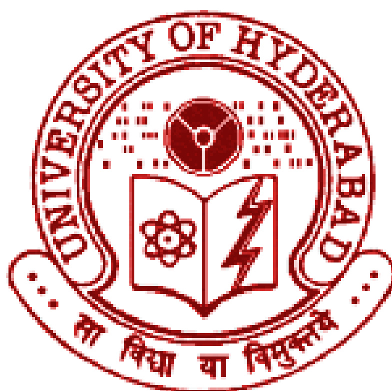
Isolation and Characterization of COX-2/5-LOX Dual Inhibitors from *Premna integrifolia:* Potential Drug Candidates for Treatment of Inflammatory disorders.

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

Raja Ram Azad



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1. **Azad R**, Babu NK, Gupta AD, Reddanna P. "Evaluation of anti-inflammatory and immunomodulatory effects of *Premna integrifolia* extracts and assay-guided isolation of a COX-2/5-LOX dual inhibitor." Fitoterapia. 2018 Nov; 131:189-199.

B. Presented in the following conferences:

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Further the student has passed the following courses towards fulfillment of coursework requirement for Ph.D.

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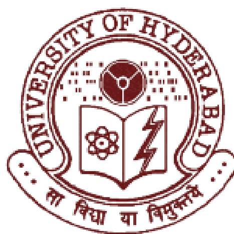

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I hereby declare that the work embodied in this thesis entitled **"Isolation and Characterization of COX-2/5-LOX Dual Inhibitors from *Premna integrifolia:* Potential Drug Candidates for Treatment of Inflammatory disorders"** has been carried out by me under the supervision of **Prof. P. Reddanna** and co-supervision of **Prof. Aparna Dutta Gupta,** this work has not been submitted for any degree or diploma of any other university earlier.

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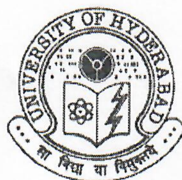

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3.	Anilkumar K, Reddy GV, **Azad R**, Yarla NS, Dharmapuri G, Srivastava A, Kamal MA, Pallu R. "Evaluation of Anti-Inflammatory Properties of Isoorientin Isolated from Tubers of *Pueraria tuberosa*". Oxid Med Cell Longev. 2017; 2017:5498054.	

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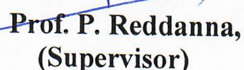

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Table of Contents

Chapter 1: General Introduction 1-22

1.1. INFLAMMATION

1.1.1. Introduction

1.1.2. Inducers of inflammation

1.1.3. Mediators of inflammation

1.1.4. Inflammatory disorders

1.2. EICOSANOIDS

1.2.1. Introduction

1.2.2. Cyclooxygenases (COXs)

1.2.3. Lipoxygenases (LOXs)

1.2.4. 5-Lipoxygenase

1.2.5. Role of COXs and 5-LOX in inflammation

1.3. NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs)

1.4. ALLERGY

1.4.1. Introduction

1.4.2. Major component of allergy:

1.4.3. Mast Cells (MCs)

1.4.4. Mast cells in host defense

1.4.5. Rat Basophillic Leukemia Cells (RBL-2H3 cells)

1.4.6. A23187, Calcium Ionophore

1.5. NATURAL PRODUCTS AS ANTI-INFLAMMATORY AGENTS

1.6. SCOPE AND OBJECTIVES OF WORK

Chapter 2: Evaluation of anti-inflammatory and immunomodulatory effects of *Premna integrifolia* extracts and assay-guided isolation of a COX-2/5-LOX dual inhibitor (CLOXIB). **23-51**

2.1 INTRODUCTION

2.2 MATERIALS AND METHODS

2.2.1 Reagents and chemicals

2.2.2 Collection of *Premna integrifolia* roots

2.2.3 Preparation of *Premna integrifolia* roots extracts

2.2.4 Fractionation of petroleum ether extract (PEE) through silica column chromatography

2.2.5 RP-HPLC column chromatography

2.2.6 Extraction and isolation of COX-1 from Ram seminal vesicles

2.2.7 Extraction and isolation of the COX-2 enzyme

2.2.7.1 Expression and isolation of COX-2

2.2.7.2 Cyclooxygenase (COX-1 and COX-2) assay

2.2.8 Purification and assay of 5-LOX

2.2.9 *In vitro* assays on RAW 264.7 cell line

2.2.9.1 Cytotoxicity Assay

2.2.9.2 Nitrite Estimation

2.2.9.3 Analysis of prostaglandin E_2, pro and anti-inflammatory cytokines

2.2.9.4 Preparation of whole cell lysates for protein expression studies

2.2.9.5 Lymphocyte proliferation assay (LPA)

2.2.9.6 *In vivo* assay-carrageenan-induced paw edema animal model

2.2.10 Statistical analysis

2.3 RESULTS

2.3.1 *In vitro* anti-inflammatory effects.

2.3.1.1 Effects of *Premna integrifolia* extracts on COX-1, COX-2 and 5-LOX activities

2.3.1.2 *In vitro* anti-inflammatory effects of various extracts of *Premna integrifolia* roots on mouse macrophage cell line, RAW 264.7

2.3.1.2.1 *Premna integrifolia* extracts showed very little or no cytotoxicity on RAW 264.7 cells

2.3.1.2.2 Effects of PEE on LPS-induced nitric oxide levels in RAW 264.7 cells

2.3.1.2.3 Effects of PEE on PGE_2 and pro-inflammatory cytokines in RAW 264.7 cells

2.3.1.2.4 Effects of PEE on the expression of iNOS, COX-2, 5-LOX, IL-1β, and TNF-α in LPS challenged RAW 264.7 cells

2.3.1.3 *In vitro* immunomodulatory and anti-inflammatory effects of PEE of *Premna integrifolia*

2.3.1.3.1 Immunomodulatory effects of *Premna integrifolia* extracts on lymphocytes

2.3.1.3.2 Effects of PEE on IL-2 production in RAW 264.7 cell line

2.3.1.4 *In vivo* effects of PEE on Carrageenan-induced paw edema in BALB/c mice

2.3.2 Isolation and characterization of COX-2/5-LOX dual inhibitor(s) from PEE of *Premna integrifolia*

2.4. DISCUSSION

Chapter 3: 52-81

(A) **Evaluation of anti-inflammatory effects of 6-hydroxy salvinolone (6-HS), a COX-2/5- LOX dual inhibitor, *in vitro* on mouse peritoneal macrophage/RAW 264.7 cell line and *in vivo* on paw edema and air pouch models of inflammation.**

(B) **Evaluation of anti-allergic effects of 6-HS on RBL-2H3 cell line *in vitro* and *in vivo* on passive systemic anaphylaxis mouse model.**

3.1 INTRODUCTION

3.2 MATERIALS AND METHODS

3.2.1 Chemicals and Reagents

3.2.2 Cell culture

3.2.3 Isolation of Mouse Peritoneal Macrophages

3.2.4 Cytotoxicity Assay of 6-HS on mouse peritoneal macrophages and RAW 264.7 cells

3.2.5 Nitrite Estimation

3.2.6 Analysis of IL-1β.

3.2.7 Analysis of the NF-κB translocation through confocal microscopy

3.2.8 mRNA expression studies in LPS challenged RAW 264.7 cells : Real time-Polymerase Chain Reaction

3.2.9 Preparation of whole cell lysate for immunoblots analysis

3.2.10 *In vitro* anti-allergic studies on RBL-2H3 cell line

3.2.11 Cytotoxicity assay in presence of 6-HS and two degranulating stimuli on RBL-2H3 cell line

3.2.12 In vitro *β*-hexosaminidase Quantification

3.2.13 *In vitro* histamine quantification

3.2.14 *In vitro* Leukotriene C_4 quantification

3.2.15 Animal Experiments

3.2.16 *Paw edema model* for acute inflammation

3.2.17 Mouse air pouch model of inflammation

3.2.18 Administration of 6-HS

3.2.19 Histopathology of mouse air pouch tissue

3.2.20 Immunoblot analysis for expression of various pro-inflammatory markers in air pouch Tissue

3.2.21 Mouse model for induction of passive systemic anaphylaxis.

3.2.22 Determination of histamine release *in vivo* animal model

3.2.23 Statistical analysis

3.3 RESULTS

3.3.1 Evaluation of anti-inflammatory effects of 6-HS *in vitro* on mouse peritoneal macrophages/RAW 264.7 cell line and RBL-2H3 cells.

3.3.1.1 Effect of 6-HS on mouse peritoneal macrophages/RAW 264.7 cell viability

3.3.1.2 6-HS reduced NO and IL-1β secretion in LPS stimulated mouse peritoneal macrophages

3.3.1.3 Effect of 6-HS on LPS induced NF-kB nuclear translocation in RAW 264.7 cell line

3.3.1.4 Effect of 6-HS on expression of pro-inflammatory markers at mRNA and protein levels in LPS stimulated RAW 264.7 cells

3.3.2 Evaluation of anti-inflammatory effects of 6-HS *in vivo* on paw edema and air pouch inflammatory mouse models.

3.3.2.1 *In vivo* effects of 6-HS on Carrageenan-induced paw edema in BALB/c mice

3.3.2.2 Evaluation of anti-inflammatory effects of 6-HS *in vivo* on mouse air pouch model

3.3.2.2.1 Inflammatory reaction

3.3.2.2.2 Leukocytes infiltration into the air pouch tissue

3.3.2.2.3 Histological observation of neutrophils infiltration in air pouch tissue

3.3.2.2.4 Effect of 6-HS on expression of pro-inflammatory proteins (COX-2, iNOS, 5-LOX, TNF-α and IL-1β) in air pouch tissue.

