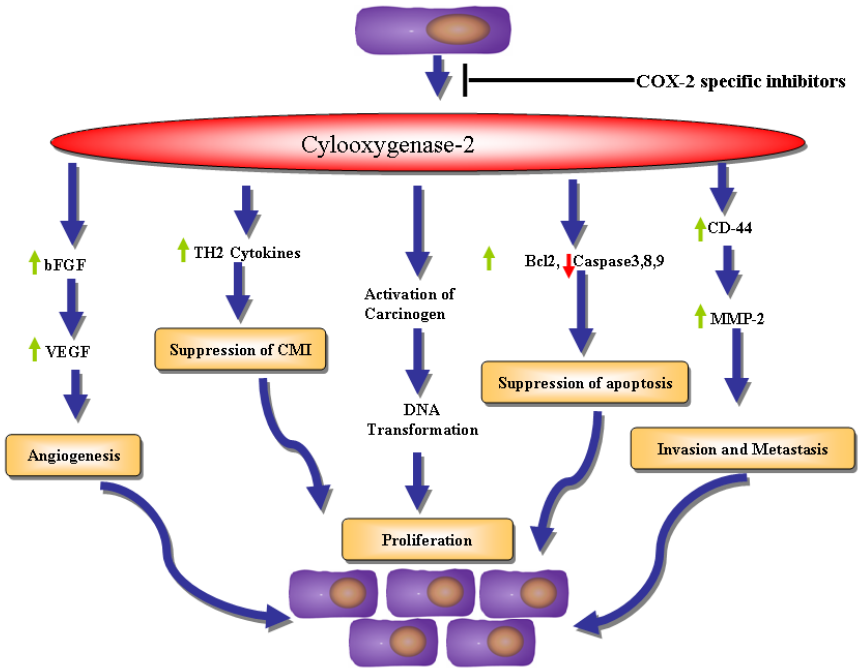


Figure 1.7. *Role of COX-2 in the initiation of cancers, the downregulation of apoptosis and the promotion of angiogenesis, invasion and metastasis.(Source: Smita et al 2009, Experimental Dermatology).*

1.5.5. Role of COX-2 in drug resistance

Development of drug resistance in cancer patients is often responsible for failure of chemotherapy and poor prognosis. The expression of P-glycoprotein 170 (multidrug resistance-MDR-1/Pgpl70) in patients undergoing chemotherapy is often associated with drug resistance because of its ability to export chemotherapeutic agents [55]. Resistance to doxorubicin in K562 cells has been attributed to over-expression of MDR-1 [55]. The role of MDR-1 in protecting cells from apoptosis has

been studied in several cellular systems [56]. Over-expression of COX-2 was reported to increase the production and function of MDR-1, an efflux pump for chemotherapeutic drugs [55]. A close association between MDR-1 and COX-2 has been reported in human hepatocellular carcinoma [57] and rat renal mesangial cells [55]. A strong correlation between expression of COX-2 and MDR-1 was also found in tumor specimens derived from breast cancer patients. In drug resistant cell lines that over-express MDR-1 there was significant expression of COX-2 [58]. It has been suggested that COX-2 inhibitors sensitize cells to chemotherapeutic drugs by a functional blockade of P-glycoprotein [59]. These studies strongly suggest that COX-2 modulates P-glycoprotein expression and is involved in the development of the MDR phenotype [60] (Figure 1.8).

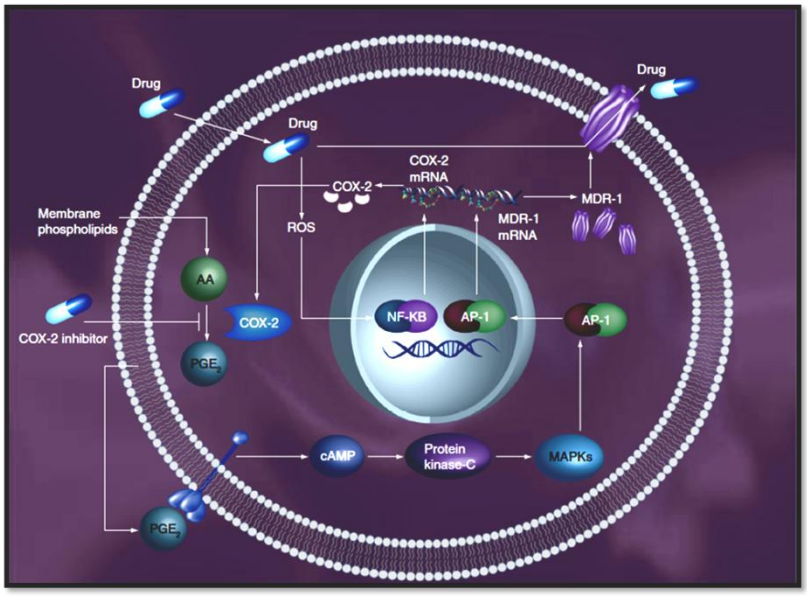


Figure 1.8. *Regulation of MDR-1 by COX-2 and the mechanism of overcoming drug resistance by COX-2 inhibitors. Source: Arunasree et al 2008, Leukaemia Research.*

1.6. Targetting Eicosanoids

1.6.1. Non steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are the most commonly used drugs since they are effective in management of pain, fever, redness, edema arising as a consequence of inflammatory mediator release [61]. They are used in inflammatory diseases like rheumatoid arthritis, juvenile arthritis, ankylosing spondilitis, psoriatic arthritis, systemic lupus, post traumatic pain, headache, upper respiratory tract infections, etc. [62, 63]. NSAIDs exhibit their action by affecting COX activity, either by covalently modifying the enzyme or by competing with the substrate for the active site [64].

The major therapeutic limitations in the use of NSAIDs are their side effects. The major side effects are the gastric complications such as mucosal ulceration, reflux esophagitis, esophageal thinning and peptic ulcers [65]. In addition, the inhibition of COX in thrombocytes leads to decreased production of thromboxane A_2. This phenomenon prolongs bleeding time and leads to inhibition of platelet aggregation [66]. With the discovery of COX-2, it became clear that many of the side effects of NSAIDs are due to inhibition of constitutive and cytoprotective COX-1. Since COX-2 expression was induced by a variety of inflammatory stimuli, growth factors, and bacterial endotoxin, it was theorized that NSAID anti-inflammatory activity reflected COX-2 inhibition, whereas NSAID adverse effects were due to COX-1 inhibition. Several pharmaceutical companies made extensive commitment to develop COX-2 selective inhibitors (“COXIBs”) as effective anti-inflammatory and analgesic drugs with potentially reduced GI toxicity as compared to non-selective NSAIDs [67].

1.6.2. Selective COX-2 inhibitors (COXIBs)

The new generation of anti-inflammatory drugs proved to selectively inhibit COX-2 activity *in vitro* and to be as efficacious as the standard NSAIDs in a number of *in vivo* models of inflammation [68, 69]. The two selective COX-2 inhibitors first approved and marketed for the treatment of osteoarthritis and rheumatoid arthritis were celecoxib [70] and rofecoxib [71]. The second-generation of selective COX-2 inhibitors including valdecoxib (Bextra) and etoricoxib (Arcoxia), with a higher COX-1 to COX-2 selectivity ratio than celecoxib and rofecoxib, are now also approved for treatment of osteoarthritis, rheumatoid arthritis [72]. Though COXIBs became block buster drugs, the voluntary withdrawal of rofecoxib by Merck due to cardiac side effects on long-term usage, raised several questions on their safety and wider application to other inflammatory disorders [73]. These cardiovascular side effects of selective COX-2 inhibitors were attributed to the altered ratio between PGI_2, a smooth muscle relaxant and platelet inhibitor, and TXA_2, a platelet activator and inducer of smooth muscle contraction [74]. The enhanced level of TXA_2 compared to PGI_2 following long term intake of rofecoxib, is a strong rationale for observed cardiovascular adverse effects [75].

1.6.3. COXIBs in the treatment of cancers

Several studies have shown that selective COX-2 inhibitors inhibit the proliferation of malignant cells *in vitro*, and retard tumor growth and reduce metastasis *in vivo* [44]. These inhibitors suppress angiogenesis and tumor growth by

inhibiting expression of angiogenic factors and vascular endothelial cell migration [76]. COXIBs stimulate apoptosis and suppress cell proliferation at low concentrations in cultured human cancers of the colon, stomach, esophagus, tongue, brain, lung, and pancreas [77]. There are different mechanisms by which COXIBs inhibit proliferation in different cells. This anti-tumor response can be mediated through Bcl-2 down regulation and subsequently apoptosis [78], or an increase in pro-apoptotic Bax protein and a decrease in Bcl-XL protein [79], Bak and Bax up regulation, mitochondrial membrane potential loss and activation of caspase-3 [80] or a significant decrease in Akt activation [81]. Addition of PGE_2 blocked the apoptotic actions of celecoxib [82] suggesting the pro-survival effects of COX-2. Celecoxib was shown to induce apoptosis in K562 cells [83] by decreasing the membrane potential, Bcl-2 down regulation, release of cytochrome C and PARP cleavage. Tilmacoxib (JTF-522), a COX-2 inhibitor, caused down regulation of pi integrin (a cell adhesion molecule), with consequent impairment of the ability of cancer cells to adhere to and migrate on the extra cellular matrix, which were steps crucial to the formation of cancer metastases [84]. In addition to COX-dependent mechanism, COX-2 inhibitors and a number of non-selective NSAIDs were known to mediate their effects by COX-independent mechanisms also [85]. In this connection recent studies indicate that NS398, inhibits invasiveness of lung cancer cells by up regulating the expression of several metastasis suppressor genes, including secreted protein acidic and rich in cysteine (SPARC), thrombospondin 1 and 3 (TSP-1&3) and tissue inhibition of matrix metalloproteinase-2 (TIMP-2) [86]. A number of studies, thus, indicate the

effectiveness of NSAIDs and COXIBs in the prevention and treatment of cancers, by both COX dependent and COX-independent mechanisms.

1.6.4. COXIBs in overcoming drug resistance

Development of drug resistance in cancer patients is often responsible for failure of chemotherapy and poor prognosis. The expression of P-glycoprotein 170 (multidrug resistance-MDR-1/Pgpl70) in patients undergoing chemotherapy is often associated with drug resistance because of its ability to export chemotherapeutic agents [55]. The role of MDR-1 in protecting cells from apoptosis has been studied in several cellular systems [56]. Over-expression of COX-2 was reported to increase the production and function of P-glycoprotein MDR-1, the product of ABC-B1, an efflux pump for chemotherapeutic drugs [55]. A close association between MDR-1 and COX-2 has been reported in human hepatocellular carcinoma [57], rat renal mesangial cells [55], in tumor specimens derived from breast cancer patients and in drug resistant cell lines that over-express MDR-1 [58]. Exogenous PGE_2 and forced COX-2 expression were shown to up regulate Bcl-2 expression levels [87, 88]. Bcl-2 inhibits the release of mitochondrial pro-apoptotic proteins to block the intrinsic apoptotic pathway, thereby conferring multiple drug resistance [89]. Furthermore, it has been demonstrated that COX-2 inhibitors sensitize cells to chemotherapeutic drugs by a functional blockade of P-glycoprotein [60]. These studies strongly suggest that COX-2 modulates P-glycoprotein expression and is involved in the development of the MDR phenotype [61]. Our group has recently shown the role of COX-2 in

mediating imatinib resistance in K562 cells and demonstrated how celecoxib sensitizes the resistant cells to imatinib [90]. Furthermore, we have demonstrated that celecoxib inhibits MDR-1 expression through COX-2 dependant mechanism in HepG2 cell line [91]. Also, we have shown that C-phycocyanin, a natural COXIB, induces apoptosis in doxorubicin resistant hepatocellular carcinoma cell line HepG2 [92]. Although the clinical significance of these findings is not clear, it is reasonable to anticipate that COXIBs may enhance the effect of chemotherapeutic drugs by reducing the expression of efflux pumps and thus overcoming drug resistance. The regulation of MDR-1 by COX-2 and the mechanism of overcoming drug resistance by COX-2 inhibitors are shown in Figure 1.8.

1.6.6. COX-2/5-LOX Dual Inhibitors (CLOXIBs)

Enhanced production of leukotrienes as a result of diversion of substrate AA towards 5-LOX pathway was also attributed to some of the side effects associated with the usage of COXIBs [93]. As a result pharmaceutical companies started their attention towards the development of COX-2/5-LOX dual inhibitors (CLOXIBs). Licofelone, a COX-2/5-LOX dual inhibitor, is one such promising candidate drug in Phase III clinical trials, which decreases the production of pro-inflammatory prostaglandins and leukotrienes and has the potential to combine good analgesic and anti-inflammatory effects with excellent GI tolerability [94]. Recent studies, however, indicate that licofelone suppresses PGE_2 formation by inhibiting mPGES-1 than COX-2 [95]. A natural compound, chebulagic acid, with COX-2/5-LOX dual

inhibition was recently isolated from *Terminalia chebula* with potent anti-inflammatory and anti-cancer effects in pre-clinical studies [96, 97]. Further well designed clinical trials of this candidate, however, are needed before a final evaluation is possible.

Scope of the study:

The biological processes underlying inflammatory diseases and cancer are fundamentally linked and chronic inflammation due to infection or to conditions such as chronic inflammatory bowel disease is associated with up to 25 percent of all cancers. Poly unsaturated fatty acids (PUFAs) liberated from the membrane phospholipids are the substrates for the enzymes cyclooxygenases (COXs) and lipoxygenases (LOXs) and the resulting metabolites, viz., the eicosonoids and the prostaglandins, are one of the important mediators in various inflammatory disorders including cancer. Therefore, drugs targeting the inhibition of these enzymes have become a major attraction in controlling several inflammatory diseases as well as cancer. Several anti-inflammatory drugs targeting COX are being used as potent anti-cancer agents. However, these are associated with various side effects. Consequently, there have been constant efforts towards identification of natural compounds as chemo-preventive agents against cancer and other inflammatory disorders. Besides being readily available in abundance, natural compounds have minimum side effects. In this context, our group has earlier identified a natural COX-2/5-LOX dual inhibitor, chebulagic acid isolated from the fruits of *Terminalia chebula*, thus offering the

potential advantage of minimizing COX-2 dependent side effects. Chebulagic acid is a benzopyran tannin present as one of the major constituents in the fruits of *Terminalia chebula*. The fruit powder of *Terminalia chebula* is used in India to treat several diseases ranging from digestive, coronary disorders to allergic and infectious diseases like cough and skin disorders. The water or ethanolic extracts of the powder are used for treating diseases associated with oxidative stress as well as the cancers. Previous studies from our lab have demonstrated that chebulagic acid is a potent anti-oxidant and shows a broad spectrum anti-proliferative effects against various cancer cell lines including HCT-15, COLO-205, MDA-MB-231, DU-145, K562. Furthermore, potent anti-inflammatory effects of this compound have been reported *in vitro* in mouse macrophage cells. However, the underlying molecular mechanisms involved in these effects are not yet completely resolved. Therefore the current study is designed to understand the molecular mechanisms mediating the anti-inflammatory and anti-proliferative properties of chebulagic acid in LPS induced acute lung injury model and human hepatocellular carcinoma model respectively.

Aims and objectives of this study

The primary aim of this work is to examine the efficacy of chebulagic acid as remedy for inflammation and cancer. The specific objectives include:

1. To study the *in vivo* anti-inflammatory activity of chebulagic acid on LPS induced acute lung injury in mice.

2. To study the i*n vitro* anti-proliferative activity of chebulagic acid and its effects on Wnt/β-catenin signalling on human hepatocellular carcinoma.

3. To elucidate the role of chebulagic acid in the regulation of multidrug resistance (MDR-1) in human hepatocellular carcinoma.

Chapter 2

Protective effects of chebulagic acid on acute lung injury induced by LPS in mice

Chapter 2

2.1. Intoduction

Acute lung injury (ALI) is characterised by acute lung inflammation involving the local recruitment and activation of polymorphonuclear leukocytes (PMNLs) [98] and release of pro inflammatory mediators such as tumour necrosis factor-α (TNF-α) and Interleukin-1β (IL-1β) [99], proteases and reactive oxygen and nitrogen species [100]. Serious ALI can lead to pulmonary edema, acute respiratory distress syndrome (ARDS) [101] and finally respiratory failure [102]. ALI is often an early symptom of multiple organ failure associated with sepsis, which is associated with elevated blood levels of endotoxin or lipopolysaccharide (LPS) derived from gram-negative bacteria. LPS is therefore considered as a major component in the induction of ALI. Detection of LPS by the host receptors is the first step in a multistep sequence which in turn is followed by the coordinated expression of inflammatory cytokines, chemokines, and adhesion molecules that direct the migration of neutrophils across the endothelial and epithelial barriers that separate the bloodstream from the pulmonary air spaces. Early response cytokines, such as TNF- α and IL-1β, can amplify this response by stimulating the NF-κB-dependent induction of pro-inflammatory mediators in cells [103]. In spite of considerable progress in elucidating these mediators, their role in the development of ALI is not well understood.

To study the pathophysiological mechanisms involved in ALI, several different animal models have been developed. In this context, previous studies have shown that a rodent model reproduces several features of ALI in humans [104],

including neutrophil influx and severe lung damage. In particular, intra-tracheal administration of LPS is a widely accepted clinically relevant model of severe lung injury. It causes acute lung inflammation and ALI, characterised by activation of alveolar machrophages, infiltration of neutrophils, lung edema and production of inflammatory mediators that resembles the inflammation and ALI seen in human ARDS [105].

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. The fruit powder of *Terminalia chebula* is used in India to treat several diseases ranging from digestive, coronary disorders to allergic and infectious diseases like cough and skin disorders [106, 107]. The water or ethanolic extracts of the powder are used for treating diseases associated with oxidative stress as well as the cancerous diseases [108, 109]. Its aqueous extract was reported to have free radical scavenging and radio-protector properties [110]. Water soluble fraction of *Terminalia chebula* fruit was reported to have strong anti-anaphylactic actions, anti-inflammatory and analgesic properties [111, 107]. Chebulagic acid (CA) is one of the main bioactive constituents of *T. Chebula* fruit powder. It has been previously shown to inhibit α-glucosidase activity [112], ROS generation from PMA stimulated leukocytes [113] and CTL mediated cytotoxicity [114]. Additionally, CA has been shown to suppress arthritis in mice [115] as well as LPS induced nitric oxide (NO) generation in RAW 264.7 mouse

macrophage cells [116]. Earlier studies from our lab have shown that CA is a potent antioxidant, COX-2/5-LOX dual inhibitor and induces anti-proliferative effects in various human cancer cell lines [96]. Further, the anti-inflammatory effects of CA in LPS induced mouse macrophage cells - RAW 264.7 were demonstrated by a mechanism involving suppression of NFκB and MAP kinase activation [97]. Based on above studies, it was suggested that CA might be applied in the treatment of inflammatory diseases, such as ALI. Despite extensive investigations into various new strategies for the treatment of ALI/ARDS, the mortality associated with ALI remains unchanged [117]. Until now, no effective treatment for these diseases has been developed [118], and CA may offer new therapeutic potential.

In the present study, the effects of intraperitoneally administered CA on intratracheally LPS-induced acute pulmonary inflammation and oxidative stress in mice were investigated. Furthermore, the effects were compared to the well known anti-inflammatory drug celecoxib.

2.2. Materials and Methods

2.2.1. Animals: Male BALB/c mice, weighing approximately 20 to 25 g, were purchased from the National Institute of Nutrition (NIN), Hyderabad, India. The mice were housed in micro isolator cages and received food and water *ad libitum*. The laboratory temperature was 24±1 °C, and relative humidity was 40– 80%. Mice were housed for 2–3 days to adapt them to the environment before experimentation. All

animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals published by the US National Institute of Health.

2.2.2. Chemicals: *Escherichia coli* lipopolysaccharide (LPS), Phosphate-buffered saline (PBS), and protease inhibitor cocktail were purchased from Sigma Chemical Company (St. Louis, USA). Celecoxib was a generous gift from Dr. Reddy Laboratories Ltd., Hyderabad, India. COX-2, iNOS, Nrf-2, TNF-α, NF-κB, p- ERK, ERK, p-p38, p38, p-JNK, JNK, β-actin and PARP antibodies were purchased from Santa Cruz Biotechnology, Inc., (CA, USA).

2.2.3. Isolation of CA by RP-HPLC: CA was isolated from the ethanolic extract of *Terminalia chebula* fruits by RP-HPLC [96].The purity of the CA obtained was greater than 98.0%.

2.2.4. Establishment of the Animal Model and Treatment Regimen: The mice were divided randomly into phosphate buffered saline (PBS) vehicle group (control group), LPS challenge group (LPS group), chebulagic acid intervention group (CA group), and celecoxib intervention group (CE group), with 8 mice per group. After fasting for 8 h, mice were anesthetized with 50 mg/kg of ketamine and 10 mg/kg of xylazine in 100 µl of PBS, and 25 µg of LPS was administered intratracheally in 50 µl PBS (1 mg/kg), to induce lung injury. Control mice were given 50 µl PBS intratracheally. The mice from CA and CE group were administrated CA at 50 mg/kg or CE at 25 mg/kg intraperitoneally, 2 h prior to LPS instillation, mice from control and LPS groups were treated with the same volume of PBS.

2.2.5. Bronchoalveolar Lavage and Cell Counting: After 12 h of LPS or PBS instillation, bronchoalveolar lavage fluid (BALF) was collected three times through a tracheal cannula with 0.5 mL of autoclaved PBS, instilled up to a total volume of 1.3 mL. BALF was immediately centrifuged at 3,000 rpm for 10 min (4°C). The supernatants of BALF were stored at −70°C until required for determination of protein content. The cell pellets were re suspended in PBS, and the total cell number was counted using a standard haemocytometer. Differences in cell numbers were examined by counting at least 200 cells on a smear prepared by Diff-Quik staining.

2.2.6. Protein Assay: Protein concentration in the supernatants of BALF was quantified using the Bradford assay. Protein was expressed as μg/mL of BALF.

2.2.7. Isolation of RNA and RT PCR Analysis: Total RNA from the lung tissues was extracted using TRIzol reagent and RT PCR was done as described elsewhere [19]. A 2 μl aliquot of the 20 μl total cDNA was used for standard PCR reaction of 28 cycles using following primers: COX-2 forward 5'-AGC CAG GCA GCA AAT CCT T-3', COX-2 reverse 5'-ATT CCC CAC GGT TTT GAC A-3'; iNOS forward 5′-GGC AAA CCC AAG GTC TAC GTT-3′, iNOS reverse 5′-TCG CTC AAG TCC AGC TTG GT-3′; TNF-α forward 5′-TGT AGC CCA CGT CGT AGC AAA-3′, TNF-α reverse 5′-GCT GGC ACC ACT AGT TGG TTG T-3′; IL-1β forward 5′-GCT TCA GGC AGG CAG TAT-3', IL-1β reverse 5′-ACA AAC CGC TTT TCC ATC T-3′; IL-6 forward 5′- GAA ATC GTG GAA ATG AG-3′, IL-6 reverse 5′-TAG GTT TGC CGA GTA GA-3'; GAPDH forward 5′-AGG TCA TCC CAG AGC TGA ACG-3′ and

GAPDH reverse 5′-CAC CCT GTT GCT GTA GCC GTA T-3′. The PCR products were visualized on 1% agarose gels with ethidium bromide, under UV light. GAPDH primers served as control.

2.2.8. Histopathological Analysis: The lung tissues were harvested, fixed in 10% formalin for 24 h, dehydrated, embedded in paraffin, and then stained with hematoxylin–eosin (H&E). Evaluations of lung edema and inflammatory cell infiltration were performed under light microscopy.