3.3.3 Evaluation of anti-allergic effects of 6-HS on RBL-2H3 cell line *in vitro* and *in vivo* on passive systemic anaphylaxis mouse model.

3.3.3.1 Effect of 6-HS on cell viability of calcium ionophore (A23187) or IgE/antigen challenged RBL-2H3 cells

3.3.3.2 Effect of 6-HS on release of β-hexosaminidase, histamine and leukotriene C_4 (LTC_4)

3.3.3.3 Effect of 6-Hydroxy salvinolone on change in body temperature after passive systemic anaphylaxis reaction

3.3.3.4 Effect of 6-HS on change in plasma histamine concentration after passive systemic anaphylaxis reaction

3.4 DISCUSSION

Chapter 4: Summary and Conclusions 82-89

4.1 Discovery and Development of anti-inflammatory drugs: Eicosanoids as the targets

4.2 Petroleum ether extract (PEE) of *Premna integrifolia* showed anti-inflammatory and immunomodulatory properties

4.3 Assay guided purification of 6-hydroxy salvinolone (6-HS) from PEE of *Premna integrifolia*

4.4 6-hydroxy salvinolone (6-HS), a COX-2/5-LOX dual inhibitor, is anti-inflammatory agent

4.5 6-HS showed anti-allergic activity *in vitro* and *in vivo*

REFERENCES 90-100

Index 101

List of Publications and Manuscripts 102

List of Figures

Figure 1-1:	**Physiological and pathological outcomes of inflammation**	1
Figure 1-2:	**Process of inflammation**	2
Figure 1-3:	**Mediators of inflammation**	6
Figure 1-4:	**Disorders of inflammation**	7
Figure 1-5:	**Arachidonic metabolism**	8
Figure 1-6:	**Machanism of action of NSAIDs**	12
Figure 1-7:	**Global asthma prevalence in adults**	13
Figure 1-8:	**Route of exposer and allergic manifestations**	14
Figure 1-9:	**Role of mast cells in allergic inflammation.**	17
Figure 2-1:	**Anti-inflammatory effects of *Premna integrifolia* roots extracts on COX-1, COX-2 and 5-LOX.**	34
Figure 2-2:	**Anti-inflammatory effect of *Premna integrifolia* root extracts on LPS challenged mouse macrophage cell line, RAW 264.7 cells.**	37
Figure 2-3:	**Immunomodulatory and anti-inflammatory effects of *Premna integrifolia* extracts.**	39
Figure 2-4:	**Effect of *Premna integrifolia* PEE on carrageenan induced paw edema in BALB/c mice.**	41
Figure 2-5A:	**Isolation of active principle(s) from *Premna integrifolia*: Schematic Presentation**	42
Figure 2-5B:	**Isolation of active principle(s) from *Premna integrifolia*: RP-HPLC analysis**	44
Figure 2-6:	**Details of (A) 1H-NMR (Proton Nuclear Magnetic Resonance)**	

	spectra; (B) C-13-NMR (Carbon-13 Nuclear Magnetic Resonance) spectra; (C) HR-MS (High Resolution Mass Spectrometry) and (D) Infrared Spectroscopy of peak H3 from RP-HPLC.	**46**
Figure 2-7:	**Characterization of COX-2/5-LOX dual inhibitor, 6-hydroxy salvinolone (6-HS), and its effects on COX-1, COX-2 and 5-LOX.**	**47**
Figure 3-1:	**Schematic representation of RBL-2H3 basophils' degranulation assay using calcium ionophore (A23187) (A) or IgE/antigen as stimuli.**	**58**
Figure 3-2:	**Effect of 6-HS on cell viability. Mouse peritoneal macrophages and RAW 264.7 cells.**	**64**
Figure 3-3:	**Effect of 6-HS on LPS induced nitric oxide and IL-1β released from mouse peritoneal macrophages.**	**65**
Figure 3-4:	**6-HS inhibits translocation of NF-κB in LPS stimulated RAW 264.7 cell line.**	**66**
Figure 3-5:	**Effect of 6-HS on expression of mRNA of (A) COX-2, (B) iNOS, (C) 5-LOX, (D) IL-1β and (E)TNF-α and protein expression (F) in LPS challenged RAW 264.7 cells.**	**67**
Figure 3-6:	**Effect of 6-HS on carrageenan induced paw edema in Balb/c mice.**	**68**
Figure 3-7:	**The photographs of air pouch tissue after 24 h treatment with saline (A), Carrageenan alone (B), Celecoxib + Carrageenan (C) and 6-HS + Carrageenan (D).**	**70**
Figure 3-8:	**Effect of 6-HS on number of cells infiltrated into the pouch tissue of either carrageenan or carrageenan + 6-HS treated mice.**	**70**
Figure 3-9:	**The photomicrographs (20x) of histological sections of the air pouch tissue after 24 h treatment with saline group (A), Carrageenan alone**	

(B), Celecoxib + Carrageenan group (C) and 6-HS+Carrageenan (D). **71**

Figure 3-10: **Effect of 6-HS on carrageenan induced protein expression in iNOS, COX-2, 5-LOX, TNF-α and IL-1β in air pouch tissue of mouse model.** **72**

Figure 3-11: **Effect of 6-HS on cell viability of calcium ionophore or IgE/antigen challenged in RBL-2H3 cells.** **73**

Figure 3-12: **Effect of 6-HS on release of β-hexosaminidase, histamine and LTC_4.** **74**

Figure 3-13: **Schematic representation of passive systemic anaphylaxis using IgE/antigen as stimuli.** **75**

Figure 3-14: **Effect of 6-HS on change in body temperature of BALB/c mice after the passive systemic anaphylaxis reaction.** **76**

Figure 3-15: **6-HS inhibits IgE mediated histamine release in passive systemic anaphylaxis in murine model.** **77**

Figure 4: **Schematic presentation showing the possible mechanisms behind the anti-inflammatory and anti-allergic properties of 6-hydroxy salvinolone (6-HS), isolated from the young roots of *Premna integrifolia*.** **89**

List of Tables

Table 2-1: **Data showing IC_{50} values (µg/mL) of extracts of *Premna integrifolia*, petroleum ether, ethyl acetate, methanol and water on 5-LOX, COX-1 and COX-2 enzymes.** **35**

Table 2-2: **Effect of different fractions of petroleum ether extracts of *Premna integrifolia* on open silica column on 5-LOX, COX-1 and COX-2 enzymes.** **43**

Table 2-3: **IC_{50} values for 5-LOX, COX-1 and COX-2 by different peaks- H1, H2, H3 and H4 obtained on RP-HPLC (Figure 2-5.B) of the fraction H from open column chromatography, as shown in Figure 2-5.A.** **45**

Table 3: **List of Primers used for Real Time PCR** **56**

List of Abbreviations

AA	:	Arachidonic acid
BSA	:	Bovine serum albumin
COX	:	Cyclooxygenase
DAPI	:	4',6-diamidino-2-phenylindole
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethyl sulfoxide
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme linked immunosorbent assay
FBS	:	Fetal bovine serum
g	:	Gram
h	:	Hour(s)
IL-1β	:	Interleukin-1β
iNOS	:	Inducible nitric oxide synthase
kDa	:	Kilo Dalton
LOX	:	Lipoxygenase
LPS	:	Lipopolysaccharide
LT	:	Leukotriene
mg	:	Milli gram
min	:	Minute(s)
mL	:	Milli liter
mM	:	Milli molar
mRNA	:	Messanger Ribonucleic acid

MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium bromide
nm	:	Nano meter(s)
NO	:	Nitric oxide
NSAIDs	:	Non-steroidal anti-inflammatory drugs
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PG	:	Prostaglandin
RP-HPLC	:	Reverse phase-High pressure liquid chromatography
SDS	:	Sodium dodecyl sulfate
Tris	:	Tris-(Hydroxymethyl) aminomethane
µM	:	Micro molar
°C	:	Degree Centigrade/ Degree Celsius

Chapter 1

GENERAL INTRODUCTION

1.1 INFLAMMATION

1.1.1 Introduction

Inflammation is the immune response of the body towards damage of its cells, tissues or organs by harmful pathogens, chemicals or physical injury [1]. It is a protective mechanism adopted by the organism to eliminate the harmful and injurious stimuli in order to initiate the healing process. When inflammation remains for longer time, chronic inflammation may lead to tissue injury and can cause physiological problems, organ dysfunction and even death. Inflammation is closely linked with immune response (Figure 1-1). The five basic symptoms of inflammation include pain, heat, swelling, redness and malfunction.

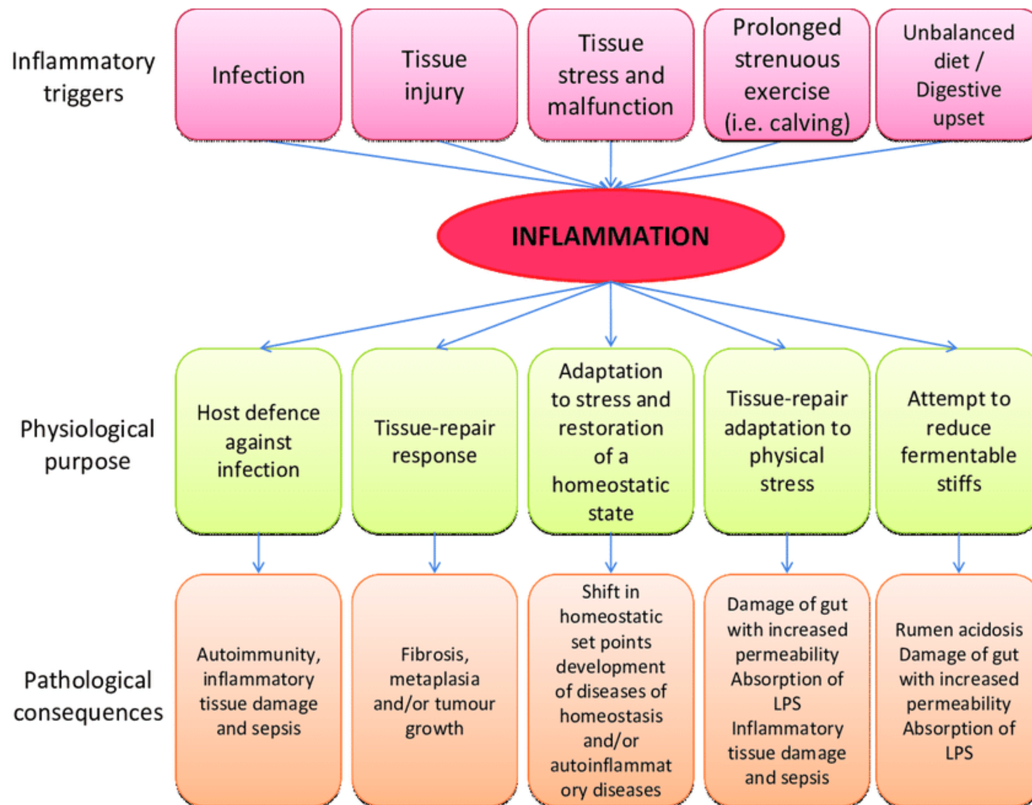


Figure 1-1: Physiological and pathological outcomes of inflammation *(Adopted from Medzhitov, Nature. 2008)*

Inflammation is marked by vasodilation, leukocytes infiltration and edema. Inflammatory vasodilatation is characterized by warmth and redness. The vasodilation facilitates delivery of inflammatory cells and soluble mediators at the site of inflammation. The leucocytes play major role in inflammatory response; they move from blood vessels to injured tissue for phagocytic activities by picking up unwanted substances such as harmful bacteria and own cellular debris. These prevent spreading of infection by forming barrier. This complex response leads to migration of cells, fluid and proteins from the blood plasma into the damaged tissues (Figure 1-2).

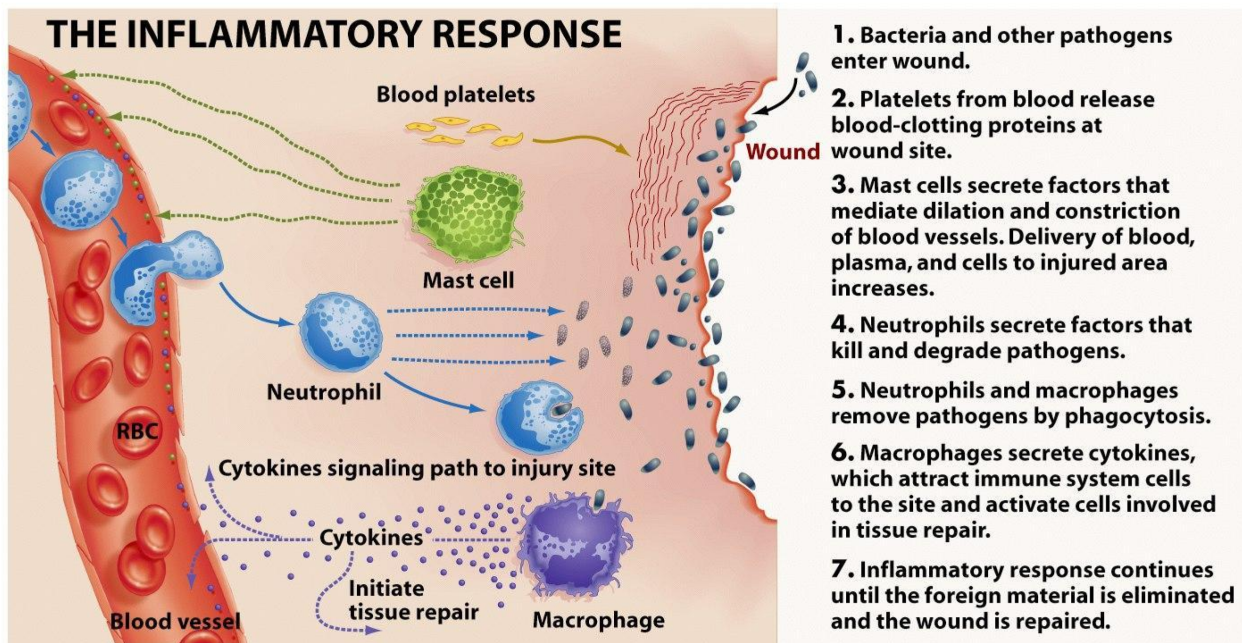


Figure 1-2: Process of inflammation *(Source: biological Science, 2005 Pearson Prentice Hall, Inc.)*

The vasodilation is the main feature of inflammatory response. It leads broadening of the blood vessels and increases flow of blood to the injured area of the tissue, due to which diffusible substances enter the site of injury. It leads to passage of inflammatory cells through thin cell wall of blood vessels or infiltrate these cells via chemo taxis, finally lead to activation of defense

mechanism by complex enzymes system of the plasma and immune system. Extent of these events depends on degree and condition of the injury and the infection. Inflammation is mainly categories in to two- acute and chronic, based on pathological features and duration. Short-duration is for acute inflammation but lead to a cascade of events that are initiated by immune system of organisms, its localized vascular system and cells within injured tissue, which results in healing of wound. Chronic inflammation is a prolonged reaction that involves tissue destruction and numerous events within body system, attempting to tissue repair. Chronic inflammation that persist for long are cause for several health issue like allergy, arthritis, artherosclerosis, autoimmune diseases, metastasis and cancer [2,3,4,5].

1.1.2 Inducers of inflammation

Inflammation can be induced by endogenous or exogenous agents.

Endogenous agents:

Endogenous substances that induce inflammation are signals produced under various physiological conditions by damaged tissues or malfunctioning tissues. These signals causing inflammation are still not well defined. These signals may be classified based on the nature and extent of tissue abnormality.

Exogenous agents

Exogenous agents can be classified mainly in two form that is microbial and non-mocrobial. Microbial factors can be distinguished into "pathogen associated molecular pattern" (PAMPs) and "virulence" factors. PAMPs are conserved molecular patterns of group of microorganisms of same class whether pathogenic or commensal [6]. The second class, the

virulence factors are not directly detected by receptors but show their adverse effects on host tissue that trigger the inflammatory response. Non-microbial exogenous agents include foreign bodies, irritants, allergens and toxic compounds.

1.1.3 Mediators of inflammation

Inflammatory agents start the synthesis of various mediators responsible for inflammation that change functioning of particular tissue and organs. These agents further affect other effectors that are involved in later stage in the inflammatory pathways. Majority of mediators involved in inflammatory responses are with similar effects with respect to vasodilation or for recruitment of the leukocytes from the blood. These mediators are mostly either plasma proteins or secreted from cells of local tissues. Inflammatory mediators are sometime preformed like histamine and serotonin or circulate in inactive form in the blood. Other mediators are directly produced during activation of inflammation. Inflammatory mediators are classified in to seven groups on the basis of their biochemical properties (Figure 1-3).

- First, lipid derived mediators are from phospholipids of the cellular membranes. Arachidonic acid (AA) is released from membrane due to action of cytosolic phospholipase A_2 ($cPLA_2$) and intracellular Ca2+ ions. AA is metabolized to form eicosanoids by the two main enzymes of arachidonic acid pathway, cyclooxygenase-1 and cyclooxygenase-2 that generate prostaglandins and thomboxanes and the lipoxygenases that convert AA to its metabolites LTs and lopoxin. The prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) are involved in vasodilation whereas PGE_2 also induces fever. Lipoxins on the other hand, play important contribution towards resolution of the inflammatory condition and tissue repairs [7].