2.2.9. GSH/GSSG ratio: The GSH (reduced glutathione) and GSSG (oxidized glutathione) contents of the tissues were estimated by the method described elsewhere [119, 120]. For GSH estimation, 0.5 mL tissue extract (10 000 *g* supernatant) was diluted by adding 4.5 mL of the phosphate–EDTA buffer (pH 8.0). The final assay mixture (2 mL) contained 100 µl of the diluted tissue supernatant, 1.8 mL of phosphate–EDTA buffer and 100 ml of the 0.1% orthophthalaldehyde (OPT) solution and incubated for 15 min. For GSSG estimation, a 0.5 mL portion of the tissue extract was incubated at room temperature with 200 µl of 0.04 M *N*-ethylmaleimide (NEM) for 30 min to interact with the GSH present in the tissue. To this mixture, 4.3 mL of 0.1 N NaOH was added. A 100 µl portion of this mixture was taken and added to 1.8 ml of 0.1 N NaOH and 100 µl of the 0.1% OPT solution and incubated at room temperature for 15 min. The solutions were transferred to a quartz cuvette for GSH and GSSG estimation. Fluorescence was measured at 420 nm with activation at 350 nm. The tissue GSH and GSSG contents were obtained from a standard curve

prepared using GSH and GSSG standards. The results were expressed as a ratio (GSH/GSSG) of reduced (GSH) and oxidized (GSSG) forms of glutathione.

2.2.10. Preparation of lung tissue extracts and immunoblot analysis: Lung tissues from the control and treated groups were minced and whole cell extracts were prepared as described elsewhere [119]. Nuclear extracts were prepared as described elsewhere [97] with minor modifications. 70 μg of protein from each treatment was resolved on 8–12% SDS–PAGE along with protein molecular weight standards, and then transferred onto nitrocellulose membranes. The membranes were blocked, incubated with the primary antibodies [119] and then treated with respective alkaline phosphatase conjugated secondary antibodies. Signals were detected by using BCIP/NBT substrate. Equal protein loading was detected by reprobing the membrane with β-actin and PARP antibodies.

2.2.11. Statistical Analysis: All values were expressed as the mean ± SEM. P-values were determined using the unpaired Student's *t*-test. P value of less than 0.05 was considered as statistically significant.

2.3. Results

2.3.1. CA decreased inflammatory cells count and total protein concentration in the BALF of LPS-induced mice: Alveolar macrophages are one of the main sources of pro-inflammatory and anti-inflammatory cytokines, and their activation plays a critical role in the development of ALI [121]. Total cell counts, differential cell counts and protein concentration in the BALF were evaluated at 12 h after LPS

administration. In this study, mice exposed to LPS exhibited massive recruitment of inflammatory cells, including neutrophils and macrophages to the airways. In contrast, pre-administration with CA at a single dose of 50 mg/kg, considerably inhibited the LPS-induced increases in the numbers of total cells, neutrophils and macrophages in the BALF [Figure 2.1 (1-3)]. Comparable results were obtained with pre treatment with 25 mg/kg dose of CE. Furthermore, after LPS administration, the protein concentration in the BALF of mice was notably increased. However, pre treatment with CA and CE at a single dose of 50 mg/kg and 25 mg/kg, respectively, effectively reduced the protein concentration in the BALF [Figure 2.1 (4)]. Thus, CA could attenuate some of the deleterious effects of LPS by reducing the accumulation of inflammatory cells and by decreasing total protein concentration in the BALF of ALI mice.

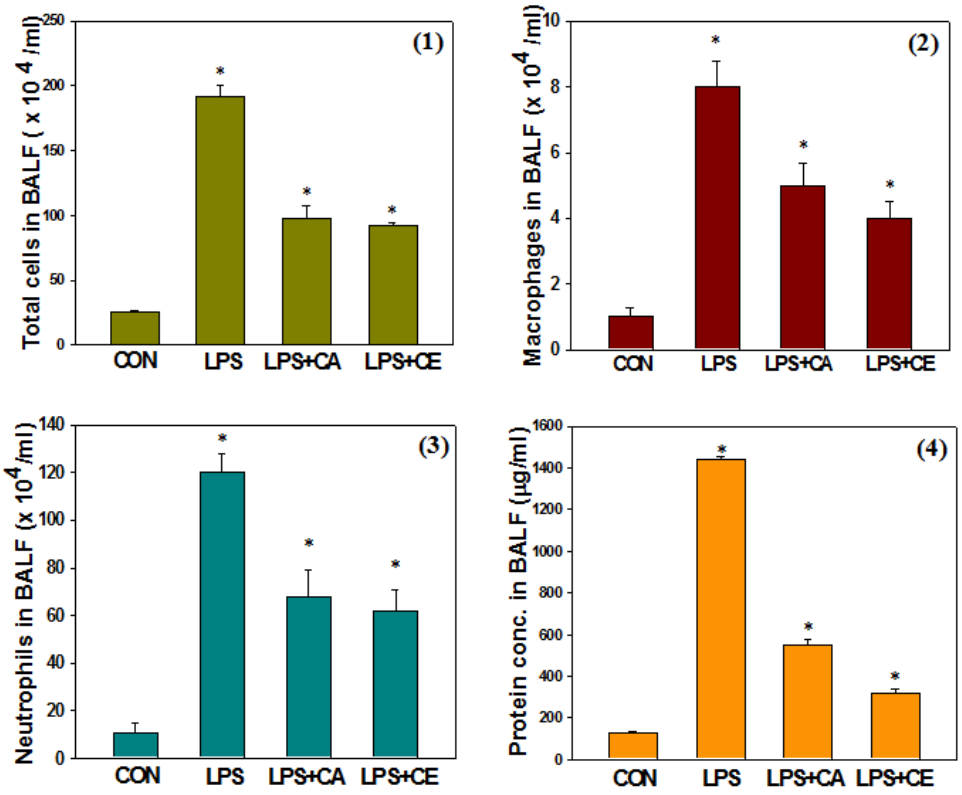


Figure.2.1. *Effects of CA on LPS-induced inflammatory cell accumulation and total protein concentration in the BALF. BALF was prepared from mice12 h after LPS instillation, and the total cell numbers (1) were counted using a standard hemocytometer. Each cell population including macrophages (2) and neutrophils (3) was examined by counting at least 200 cells on a smear prepared by diff-quick staining and total protein concentration was determined by BSA method (4). Bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with untreated control cells.*

2.3.2. Effect of CA on LPS induced pulmonary histopathological changes: The effect of CA on the lung histopathology of mice was determined 12 h after LPS administration by histochemical staining with H & E. As shown in Figure 2.2, the lungs of mice exposed to LPS showed pro-inflammatory alterations, characterized by alveolar wall thickening, massive infiltration of inflammatory cells into the lung interstitium and alveolar space as well as signs of tissue injury.

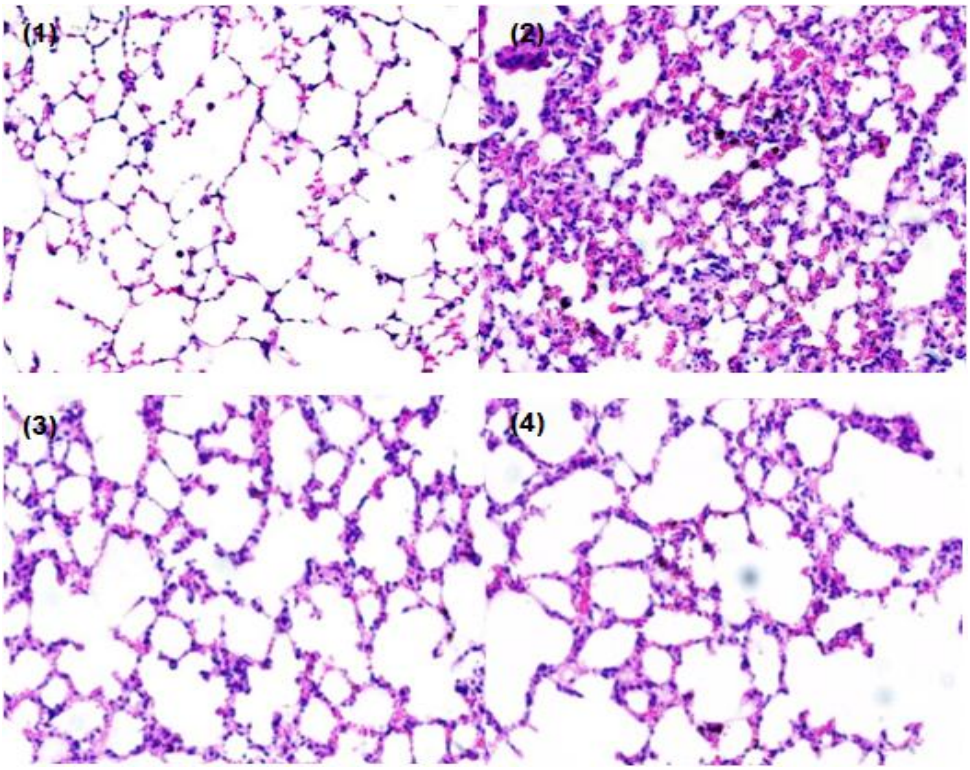


Figure 2.2. *Histological assessment of the effect of CA on LPS-induced ALI. 12 h after LPS instillation, the lung tissues were inflated and fixed with 10% buffered formalin, samples were embedded in paraffin, and then stained with H&E (20 X). (1) Control group,(2) LPS group, (3) LPS + CA group and (4) LPS + CE group.*

In contrast, pre-treatment with a single dose of CA (50 mg/kg), 2 h before LPS challenge, markedly abated LPS induced inflammatory cell infiltration and prevented alveolar wall thickening which was comparable with effects of 25 mg/kg dose of CE. These results corroborated our findings in BALF which confirmed that the protective effect of CA on ALI induced by LPS is related to attenuation of inflammatory cell sequestration and migration into the lung tissue.

2.3.3. CA reduced oxidative stress induced by LPS: Oxidative stress plays an important role in the development of LPS-induced ALI. The GSH/GSSG ratio is used to evaluate oxidative stress status in biological systems and alterations of this ratio under conditions of intracellular stress leads to oxidation and damages of lipids, proteins and DNA by ROS [122]. To evaluate the effects of CA on LPS induced oxidative stress, we estimated the GSH/GSSG ratio in the lung tissues, 12 h after LPS administration. As shown in Figure. 2.3, LPS challenge considerably ameliorated (by 2 fold) the GSH/GSSG ratio compared with the control group indicating that LPS altered the antioxidant capacity and thiol redox status in the lungs of mice. However, this ratio was restored effectively with CA and CE pre-treatments suggesting that CA being a natural antioxidant modulated LPS induced oxidative stress by elevation of tissue glutathione redox status.

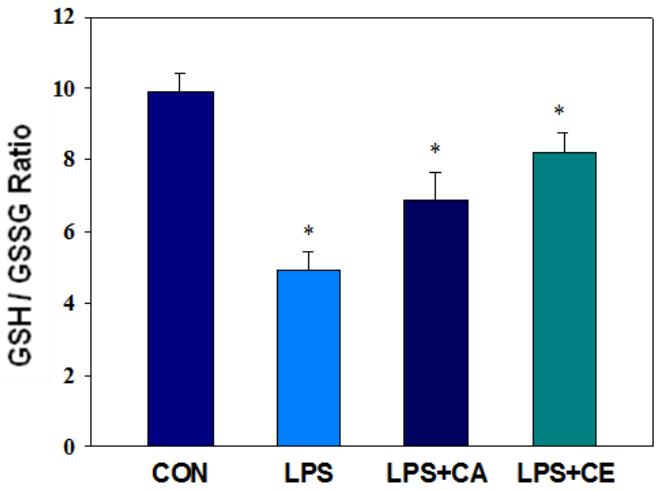


Figure.2.3. *Effect of CA on LPS induced oxidative stress. Ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) in different treatment groups. Bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with untreated control cells.*

2.3.4. CA attenuated LPS-induced iNOS and COX-2 protein and mRNA expressions in the lungs of ALI mice: The enzymatic products of iNOS and COX-2 play critical role in inflammatory diseases such as sepsis and arthritis [123]. Inhibition of COX-2 and iNOS expressions, therefore, is important for alleviating inflammation as well as for the prevention of cancer [124]. Also, their inhibition may constitute an effective new therapeutic strategy for the treatment of inflammation and the prevention of inflammatory reactions and diseases. In the present study, the levels of COX-2 and iNOS proteins in the lung tissues of ALI mice were estimated by western blots and the results showed that both iNOS and COX-2 protein levels were markedly up-regulated in response to LPS treatment. However, pre-treatment with both CA (50mg/kg) and CE (25 mg/kg) suppressed the expression of COX-2 protein and drastically inhibited the expression of iNOS protein [Figure 2.4 (3, 4)].

Moreover, RT-PCR analysis showed that the expression of iNOS and COX-2 mRNAs correlated well with their protein levels [Figure 2.4 (1, 2)]. This in turn, indicates that the protective effects of CA in alleviating inflammation induced by LPS are mediated through inhibition of expression of these pro-inflammatory enzymes in the lungs of ALI mice. The above concept is supported by our previous studies where CA effectively decreased LPS induced inflammation in mouse macrophage cells (RAW 264.7) by decreasing the expression of COX-2 and iNOS genes [97].

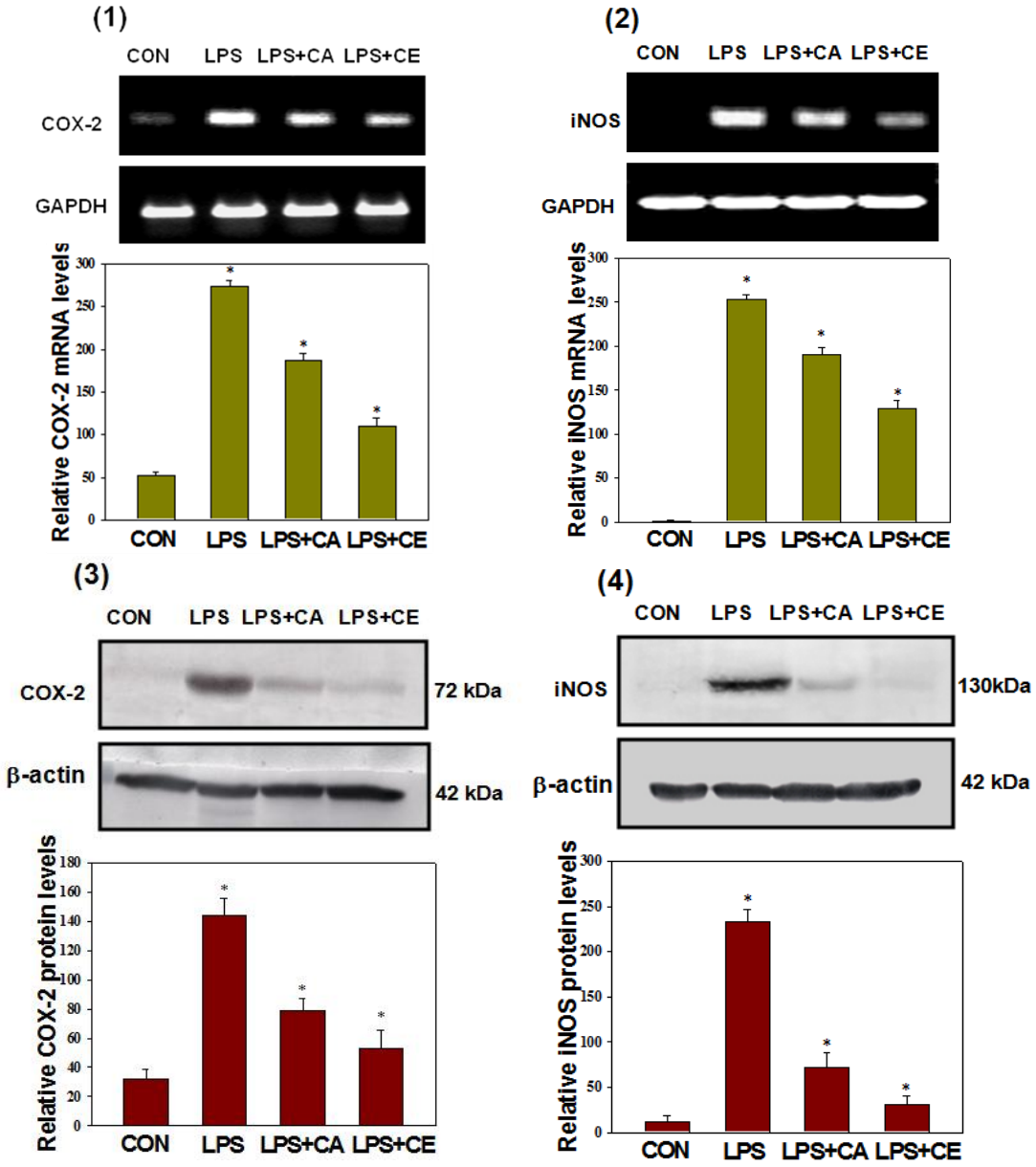


Figure 2.4. *Effect of CA on the expression of pro-inflammatory enzymes in the lung tissues of LPS induced mice. RT-PCR analysis showing the effect of CA on (1) COX-2 and (2) iNOS mRNAs in LPS-induced mice and western blot analysis showing the effects of CA treatment on expression of COX-2 (3) and iNOS (4) proteins in LPS-induced mice. The relative band intensities were measured by quantitative scanning densitometry. Bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with untreated control cells.*

2.3.5. CA ameliorated the expression of pro inflammatory cytokines induced by LPS in the lungs of ALI mice: Previous studies have indicated that the cytotoxic effect of LPS is mediated through the induction of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6, most notably TNF-α that is known to have a key role in inflammatory processes [125]. So, we further investigated the effects of CA on LPS-induced mRNA expression of IL-1β, IL-6 and TNF-α by RT-PCR and TNF-α protein expression by Western blot. As shown in the Figure 2.5 (1, 2, 3), CA effectively reduced LPS induced IL-1β, IL-6 and TNF-α mRNA expression as well as protein expression of TNF-α in the lungs of ALI mice [Figure 2.5 (4)]. Treatment with CE showed similar effects. These results are in good agreement with our and other previous reports showing that inhibition of inflammatory cytokines to be involved in ameliorating LPS induced inflammatory responses [97, 126]. This strengthens our argument that CA mediated anti-inflammatory effects in ALI are attributed to the reduced expression of pro-inflammatory cytokines.

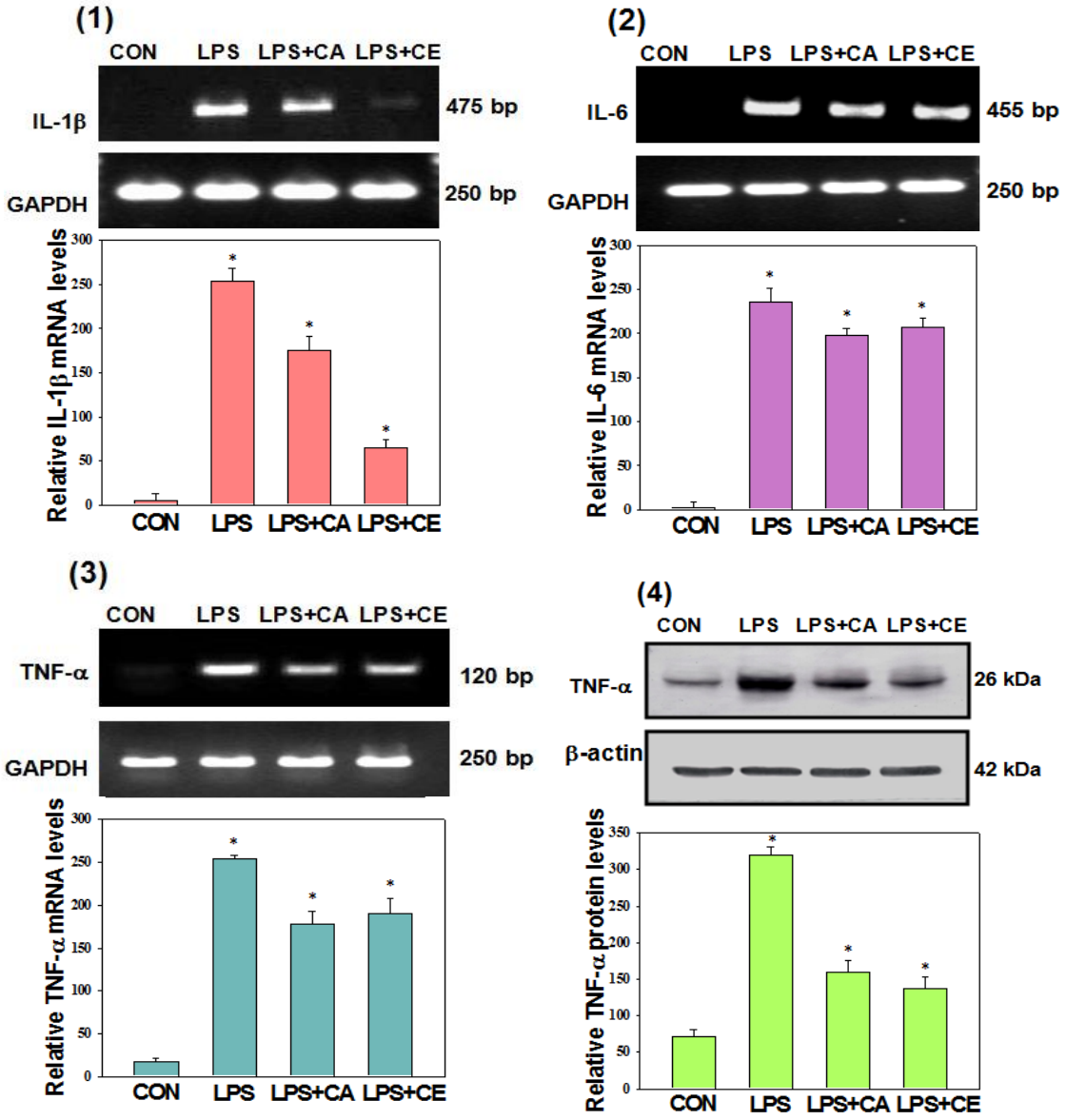


Figure 2.5. *Effect of CA on LPS induced expression of pro-inflammatory cytokines in the lungs of ALI mice. RT-PCR analysis showing the effect of CA on IL-1β (1), IL-6 (2), TNF-α (3) mRNAs and western blot analysis showing the effect of CA treatment on expression of TNF-α protein (4) in LPS-induced mice. The relative band intensities were measured by quantitative scanning densitometry. Bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with untreated control cells.*

2.3.6. CA treatment inhibited LPS-induced nuclear translocation of NF-κB in the lungs of ALI mice: NF-κB is known to play a critical role in the regulation of cell survival genes and to coordinate the expressions of pro-inflammatory enzymes and cytokines, such as iNOS, COX-2, TNF-α , and IL-6 [127]. Since NF-κB modulates the expression of these pro inflammatory mediators, our findings suggest that CA mediated inhibition of their expression in the LPS induced lungs may be due to blocking of the NF-κB signaling pathway. So, we next examined the effect of CA on NF-κB activity. NF-κB activity is associated and tightly controlled by an inhibitory subunit, IκB, which is present in the cytoplasm in an inactive form. However, once IκB is phosphorylated, it targets its proteolysis and allows NF-κB translocation to the nucleus, where it activates the transcription of NF-κB-responsible genes. We examined NF-κB translocation to the nucleus by checking the levels of p50 and p65 subunits in the nuclear fractions by Western blots. Negligible levels of p50 or p65 proteins were detected in control, but 12 h after treatment with LPS, a robust increase in their nuclear translocations was observed. However, pre-treatment with CA at a dose of 50 mg/kg caused a considerable decrease in the p50 and p65 levels in nuclear fractions of LPS induced lung tissue [Figure 2.6]. Poly ADP ribose polymerase (PARP) was used as an internal control in these experiments.

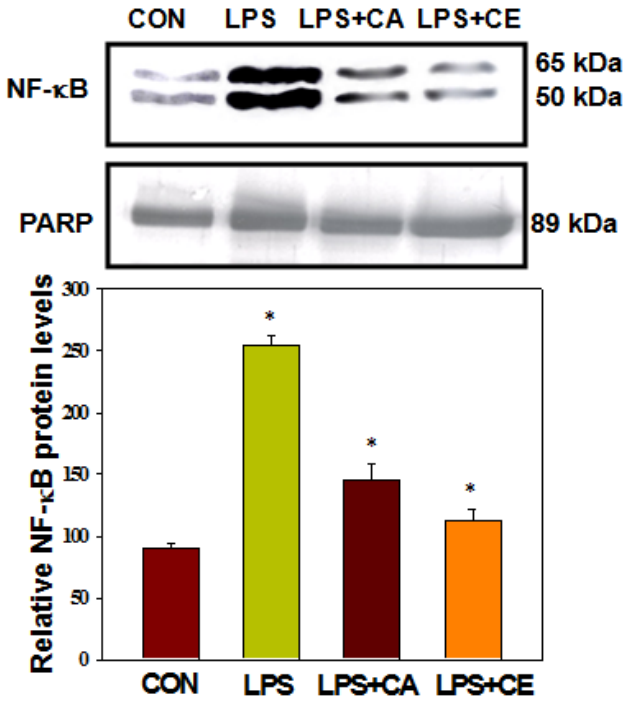


Figure 2.6. *Effect of CA on nuclear translocation of NF-κB protein in LPS induced mice. Western blot analysis showing the effect of CA on expression of NF-κB p65 and p50 proteins in the nuclear extracts of LPS-induced mice tissues. The relative band intensities were measured by quantitative scanning densitometry, Bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with untreated control cells.*

2.3.7. CA treatment attenuated LPS-induced MAP kinase phosphorylation in the lungs of ALI mice: MAP kinases play a critical role in the regulation of cell growth and differentiation and they control cellular responses to cytokines and stress [128]. In addition, they play a critical role in the modulation of NF-κB activity [129]. To investigate the molecular mechanism of NF-κB inhibition by CA, we investigated the effects of CA on the phosphorylation of MAP kinases in the lungs of LPS-stimulated mice. These studies revealed increase in the phosphorylation of p38, ERK1/2 and

JNK in the mice administered with LPS alone. However, mice pre-treated with CA at a single dose of 50 mg/kg or CE at a dose of 25 mg/kg for 2 h before LPS treatment, showed a marked reduction in the phosphorylated levels of these proteins in LPS stimulated mice [Figure 2.7(1, 2 and 3)]. Furthermore, no changes in the expression of non-phosphorylated ERK, JNK and p38 kinase were observed in any of the groups. The above study explains that suppression of phosphorylation of MAP kinases is involved in the inhibitory effects of CA on LPS-stimulated NF-κB activation in ALI mice.

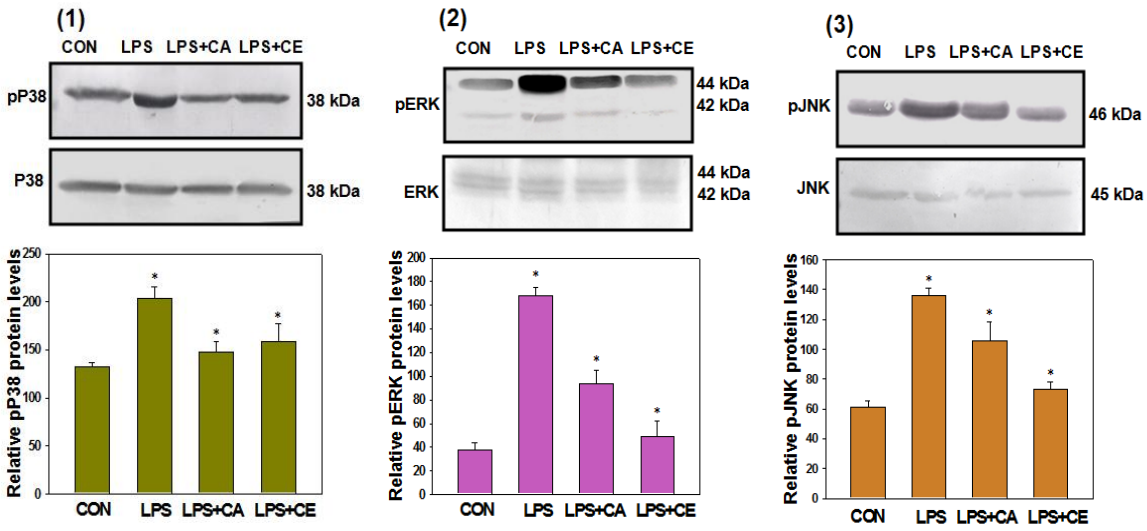


Figure 2.7. *Effect of CA on the phosphorylation of MAP kinases in LPS induced mice. Western blot analysis showing the effect of CA treatment on p-P38 (1), p-ERK (2) and p-JNK (3) proteins in LPS induced ALI mice. The relative band intensities were measured by quantitative scanning densitometry; Bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with untreated control cells.*

2.3.8. CA treatment induced Nrf-2 activation in the lungs of LPS induced mice:

Activation of Nrf2-antioxidant signaling is known to attenuate NF-κB mediated inflammatory response. Also, LPS induced NF-κB activation could be attenuated by diverse Nrf-2 activators [130]. Therefore, we checked whether CA could activate Nrf-2 in the lungs of LPS induced mice. Activation of Nrf-2 was determined by estimating the nuclear translocation of Nrf-2 protein in the different treatment groups. As illustrated in Figure 2.8, CA treatment caused a marked increase in the nuclear levels of Nrf-2 protein compared to that of LPS administered group. A comparable increase in Nrf-2 nuclear levels was also observed in CE treated group. These results illustrate that CA attenuates NF-κB mediated pro-inflammatory effects through activation of Nrf2-antioxidant signalling.

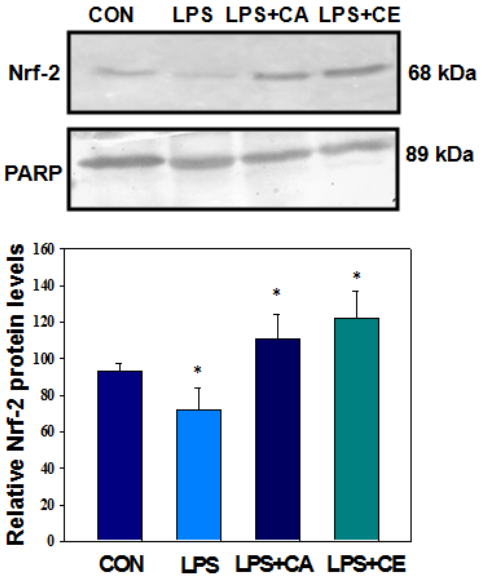


Figure 2.8. *Effect of CA on nuclear translocation of Nrf-2 protein in the lungs of LPS induced mice. Western blot analysis showing the effect of CA on expression of Nrf-2 protein in the nuclear extracts of LPS-induced mice. The relative band intensities*

*were measured by quantitative scanning densitometry, Bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with untreated control cells.*

2.4. Discussion

The lack of effective pharmacological interventions remains a major impediment in the treatment of inflammatory diseases of the lung, especially acute respiratory distress syndrome (ARDS). ALI and its severest form (ARDS) are associated with the development of multiple organ dysfunction syndrome (MODS), which plays a pivotal role in the death of patients with multiple transfusions, shock, sepsis and ischemia reperfusion [131, 132]. Lung tissue contains the largest epithelial/endothelial surface area in the body and is sensitive to oxidant injury by inhalation of high concentrations of oxygen or circulating oxidants and xenobiotics [133]. LPS is known to induce the infiltration of inflammatory cells, overproduction of inflammatory mediators, tissue edema, and injury [134]. LPS-mediated activation of macrophages leads to the production of various cytokines such as TNF-α, IL-1β, and IL-6 [135]. The production of these cytokines may result in ALI/ARDS [136]. LPS-induced ALI exhibits some relevant characteristics of the disease and therefore, could serve as a preliminary model for ARDS. Intra-tracheal administration of LPS is a widely accepted clinically relevant model of severe lung injury. It causes acute lung inflammation and ALI, characterised by activation of alveolar machrophages, infiltration of neutrophils, lung edema and production of inflammatory mediators that resembles the inflammation and ALI seen in human ARDS [105].

Fruits of *Terminalia chebula* have been used in various Ayurvedic preparations for the treatment of various diseases. Chebulagic acid (CA) is one of the main bioactive constituents of *T. Chebula* fruit powder. It has been reported to possess several biological activities [112-116]. In our earlier studies we have shown that CA is a potent antioxidant, COX-2/5-LOX dual inhibitor and induces anti-proliferative effects in various human cancer cell lines [96]. Further, the anti-inflammatory effects of CA in LPS induced mouse macrophage cells - RAW 264.7 by a mechanism involving suppression of NFκB and MAP kinase activation has also been demonstrated [97]. In view of the above data it is hypothesised that CA could form a potential candidate for the prevention/treatment of ALI. In the present study, for the first time, we explored the effect of CA on LPS-induced ALI in mice. The data presented here demonstrates that CA exerts potent anti-inflammatory effects in mice during ALI induced by LPS.

In ALI, the deregulation of apoptotic pathways is thought to play an important role in the pathogenesis [137], and the predominant inflammatory cells involved are neutrophils and macrophages [138, 139]. Recent studies showed that neutrophilic granulocytes are important inflammatory cells implicated in the exacerbation of the inflammatory response [140], and apoptosis of neutrophilic granulocytes in lung tissue is delayed during ALI. Delayed apoptosis may lead to prolonged release of neutrophilic granulocyte products and direct tissue injury, facilitating the development of ALI [141]. Alveolar macrophages are one of the main sources of pro-inflammatory and anti-inflammatory cytokines, and their activation is critical in the development of

ALI [142], since metabolically activated macrophages may cause respiratory failure.

In the present study, mice exposed to LPS exhibited massive recruitment of inflammatory cells, including neutrophils and macrophages to the airways. In contrast, pre-administration of CA significantly inhibited the LPS-induced increases in the numbers of total cells, neutrophils and macrophages in the BALF. Consistent with histological analysis of the lung, there was substantial infiltration of inflammatory cells in mice with LPS-induced ALI. CA treatment reduced the infiltration of inflammatory cells significantly in the lung tissue and this in turn corroborated our findings in the BALF. As another index of ALI by LPS, we measured the total protein content in the BALF, which indicates epithelial permeability and pulmonary edema [143]. Mice exposed to LPS showed a high protein content in the BALF. LPS-induced increase in total protein in the BALF was reduced with prior treatment of CA. These findings confirm the protective effect of CA on LPS induced ALI and appears to be related to attenuation of inflammatory cell sequestration and migration into the lung tissue as well as decrease in epithelial permeability. These effects of CA were comparable to that of CE, a known anti-inflammatory drug in the market.

Oxidative damage is a major cause of lung injury during ARDS. It has been proposed that antioxidant defences may fail in ALI/ARDS and make the lung tissue susceptible to oxidant damage. One of the major pathological consequences of ALI is ARDS, which is mainly caused by oxidative damage due to inflammatory responses [144]. Glutathione is known to be a major low molecular weight scavenger of free

radicals in the cytoplasm. The reduced glutathione/oxidized glutathione (GSH/GSSG) ratio is used to evaluate oxidative stress in biological systems, and alterations of this ratio have been demonstrated in several pathological disorders [122]. N-acetylcysteine (NAC) is a synthetic antioxidant that has been shown to protect lung tissue against oxidative damage in different experimental animal models [145]. In the present study, decreased ratio of GSH/GSSG in the lung tissue after LPS administration indicates that LPS induces oxidative stress in the lungs. However, pre-treatment with CA significantly restored the GSH/GSSG ratio, thus suggesting that CA modulated the anti-oxidant defence system in the lungs and reduced the LPS-induced oxidative stress. During the inflammatory response, neutrophils undergo a respiratory burst and produce reactive oxygen species (ROS). Overproduction of ROS is highly toxic to host tissues, and their interactions with various cellular macromolecules can result in severe pathophysiological consequences [146]. Being an anti-oxidant CA could have blocked the production of ROS induced by LPS and thus sparing tissue glutathione levels.

The pro-inflammatory cytokines, prostaglandins, and NO produced by activated macrophages play critical role in inflammatory diseases such as sepsis and arthritis [123, 147]. COX-2 and iNOS proteins have been reported to be closely associated with cutaneous inflammation, cell proliferation and skin tumor promotion and these can be rapidly induced by pro-inflammatory mediators [129]. Reports suggest that the inhibition of COX-2 and iNOS expression is important for alleviating inflammation as well as for the prevention of cancer [124]. Therefore, the inhibition

of COX-2 and iNOS expressions may constitute an effective new therapeutic strategy for the treatment of inflammation and the prevention of inflammatory reactions and diseases. In the present study, it was found that CA effectively inhibited the expression of iNOS and COX-2 proteins that were markedly up-regulated in response to LPS treatment. Moreover, RT-PCR analysis showed that the expression of iNOS and COX-2 mRNAs correlated well with their expression at protein levels.

It has been reported that cytokines, such as TNF-α, IL-6 and IL-1β are known to have a key role in inflammatory processes [125]. These pro-inflammatory cytokines were reported to be secreted by activated alveolar macrophages in patients with ALI/ARDS [148]. This mimics the clinical situation, where neutrophilia and elevated cytokine levels in the lungs are typical clinical findings among patients with ALI. In the present study, CA significantly inhibited the LPS induced expression of IL-1β, IL-6 at mRNA levels and suppressed TNF-α expression at both protein and mRNA levels. This blocking of cytokine expression, induced by LPS, by CA could be responsible for reduced generation of ROS and maintenance of redox homeostasis. Therefore, CA may protect against LPS-induced ALI by decreasing the expression of these pro-inflammatory cytokines as well as pro-inflammatory enzymes. This is consistent with earlier reports where CA decreased the expression of pro-inflammatory cytokines and enzymes *in vitro* [97].

NF-κ B is known to play a critical role in the regulation of cell survival genes, and to coordinate the expression of pro-inflammatory enzymes and cytokines, such as

iNOS, COX-2, TNF-α and IL-6 [127] involved in the pathogenesis of ALI. Since NF-κB modulates the expression of these pro-inflammatory mediators, it is quite possible that CA mediated inhibition of their expression in the LPS induced lungs may be due to blocking of the NF-κB signaling pathway. Hence, we next examined the effect of CA on NF-κB activity. NF-κB is associated and tightly controlled by an inhibitory subunit, IκB, which is present in the cytoplasm in an inactive form. However, phosphorylation of IκB targets its proteolysis and allows NF-κB translocation to the nucleus, where it activates the transcription of NF-κB-responsible genes. We found that CA significantly inhibited the nuclear translocation of NF-κB induced by LPS. Similar inhibition of nuclear translocation of p50 and p65 was observed with CE treatment (25 mg/kg). Several natural compounds have been reported to down regulate the activation of NF-κB and thus alleviate LPS induced inflammation in ALI mice [149]. Consistent with these reports, CA treatment decreased NFκ-B activity by suppressing the nuclear translocation of p65 and p50 subunits, thereby resulting in the inhibition of LPS induced expression of iNOS, COX-2 and pro-inflammatory cytokines in the lungs of ALI mice.

MAP kinases play a critical role in the regulation of cell growth and differentiation, and they control cellular responses to cytokines and stress [128]. In addition, they play a critical role in the modulation of NF-κB activity [129]. To investigate the molecular mechanism of NF-κB inhibition by CA, in the present study, we investigated the effects of CA on the phosphorylation of MAP kinases in the lungs

of LPS-stimulated ALI mice. Treatment with CA substantially inhibited JNK, ERK and p38 phosphorylation induced by LPS. Our results are quite in agreement with previous studies showing inhibition of MAPK to be involved in attenuation of LPS induced lung injury [150]. These results suggest that suppression of phosphorylation of MAP kinases might be involved in the inhibitory effect of CA on LPS-stimulated NF-κB activation in ALI mice.

Activation of Nrf2-antioxidant signaling is known to attenuate NF-κB mediated inflammatory response. Also, LPS induced NF-κB activation could be attenuated by diverse Nrf2 activators [130]. So we next checked whether CA had any effect on the activation of Nrf-2. Our studies revealed that CA treatment considerably increases the nuclear levels of Nrf-2 protein in the lungs of LPS stimulated mice. Previous studies have demonstrated the importance of Nrf-2 in protection against ALI in mice [151]. Similarly, curcumin, a natural antioxidant, was demonstrated to attenuate liver injury in rats through induction of Nrf-2 signalling [152].These results suggest the involvement of Nrf2-antioxidant signalling in CA mediated attenuation of NF-κB activity and thus the pro-inflammatory effects of LPS.

In conclusion, our studies suggest that CA, a natural antioxidant exerts potent anti-inflammatory effects in a mouse model of ALI. These effects were mediated by inhibiting LPS induced gene expression of various pro-inflammatory mediators including TNF-α, IL-6, IL-1β, iNOS and COX-2. This inhibition was found to be mediated by activation of Nrf-2 and suppression of NF-κB activation as well as

through inhibition of phosphorylation of MAP kinases. Therefore, we conclude that CA could form a potential candidate for prevention of inflammatory diseases like ALI. Further pre-clinical and clinical studies, however, are required to evaluate the safety and efficacy of CA.

Chapter 3

Anti-proliferative properties of chebulagic acid in human hepatocellular carcinoma cells: Studies on Wnt/β-catenin signalling

3.1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant human tumours worldwide [153-156]. The major risk factors for HCC include chronic Hepatitis B and C viral infections, chronic alcohol consumption, environmental carcinogens and inherited genetic disorders [157-160]. The genetic alterations or chromosomal aberrations that may occur during the process of inflammation, regeneration and cirrhosis lead to the development of HCC [161, 162]. The molecular mechanisms contributing to hepatocarcinogenesis are largely unknown. However, activation of Wnt/β-catenin signalling pathway has been reported to be commonly associated with the development of HCC, colorectal cancer and various other types of cancer [163, 164].

Wnt/β-catenin signalling pathway plays a crucial role in the regulation of cells fate and proliferation during normal development including embroyogenesis, organogenesis and epithelial-mesenchymal interaction [165-169]. However, deregulation of this signalling pathway may lead to many cancers including HCC, colorectal cancer, melanoma and head & neck carcinoma [165-169]. Studies have reported that β-catenin is an essential component of Wnt signalling, which plays important role in regulation of cell proliferation, differentiation and movement [163]. β- catenin is a multifunctional protein and is shown to play an important role in intercellular adhesion, cell growth, survival and differentiation [164]. In normal epithelial cells, β-catenin is localized in the plasma membrane, where it forms a

complex with E-cadherin and α-catenin at the sites of adherent junctions. Excess β-catenin is phosphorylated at its four N-terminal serine-threonine residues by a multiprotein complex that contains the adenomatous polyposis coli (APC) tumor suppressor, the scaffold protein axin, the glycogen synthase kinase-3β (GSK-3β) and casein kinase 1 that targets it for degradation by ubiquitin-proteosome pathway [163]. Binding of Wnt ligand to its cell surface receptors of the Frizzled (Fz) and the low density lipoprotein receptor (LRP) families inhibits the action of this degradation complex by inhibiting GSK-3β activity. This in turn leads to the stabilization and translocation of β-catenin to the nucleus where it interacts with transcription factors TCF/LEF and activates the transcription of target genes governing cancer-relevant processes, including Myc and cyclin D1 [170-174]. One of the frequent observations in many types of cancer is the deregulation of Wnt/β-catenin signalling pathway and thus it is suggested as an early event in cancer [170-173, 175].

There is growing evidence on the involvement of Wnt/β-catenin pathway in various aspects of liver biology [176]. Studies in transgenic mice model have reported that high expression of Wnt1 could be a major mechanism of nuclear accumulation of β-catenin, which then contributes to c-Myc/E2F1 driven hepatocarcinogenesis [177]. It has been implicated that aberrant deregulation of Wnt signalling is a major mechanism of liver tumourigenesis [178, 179]. β-catenin was reported to be mutated and increasingly expressed in nucleus in different human HCC [180, 181]. In some reports overexpression and mutations of β-catenin have been related to early-stage

HCC [182, 183] while in others it has been related to cancer progression [184]. Given these observations, effective antagonists of Wnt/β-catenin signalling might be attractive candidates for developing effective therapies for HCC.

Recently, there have been concerted efforts to develop natural products as cancer preventive and/or therapeutic agents. Fruits of *Terminalia chebula* have been used in various Ayurvedic preparations for the treatment of various diseases. Their fruit powder is used as one of the main constituents of Triphala, a well known Ayurvedic medicine used for the treatment of allergies and other common health disorders. Chebulagic acid (CA) is a benzopyran tannin present as one of the main bioactive constituents of *T. Chebula* fruit powder. CA has been found to be hepatoprotective [113], immunosuppressive [114] and a potent alpha-glycosidase inhibitor [112]. A broad spectrum anti-proliferative effects of CA have been recently shown in various cancer cell lines including HCT-15, COLO-205, MDA-MB-231, DU-145, K562 [95]. Detailed studies on the molecular mechanisms of its action on COLO-205 cells revealed that the molecule induces apoptotic effects [96]. Furthermore, potent anti-inflammatory effects of CA have been reported *in vitro* [97]. In the present study, we determined the effects of CA on proliferation of liver cancer cells and on modulation of key components of Wnt/β-catenin signaling. The specific objectives of the present study are:

- To evaluate the anti-proliferative properties of CA on human hepatocellular carcinoma cell lines.

- To study the effects of CA on modulation of key components of Wnt/β-catenin signaling.

3.2. Materials and methods:

3.2.1. Plant material: Dried fruits of TC (locally known as *Karakkaya*) collected from Adilabad forests, were procured from the local vendors of traditional herbs and nuts of Adilabad, Andhra Pradesh, India.

3.2.2. Isolation of CA by RP-HPLC: The lyophilized ethanolic extract of dried TC fruits was re dissolved at 20 mg/mL in absolute alcohol and subjected to reverse phase HPLC (RP-HPLC) by employing C18 column (Shimpack column with dimensions 250×4.6mm and particle size 5 μm) with 1 mL/min flow rate and the eluants were monitored at 280 nm. The mobile phase consisted of a complex gradient of solvent A [water: acetic acid (1000:1)], and solvent B [acetonitrile:acetic acid (1000:1)]. All the fractions were collected and CA was identified by comparing LC-MS, IR and NMR spectra [96, 97]. The purity of the CA so obtained was greater than 98.0% by HPLC.

3.2.3. Cell Culture: The human hepatoma cell lines HepG2 and Hep3B were obtained from National Centre for Cell Science (NCCS), Pune, India and were maintained in DMEM supplemented with 10% fetal bovine serum (v/v) (DF10; Invitrogen, Carlsbad, CA) in an atmosphere containing 5% CO_2 at 37 °C.

3.2.4. Reagents: CA was dissolved in Dulbecco's Modified Eagle Medium (DMEM) at 10 mM concentration before use. Final concentrations of CA ranged from 1-500

μM. Phosphate buffered saline (PBS), Fetal Bovine Serum (FBS), Penicillin and Streptomycin were purchased from Gibco BRL (CA, USA). DMEM was purchased from HiMedia laboratories (India), MTT [3-(4, 5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was purchased from Sigma Chemical Company (St. Louis, USA). Antibodies were purchased from Santa Cruz Biotechnology (CA, USA) and Epitomics (GeneX India Bioscience Pvt. Ltd., India).

3.2.5. Cell Proliferation Assay: Cell proliferation was assessed by MTT assay [185]. Briefly, cells ($5x10^3$ cells per well) were seeded in 96 well plates and incubated in the presence or absence of various concentrations of CA for 48 h in a final volume of 100 μl. 20 μl of MTT (5 mg/mL in PBS) was then added to each well and incubated for an additional 4 h at 37 ^oC. The purple-blue formazan crystals were dissolved in 100 μl of DMSO and the optical density was quantified at 570 nm on Microtiter plate reader (μ Quant Bio-tek Instruments, Inc.). Each experiment was conducted in triplicates.