- Second, inflammatory cytokines IL-1β, IL-6, TNF-α and others are produced mostly by macrophages and mast cells. These cytokines play crucial roles in inflammation, including modulation of leukocytes, initiation of acute phase inflammatory response.

- Third, the chemokine's that are produced by several cell types in response to initiation of inflammation. These are also responsible for migration of leukocytes towards the affected tissues.

- Fourth, C3a, C4a and C5a are the complement fragments mainly involved in vasodilation during inflammatory response and are part of complement activation. Among other complement fragments, C3a, C4a and C5a are also involved in recruitment of monocytes, granulocytes and involved in degranulation of mast cells.

- Fifth, Proteolytic enzymes like elastin, cathepsins have various roles in inflammation. These cause degradation of basement-membrane proteins and extracellular matrix. These proteases also have roles in host defense, leukocyte migration and tissue remodeling.

- Sixth, vasoactive amines like serotonin and histamine are released on degranulation of mast cells, basophils and platelets. These amines show complex effects on vascular system with respect to permeability [8]. The immediate release of these amines by mast cells in sensitized organism may result in serious health issue such as anaphylactic shock.

- Seven, the vasoactive peptides are generally stored in vesicles mean for secretion in active state and inactive precursors like fibrin, fibrinopeptide A and fibrinopeptide B, which are released in the extracellular fluid and become active after action of enzymes as per requirement.

Among these eicosanoids, the oxymetabolites formed via the cyclooxygenase and lipoxygenase pathways, form the key mediators of inflammation.

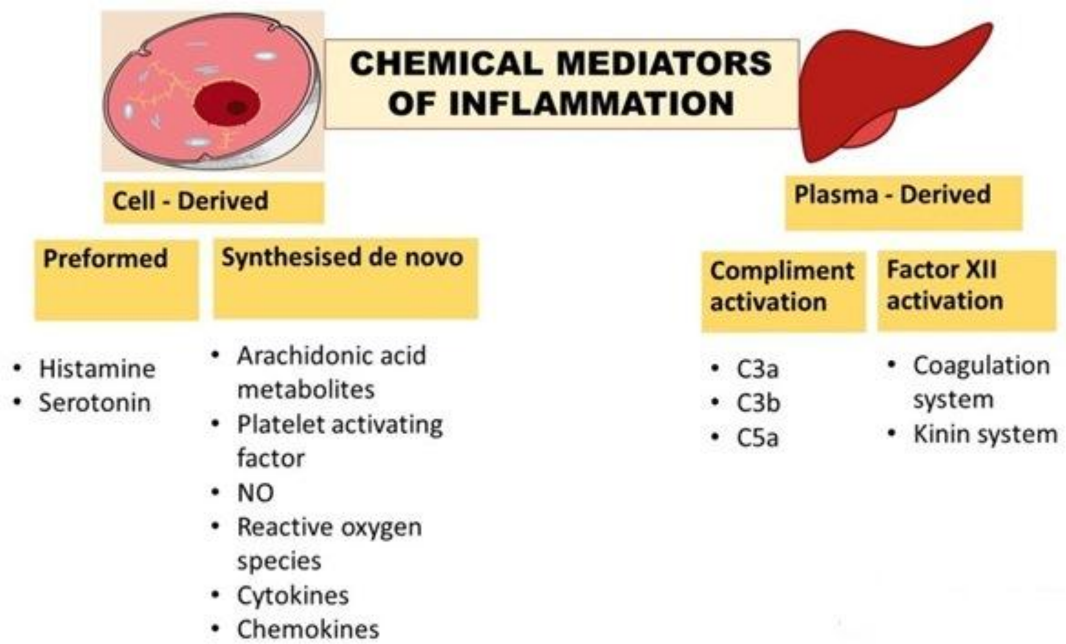


Figure 1-3: Mediators of inflammation *(Source: ilovepathology.com)*

1.1.4 Inflammatory disorders

Inflammatory disorders are very common and are due to uncontrolled inflammatory responses. Primarily, abnormalities associated with inflammatory disorder are mainly due to failure of immune system. Some time, the immune system itself becomes a cause for inflammatory disorders like allergic reactions, asthma and many immune disorders. Non-immune diseases like cancer, arthritis, atherosclerosis and heart diseases also have origin in inflammatory

processes. Inflammatory disorders may also result from mutation in proteins involved in normal physiological processes. Some of the inflammatory disorders are shown in figure 1-4.

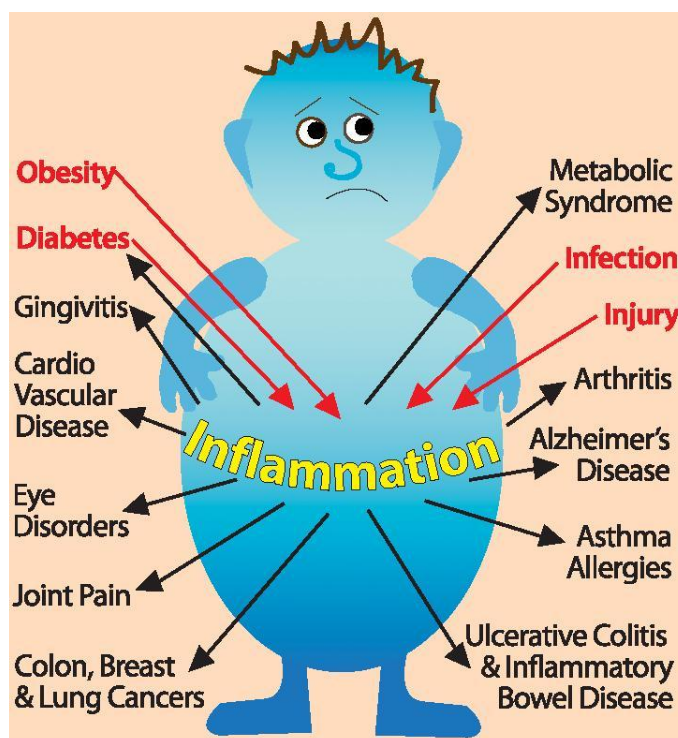


Figure 1-4: Disorders of inflammation *(Source: @ 2018 My Remedy).*

1.2 EICOSANOIDS

1.2.1 Introduction

Eicosanoids are biologically active lipid mediators that are involved in homeostasis as well as in the mediation of various pathological conditions like inflammation, allergy, asthma etc. Their biosynthesis is generally initiated by the action of PLA_2 that leads to the release of arachidonic acid from cell membrane phospholipids. The release of AA is oxygenated to prostaglandins, thromboxane and leukotrienes due to the action of two important enzymes of AA metabolic pathway- cyclooxygenase (COX) and lipoxygenase (LOX). Besides COXs and LOXs

enzymatic pathway, arachidonic acid may be oxygenated via epoxygenase enzyme pathwat through cytochrome P450s that produce epoxyeicosatrienoic acids (EETs). During inflammation, eicosanoid production increases considerably as a result of the induction of key enzymes involved in their biosynthesis (Figure 1-5). Eicosanoids are the most actively studied among all the factors that contribute to inflammation [9,10].

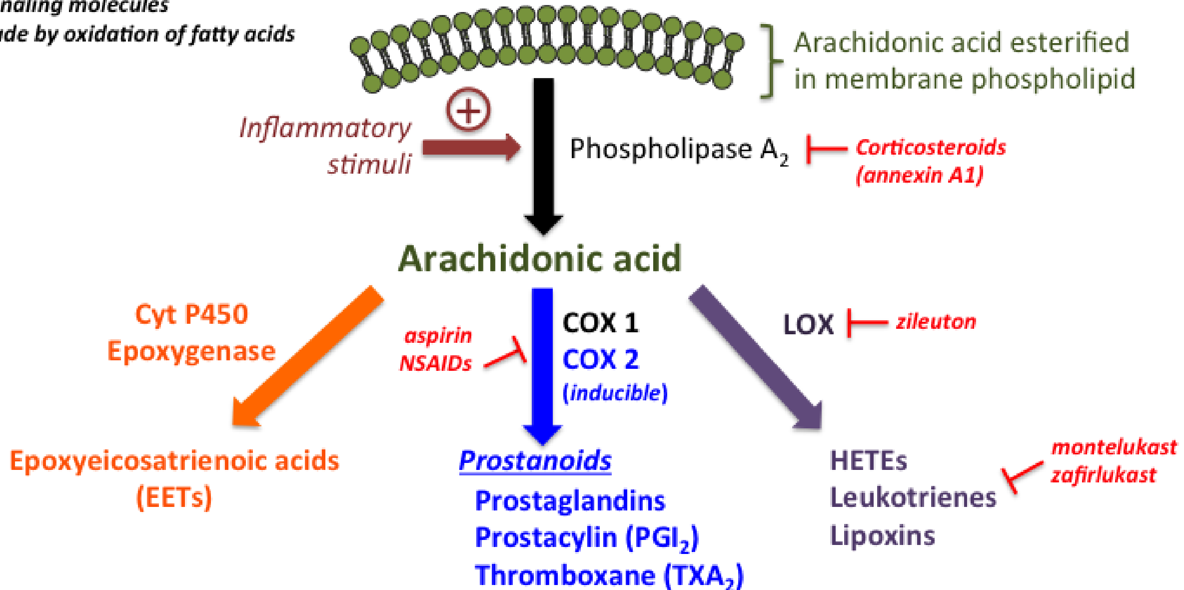


Figure 1-5: Arachidonic metabolism *(Source: TMedWeb)*

1.2.2 Cyclooxygenases

Cyclooxygenases (COXs), are the important enzymes that are responsible for rate limiting in the pathway of AA metabolism during the biosynthesis of prostanoids [11]. These COXs convert arachidonic acid to PGH_2 which in turn get metabolized to other prostaglandins, thromboxanes and prostacyclin depending upon tissue specificity, collectively called prostanoids. The prostanoids are important biological active molecules that show variety of