3.2.6. Thymidine Incorporation Assay: Equal number of cells ($5x10^3$) were seeded in 96 well plates and incubated with CA as described above. [^3H] Thymidine (BARC, Mumbai, India) was added to each well for 24 h at 0.5 μCi/well. The culture medium was then removed, washed twice with PBS, and the proteins were precipitated with 5% trichloroacetic acid. The supernatant was removed and after washing with ethanol, the cells were solubilized with 0.2 N NaOH and placed in scintillation vials. The level of radioactivity was determined using a liquid scintillation counter (Beckman Coulter, Fullerton, CA).

3.2.7. Morphological Study: Equal number of cells per well were seeded in 60 mm dishes and incubated with appropriate concentrations of CA for 48 h. After 48 h, morphological differences in the control and treated cells were assessed under inverted phase contrast microscope.

3.2.8. Apoptosis Assay: Cells were seeded on coverslips in 35 mm dishes until 50-60 % confluent and incubated with CA as described above. Apoptotic cells were detected by the terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) staining using the fluorescence method with the APO-BrdU TUNEL assay kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

3.2.9. Quantification of apoptosis by flow Cytometry: Flow Cytometry analysis using propidium iodide was performed to quantitate apoptosis. After treatment with CA for 48 h, cells were stained as mentioned elsewhere [186]. Cells that were less intensely stained than G1 cells (sub G0/G1 cells) in flow cytometric histograms were considered apoptotic. The red fluorescence of individual cells was measured with a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). A minimum of 10,000 events were counted per sample.

3.2.10. RNA Isolation and Real Time PCR Analysis: Total cellular RNA was obtained by homogenizing cells using Trizol reagent (Invitrogen, Carlsbad, CA). The sequences of primer pairs were as follows: Human β-catenin, 5'-TTGTTCAGCTTCTGGGTTCA-3' (sense) and 5'-ATACCACCCACTTGGCAGAC-3' (antisense); β-actin, 5'-AGGCATCCTCACCCTGAAGTA-3' (sense) and 5'-

CACACGCAGCTCATTGTAGA-3' (antisense). RT-PCR was performed according to Komoroski *et al* [180] on a ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) and results normalized to β-actin. The relative gene expression of β-catenin was calculated using the comparative $2^{-\Delta\Delta CT}$ method [188].

32.2.11. Western Blot Analysis: To prepare the whole cell extract, cells were pelleted after 24 h of CA treatment, washed with PBS and suspended in a lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% NP 40, 0.5% deoxy cholic 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^{o} C, the mixtures were centrifuged (10,000 g) for 10 min, and the supernatants were collected as the whole-cell extracts [189]. Nuclear extracts were prepared as described elsewhere [97] with minor modifications. The protein content was determined according to the Bradford method. An equal amount of total cell lysate (100 μg) was resolved on 8-12% SDS-PAGE gels along with protein molecular weight standards, and then transferred onto Nitrocellulose membranes The membranes were blocked with 5% w/v non-fat dry milk and then incubated with the following primary antibodies: active-β-catenin (ser37/thr41-hypophosphorylated-form) (1:200); GSK-3β-P (1:1000); cyclin-D1 (1:1000); TCF-4 (1:200); LEF-1 (1:200), c-Myc (1:500), COX-2 (1:1000), β-tubulin (1:1000) and Lamin B1 (1:200) in 10 mL of antibody-diluted buffer (Tris buffered saline and 0.05% Tween-20 with 1% milk) with gentle shaking at 4 ^{o}C for 8-12 h and then incubated with respective ALP conjugated secondary antibodies. Signals were detected using BCIP/NBT western blot detection reagents.

3.2.12. Immunofluorescence Microscopy: Equal number of cells were seeded on coverslips in 35mm dishes, allowed to grow till 60% confluency and then treated with CA for 24 h. After 24 h, cells were washed with 1X PBS, fixed with 4% paraformaldehyde for 15-20 min and permeabilized with 1:3 ratio of acetone and methanol at -20 ^{o}C for 20 min. Staining was done as described in [190]. The coverslips were then dried and mounted on glass slides with a drop of prolong gold antifade reagent with DAPI (Invitrogen, India) and viewed on Nikon Fluorescence microscope and images were taken with Olympus CCD camera.

3.2.13. Statistical Analysis: All values were expressed as the mean ± SEM. P-values were determined using the unpaired Student's *t*-test. P value of less than 0.05 was considered as statistically significant.

3.3. Results

3.3.1. Effect of CA on viability of HCC cells: In the present study we employed two widely used human hepatocellular carcinoma cell lines, HepG2 and Hep3B to examine the anti proliferative effects of CA. HepG2 and Hep3B cells were treated with different doses of CA (1-500 μM) for 48 h and cell viability was determined by the MTT assay. Under these conditions, a dose-dependent decrease in the cell viability was observed in both HepG2 and Hep3B cells with CA treatment for 48 h. In case of HepG2 cells, 25–100 μM doses of CA caused 30–62% (P <0.05) cell growth inhibition (Figure 3.1 A) following 48 h of treatment. Similar cell growth inhibitory effects of CA were also evident in case of Hep3B cells accounting for 15– 56% (P

<0.05–0.01) with 10- 100 µM doses of CA (Figure 3.1 B). The GI_{50} values for CA were found to be 50µM and 80 µM for HepG2 and Hep3B cells, respectively.

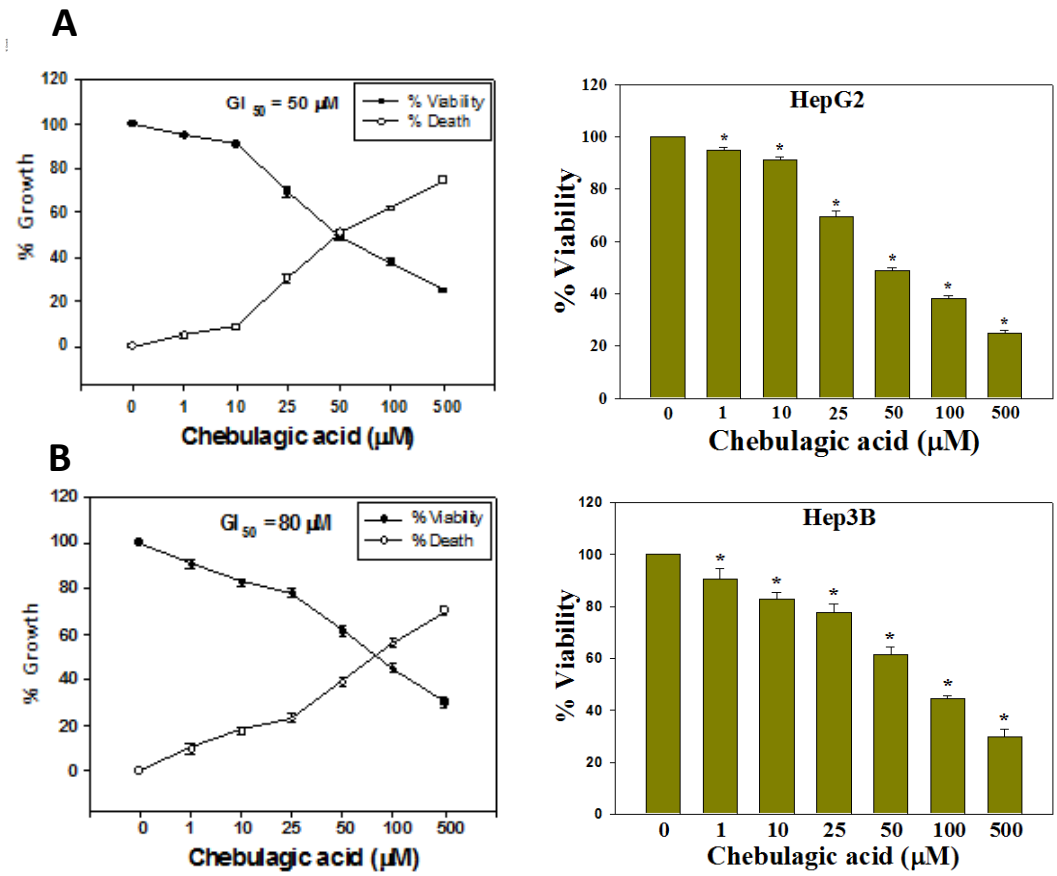


Figure 3.1. Effect of CA on the proliferation of HCC cells: *HepG2 (A) and Hep3B (B) cells were incubated with different concentrations (as shown in graph) of CA for 48 h and the cell viability was examined by MTT assay. Dose dependent growth inhibition was observed in both the tested cell lines. The values represent the mean ±SD of three independent experiments. * denotes statistical significance over control (P < 0.05).*

3.3.2. Effect of CA on proliferation of HCC cells: HepG2 and Hep3B cells were treated with different doses of CA (1-500 µM) for 48 h and cell proliferation was

assessed by Thymidine incorporation assay. Treatment with CA significantly inhibited Thymidine incorporation as shown in Figure 3.2 and a 50 % decrease was observed at about 50 µM in HepG2 cells and at about 80 µM in Hep3B cells indicating decreased cell proliferation induced by CA treatment. Further experiments were carried out on HCC cells at their GI_{50} concentrations.

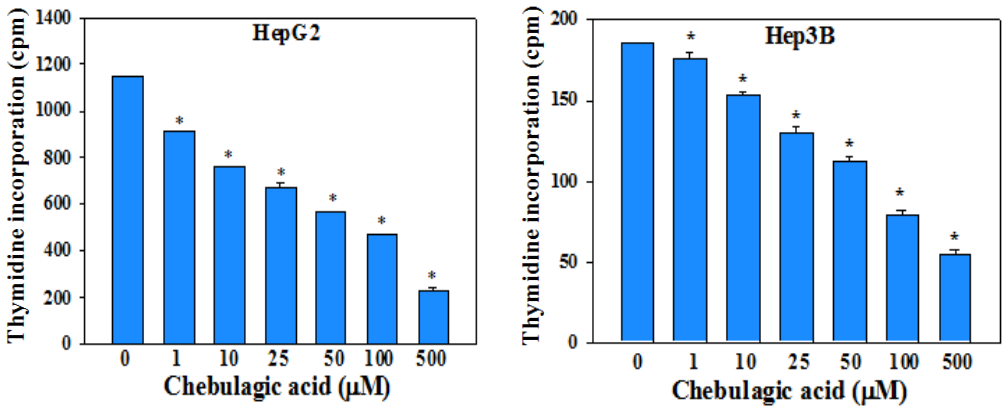


Figure 3.2. Effect of CA on the proliferation of HCC cells. *HepG2 and Hep3B cells were treated with various concentrations of CA for 48 h and the percentage of cell proliferation was determined by Thymidine incorporation assay. A significant dose dependent decrease in cell proliferation in response to CA was observed in both HepG2 (left) and Hep3B (right) cells The values represent the mean ±SD of three independent experiments. * denotes statistical significance over control (P < 0.05).*

3.3.4. Phase contrast microscopic studies: Phase-contrast microscopic pictures of Hep3B and HepG2 cells treated with or without CA for 48h were taken to observe the altered morphological features. Cells grown in the absence of CA were very healthy however treatment with CA at 50µM and 100µM showed extensive vacuolization,

membrane blebbing and various round detached cells from the substratum in Hep3B and HepG2 cells thus indicating cell death induced by CA (Figure 3.3).

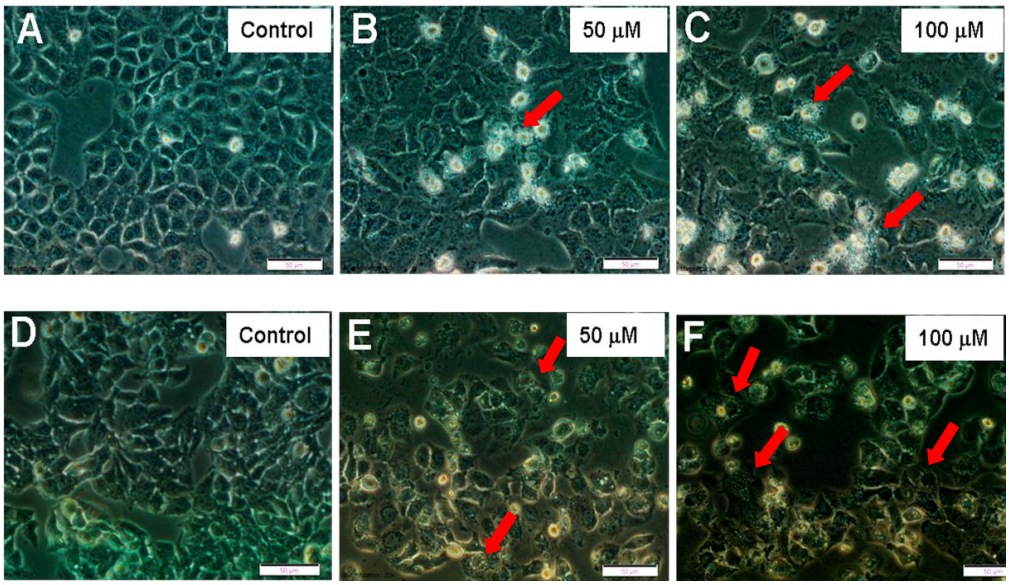


Figure 3.3. Phase contrast microscopy. *Pictures of Hep3B (A-C) and HepG2 (D-F) cells treated with media alone (control), or with media containing varying concentrations of CA for 48 h, showing dose-dependent alteration in cellular morphology. Magnification 40 X.*

3.3.5. *In situ* apoptosis detection (TUNEL Assay): Given the potent anti-proliferative effects of CA, we were interested in determining the mode of cell death in HCC cells. To test whether the cell death was by induced apoptosis TUNEL assay was performed in CA treated HCC cells by using fluorescence microscope. In this study, HepG2 and Hep3B cells were treated with 50 µM and 80 µM of CA respectively for 48h. After 48 h, following TUNEL staining, several fragmented nuclei (distinguishing feature of apoptosis) were observed in both the CA treated hepatoma cells (Figure 3.4, 3.5) compared to that of the untreated controls.

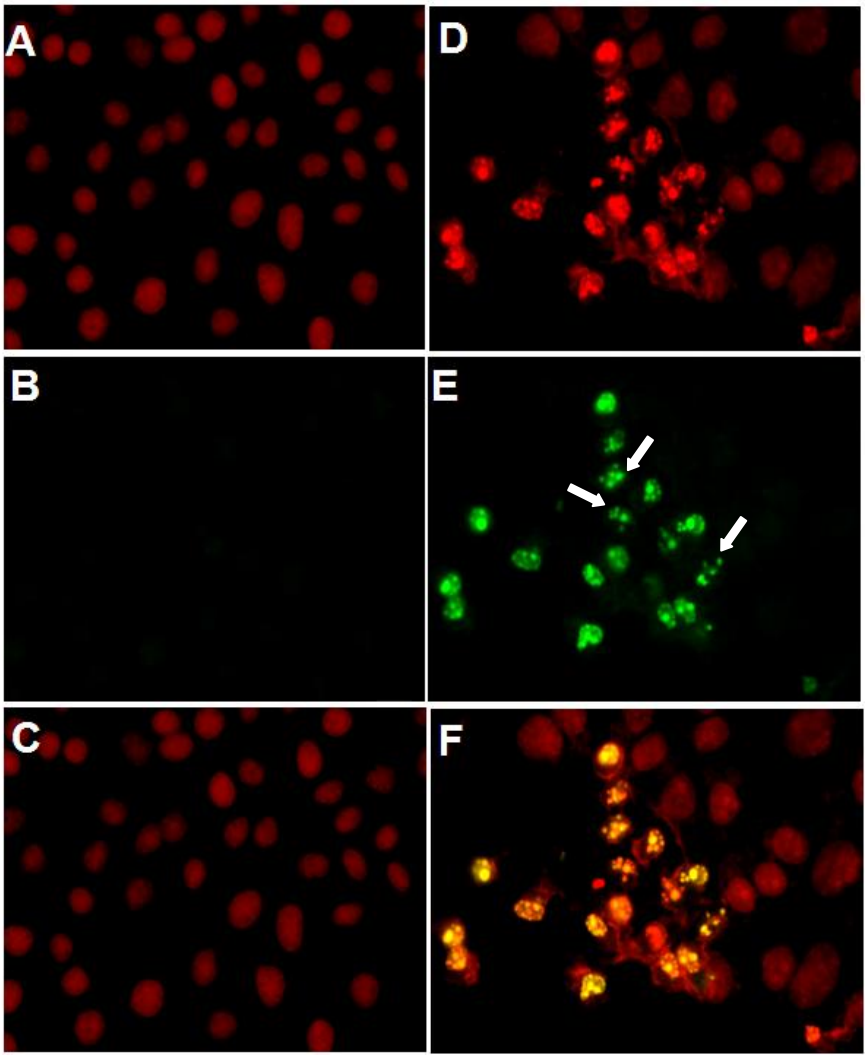


Figure 3.4. TUNEL assay for CA induced apoptosis in HepG2 cells. *Immunofluorescense micrographs showing results of TUNEL assays with HepG2 cells treated either with medium alone as control (panels A–C) or 50 μM of CA for 48 hours (panels D–F). Panels A, D, nuclear counter stain with propidium iodide; Panels B, E, TUNEL staining; Panels C, F, merged images.*

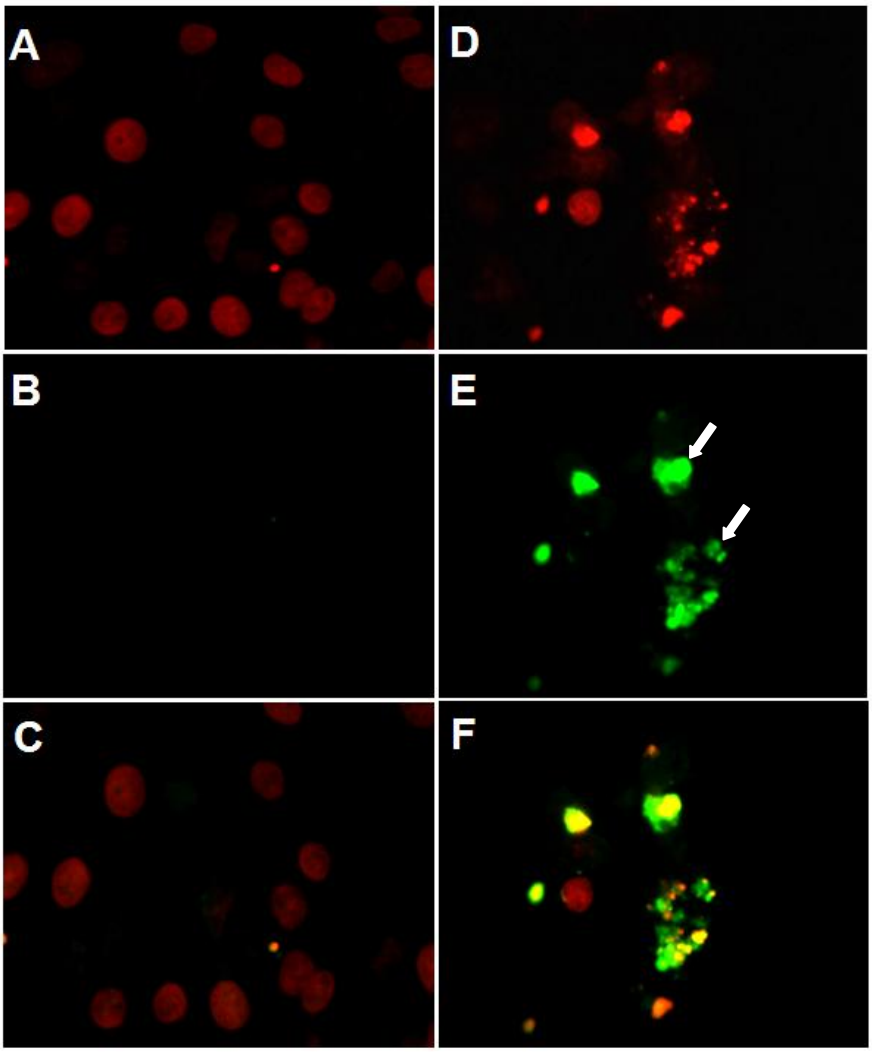


Figure 3.5. TUNEL assay for CA induced apoptosis in Hep3B cells. *Immunofluorescense micrographs showing results of TUNEL assays with Hep3B cells treated either with medium alone as control (panels A–C) or 80 μM of CA for 48 h (panels D–F). Panels A, D, nuclear counter stain with propidium iodide; Panels B, E, TUNEL staining; Panels C, F, merged images.*

3.3.6. Quantification of apoptosis by Flow cytometry: The induction of apoptosis in treated HCC cells was further quantified by flow cytometric analysis of DNA content. Loss of DNA is a typical feature of apoptotic cells. In the present study, HepG2 and Hep3B cells treated with CA at 50 and 80 μM respectively for 48h were taken for

FACS analysis. Typical sub-diploid apoptotic peaks were observed in both HepG2 and Hep3B cells treated with CA for 48h (Figure 3.6). In control only 4 % and 5 % of the cells showed hypo diploid DNA (sub G0⁄G1 peak) in HepG2 and Hep3B cells, respectively. This value of hypo diploid DNA increased to 48 % and 51 % with CA treatment for 48 h in HepG2 and Hep3B cells respectively. Increase in hypo diploid apoptotic cells in response to CA treatment demonstrates that the cells are undergoing apoptosis.

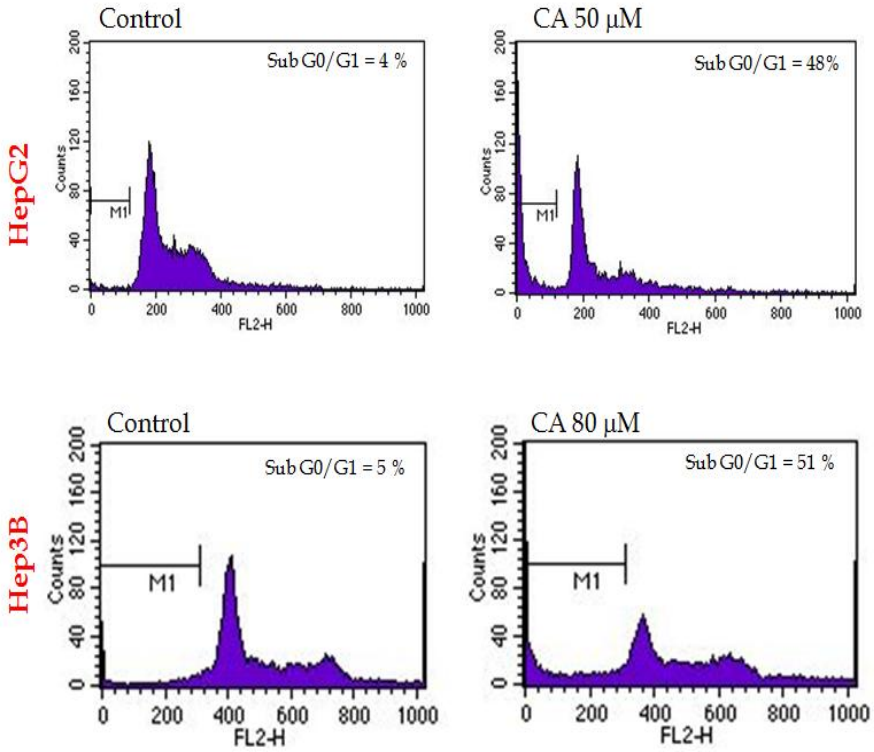


Figure 3.6. Quantification of Apoptosis by Flow Cytometry. *HepG2 and Hep3B cells were treated with medium alone (control), or with 50 and 80 µM CA respectively, for 48 h, fixed in 1 mL of 70% ethanol with 0.5% Tween-20 at 4˚C for 30 min and suspended in PBS. The cells were then stained with propidium iodide solution for 1 h and analysed for DNA content by flow cytometry. Data represent the result from one of three similar experiments.*

3.3.7. CA inhibits β-catenin protein levels: Several reports have shown that β-catenin is mutated and incongruously expressed in nucleus in 26-34% of human HCCs [178, 191] and its accumulation has been shown to be primarily involved in the pathogenesis of hepatic tumours [176]. β-catenin activation and cytoplasmic/nuclear localization have been associated with increased proliferation and survival of hepatocytes in normal physiology and tumor cell survival/ proliferation in HCC [192, 193]. Given that β-catenin/TCF signalling and its gene products are known to regulate cell proliferation and apoptosis in HCC, we have investigated the effect of CA on β-catenin/TCF signalling. Uncomplexed cytosolic β-catenin (free β-catenin) is the active form of β-catenin that translocates to the nucleus of the cell where it alters the levels of TCF/LEF family, leading to the transcription of Wnt target genes [194, 195]. To test whether the active form of β-catenin, which mediates target gene activation, was decreased with CA treatment we performed western blot analysis with a monoclonal antibody specific for the activated form of β-catenin (lacking phosphorylation at ser37/thr41 residues). Decrease in the activated or Ser37/Thr41- hypophsophorylated-β-catenin protein expression was observed at 24 h of CA treatment in both hepatoma cell types (Figure 3.7).