action. These enzymes show two different types of activity: cyclooxygenase activity and peroxidase activity [12,13]. COXs known to exist in two forms: COX-1 and COX-2. COX-1 enzyme is with "house-keeping" functions like vascular homeostasis, gastric protection and renal water reabsorption [14]. COX-2, on the other hand, is undetectable at normal condition but is induced under stress or stimuli such as inflammatory cytokines, growth factors, mitogenic or hormonal stimuli [15,16]. COX-1 and COX-2 enzymes share nearly 60% of primary sequence identity and their X-ray crystal structures are virtually superimposable [17]. Kinetic profiles of these enzymes show not identical but similar reaction mechanism. These isozymes show significant differences in pharmacological activities with independent role in cell physiology [18,17]. COX isoforms in purified form, show almost identical catalytic properties toward AA. These isoforms share all critical amino acids required for prostaglandin H2 synthesis from arachidonic acid. Both enzymes are glycoproteins [19], and show association with membranes of nucleus and endoplasmic reticulum. [20].

1.2.3 Lipoxygenases (LOXs)

Lipoxygenases are another important enzymes in AA metabolic pathway that consist of a family of non-heme iron containing dioxygenases. These add oxygen to unsaturated fatty acid which have 1-cis, 4-cis-pentadiene structures like arachodonic acid. Lipoxygenases catalyse AA to hydroperoxyeicosatetraenoic acids (HPETEs), leukotrienes (LTs) and lipoxins. Five major active LOXs are found in human: 5-LOX, 12R LOX, 12S LOX, 15 LOX 1 and 15 LOX 2. Roles of LOXs metabolites in human pathogenesis of psoriasis, asthma and cancer are well known. Among LOX metabolites, leukotrienes (LTs) play an important role in the inflammatory process [21].

1.2.4 5-Lipoxygenase

5-lipoxygenase is the main and initiating enzyme responsible for leukotrienes biosynthesis [22]. It is an important target for drugs in a number of diseases like allergy and asthma. It converts AA to leukotrienes. The important active metabolites of the 5-LOX are non-peptido (LTB_4) and peptido (LTC_4, LTD_4 and LTE_4) leukotrienes. Leukotrienes are very potent metabolites that mediate inflammation. The biological targets of LTB_4 are mainly inflammatory cells. It is highly potent activator of leukocytes that stimulate these cells to get adhere to vascular endothelium and also help in chemotactic responses [23]. LTB_4 also has been cause for numbers of inflammatory diseases in human. LTC_4 and LTD_4 are agents that act on smooth muscles leading to contraction. These show inflammation like responses by increasing vascular permeability and also by showing alternation in blood flow. LTs are involved in number of diseases like asthma, ulcerative colitis, psoriasis, gout etc.

1.2.5 Role of COXs and 5-LOX in inflammation

COXs and LOXs, key enzymes in eicosanoids biosynthesis, are the most studied among all other factors contributing to inflammation [10]. Eicosanoids, the oxy-metabolites of COXs and LOXs, mediate inflammatory responses in a receptor coupled mechanism. Thromboxane A_2 (TXA_2), formed in platelets-for example, aggregates platelets and lead to constriction of blood vessels. Prostacyclin (PGI_2) formed from the blood vessel wall, on the other hand, prevents platelet aggregation and cause dilation of vessels. Prostaglandin E_2 (PGE_2) is a potent vasodilator along with the ability to potentiate bradykinin that mediates pain and fever. Altogether, eicosanoids play important roles in inflammatory reaction at all steps [24]. Leukotrienes, metabolites of LOX pathway, are produced in all inflammatory cells except lymphocytes and are

shown their roles in pulmonary fibrosis, atherosclerosis and cancer [25,26]. Leukotriene C_4 and its downstream products like LTD_4 and LTE_4 are responsible for vascular permeability. By the action of carboxy peptidase, LTC_4 get converted in to least potent LTF_4 [27]. Leukotriene B_4 being one of the most effective chemotactic molecule among all inflammatory mediators.

In view of their key role in the mediation of inflammation, COXs and LOXs have become the targets for development of drugs for inflammation. Among these, non-steroidal anti-inflammatory drugs (NSAIDs) are largely used marketed drugs for the treatment of inflammatory disorders.

1.3 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs)

First NSAIDs developed in 1897 by the Felix Hoffmann (German chemist) and Bayer Company was acetylsalicylic acid and later was named Aspirin by Heinrich Dresser. Development of other NSAIDs started from 1950 onwards. NSAIDs reduce fever, pain, decrease inflammation and prevent blood clots. Side effects of NSAIDs depend largely on the specific drug that causes an increased risk of gastrointestinal ulcer and bleeding from it, kidney disease and cardiovascular disorders [28]. NSAIDs became popular because they target various range of inflammatory disorders [29]. NSAIDs, though are potent in alleviating inflammatory symptoms, they are associated with side effects such as gastric ulceration, kidney failure etc. COX-2 discovery made clear that unwanted side effect of NSAIDs are because of inhibition of COX-1, which is cytoprotective. This discovery also led for the synthesis of COX-2 inhibitors. Need for development of preferential/specific inhibitors for COX-2 was their similar therapeutic actions but without unwanted side effects as of non-selective COXs inhibitors [30,31]. Though the

COX-2 inhibitors are devoid of unwanted gastrointestinal side effects, but are associated with cardiac side effects due to prolong usage in condition like arthritis [32].

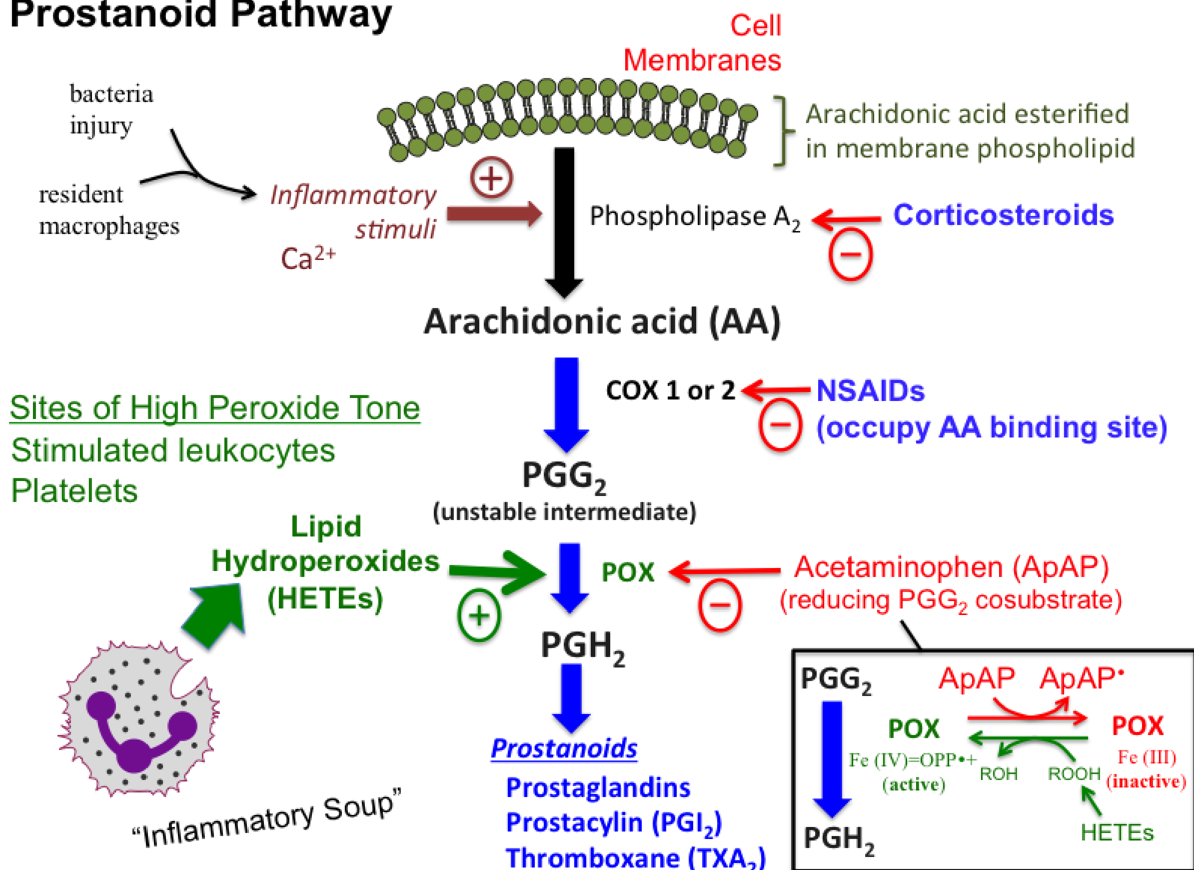


Figure 1-6: **Mechanism of action of NSAIDs** *(Source: TMedWeb)*

1.4 ALLERGY

1.4.1 Introduction

Tropical diseases such as allergies, asthma, eczema are increasing at alarming rate in industrialized countries around the world in last fifty years. Nearly, 100 million people around the world are affected by one or other type of familiar atopic diseases that create large burden on

economy (Figure 1-7). Allergy is one of the tropical disease, affecting 25% population worldwide.

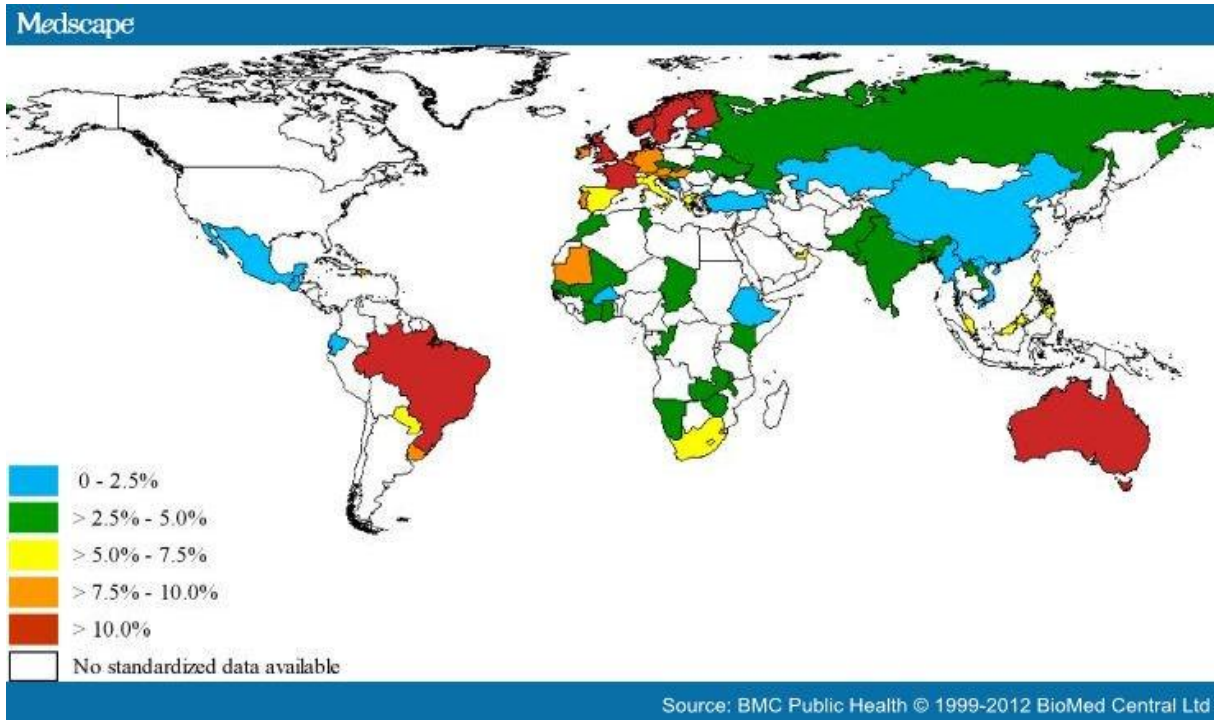


Figure 1-7: Global asthma prevalence in adults *(Source: © 1994-2019 by WebMD LLC)*

Clemens Von Pirquet in 1906 coined the term "allergy", that shows sign and symptom of reactivity or hypersensitivity reaction to some individual, when exposed to certain foods (milk, eggs, fish, peanut, etc.), some medicines like NSAIDs [33,34], insect venoms, animal dander (from skin and fur) and non-infectious substances such as pollens, grass etc. (Figure 1-8).

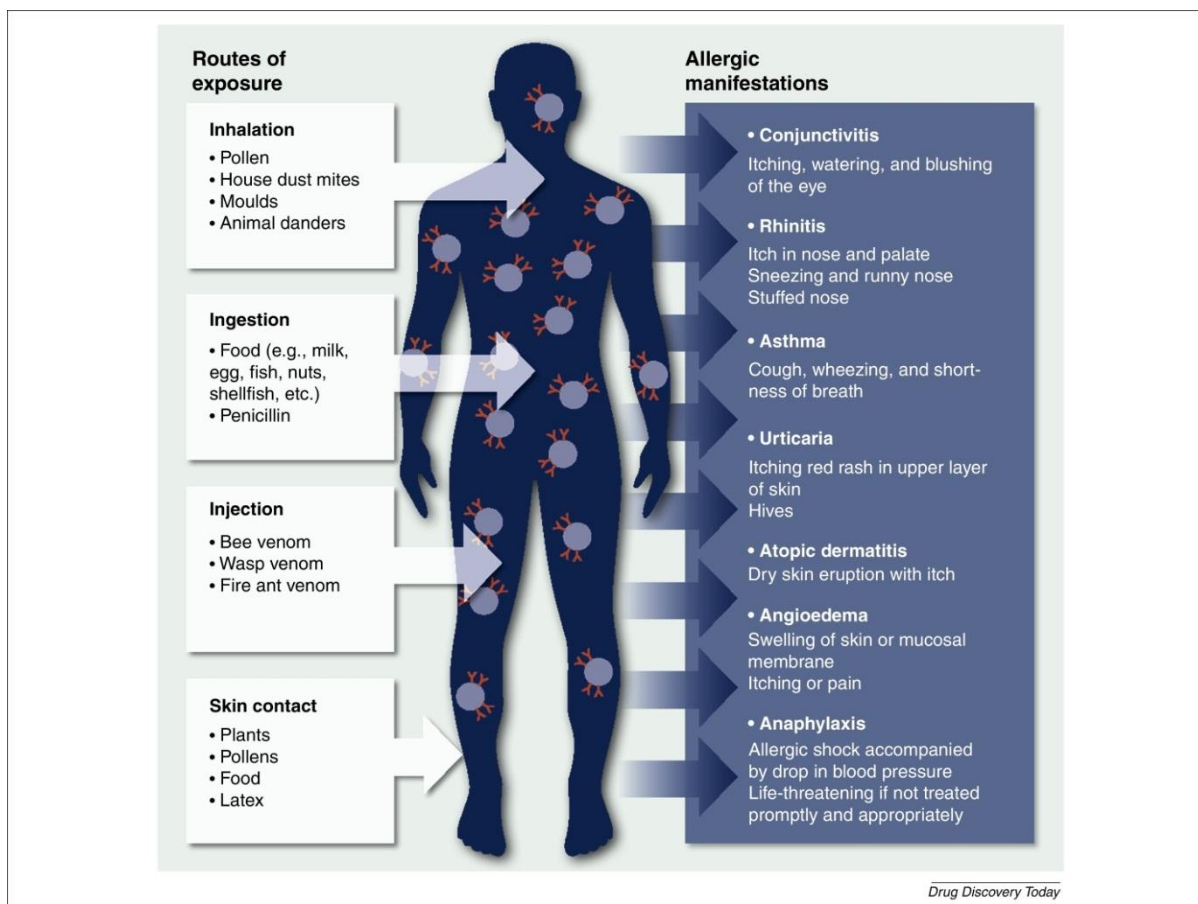


Figure 1-8: **Route of exposer and allergic manifestations** *(Source:Drug Discovvery Today, Vol. 21, page 26-37)*

Asthma is non-communicable chronic inflammatory disease, that is characterized by shortness of breath, chest tightness, wheeze and cough that differ with time and in intensity.

Worldwide as much as 339 million people are affected with asthma (The global asthma report 2018). When we count for other allergy related diseases like dermatitis (eczema) and hay fever, nearly 20 to 25% of people from industrialized nations are affected. Survey results highlight, major cause as poor management and treatment of asthma, and unavailability or unaffordability of medicines as most effective β_2 –agonist and corticosteroids are expensive for majority of people.

1.4.2 Major component of allergy:

Antigen: Anything that elicits immune system to produce antibodies which in return destroy antigen (foreign protein, drug or micro-organism).

Antibody: Lymphocytes produce protein whose function is to neutralize toxin, kill invading micro-organism and destroy foreign antigens.

Mast Cells: Contain fine packet of powerful chemicals that once released initiate inflammation and destroy micro-organisms.

Lymphocytes: These are circulating WBC which produce antibodies and are involved in immune function.

Macrophages: These are immune cells involved in engulfing and destroying foreign proteins/ bacteria present in the circulatory system.