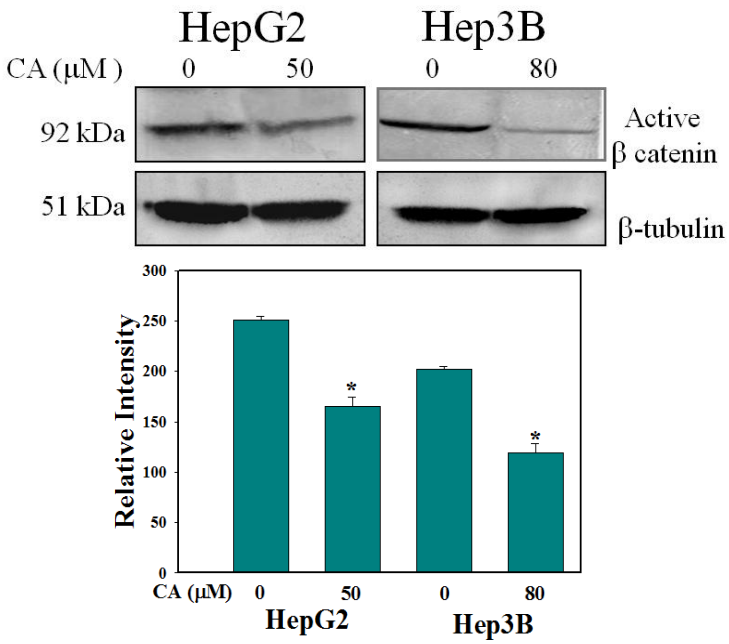


Figure 3.7: Effect of CA on activated β-catenin protein. Western *blot analysis of protein extracts from HepG2 and Hep3B cells treated with medium alone or CA (50μM for HepG$_2$ and 80 μM for Hep3B) for 24 hours with antibodies for active β-catenin. β-tubulin was used as an internal loading control. The relative band intensities were measured by quantitative scanning densitometry, bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with untreated control cells.*

3.3.8. CA Decreased Nuclear Localization and Increased Membrane Localization of β-Catenin: Next we checked the effect of CA on the membrane localization of β-catenin protein in the HCC cells by taking immunofluorescent microscopic pictures of HepG2 and Hep3B cells treated with CA for 24 h. Our immunofluorescense studies demonstrate that treatment with CA increases the membrane localization as well as decreases the nuclear levels of β-catenin when compared to untreated cells where most of the β-catenin was found to be localized in the nucleus thereby activating the β-catenin target genes(Figure 3.8 & 3.9).

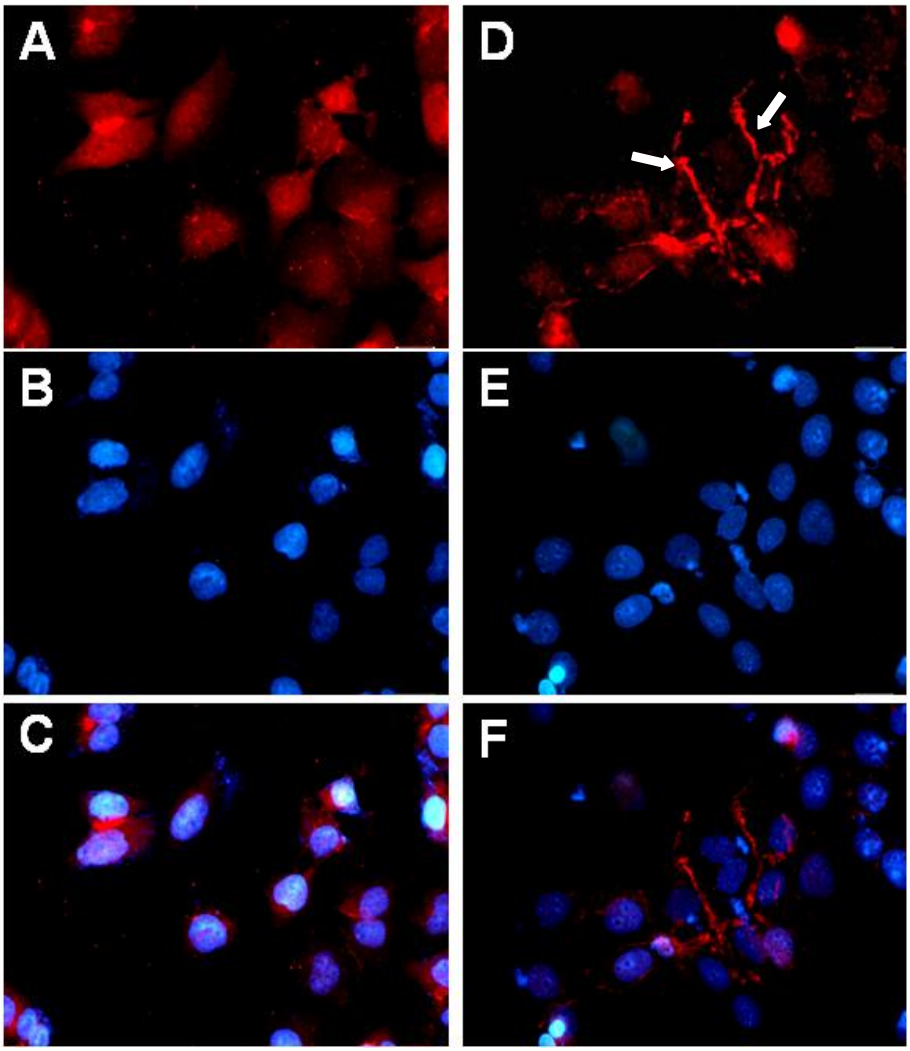


Figure 3.8. Immunofluorescense microscopy for β-catenin localization in HepG2 cells treated with CA. *Results showing increased membrane localization and decreased nuclear localisation of β-catenin in CA treated HepG2 cells compared to control (only medium treated) cells showing increased nuclear and decreased membrane localisation of β-catenin. Cells were treated with medium alone (panels: A-C) or 50 μM of CA for 24 h (panels: D-F). A and D, β-catenin immunestaining; B and E, DAPI staining; C and F, merged images.*

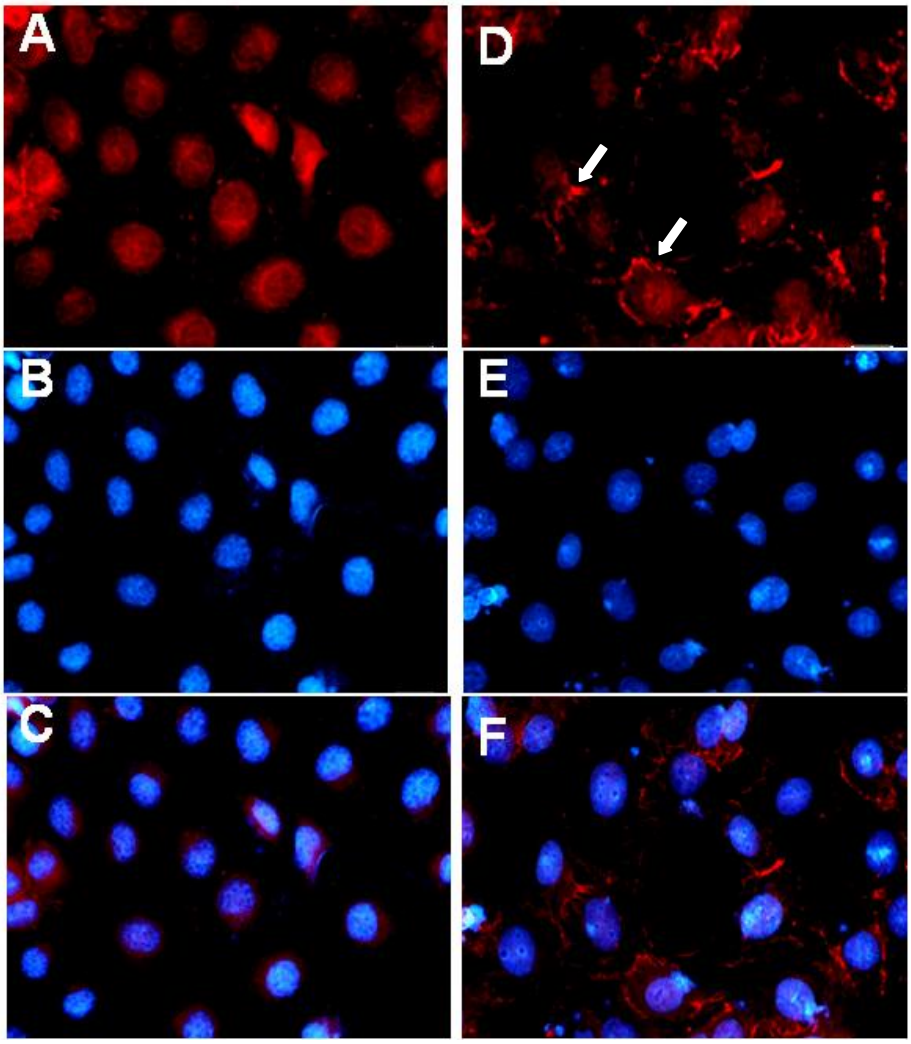


Figure 3.9. Immunofluorescense microscopy for β-catenin localization in Hep3B cells treated with CA. *Results showing increased membrane localization and decreased nuclear localisation of β-catenin in CA treated Hep3B cells compared to control (only medium treated) cells showing increased nuclear and decreased membrane localisation of β-catenin. Cells were treated with medium alone (panels: A-C) or 80 μM of CA for 24 h (panels: D-F). A and D, β-catenin immunostaining; B and E, DAPI staining; C and F, merged images.*

3.3.9. CA inhibited the expression of downstream transcription factors activated by β-catenin: Since cytosolic free β-catenin translocates to the nucleus to activate transcription factors like TCF-4/LEF-1, we then examined the expression levels of TCF-4 and LEF-1 in the nuclear fractions of HCC cells treated with CA for 24 h. As expected, a concomitant decrease in the levels of both TCF-4 and LEF-1 was observed in both the hepatoma cells (Figure 3.10).

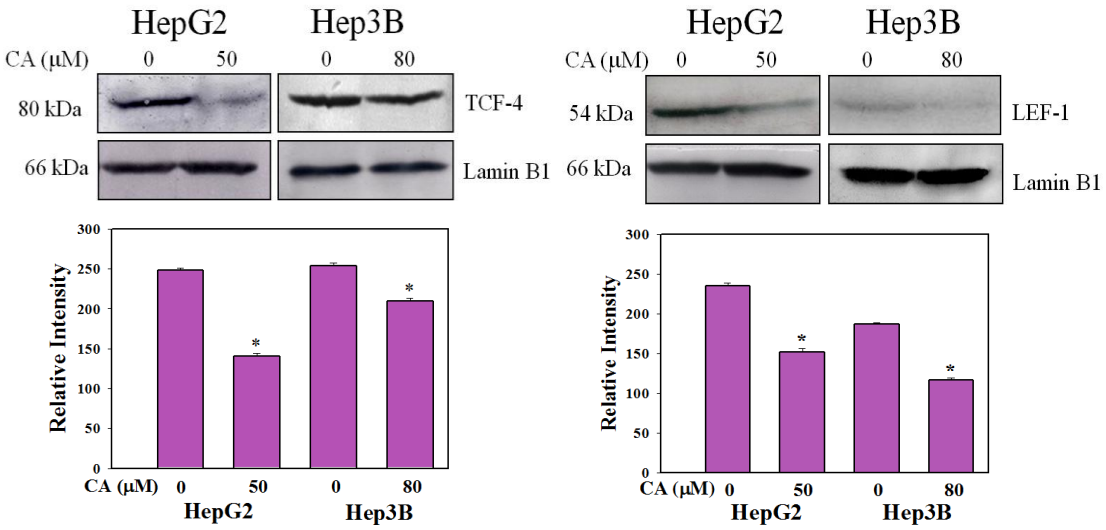


Figure 3.10. Effect of CA on TCF-4 and LEF-1 proteins. *Western blot analysis of protein extracts from HepG2 and Hep3B cells treated with medium alone or CA (50 μM for HepG2 and 80 μM for Hep3B) for 24 hours with antibodies for TCF-4(left) and LEF-1(right) proteins. Lamin B1 was used as internal loading control for nuclear extract. The relative band intensities were measured by quantitative scanning densitometry, bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with untreated control cells.*

Thus, a considerable decrease in the levels of β-catenin and expression of its target transcription factors, TCF-4 and LEF-1 were observed in response to CA treatment for 24 h and these changes preceded the biological responses like inhibition of cell survival, proliferation and apoptosis observed at 48 h of CA treatment.

3.3.10. CA Inhibits Proliferation Associated β-Catenin Target Genes: Now the question is whether these anti-proliferative and pro-apoptotic effects of CA are mediated through proliferation-associated β-catenin/TCF target genes such as cyclin D1, c-Myc, and COX-2. Further, we examined the levels of cyclin D1, c-Myc and COX-2 protein, key downstream targets of TCF-4/LEF-1, which are critical for tumour cell survival and proliferation [196, 197]. Treatment with CA significantly suppressed the expression of these β-Catenin target proteins like cyclin-D1, COX-2 and c-Myc at 24 h (Figure 3.11A-C). Taken together, our findings strongly suggest that CA suppressed the expression of several proliferation associated Wnt-target genes such as cyclin D1, c-Myc and COX-2, possibly giving rise to diminished proliferation and survival of HCC cells.

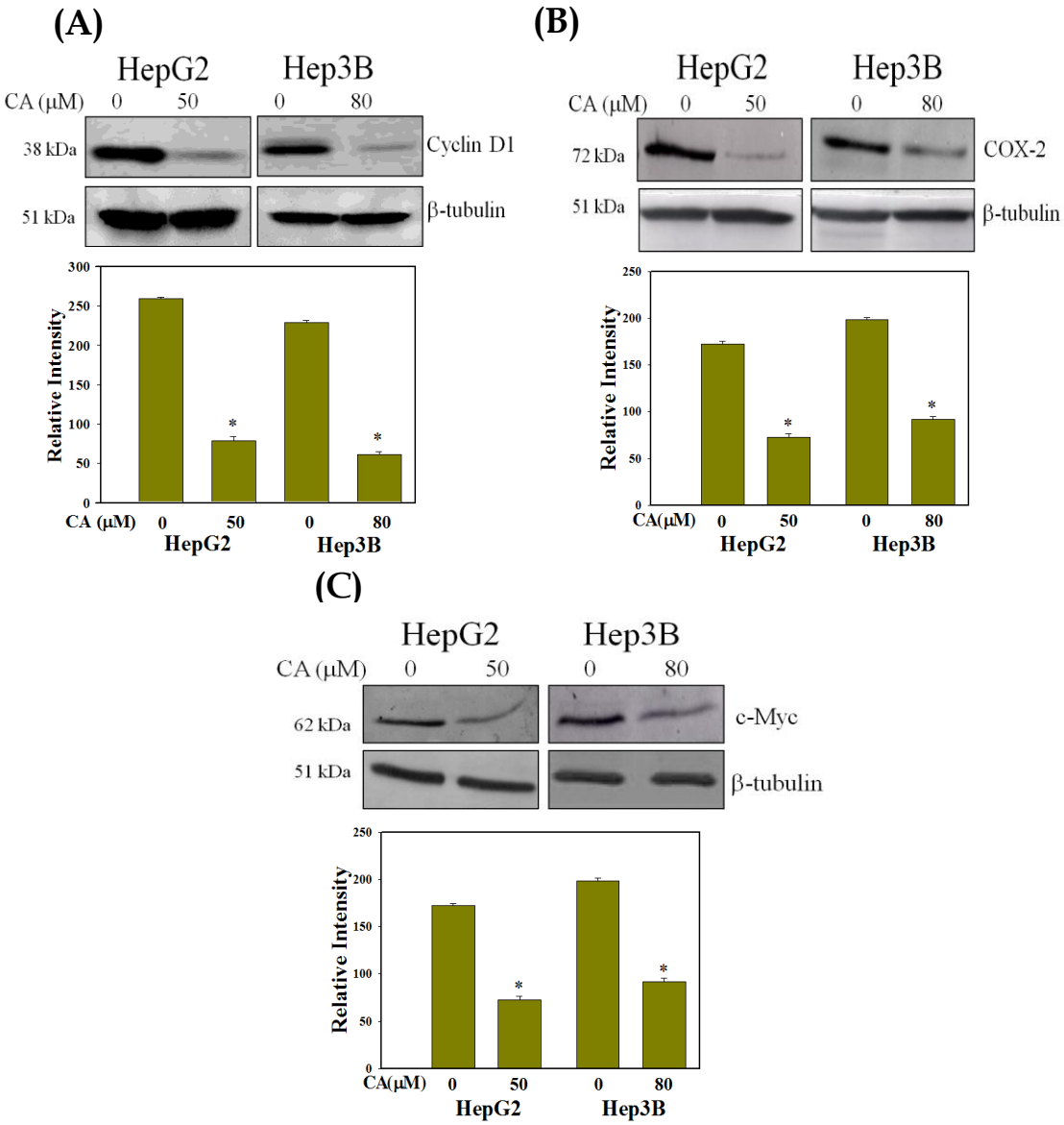


Figure 3.11. Effect of CA on proliferation associated β-catenin target proteins. *Western blot analysis of protein extracts from HepG2 and Hep3B cells treated with medium alone or CA (50 μM for HepG2 and 80 μM for Hep3B) for 24 hours with antibodies for Cyclin D1 (Panel A), COX-2 (Panel B) and c-Myc (Panel C). β-tubulin was used as an internal loading control. The relative band intensities were measured by quantitative scanning densitometry; bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with untreated control cells.*

3.3.11. Mechanism of β-catenin down regulation by CA: To test the mechanism of decrease in β-catenin protein levels by CA we examined the expression of CTNNB1 gene for β-catenin by real-time-PCR at 24h. A drastic 5 fold and a significant 2 fold decrease in mRNA levels of β-catenin was observed in HepG2and Hep3B cells respectively at 24 h of CA treatment when compared to untreated control (Figure 3.12 A).

A well described mechanism of β-catenin regulation involves its phosphorylation by GSK-3β at serine/threonine residues which targets it for ubiquitin mediated degradation [163]. GSK-3β regulates the stability of β-catenin by phosphorylation of its serine residues critical for its ubiquitination and thereby degradation [198]. When GSK-3β is inactive, β-catenin escapes from its phosphorylytic degradation and accumulates in the cytosol and then translocates to the nucleus resulting in activation of other target genes of the Wnt signalling pathway [199]. Thus, we studied the expression levels of Ser9-phosphorylated GSK3β (inactive) in both HepG2 and Hep3B cells. As shown in Figure 3.12 B, a significant decrease in its expression was observed at 24 h of CA treatment consequently implying GSK3β activation for phosphorylation and degradation of β-catenin in HCC cells.

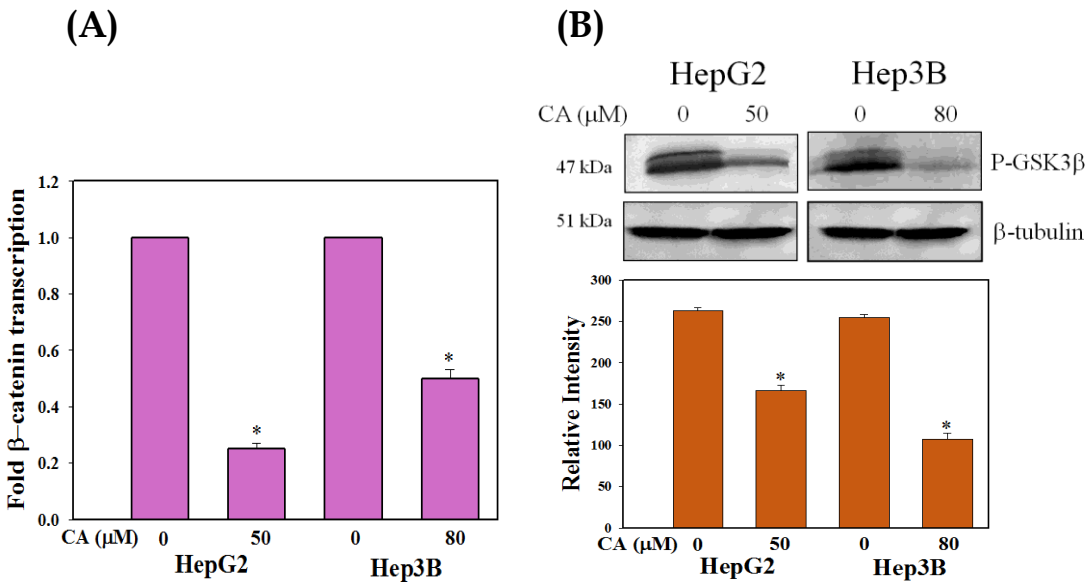


Figure 3.12. Mechanisms of down-regulation of β-catenin by CA. *(A) Expression levels of the CTNNB1 gene by real time PCR. Results from HepG2 and Hep3B cells treated with medium alone (Cont) or CA as described above for 24 hours. Results represent mean ± S. D. of three experiments. *P < 0.05 compared with untreated control cells. (B) Western blot analysis with the phosphorylated Ser9-GSK-3β antibody of protein extracts from HepG2 and Hep3B cells treated with medium alone or CA for 24 hours. β-tubulin was used as an internal loading control. The relative band intensities were measured by quantitative scanning densitometry bars indicate the mean ± S. D. (n = 3); *P < 0.05 compared with untreated control cells*

3.4. Discussion

Aberrant deregulation in multiple molecular signaling pathways including mutations in several genes result in the development of hepatocellular carcinoma

[200]. Recently several lines of evidence have implicated that aberrant deregulation of Wnt signaling is a major mechanism of liver tumourigenesis [178, 179]. β-catenin was reported to be mutated and incongruously expressed in nucleus in 26-34% of human HCCs [178 ,191] and its accumulation has been shown to be primarily involved in the pathogenesis of hepatic tumours [176]. β-catenin activation and cytoplasmic/nuclear localization have been associated with increased proliferation and survival of hepatocytes in normal physiology and tumour cell survival/ proliferation in HCC [192, 193]. Previous reports have correlated well with the successful knockdown of β-catenin using antisense oligonucleotides or drugs with diminished survival and proliferation of tumour cells [200-203]. Given these observations, identification of effective antagonists of the Wnt/β-catenin signaling, hold great promise for the successful management of hepatocellular carcinoma. For the past several years, intense efforts are being made to search for Wnt/β-catenin signaling antagonists among natural products. Our study provides the first experimental evidence for the anti cancer effects of CA on hepatocellular carcinoma cells by regulating the Wnt/β-catenin pathway.