1.4.3 Mast Cells (MCs)

Mast cells play an important role in host defense and innate immunity [35]. These were discovered a century before, since then, research brought broad understanding regarding its phenopytes and its functional characteristics that helped its role in innate immunity. The last three decades, research on MCs, established their role in the initiation of adaptive immunity. MCs are multifunctional effector cells of immune system, which are primary involved in allergic reactions and play critical roles in a varieties of homeostatic conditions such as parasite killing, angiogenesis, bone remodelling and pathological conditions like allergic inflammation and asthma [36].

Mast cells found in all tissues and organs, are involved in inflammatory responses such as allergy and anaphylaxis besides their role in innate immunity and auto-immunity. Histamine is the best characterized inflammatory mediator released from mast cells and most potent vasoactive mediator released that plays an important role in the acute phase of immediate hypersensitivity. In general, mast cells research enlightened our current understanding about pathophysiology of allergic inflammatory diseases and in return such studies have also increased our knowledge of mast cell biology.

1.4.4 Mast cells in host defense

Mast cells are in general located very close to sites that are exposed to external environment such as gastrointestinal tract, respiratory airways and under the skin. At these sites, mast cells encounter antigen or non-antigenic components, therefore play important role in defense and activation of innate immunity.

Generally, mast cells have been understood as playing a role in immediate hypersensitivity events but their role is beyond accepted role in allergic diseases, as they play more extended role in innate immune responses (Figure 1-9).

In view of their key role in the mediation of allergy and asthma, emphasis has been laid on understanding the mechanism behind their pathophysiological role. The model cell line generally employed in these studies includes invariably RBL-2H3 cell line.

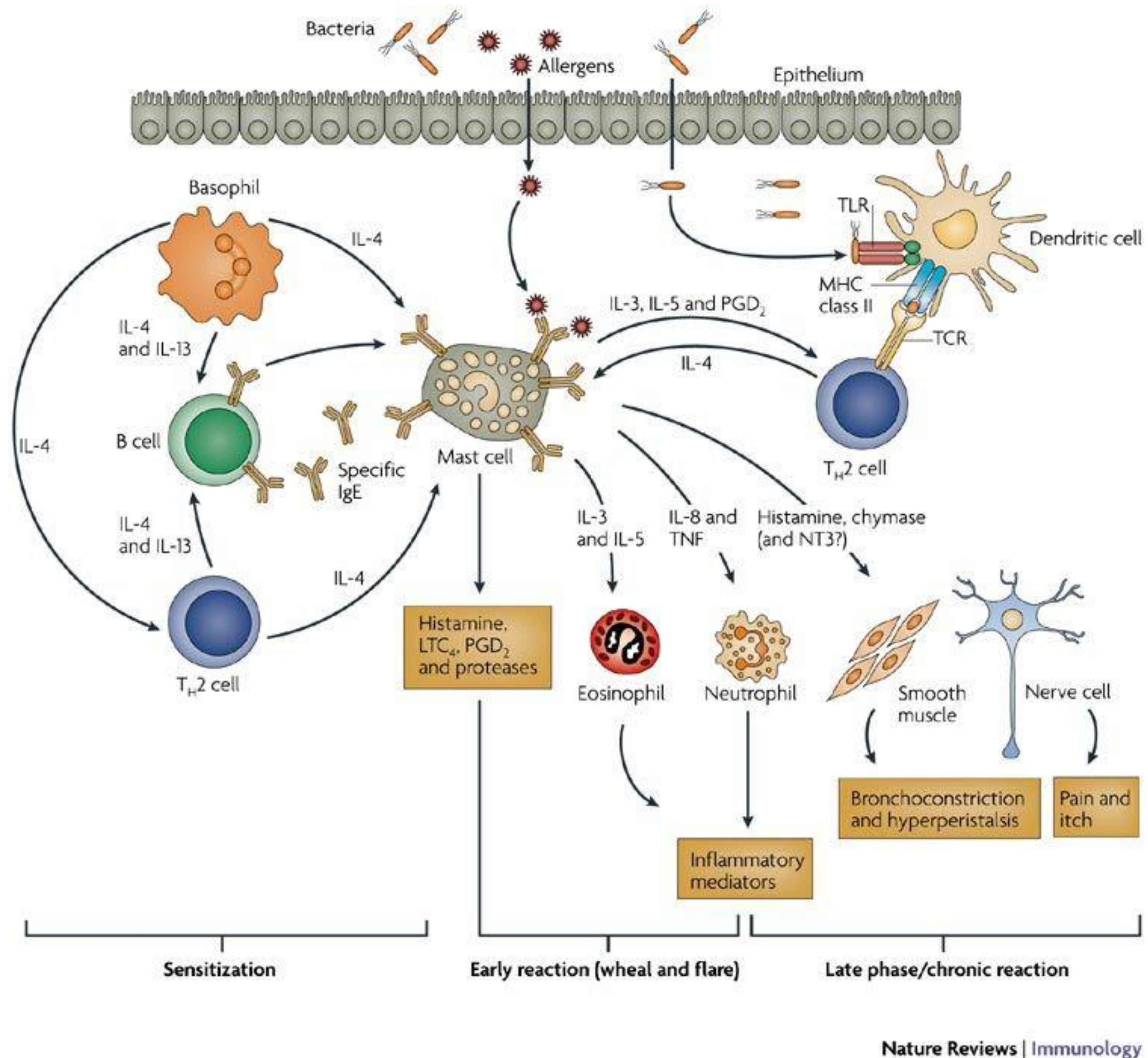


Figure 1-9: Role of mast cells in allergic inflammation

1.4.5 Rat Basophillic Leukemia Cells, (RBL-2H3 cell line)

In 1973, β-chlorethylamine a carcinogen was used in rat to induce leukemia that lead to development of rat basophillic leukemia (RBL) cell line. It was labeled as RBL-1 and was considered a major breakthrough in studying the mast cell as model. RBL-1 cell line expressed FcεRI for IgE and also has characteristics of mast cells, but lack secretory nature, therefore was

able to use in degranulation studies. To overcome non-secretory nature of RBL-1, several sub-clones were developed. In 1976, RBL-IV (HR+), a sub clone was developed, that has histamine secretory characteristics-RBL-2H3 clone [37].From that time, this RBL-2H3 cell line has been used for studying interaction between FcεRI and its ligands. RBL-2H3 single cell bear nearly 5,00,000 FcεRI receptors on its outer surface [38,39]. RBL-2H3 cells have almost similar granular content as of mast cells, therefore, commonly used as model system to study IgE mediated mast cells degranulation [40]. Its receptor cross-linking activation signaling pathways are almost same as that of mast cells and basophils [41]. Hence, RBL-2H3 cell line has become a common research model system to study biological characteristics of primary mast cells and also has potential application in clinical research [42,43].

1.4.6 A23187 Calcium Ionophore

A23187 is known as Calcium Ionophore. It is a polyether carboxylic acid class of antibiotics. It allows divalent cations to cross cell membranes that are normally not permeable for them. It is used in laboratories to increase intracellular Ca^{2+} level in intact cells [44]. Number of biological events like secretion of PGs, LTs and interferon production have been shown to be modified by A23187. It has been used commonly in research for exocytosis to bypass molecular events that comes in to play with the excitation of the FcεRL receptor. A23187 generally induces the degranulation and release of histamine from mast cells.

1.5 NATURAL PRODUCTS AS ANTI-INFLAMMATORY AGENTS

Medicinal plants have been playing an important role in human health since ancient time. Despite great advancement in recent decades in modern allopathic system of medicines, still plants derived medicines have greater contribution to human and animal health [45]. It is a fact that plant derived natural products are contributing greater for development of modern drugs [46]. It has been reported that 25% plant derived drugs are prescribed worldwide [47]. World Health Organization (WHO) considered 252 drugs from plant origin as basic and essential. In recent times, more interest for researchers is in correlating phytochemical constituents obtained from plants with their pharmacological activities [48].

In the last decade, there have been persistent search for the unique anti-allergic and anti-inflammatory molecules from natural source like the medicinal plant has been intensified. It has been recognized that majority of chronic condition related diseases are due to persistent result of inflammation [49]. Growing interests among researchers have been generated for phyto-constituents that have a potential roles to modulate inflammatory responses [50], that includes terpenoids, phenolics and alkaloids [51]. Efforts have been focused on the mode of action of phytochemicals, their characterization and establishing them as potential therapeutic agents in the treatment of inflammatory disorders. Similarly, it is important to develop unique molecule (s) from nature for the treatment of allergy that is cause due to various chemicals mediators released during diseases condition like histamine, inflammatory cytokines, leukotrienes during activation of mast cells. Presently, the therapeutic molecules for treatment of allergy are anti-histaminic drugs, steroids and immune-suppressants. However, the currently available anti-allergic agents are associated with many side effects. Hence, the pharmaceutical companies are in search of novel therapeutic molecules without side effects. In this connection, the search for natural

products needed for the treatment of diseases related to inflammation and allergy assumed importance [52]. Plants are still important natural sources of structurally divergent chemical molecules that may help in development of unique and innovative drugs [53]. There are several natural products of plants origin in clinical trials and at various stages of development. It has been reported that a major initiative was taken for discovery of unique anti-allergic and anti-inflammatory agents that resulted in invention of several patented formulations [54]. Molecules derived from plants or from any natural source are extremely important source for new molecules or its derivatives, as these products represents privileged molecular structures that have been selected by evolutionary mechanism since millions of years [55,56,57].