In the present study we employed two widely used human hepatocellular carcinoma cell lines, HepG2 and Hep3B to examine the anti-proliferative effects of CA. We observed that CA effectively inhibited survival and proliferation of HCC cell lines in a dose dependant manner with more intense effects on HepG2 cells (GI_{50} 50 μM) compared to Hep3B cells (GI_{50} 80 μM) (Figure 3.1, 3.2). Resistance against

apoptosis is critical for survival and contributes to drug resistance in many cancers, including HCC [204]. Given the potent anti-proliferative effects of CA, we were interested in determining whether CA also induces apoptosis in HCC cells. Induction of apoptosis in both HepG2 and Hep3B cells was demonstrated by TUNEL assay and quantified by Flow Cytometry analysis of DNA content. In TUNEL assay several apoptotic and fragmented nuclei (distinguishing feature of apoptosis) were observed 48 h after treatment of HCC cells with CA. Flow Cytometry analysis revealed typical sub-diploid apoptotic peaks in both HepG2 as well as Hep3B cells treated with CA.

Given that β-catenin/TCF signalling and its gene products are known to regulate cell proliferation and apoptosis in HCC, we hypothesised that modulation of Wnt/β-catenin signaling may be one of the mechanisms implicated in suppression of cell proliferation in liver cancer by CA. Hence, we investigated the effect of CA on β-catenin/TCF signalling. Uncomplexed cytosolic β-catenin (free β-catenin) is the active form of β-catenin that translocates to the nucleus of the cell where it activates transcription factors of the TCF/LEF family, leading to the transcription of Wnt target genes [194, 196]. To test whether the active form of β-catenin, which mediates target gene activation, was altered with CA treatment, western blot analysis with a monoclonal antibody specific for the activated form of β-catenin (lacking phosphorylation at ser37/thr41 residues) was performed. CA significantly decreased the levels of active β-catenin in both the hepatoma cells at 24 h. Moreover, our immunofluorescense studies indicate that treatment with CA increases the membrane localization of β-catenin when compared to untreated cells where most of the β-

catenin was found to be localized in the nucleus. These findings are consistent with previous studies where natural product like curcumin suppressed β-catenin/TCF signaling by reducing the nuclear β-catenin levels [205].

Active β-catenin translocates to the nucleus to activate transcription factors like TCF-4/LEF-1 [170-174]. Consistent with decreased β-catenin levels, a considerable decrease in the protein levels of TCF-4 and LEF-1 were observed in both HepG2 and Hep3B cells at 24 h. Thus, a considerable decrease in the levels of β-catenin and expression of its target transcription factors, TCF-4 and LEF-1 were observed in response to CA treatment for 24 h and these changes preceded the biological responses like inhibition of cell survival, proliferation and apoptosis observed at 48 h of CA treatment. Our results are in good agreement with previous reports showing that inhibition of β-catenin signalling pathway by using antisense oligonucleotides, antibody or drugs induces anti-proliferative effects in HCC [202, 203, 206]. This once again strengthens our argument that CA mediated anti-proliferative effects in HCC are through inhibition of β-catenin signalling pathway. Similar observation has also been found in breast and colon cancer cells treated with natural drugs like curcumin, quercetin etc. [205, 207].

Now the question is whether these anti-proliferative and pro-apoptotic effects of CA are mediated through proliferation-associated β-catenin/TCF target genes such as cyclin D1, c-Myc, and COX-2. Further, we examined the levels of cyclin D1, c-Myc and COX-2 protein, key downstream targets of TCF-4/LEF-1, which are critical

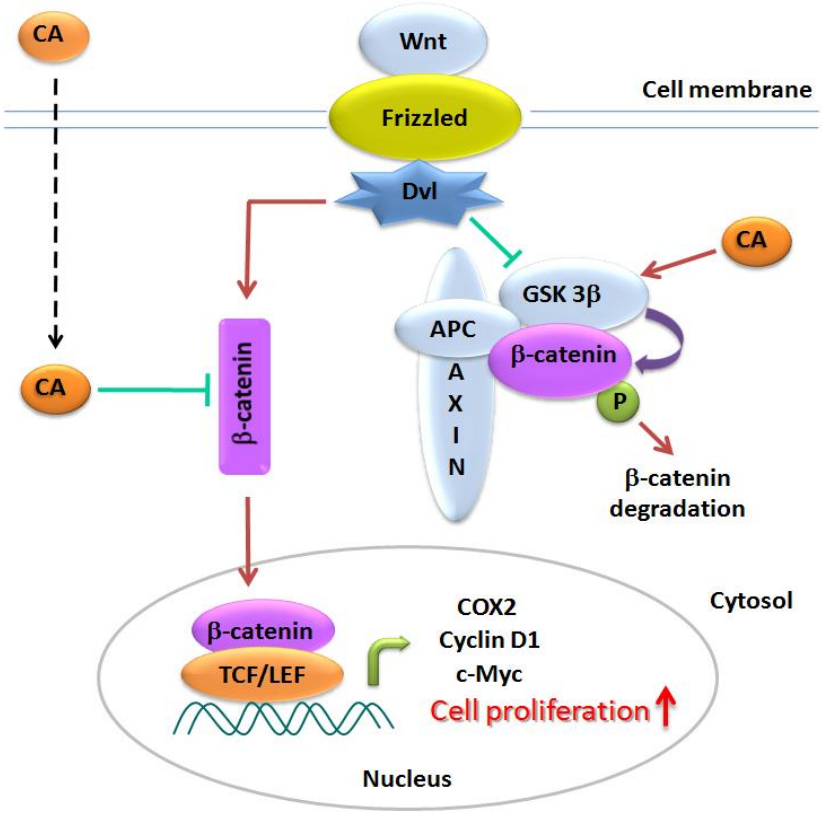
for tumour cell survival and proliferation [196, 197]. Treatment with CA significantly suppressed the expression of these β-Catenin target proteins like c-Myc, cyclin-D1 and COX-2 at 24 h. Taken together, our findings strongly suggest that CA suppressed the expression of several proliferation associated Wnt-target genes such as cyclin D1, c-Myc and COX-2, possibly giving rise to diminished proliferation and survival of HCC cells. Again, these findings are in agreement with the earlier studies showing β-catenin inhibition to be associated with decreased survival and proliferation of tumour cells via inhibition of cell proliferation associated genes [202, 203, 205, 207].

A well-described mechanism of β-catenin regulation involves its posttranslational modification by phosphorylation at serine/threonine residues by GSK-3β, critical for its ubiquitination and thereby degradation [163, 198]. When GSK-3β is inactive, β-catenin escapes from its phosphorylytic degradation and accumulates in the cytosol and then translocates to the nucleus resulting in activation of other target genes of the Wnt signalling pathway [199]. Thus, we studied the expression levels of Ser9-phosphorylated GSK3β (inactive) in both HepG2 and Hep3B cells. A drastic decrease in the levels of inactive form of GSK-3β (phosphorylated at serine 9) was noticed in both the hepatocarcinoma cells at 24 h of treatment. The above concept is supported by the decrease in the levels of ser37/thr41-hypophosphorylated- β-catenin (active form) in response to CA treatment.

In addition to the post-translational modification, transcriptional alteration also plays a key role in the regulation of β-catenin. Hence, in the present study

transcriptional alterations in β-catenin gene were examined in HCC cells in response to CA treatment. Our study demonstrates the transcriptional inhibition of β-catenin by CA as a second mechanism of β-catenin down-regulation. This was observed in both cell types and appears to be the mechanism of marked β-catenin suppression in both cells. Whether, CA directly inhibits β-catenin transcription or acts via inhibition of another transcription factor is not yet known. However, a possible mechanism could be via PPAR-γ transactivation. Gerhold *et al* identified β-catenin as a negative downstream target of PPAR-γ and that PPAR-γ agonists diminished γ-catenin expression [208]. In addition, PPAR-γ activation is known to induce GSK3β mediated β-catenin degradation as well [209]. The proposed mechanism of action of CA in HCC cells is presented in scheme 3.1.

In summary, we have identified a natural compound, CA as an effective inhibitor of Wnt/β-catenin signalling in two HCC cells. Furthermore, CA inhibited hepatoma cells proliferation, survival and induced apoptosis by increasing the sub G0/G1 cell population and by down-regulating Wnt target genes including cyclin D1, c-Myc and COX-2. In conclusion, our results suggest that development of therapeutic agents like CA that target β-catenin signalling may be an attractive approach towards treatment of various cancers with abnormally regulated β-catenin expression such as HCC.



Scheme 3.1. Schematic diagram illustrating modulation of Wnt/β-catenin signalling by CA. *CA significantly inhibited the expression of β-catenin and also increased the activity of GSK3β resulting in the degradation of β-catenin. This in turn resulted in decreased expression of COX-2, cyclin-D1 and c-Myc, downstream target genes of β-catenin.*

Chapter 4

Chebulagic acid overcomes doxorubicin resistance in hepatocellular carcinoma: Studies on molecular mechanisms

4.1. Introduction

Over the last two decades, considerable efforts have been made in the treatment of patients with hepatocellular carcinoma (HCC). A significant percentage of these patients fail to attain complete remission or very often they relapse due to occurrence of multidrug resistance (MDR). Multidrug resistance is a phenomenon of resistance of tumours to many structurally unrelated therapeutic drugs. Development of MDR is one of the major limitations in the treatment of HCC. Several molecular mechanisms involving overexpression of membrane efflux pumps, p^{53} mutations, up-regulation of BCL2, DNA repair or cellular detoxifying enzymes give rise to MDR [210]. Overexpression of membrane associated P-glycoprotein (P-gp/MDR-1/ABCB1), a product of MDR gene family was the first discovered and probably still is the most widely observed mechanism in clinical MDR [211, 212]. Multidrug resistance protein-1 (MDR-1) is an ATP dependant transmembrane protein consisting of 12 transmembrane domains that form a drug-binding pore and two ATP-binding sites belonging to the ATP-binding cassette (ABC) transporter family. It mediates resistance to various classes of chemotherapeutic agents including vinblastine, vincristine, daunorubicin, doxorubicn, colchicine, paclitaxel, etoposide, actinomycin-D, docetaxel, etoposide, teniposide, bisantrene, homoharringtonine and gleevec by actively extruding the drugs from the cells thereby reducing their intracellular concentrations [213]. It is apparent in inflammation associated processes like cholestasis [214] as well as in liver regeneration [215].

COX-2 has been reported to play a major role in the regulation of MDR. Simultaneous overexpression of the COX-2 and MDR1, reported in the regenerative nodules of cirrhotic livers as well as in well-differentiated hepatocellular carcinoma [216, 217, 96], suggests a possible role for COX-2 in MDR. In view of the above findings, the present study is undertaken to test whether CA, a COX-2/5-LOX dual inhibitor, can overcome drug resistance in HepG2 cancer cells.

Intense efforts are underway towards identification of chemo sensitizers (MDR reversers or modulators) that can modulate MDR-1 mediated multidrug resistance. Such chemosensitizers include calcium channel blockers, calmodulin antagonists, steroids, cyclin peptides and drug analogs [218]. However, their side effects and dose limiting cytotoxicity, limits their use for systemic chemotherapy [219, 220]. In view of this problem, several plant derived compounds are gaining importance as potential cancer therapeutics. The chemical constituents of several medicinal plants with diversified pharmacological properties have potential for prevention/treatment of several human cancers [221, 222]. Many dietary chemo preventive phytochemicals and polyphenols have been reported to modulate the function of MDR-1 [223-227]. Chebulagic acid (CA) a benzopyran tannin present as one of the major components in the fruits of *Terminalia chebula*, is one such compound. We have reported earlier that CA is a COX-2/5-LOX dual inhibitor [96] and induces apoptosis in colon cancer cells *in vitro* [97]. Recently we have shown that CA attenuates LPS-induced inflammation by suppressing NF-κB and MAPK activation in RAW 264.7 macrophages [97]. These studies provide a rationale for the

potential use of CA in cancer treatment. However, no attention has been paid on the potential use of CA in combination treatments involving commercial chemotherapeutic drugs. Also, the effects of these interactions on the expression and function of the drug transporters have not been studied. Here, we demonstrate for the first time, that CA increased the sensitivity of doxorubicin (Dox) in human hepatocarcinoma cell line (HepG2) via downregulation of MDR1 expression and also elucidated the mechanisms underlying these effects.

Objectives

The specific objectives of the present study are:

- To evaluate the effect of CA on the cytotoxicity of Dox in human hepatocellular carcinoma cell line, HepG2.
- To study the effect of CA on MDR-1 expression in HepG2 cells.
- To understand the molecular mechanisms involved in CA mediated effects.

4.2. Materials and Methods

4.2.1. Reagents: CA was isolated from ethanolic extract of dried fruits of *T.chebula* by RP-HPLC and dissolved in DMEM at 10 mM concentration before use. Dox was a generous gift from Dabur Pharma, India. PBS, DMEM and foetal bovine serum (FBS) were purchased from Gibco BRL (California, USA). Nitrocellulose membrane was from Millipore (Bangalore, India). Phosphatase inhibitor was purchased from Sigma-Aldrich (Bangalore, India). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl

tetrazolium bromide], was purchased from Sigma Chemical Company (St. Louis, USA). Primary antibodies to Akt, p-Akt, JNK, p-JNK, ERK, p-ERK, P38, p-P38, COX-2 and MDR1, PGE_2 were from Cayman Chemical Co. USA and siRNA for COX-2 were procured from Santa Cruz Biotechnology Inc. (California, USA) and β-actin was from Epitomics (USA). All other chemicals and reagents were purchased from local companies and are of molecular biology grade.

4.2.3. Cell culture and treatment: The human hepatoma cell line HepG2 was obtained from National Centre for Cell Science (NCCS), Pune, India and maintained in DMEM supplemented with 10% heat inactivated FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine and maintained in a humidified atmosphere with 5% CO_2 at 37°C. The cells were sub-cultured twice each week and the exponentially growing cells were used in all treatments. CA and Dox dissolved in DMEM and DMSO respectively were used in the treatments. 10 mM stock of CA and 20 mM stock of Dox were employed in this study. At the time of treatment, working solutions were diluted accordingly in DMEM. Final concentrations of CA ranged from 1 to 250 µM and that of Dox ranged from 10nM to 100 µM. The drugs were added to the cells, 12 h after the sub-culture. The final concentration of the vehicle (DMSO) never exceeded 0.1%. HepG2 cells exposed to 0.1% DMSO served as controls.

4.2.4. Cell proliferation assay: Cytotoxicity of CA and Dox in HepG2 cells was determined by cell proliferation assay using the 3-(4,5- dimethylthiazol-2-yl)-2,5-

diphenyl tetrazolium bromide (MTT) staining as described elsewhere [185]. Briefly, $5x10^3$ cells were incubated in 96-well plates in the presence or absence of CA (1, 10, 25, 50, 100 and 250 μM) or Dox (100 nM, 1 μM, 10 μM, 25 μM, 50 μM and 100 μM) for 24 and 48 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 crystals were 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 Microtiter plate reader (m Quant Bio-tek Instruments, Inc.). Each experiment was carried out in triplicates. Mean ±SE was calculated and reported as the percentage of growth vs. control.

4.2.5. Analysis of interactions: The level of interaction between CA and Dox was assessed by measuring combination-index (CI), a quantitative representation of pharmacological interaction between two drugs [228]. Briefly, variable ratios of drug concentrations (10-50 μM CA and 10-1000 nM Dox) were used in several different combinations for the treatment of HepG2 cells for 48 h. Cell growth inhibition was determined using the MTT assay, as previously described. The anti-proliferative data obtained were analyzed using mutually exclusive equations to determine the CI. The CI value of 1 indicates an additive effect, whereas a CI < 1 or >1 indicates synergism and antagonism, respectively. The CI values were calculated at x % cell growth inhibition, as:

$$CI = D_1 / Dx_1 + D_2 / Dx_2$$

Where, Dx_1 and Dx_2 are the doses of drug 1, for example, the CA and drug 2, for example, the Dox alone that gives *x*% cell growth inhibition, whereas D_1 and D_2 are the doses of drug 1 and drug 2 in combination that also inhibits cell growth by *x*% (i.e., isoeffective). Dx_1 and Dx_2 can be readily calculated from the Median-effect equation of Chou *et al* [228].

The dose-reduction index (DRI) defines the extent (folds) of dose reduction possible in a combination, for a given degree of effect, compared with the dose of each drug alone: $DRI_1 = Dx_1 / D_1$ and $DRI_2 = Dx_2 / D_2$.

4.2.6. Intracellular drug accumulation assays: HepG2 cells (2×10^5 cells/well) were seeded on 6-well plates and incubated overnight after which CA was added for 24 h. After 24 h, to determine intracellular drug accumulation, Dox was added to each well for another 3 h. Subsequently, the culture medium was removed and cells were washed three times with PBS. The final Dox accumulated in HepG2 cells was visualized and analyzed using fluorescence microscope (Max Excitation λ 480 nm) and Flow Cytometry (FL2 filter), respectively.

4.2.7. RT-PCR analysis: HepG2 cells were seeded at a density of 5 x 10^6 in 90 mm culture dishes. Cells were treated with 50 μM of CA or PGE_2 (6 μg/mL) for 48 h. Cells were harvested and total RNA was extracted using TRIzol reagent from control and treated HepG2 cells. cDNA was synthesized using oligo (dT), dNTP mixture, RevertAid H Minus M-MuLV Reverse Transcriptase with 5 μg total RNA, isolated

from HepG2 cells. A 2 µl aliquot of the 20 µl total cDNA was used for standard PCR reaction of 28 cycles using the COX-2 FP: 5'-TCA AAT GAG ATT GTG GGA AAA TTG GT-3', RP: 5'-AGA TCA TCT TTG TCT GAG TAT TTT-3' and MDR1 FP: 5'-TGA CTA CCA GGC TCG CCA A-3', RP: 5'-TAG CGA TCT TCC CAG CAC CTT-3' primer sets. The PCR products were visualized on 1% agarose gels with ethidium bromide, under UV light. The GAPDH primers served as control.

4.2.8. Transfection of COX-2 siRNA: HepG2 cells (1 x 10^6 cells/well) were seeded into 60mm petridishes. After overnight incubation, cells were transfected with siRNA for COX-2 at concentration of 100 nM for 48 h. Total RNA was isolated and RT-PCR analysis was performed to measure the expression of MDR1 and COX-2 in control and COX-2 siRNA transfected cells.

4.2.9.Western blot analysis: HepG2 cells were seeded in 100 mm dishes at a density of 5 x 10^6 and incubated with 50 µM of CA and PGE_2 (6 µg/mL) for 48h. After 48 h, whole cell extracts were prepared as mentioned elsewhere [189] and protein content was determined according to the Bradford method [229]. Nuclear extracts were prepared as described elsewhere [97] with minor modifications. An equal amount of protein (100 µg) was resolved on 8-10% SDS–PAGE gel along with protein molecular weight standard, and then transferred onto nitrocellulose membranes. 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 (1X Tris-buffered saline and

0.05% Tween-20 with 1% milk) with gentle shaking at 4 °C for 8–12 h and then incubated with respective ALP conjugated secondary antibodies. Signals were detected using BCIP/NBT western blot detection reagents. β-actin was used as loading control.

4.2.10. Statistical analysis: Data were presented as the mean ± standard error (SE) from three separate experiments. P-values were determined using the unpaired Student's *t*-test. P value of less than 0.05 was considered as statistically significant.

4.3. Results

4.3.1. Effect of CA and Dox on HepG2 cell growth: The effects of CA and Dox on the proliferation of HepG2 cells were determined using MTT assay. HepG2 cells were treated with different doses of CA (1-250 μM) and Dox (10 nM - 100 μM) for 24 and 48 h. A dose-dependent decrease in the cell viability was observed with both CA and Dox treatments with IC_{50} value of 50 μM and 3 μM for CA and Dox respectively at 48 h exposure (Figure 4.1 A, B).

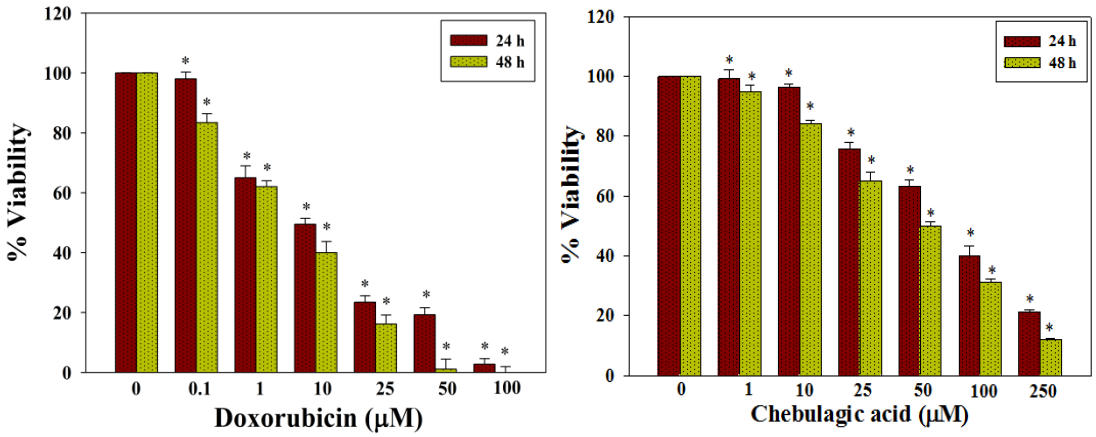


Figure 4.1. Cytotoxic effects of Dox and CA in HepG2 cells*. Cells were plated at a density of 5x10^3 in 96-well culture plates and were treated with various concentrations of Dox (A) or CA (B) and measured for viability at 24 and 48 h after treatment by using MTT assay. The values represent the mean ±SD of three independent experiments. * denotes statistical significance over control (P < 0.05).*

4.3.2. CA increases the sensitivity of HepG2 cells towards Dox induced cytotoxicity: It is essential to comprehend the effects of CA on the efficacy of Dox prior to using CA as an adjunct agent to chemotherapy. Consequently, we determined the effect of CA on Dox induced cytotoxicity. In order to test the combination effects, HepG2 cells were treated with varying concentrations of Dox (10 nM, 100 nM and 1 μM) and varying concentrations of CA (10 μM, 25 μM 50 μM) for 48 h and cell proliferation was determined by MTT assay. As shown in Figure 4.2, in HepG2 cells a combination of 50 μM of CA and 100 nM Dox for 48 h resulted in 78 % growth inhibition when compared to either agent alone showing 50% (CA) and 30 % (Dox) growth inhibition. Similarly a combination of 25 μM CA with 100 nM Dox resulted in 50 % growth inhibition compared to either agents alone showing 35 % (CA) and 30

% (Dox) growth inhibition respectively and thus reducing the IC_{50} value of Dox by 25 fold. An increased cell growth inhibition was also observed in several other combinations ranging from 10, 25 and 50 μM of CA and 10, 100 and 1000 nM of Dox, as compared to individual doses of CA and Dox (Figure 4.2).

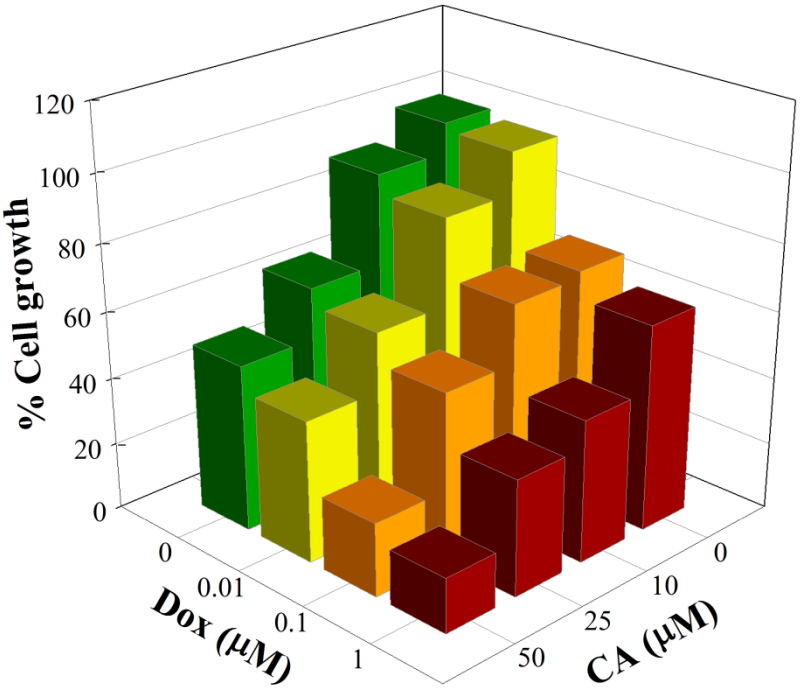


Figure 4.2. Enhanced growth inhibitory effect of CA–Dox combination on human hepatocarcinoma cells*. Exponentially growing HepG2 cells (5x10^3) were seeded in 96 well plates and treated with the indicated concentrations of Dox alone and various combinations of Dox and CA for 48 h. After 48 h of these treatments the number of viable cells in replica plates were estimated by MTT assay and expressed as the percentage of the control value. The values represent the mean ± SD of three independent experiments.*

All the data shown in Figure 4.2 were next analyzed for a possible synergism between different drug combinations of CA and Dox by plotting CI-isobologram

[228]. As shown in Figure 4.3 most of the CA plus Dox combinations showed synergistic effects in terms of HepG2 cell growth inhibition with CI values < 1. The strongest synergistic effect (CI<0.5), however, was evident at a CA dose of 25 μM plus 1 μM Dox. Other effective combinations where the CI values were almost equal to 0.5 included 10, 25 and 50 μM CA plus 100nM Dox (CI values 0.51, 0.55, 0.54 respectively); 50 μM CA plus 1 μM Dox (CI value 0.51) and 25 μM CA plus 10 nM Dox (CI value 0.56). Combination of 10 and 50 μM CA plus 10 nM Dox, however, showed antagonistic effect with CI values more than 1 (2.8 and 1.58 respectively).

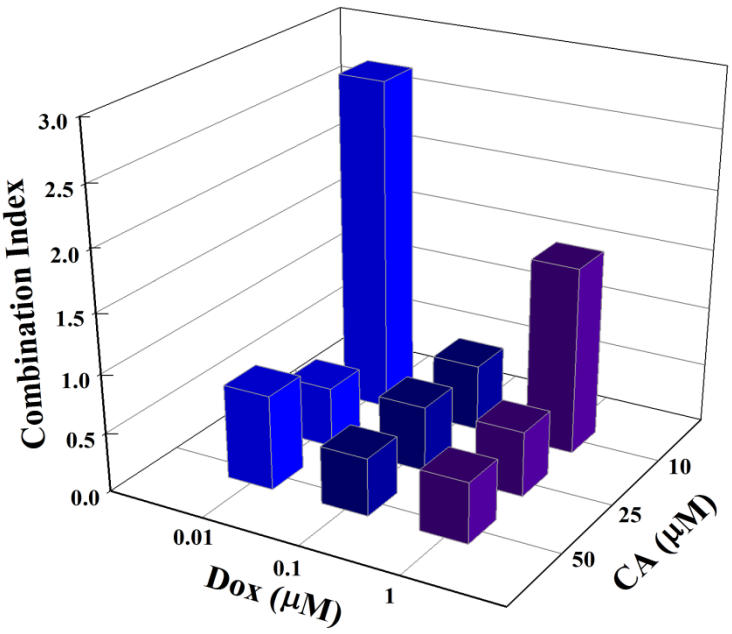


Figure 4.3. Plot of combination index for various combinations of CA and Dox. *A median effect analysis was performed as described in section 2 and a combination index was derived for each combination. A combination index <1, 1 or >1 indicates a synergistic, additive, or antagonistic interaction.*

Synergism between therapeutic agents allows for a reduction in their doses while maintaining their therapeutic efficacy and thus minimizing their toxic effects.

Thus, we calculated the dose-reduction index (DRI) for various combinations of CA and Dox in HepG2 cell lines. A DRI > 1 correlates with a synergistic interaction. As shown in Figure 4.4, most of the CA plus Dox combinations caused a reduction in the dosage of Dox with DRI values >1 confirming synergism. The highest DRI was evident at a CA dose of 50 µM plus 10 nM Dox which reduced the dosage of Dox by 354 fold. Other effective combinations were CA 50 µM plus 100 nM Dox and CA 25 µM plus 10 nM Dox which reduced the dosage of Dox by 105 and 97 fold respectively. Summary of the combination indices and dose reduction indices for the various CA-Dox combinations are presented in Table 4.1.

Table 4.1. *Combination index (CI) and Dose Reduction Index (DRI) values for various combinations of CA and Dox in HepG2 cells.*

Drug combination CA + Dox	CI Values	DRI values for Dox
10 µM + 10 nM	2.8	0.1
10 µM + 100 nM	0.56	0.052
10 µM + 1 µM	1.58	1.51
25 µM + 10 nM	0.51	97.3
25 µM + 100 nM	0.55	20
25 µM + 1 µM	0.54	4.76
50 µM + 10 nM	0.77	354
50 µM + 100 nM	0.48	104.7
50 µM + 1 µM	0.514	12.76

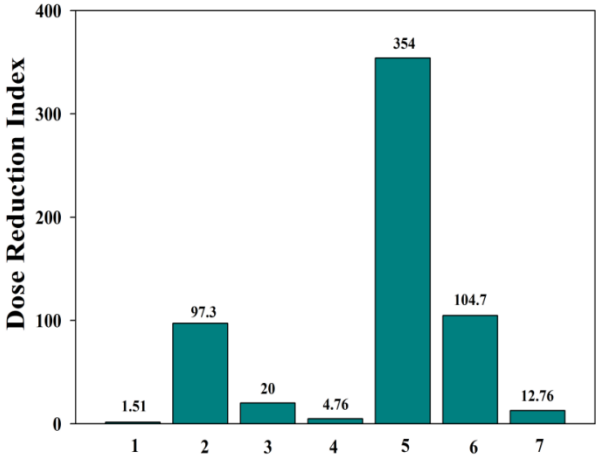


Figure 4.4. Plot of dose reduction index for various combinations of CA and Dox. *CA allows dose reduction when combined with Dox. The dose-reduction index was calculated as described in Section 2. A marked dose reduction was observed with a combination of 50 μM of CA with 10 and 100 nM of Dox and with 25μM of CA with 10 nM of Dox. A much lower dose-reduction for Dox was also observed with other combinations of CA + Dox: (1) 10 μM + 1 μM; (2) 25 μM + 10 nM; (3) 25 μM + 100 nM; (4) 25 μM + 1 μM; (5) 50 μM + 10 nM; (6) 50 μM + 100 nM; (7) 50 μM + 1 μM.*

4.3.3. CA treatment increased Dox accumulation in HepG2 cells: The cytotoxicity of drugs could be augmented by mechanisms that lead to reduced efflux and thereby increase in its intracellular concentrations in the cancer cells. We thus determined the effect of CA on accumulation of Dox in HepG2 cells. Increased accumulation of Dox in HepG2 cells pre-treated with CA was demonstrated by fluorescence microscopy analysis (Figure 4.5) and by FACS (Figure 4.6). As observed by fluorescence microscopy, HepG2 cells treated with CA (50 μM) for 24 h followed by incubation with 5, 2.5 and 1 μM Dox for 3 h, showed increase in Dox fluorescence, thus implicating enhanced accumulation of Dox in the presence of CA compared to cells

without CA treatment. Similarly, FACS analysis with FL2 filter for HepG2 cells treated with increasing doses of CA (5, 25 and 50 µM) for 24 h followed by incubation with 5 µM Dox for 3 h showed concentration dependent increase in Dox accumulation.

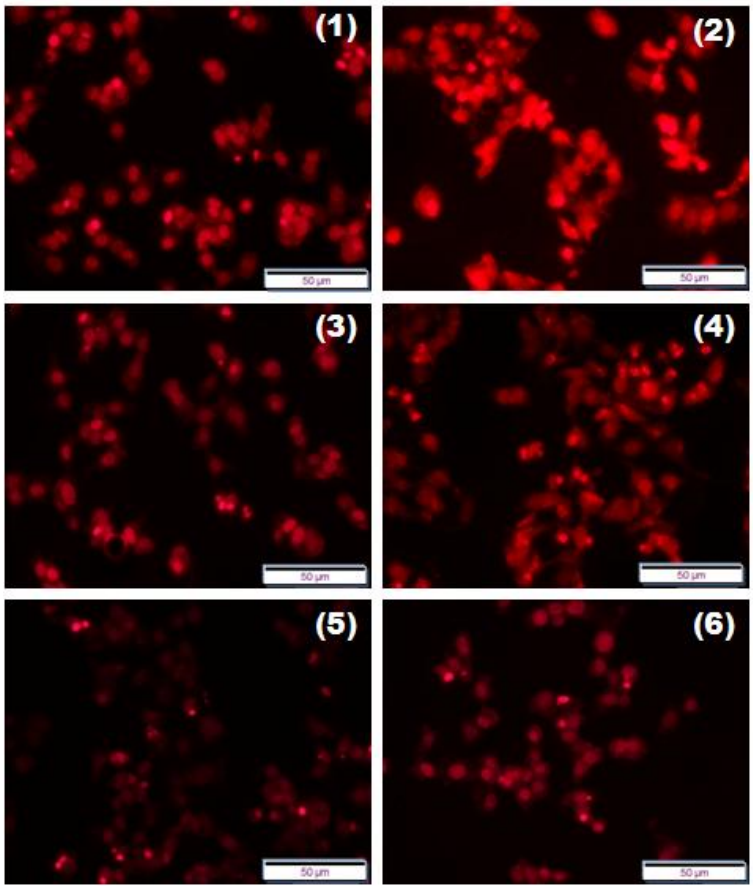


Figure 4.5. Fluorescence microscopic analysis showing increased accumulation of Dox in presence of CA. *HepG2 cells were treated with 50 µM of CA for 24 h and then incubated with 5, 2.5 or 1µM Dox for 3 h. The fluorescence of Dox retained in HepG2 cells was visualized by Fluorescence microscopy at excitation wavelength of 480 nm. (1) Dox 5 µM + 0 µM CA; (2) Dox 5 µM + 50 µM CA; (3) Dox 2.5 µM + 0 µM CA; (4) Dox 2.5 µM + 50 µM CA; (5) Dox 1 µM + 0 µM CA; and (6) Dox 1 µM +50 µM CA.*

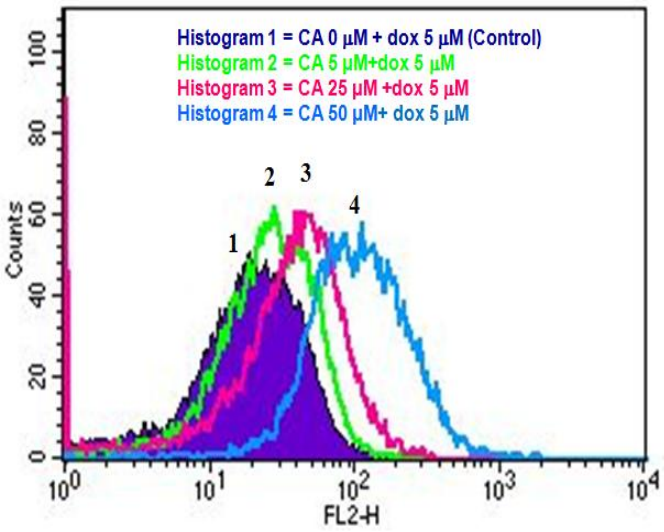


Figure 4.6. FACS analysis showing dose dependant increase in Dox accumulation with increasing doses of CA. *HepG2 cells were treated with 5 µM, 25 µM and 50 µM of CA for 24 h and then incubated with 5 µM doxorubicin for 3 h. The fluorescence of doxorubicin retained in HepG2 cells was quantified by using FL2 filter. Histogram 1 (Dox retained in control cells exposed to 5µM Dox) overlaid with histogram 2, 3 and 4 (Dox retained in cells treated with 5, 25 and 50 µM CA respectively, and exposed to 5 µM Dox).*

4.3.4. CA inhibits MDR1 expression in HepG2 cells: To assess the mechanism by which CA increased Dox sensitivity and decreased its efflux from the HepG2 cells, we examined the levels of MDR-1 which is known as one of the major factors for the development of drug resistance in experimental models of hepatoblastoma cells [230, 231]. Treatment of HepG2 cells with CA (50 µM) for 48 h resulted in reduction of MDR1 at both mRNA (Figure 4.6A) and protein (Figure 4.6B) levels. Treatment of

cells with PGE_2 a downstream metabolite of COX-2 at a dose of 6 μg/mL, on the other hand, induced the expression of MDR1, compared to untreated controls.

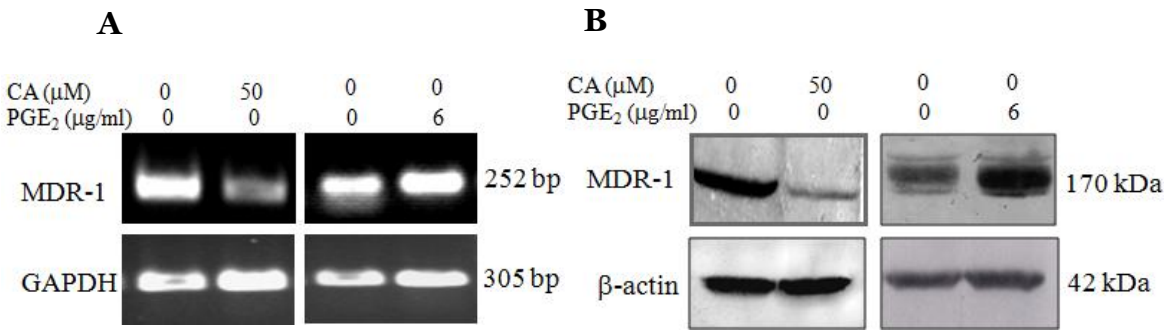


Figure 4.6. Effect of CA on MDR1 expression. *(A) RT-PCR analysis showing the effect of CA and PGE_2 on MDR1 mRNA expression in HepG2 cells. GAPDH was used as an internal control. (B) Western blot analysis for CA and PGE_2 mediated effect on MDR1 protein expression in HepG2 cells. β-actin was used as an internal control to monitor equal loading.*

4.3.5. CA down regulates COX-2 expression in HepG2 cells: In view of the fact that COX-2 has a role in the regulation of MDR-1, we checked the effect of CA on COX-2 expression. Treatment of HepG2 cells with CA (50 μM) resulted in the inhibition of COX-2 expression at mRNA (Figure 4.7A) and protein (Figure 4.7B) levels at 48h. Addition of PGE_2 (6 μg/mL), on the other hand, induced the expression of COX-2 compared to untreated controls.

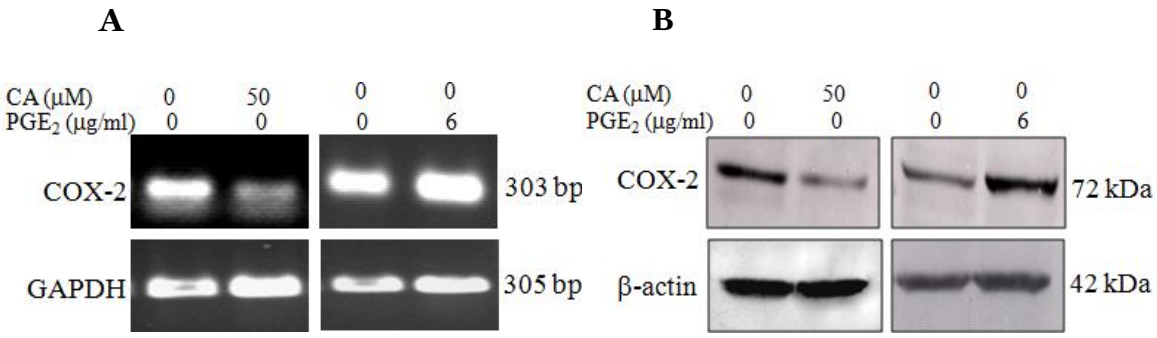


Figure 4.7: Effect of CA on COX-2 expression in HepG2 cells. *(A) RT-PCR analysis of CA and PGE_2 mediated effect on COX-2 mRNA expression in HepG2 cells. GAPDH was used as an internal control. (B) Western blot analysis of CA and PGE_2 mediated effect on COX-2 protein expression in HepG2 cells. β-actin was used as an internal control to monitor equal loading.*

4.3.6. Reduced expression of MDR1 by COX-2 siRNA: To further understand the role of COX-2 in the regulation of expression of MDR-1, the effect of COX-2 depletion on MDR1 expression was tested by the transfection of HepG2 cells with COX-2. Transfection of cells with COX-2 siRNA knockdown reduced the expression of COX-2 as well as MDR1 (Figure 4.8). These results show that CA regulates MDR1 expression by inhibition of COX-2 enzyme as well as by downregulation of its expression.

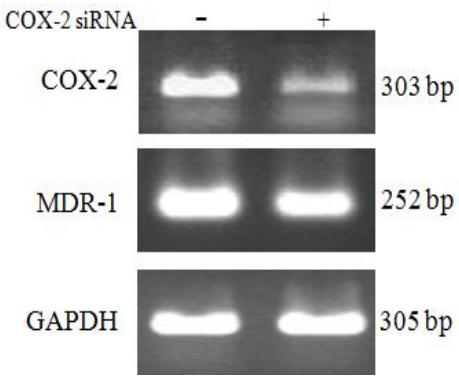


Figure 4.8. Effect of COX-2 knockdown on MDR1 expression in HepG2 cells. *RT-PCR analysis of COX-2 knockdown mediated effect on MDR1 and COX-2 mRNA expression in HepG2 cells.*

4.3.7. CA induced down regulation of MDR1 expression is mediated by signal transduction pathways involving Akt/NF-κB and MAPK: In view of the fact that MAP kinases and Akt play an important role in the development of multi-drug resistance [232, 233], the effect of CA on these signaling mediators was checked. Treatment of HepG2 cells with CA decreased the phosphorylated levels of Akt, JNK, ERK and p38 (Figure 4.9). These MAP kinases and AKT inturn activate NF-κB, a downstream transcription factor which plays a major role in the regulation of cell survival and proliferation and is also a positive regulator of MDR1. NF-κB activity is associated and tightly controlled by an inhibitory subunit, IκB, which is present in the cytoplasm in an inactive form. However, once IκB is phosphorylated, it targets its proteolysis which in turn allows NF-κB translocation to the nucleus, where it activates the transcription of NF-κB-responsible genes. So we next examined NF-κB translocation to the nucleus by checking the levels of p50 and p65 subunits in the nuclear fractions by Western blots. Nuclear levels of both NFκB, p50 and p65 subunits were reduced with CA treatment compared to that of untreated control (Figure 4.10).

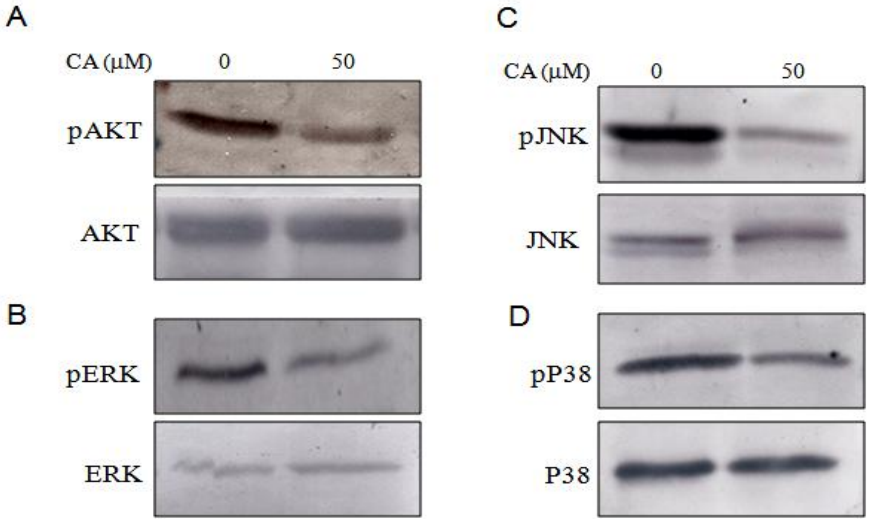


Figure 4.9. Effect of CA on phosphorylation of Akt, ERK, JNK and p38 in HepG2 cells. *Cells were treated with CA (50 μM) for 48 h and aliquots of cell lysates were resolved by SDS-PAGE and analyzed for p-Akt, Akt (A); p-JNK, JNK (B); p-ERK, ERK (C) and p-P38, P38 (D) protein expression by western blotting.*

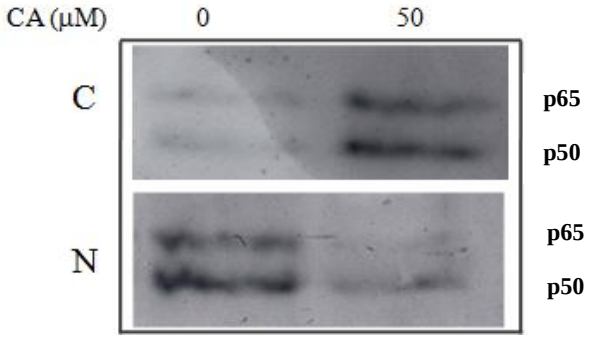


Figure 4.10. Effect of CA on nuclear translocation of NF-κB p65 and p50 subunits in HepG2 cells. *Western blot detection of NF-κB p65 and p50 subunits in the nuclear (N) and cytoplasmatic (C) extracts from HepG2 cells treated with 50 μM of CA for 48 h.*

4.4. Discussion

Drug resistance of tumor cells is recognized as the primary cause of failure of chemotherapeutic treatment of most human tumors [234]. P-glycoprotein a drug transporter protein encoded by MDR1 has been recognized as one of the major causes for the acquisition of the multidrug resistant phenotype of cancer cells [235]. The role of MDR1 in protecting cells from apoptosis induced by chemotherapy has been demonstrated in several cellular systems [236]. Potential circumvention of this problem requires identification of novel molecules that overcome MDR by inhibiting or suppressing the expression of MDR transporters [237-239]. Recent studies show that the activation of cyclooxygenase system might play a significant role in the development of MDR-1 mediated drug resistance [91, 119, 44]. Selective COX-2 inhibitors have been shown to have strong chemopreventive actions against colon cancers in animals and patients with familial adenomatous polyposis [240, 241]. The role of COX-2 inhibitors in overcoming the p-glycoprotein mediated drug resistance has been reported in epileptic brain of rats [242]. Recent studies from our laboratory have shown that celecoxib, a selective COX-2 inhibitor, enhanced the accumulation of Dox and down regulated the levels of MDR1 in HepG2 cells in a dose dependent manner [91].

In this study we show that CA, a COX-2/5-LOX dual inhibitor enhances the cytotoxic effect of Dox in human hepatocellular carcinoma (HepG2) cells by modulating MDR-1. Many synthetic MDR modulators including first and second generation reversal agents comprising drugs like verapamil, cyclosporine A,

quinidine or analogues of the first-generation drugs like dexverapamil, valspodar, cinchonine have been effective in overcoming MDR [212]. However, their harmful effects on health such as immunosuppression and cardiomyopathy and their predominantly negative therapeutic outcomes in *in vivo* studies due to dose restrictive toxicity limit their clinical success in cancer chemotherapy [220]. In view of this problem, there has been an increased interest in the use of natural compounds for the treatment of cancer patients, who constitutively express P-glycoprotein and are resistant to many synthetic chemotherapeutic agents. Many phytochemicals have been shown to counteract the cardiotoxic side effects of cancer chemotherapy [243]. While the current use of these phytochemicals as drugs is at minimal, intense rise in the undesirable side effects of synthetic chemotherapeutic drugs has promoted research on natural products to combat drug resistance. The multifactorial nature of hepatocellular carcinoma drug resistance and the role of plant polyphenols in targetting some of the principal tumor targets to overcome drug resistance has been discussed in a review by Natale D'Alessandro [244]. Various natural products well known for their antioxidant and anti-inflammatory properties have been proven as effective MDR modulators [245-247, 223, 224]. Recent reports bring to light that combination chemotherapy is a better approach for cancer therapy than single agent. In this connection natural products with known anticancer properties could be the first choice for the combination chemotherapy as they are known to reduce the systemic toxicity of the chemotherapeutic agents [248-250]. Consistent with the above background we have used CA, a benzopyran tannin present in the fruits of medicinal

plant *Terminalia chebula*, for the current study. We hypothesized that CA could be useful in enhancing the efficacy of cancer chemotherapeutic agents in liver cancer treatment. Earlier investigations from our laboratory indicate that CA is a COX-2/5-LOX dual inhibitor with anti-inflammatory and anticancer properties [96, 97].

In this study we showed strong synergistic therapeutic effect of CA in combination with Dox in HepG2 cells. We found that *in vitro* therapeutic effect of Dox in terms of 50 % cell growth inhibition (IC_{50}) at 3 μM dose could be achieved at its one-thirtieth concentration (100 nM) in combination with 25 μM CA in HepG2 cells. When analyzed by the isobologram method [228], the CI value for this combination was 0.55, thus indicating synergism. A plot of CI isobologram for CA-Dox at various other combinations showed strong synergistic effects with combination index values of less than 0.8 (Figure 4.3). Similar synergistic effect of grape seed extract with Dox was reported in human breast carcinoma cell lines [249] and by tannic acid in human cholangiocarcinoma [250]. Furthermore, calculation of the dose reduction index (DRI) showed a significant decrease in the dosage of Dox in the presence of CA with strongest effect observed at a combination of 50 μM CA with 10 nM Dox, where the dosage of Dox to cause 70 % growth inhibition reduced by 354 folds when compared to Dox alone. These results showing a strong synergistic therapeutic effect of CA and Dox in HepG2 cells, validate our hypothesis that combination of a natural product like CA might reduce the dosage of the conventional chemotherapeutic agents like Dox in liver cancer traetment, minimizing their toxic effects and thereby enhancing their therapeutic efficacy.

The enhanced efficacy of Dox in the presence of CA may partly be due to increase in the intracellular concentration of Dox, as the major factor in the resistance of cancer cells is reduced drug accumulation [251]. Consistent with this notion, we evaluated the effect of CA on intracellular accumulation of Dox in HepG2 cells. As expected, treatment of HepG2 cells with CA showed a dose dependant increase in the accumulation of Dox, which could be responsible for enhanced sensitivity of HepG2 cells to Dox. Similar increase in the accumulation of rhodamine 123 and Dox with other natural antioxidants was shown earlier in human cervical and breast cancer cell lines [252, 223]. Additional studies identifying the mechanism of the observed effect showed that CA downregulated the expression of MDR-1 and COX-2 at both mRNA and protein levels. This down regulation of MDR1 by CA appears to be dependent on COX-2. The fact that CA, a COX-2/5-LOX dual inhibitor, inhibited the expression of MDR-1, PGE_2 a product of COX-2 increased the expression of MDR-1 at both RNA and protein levels, further confirms the role of COX-2 in the regulation of MDR-1 in HepG2 cells. Similar downregulation of MDR-1 by celecoxib (a COX-2 inhibitor) and its upregulation by PGE_2 has been reported in our ealier studies [91]. Similar results were shown in rat glomerular mesangial cells, where in the transfection of COX-2 expression vector resulted in increased expression of MDR1 and its expression was decreased with NS-398 treatment [253]. In addition, inhibition of EGF-induced MDR1 mRNA over-expression by structurally different cyclooxygenase inhibitors (Indomethacin, Meloxicam, NS-398), leading to the accumulation rhodamine 123 in rat primary hepatocyte cultures has also been reported [254]. The

role of COX-2 in the regulation of MDR1 expression was further supported by the observed decrease in the expression of MDR-1 by COX-2 siRNA.

Signaling pathways that govern cell proliferation, survival and oncogenesis involving MAPK and PI3K-Akt pathways have been shown to play an important role in the development of multidrug resistance [232, 233]. Hence, we checked the effect of CA on these signaling mediators. Our data clearly show inactivation of MAPK and Akt pathways in parallel with inhibition of MDR1 with CA treatment in HepG2 cells. The observed decrease in the COX-2, phosphorylation of ERK, p38, JNK and Akt in HepG2 cells treated with CA may have effect on the translocation of NF-κB to the nucleus. Hence, further studies were taken up on NF-κB one of the key transcription factor regulating the expression of drug transporters [255]. Western blot analysis, showed a marked inhibition in the nuclear translocation of the p65 and p50 subunits of NF-κB with CA treatment. Inhibition of p65 translocation has been implicated in enhancing chemosensitivity of chemotherapeutic drugs [256-258]. Similar down regulation of MAPK and Akt/NF-κB activity in MDR expressing cancer cell lines by natural antioxidants with anti-inflammatory properties was reported [259, 260].

In summary our results clearly demonstrate that CA potentiates the sensitivity of Dox in HepG2 cells by regulating the expression of MDR1 in a COX-2-dependent manner. Moreover, NFκB mediated signal transduction pathway involved in the regulation of MDR1, was found to be inhibited by CA treatment. Further studies revealed the inhibition of phosphorylation of Akt and MAP kinases to be involved in CA mediated effects. Schematic representation showing the proposed mechanism

involved in the regulation of MDR1 expression by COX-2 and site of interference by CA is presented in Figure 4.11.

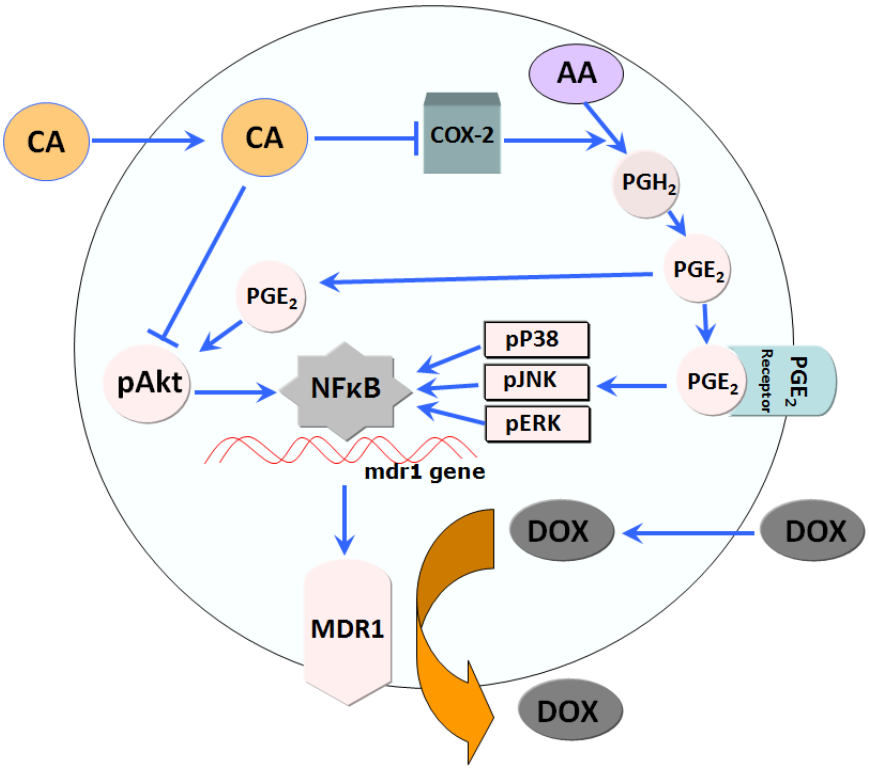


Figure 4.11. *Schematic representation showing the proposed mechanism involved in the regulation of MDR1 expression by COX-2 and site of interference by CA.*

Summary & Conclusions

Eicosanoids, a family of oxygenated metabolites of eicosapolyenoic fatty acids such as arachidonic acid, formed via the lipoxygenase (LOX), cyclooxygenase (COX) and epoxygenase (EPOX) pathways, play an important role in the regulation of various pathophysiological processes, including inflammation and cancer. Cyclooxygenase-2 (COX-2), the inducible isoform of COX, has emerged as the key enzyme regulating inflammation and promises to play a greater role in cancer. Though non-steroidal anti-inflammatory drugs (NSAIDs) are in use for centuries, the COX-2 selective inhibitors (COXIBs) have emerged as potent anti-inflammatory drugs with less gastric side effects. As COX-2 plays a major role in neoplastic transformation and cancer growth by down regulating apoptosis and promoting angiogenesis, invasion and metastasis, COXIBs have a potential role in the prevention and treatment of cancers. Recent studies indicate their possible application in overcoming drug resistance by down regulating the expression of MDR-1. The cardiac side effects of some of the COXIBs, however, have limited their application in treating various inflammatory disorders and warranted the development of COX-2 inhibitors without side effects. Enhanced production of leukotrienes as a result of diversion of substrate AA towards 5-LOX pathway was also attributed to some of the side effects associated with the usage of COX-2 inhibitors. As a result pharmaceutical companies started their attention towards the development of COX-2/5-LOX dual inhibitors (CLOXIBs). Licofelone, a COX-2/5-LOX dual inhibitor, is one such promising candidate drug, that has passed in Phase III clinical trials, which decreases the production of pro-inflammatory prostaglandins and leukotrienes and has the

potential to combine good analgesic and anti-inflammatory effects with excellent GI tolerability. Recent studies, however, indicate that licofelone suppresses PGE_2 formation by inhibiting mPGES-1 than COX-2.

The natural products isolated from the medicinal plants are proving to be good alternatives to the synthetic chemicals, with little or no side effects. We have earlier reported the anti-cancer effects of natural compounds like c-Phycocyanin (COX-2 inhibitor) and betanin (5-LOX inhibitor). Chebulagic acid (CA), a natural compound with COX-2/5-LOX dual inhibition properties, was recently isolated from *Terminalia chebula*. CA was shown to exhibit potent anti-inflammatory and anti-cancer effects in pre-clinical studies, thus offering the potential advantage of minimizing COX-2 dependent side effects. These studies provide a rationale for the potential use of CA in the treatment of cancer and other inflammatory disorders. The present study was undertaken to evaluate the efficacy of CA in overcoming alveolar lung injury in animal models. Also studies were undertaken to evaluate its anti-cancer effects *in vitro*.

- **Chebulagic acid ameliorates acute lung injury induced by LPS:** In this study, anti-inflammatory properties of CA were studied *in vivo*, in LPS-induced acute lung injury (ALI) model in mice to develop the potentially therapeutic compounds for the treatment of pulmonary inflammation. Acute lung injury (ALI) is characterised by acute lung inflammation involving the local recruitment and activation of polymorphonuclear leukocytes (PMNLs) and release of pro inflammatory mediators

such as tumour necrosis factor-α (TNF-α) and Interleukin-1β (IL-1β), proteases and reactive oxygen and nitrogen species. Serious ALI can lead to pulmonary edema, acute respiratory distress syndrome (ARDS) and finally respiratory failure. Acute pulmonary inflammation was induced by intratracheal instillation with LPS for 12 h, as it is a widely accepted clinically relevant model of severe lung injury. Alveolar macrophages and neutrophils are the main sources of pro-inflammatory and anti-inflammatory cytokines, and their activation is critical in the development of ALI. We found that pre-treatment with a single dose of 50 mg/kg of CA, 2 h prior to LPS instillation, significantly reduced the LPS-induced inflammatory cells, including neutrophils and macrophages in bronchoalveolar lavage fluid (BALF). Consistent with histological analysis of the lung CA treatment successfully ameliorated lung inflammation and reduced the infiltration of inflammatory cells induced by LPS in the lung tissue. As another index of ALI by LPS, we measured the total protein content in the BALF, which indicates epithelial permeability and pulmonary edema. We observed that CA markedly prevented LPS-induced increase in total protein in the BALF. These findings confirm that the protective effect of CA on ALI induced by LPS is related to an attenuation of inflammatory cell sequestration and migration into the lung tissue as well as decrease in epithelial permeability.

Oxidative damage is a major cause of lung injury during ALI/ARDS. Glutathione is known to be a major low molecular weight scavenger of free radicals in the cytoplasm. In this study, CA was quite effective in reducing the LPS induced oxidative stress in the lung tissue by modulating the anti-oxidant defences in the lungs

through elevation of tissue glutathione redox status. The pro-inflammatory cytokines, prostaglandins, and NO produced by activated macrophages play critical role in inflammatory diseases such as sepsis and arthritis. In the present study, we found that CA significantly inhibits the LPS induced expression of various pro-inflammatory markers like IL-1β, IL-6, TNF-α, iNOS and COX-2.

In order to understand the signaling mechanisms involved in the anti-inflammatory properties shown by CA in LPS induced ALI, further studies were undertaken on NF-κB that controls many pro-inflammatory genes. CA treatment effectively decreased NFκ-B activity by suppressing the nuclear translocation of p65 and p50 subunits, thereby resulting in the inhibition of LPS induced expression of pro-inflammatory cytokines and enzymes in the lungs of ALI mice.

MAP kinases play a critical role in the regulation of cell growth and differentiation, and they control cellular responses to cytokines and stress. In addition, they play a critical role in the modulation of NF-κB activity. To investigate the molecular mechanism of NF-κB inhibition by CA, in the present study, we investigated the effects of CA on the phosphorylation of MAP kinases in the lungs of LPS-stimulated ALI mice. Treatment with CA substantially inhibited JNK, ERK and p38 phosphorylation induced by LPS.

Further studies revealed CA mediated activation of Nrf-2, which plays an important role in protecting cells from oxidative stress, in the lungs of LPS induced mice. These studies reveal that CA attenuates LPS-induced pulmonary inflammation

through activation of Nrf-2 with simultaneous inhibition of NF-κB activation and MAP kinase phosphorylation. These studies indicate the potential use of CA in ALI. Further, in depth preclinical and clinical trials, however, are required to evaluate its potential as the anti-inflammatory drug without side effects.

• **Chebulagic acid exhibits anti-proliferative properties in human hepatocellular carcinoma cells: Involvement of Wnt/β-catenin signalling:** In order to evaluate the anti-proliferative effects of CA, further studies were taken *in vitro* on human hepatocellular carcinoma cell lines, HepG2 and Hep3B. Recently several lines of evidence have implicated that aberrant deregulation of Wnt signaling is a major mechanism of liver tumourigenesis and β-catenin was reported to be mutated and incongruously expressed in nucleus in 26-34% of human HCCs. Identification of effective antagonists of the Wnt/β-catenin signaling thus hold great promise for the successful management of hepatocellular carcinoma. For the past several years, intense efforts are being made to search for Wnt/β-catenin signaling antagonists among natural products. Our study provides the first experimental evidence for the anti-cancer effects of CA on hepatocellular carcinoma cells by regulating the Wnt/β-catenin pathway.

In the present study, CA effectively inhibited survival and proliferation of HCC cell lines in a dose dependant manner. Induction of apoptosis in both HepG2 and Hep3B cells was demonstrated by TUNEL assay and quantified by Flow Cytometry analysis of DNA content. TUNEL assay showed several apoptotic and

fragmented nuclei and Flow Cytometry analysis revealed typical sub-diploid apoptotic peaks in both HepG2 as well as Hep3B cells in response to CA treatment.

Given that β-catenin/TCF signalling and its gene products are known to regulate cell proliferation and apoptosis in HCC, we hypothesised that modulation of Wnt/β-catenin signaling may be one of the mechanisms implicated in suppression of cell proliferation in liver cancer by CA. Uncomplexed cytosolic β-catenin (free β-catenin) is the active form of β-catenin that translocates to the nucleus of the cell where it activates transcription factors of the TCF/LEF family, leading to the transcription of Wnt target genes. CA caused a considerable decrease in the protein levels of active β-catenin, through activation of GSK-3β and by transcriptional inhibition of β-catenin gene. Likewise, CA also increased the membrane localization and decreased the nuclear levels of β-catenin protein in both the hepatoma cells. An associated inhibition in the levels of β-catenin dependent TCF-4 and LEF-1 proteins, downstream transcription factors targeted by β-catenin, was also observed. Lastly, we observed that these cytotoxic effects correlated well with the decreased expression of cyclin-D1, c-Myc and COX-2, downstream target gene products of Wnt/β-catenin signaling. In conclusion, our data demonstrate the efficacy of CA in inhibition of cell proliferation and induction of apoptosis, through modulation of Wnt/β-catenin pathway in human liver cancer cells.

- **Chebulagic acid overcomes doxorubicin resistance in hepatocellular carcinoma: Studies on molecular mechanisms:** In this part, the anti-cancer

properties of CA were checked by studying its effects on multidrug transporter protein MDR-1, which is one the major drug transporter proteins that actively effluxes the drug from the cells, thereby causing resistance to various drugs. This study has been done in human hepatocellular carcinoma cell line, HepG2. Development of multidrug resistance is one of the major limitations in the treatment of hepatocellular carcinoma. MDR1 has been recognized as one of the major causes for the acquisition of the multidrug resistant phenotype of cancer cells. Potential circumvention of this problem requires identification of novel molecules that overcome MDR by inhibiting or supressing the expression of MDR transporters. In this study, we showed that CA enhances the cytotoxic effect of Dox, a standard anti-cancer drug in HepG2 cells by modulating MDR-1.

CA increased the accumulation of Dox in a concentration dependant manner and also enhanced the cytotoxicity of Dox in HepG2 cells by 20 folds. Quantitation of interaction by calculating Combination Index (CI) showed a strong synergistic interaction between CA and Dox in terms of cell growth inhibition. Calculation of dose reduction index (DRI) for CA-Dox combinations also showed a significant decrease in the dosage of Dox in the presence of CA.

Additional studies showed that CA downregulates the expression of MDR-1 and COX-2 at both mRNA and protein levels. This down regulation of MDR1 by CA appears to be dependent on COX-2. The fact that CA, a COX-2/5-LOX dual inhibitor, inhibited the expression of MDR-1, PGE_2 a product of COX-2 increased the expression of MDR-1 at both mRNA and protein levels, further confirms the role

of COX-2 in the regulation of MDR-1 in HepG2 cells. The role of COX-2 in the regulation of MDR1 expression was further supported by the observed decrease in the expression of MDR-1 by COX-2 siRNA.

Signaling pathways that govern cell proliferation, survival and oncogenesis involving MAPK and PI3K-Akt pathways have been shown to play an important role in the development of multidrug resistance. Hence, we checked the effect of CA on these signaling mediators. Our data clearly show inactivation of MAPK and Akt pathways in parallel with inhibition of MDR1 with CA treatment in HepG2 cells.

Further studies were taken up on NF-κB, one of the key transcription factor regulating the expression of drug transporters. Western blot analysis, showed that CA marked inhibition of nuclear translocation of the p65 and p50 subunits of NF-κB. The present study thus demonstrates the anticancer properties of CA and its efficacy to overcome MDR-1 mediated drug resistance in HepG2 cells through COX-2 dependant mechanism.

In conclusion, through *in vitro* and *in vivo* studies, the present study demonstrates the anti-inflammatory and anti-cancer properties of CA. Also CA was effective in overcoming MDR-1 mediated doxorubicin resistance in HepG2 cells. Further, the molecular mechanisms and signal transduction pathways involved in these anti-inflammatory and anti-cancer effects of CA were elucidated.

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PUBLICATIONS

1. **Chandrani Achari**, GV Reddy, TC Reddy, P Reddanna. (**2011**) Chebulagic acid synergizes the cytotoxicity of doxorubicin in human hepatocellular carcinoma through COX-2 dependant modulation of MDR-1. ***Medicinal Chemistry*** **(In press).**
2. **Chandrani Achari,** GV Reddy, TC Reddy, P Reddanna. **(2011)**Anti-tumor activity of a natural compound, Chebulagic Acid, as Wnt/β-catenin antagonist against human hepatocellular carcinoma cells. **(Communicated).**
3. **Chandrani Achari**, TC Reddy, P Reddanna (**2011**) Protective effects of chebulagic acid on acute lung injury induced by LPS in mice. **(Communicated).**
4. S Agarwal, **Chandrani Achari**, D Praveen, KR Roy, GV Reddy, P Reddanna (**2009**) Inhibition of 12-LOX and COX-2 reduces the proliferation of human epidermoid carcinoma cells (A431) by modulating the ERK and PI3K-Akt signalling pathways. ***Experimental dermatolog*** 18 (11): 939-946.
5. **Chandrani Achari**, Geetika Gupta, P. Reddanna (**2011**) Synergistic anticancer effects of baicalein and chebulagic acid in human breast carcinoma cells **(Manuscript under preparation).**
6. KR Roy, GV Reddy, S Agarwal, **Chandrani Achari**, L Maitreyi, S Vali, P Reddanna (**2010**) Celecoxib inhibits MDR1 expression through COX-2-dependent mechanism in human hepatocellular carcinoma (HepG2) cell line, ***Cancer Chemotherapy and Pharmacology*** 65: 903- 911.
7. NP Reddy, P Aparoy, TC Reddy, **Chandrani Achari**, P. Ramusridhar, P Reddanna. (2010) Design, synthesis and biological evaluation of prenylated chalcones as 5-LOX inhibitors. ***Bioorganic & Medicinal Chemistry*** 18:5807–5815.

8. TC Reddy, **Chandrani Achari**, P Reddanna (**2011**) Inhibitory effect of Gallic acid, an active constituent of Terminalia bellerica fruits on Carrageenan-induced Acute Inflammation., **(Manuscript under preparation).**
9. NP Reddy, TC Reddy, **Chandrani Achari**, P Reddanna (**2011**) Design, synthesis and biological evaluation of 1-Aryl-4-benzyloxybut-2-yn-1-ol, **(Manuscript under preparation).**
10. TC Reddy, DB Reddy, A. Aparna, KM Arunasree, **Chandrani Achari**, P. Aparoy , GV Reddy, V. Lakshmipathi, A. Subramanyam, P. Reddanna (**2011**) Gallic acid suppresses the growth and induces apoptosis in human leukemia K562 cells by downregulation of COX-2, inhibition of BCR/ABL kinase and NF-κB **(Under revision).**

Conference attended and Poster Presentation:

1. **Chandrani Achari**, Kishore, Geetika Gupta, Gorla Venkateswara Reddy, Pallu Reddanna "Development of Anti-inflammatory Drugs: Past, Present & Future", XXXIII All India Cell Biology Conference & International Workshop on Cell Cycle Regulation, School of Life Sciences, University of Hyderabad, December 10-13, 2009.
2. Gorla Venkateswara Reddy, **Chandrani Achari,** Pallu Reddanna "Role of reactive oxygen species and cyclooxygenase-2 in multidrug resistance" BIOQUEST 2009, School of Life Sciences, University of Hyderabad, January 24, 2009.

3. Smita Agarwal, **Chandrani Achari**, Karnati R. Roy, G. V. Reddy, P. Reddanna "Expression of enzymes involved in eicosanoid synthesis in Skin Cancer Cell line (A431): Effect of 12-LOX and COX-2 inhibition", BIOQUEST-2008, School of Life Sciences University of Hyderabad, March 15-16, 2008.
4. Smita Agarwal, **Chandrani Achari,** Karnati R. Roy, G. V. Reddy and P. Reddanna "Baicalein, the 12-Lipoxygenase inhibitor, and Celecoxib, the selective COX-2 inhibitor induce apoptosis in Skin cancer cell line (A431)", SBC Conference, S. V. University, Tirupati, November 25-27, 2007.
5. Participated in Indo German Workshop on Molecular Epidemiology of Infectious Diseases, University of Hyderabad, November 28-30, 2008.
6. Participated in International Conference on Novel updates in Reproductive Biology And Comparative Endocrinology And The 27th Annual Meeting of The Society For Reproductive Biology And Comparative Endocrinology (SRBCE-XXVII), University of Hyderabad, January 19-21, 2009.
7. Participated in 13th Human Genome Meeting "Genomics and the Future of Medicine", HICC, Hyderabad, September 27-30, 2008.
8. Participated in International Conference on Advances in Free Radicals Research, Natural Products, Antioxidants and Radioprotectors in Health & Ninth Annual Meeting of The Society of Free Radical Research India, Hotel Mariott Courtyard, Hyderabad, January 11-13, 2010.