In present time, trend towards complementary and alternative medicines is increasing. Researchers and innovators have found plants as the best starting source for drug discovery. Extensive research in last decade has revealed the importance of many phytochemicals that have efficacy against various diseases and their further development could result in therapeutic molecules without side effects.

1.6 SCOPE AND OBJECTIVES OF THE PRESENT WORK

Inflammation is a crucial part of the self-immune response to maintain cellular homeostasis. Uncontrolled inflammation leads to various disorders that are mediated via AA pathway by up-regulation of pro-inflammatory enzymes like iNOS, LOXs, and COXs [58,59,60] and pro-inflammatory cytokines tumor necrosis factor- α (TNF-α) and IL 1β [61]. Non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit COXs enzymes have become the most widely used drugs to treat inflammation and pain worldwide [62]. Acetylsalicylic acid (Aspirin) is the

first COX inhibitor, used for treatment of inflammation. Use of NSAIDs is often limited due to their gastrointestinal side effects. To overcome NSAIDs side effects, selective COX-2 inhibitors (COXIBs) were developed such as celecoxib and rofecoxib [63,15,64]. Though COXIBs are devoid of gastrointestinal side effects, they were found to be associated with increased cardiovascular risk, on long term use [65]. As a result, there is a growing demand for safer but efficacious anti-inflammatory drugs [62]. It has been experimentally observed that inhibition of COXs pathways in the case of aspirin-induced asthma resulted in a significant increase in the 5-LOX product, LTE_4. Thus, COXs/5-LOX dual acting anti-inflammatory drugs may be more effective in the treatment of chronic inflammatory diseases like rheumatoid arthritis. In fact recently one such COX-2, 5-LOX dual inhibitor (CLOXIB), licofelone, has been approved for treatment of inflammatory disorders [66].

Currently, licofelone is the only COXs/5-LOX dual inhibitor in the market, developed by Merckle GmbH, together with Alfa Wassermann and Lacer. Further, a number of COX/LOX dual inhibitors are at different stages of development which could form the next generation anti-inflammatory as well as anti-cancer drugs without side effects.

The present study is also designed to isolate COXs/5-LOX dual inhibitors (CLOXIBs) from natural sources. *Premna integrifolia*, one among the five plants species of Brihatpanchamoola, is widely employed in Ayurvedic preparations for the treatment of inflammatory disorders. Hence in the present study an attempt is made to isolate and identify the COX-2 and/or 5-LOX dual inhibitor(s) from *Premna integrifolia* and evaluate their efficacy through *in vitro* and *in vivo* studies. Also attempt is made to understand the molecular signaling pathways involved in these anti-inflammatory effects. **In the light of the above, the objectives are:**

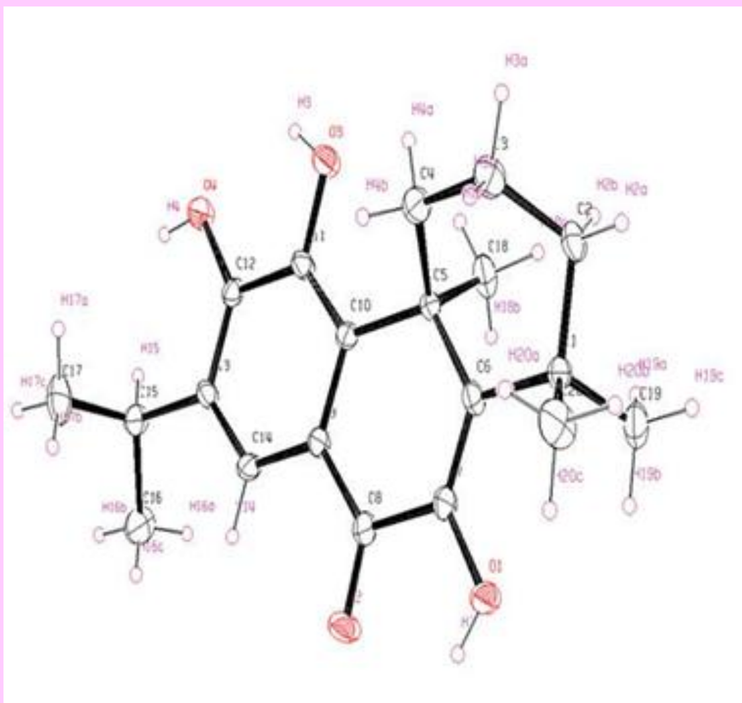
- **1. Evaluation of anti-inflammatory and immunomodulatory effects of *Premna integrifolia* extracts and assay-guided isolation of a COX-2/5-LOX dual inhibitor (CLOXIBs).**

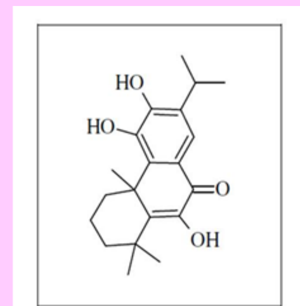
- **2(a). Evaluation of anti-inflammatory effects of CLOXIB(s), a COX-2/5-LOX dual inhibitor identified above, *in vitro* on mouse peritoneal macrophage/RAW 264.7 cell line and *in vivo* on air pouch and paw edema models of inflammation.**

- **3(b). Evaluation of anti-allergic effects of CLOXIB(s) isolated above on RBL-2H3 cell line *in vitro* and *in vivo* on passive anaphylaxis mouse model.**

Chapter 2

EVALUATION OF ANTI-INFLAMMATORY AND IMMUNOMODULATORY EFFECTS OF *PREMNA INTEGRIFOLIA* AND ASSAY-GUIDED ISOLATION OF COX-2/5-LOX DUAL INHIBITORS (CLOXIBs)





6-Hydroxy salvinolone

2.1 INTRODUCTION

Premna integrifolia is a large shrub which belongs to the family Verbenaceae and is commonly known as Agnimantha or Arani [67]. This plant grows in the dry parts of India, Sri Lanka, Bangladesh and South-east Asia. All parts of this plant like roots, stem, barks and leaves have medicinal value and roots of *Premna integrifolia* are one of the active ingredients in Ayurvedic formulation, "Dasmula" [68] which is used in the treatment of various disorders in traditional systems of medicine [69]. The roots are widely used in the treatment of inflammation, diabetes, swellings, bronchitis, dyspepsia, fever, liver diseases, constipation, piles etc [70,71]. Also this plant is widely used by the traditional medicine practitioners as hepatoprotective, cardiotonic, carminative, antibiotic, and antitumor agents [72]. The reported pharmacological properties of this plant are its antidiabetic [73], anti-atherosclerotic [74], analgesic, hypolipidemic and antimicrobial [75] and antioxidant [76] activities. Phytochemical study of *Premna integrifolia* showed the presence of flavonoid luteolin [77], sterols, alkaloids premnine, triterpenes, ganikarine and premnazole.

The non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat pain and inflammation and they act by blocking the formation of pro-inflammatory prostaglandin E_2 (PGE_2). NSAIDs are non-selective inhibitors of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), enzymes which are involved in the production of prostaglandins [78]. However, the use of NSAIDs are reported to cause several side effects that include gastrointestinal complications, bleeding, platelet dysfunction, perforation of the esophagus, stomach, duodenum and mucosal lesions, kidney disorders [79,80] and liver disorders, headaches, allergic reactions, etc. These side effects were attributed to the inhibition of COX-1,

which is cytoprotective, in addition to COX-2 which is involved in all inflammatory disorders. To overcome these problems, the selective COX-2 inhibitors (COXIBs) were developed. Though COXIBs are without gastric side effects, they tend to enhance cardiac-related complications on long term use [81]. The cardiac side effects of COXIBs are due to activation of 5-lipoxygenase (5-LOX), that mediates allergy and asthma. Due to the above reasons COX-2/5-LOX dual inhibitors (CLOXIBs) are emerging as the front runners of the next generation anti-inflammatory drugs. COX-2 and 5-LOX involved in arachidonic acid metabolic pathways are associated with various inflammatory and allergic disorders [82]. The COX-2/5-LOX dual inhibitors showed least side effects when compared with NSAIDs and COXIBs [83,84]. In this scenario, the natural plant extracts/compounds with COX-2/5-LOX dual inhibition may have an added safety benefit.

In the present study, four different solvent systems were used to extract the roots of *Premna integrifolia*, out of which PEE showed the most potent inhibition of both COX-2 and 5-LOX enzymes and it also showed immunomodulatory effect on human lymphocytes. PEE also showed potent anti-inflammatory effects *in vitro* on RAW 264.7 cells as well as *in vivo* on mouse paw edema model of inflammation. In the quest to isolate COX-2/5-LOX dual inhibitor(s), the PEE was subjected to partial purification on an open silica column and most active fraction towards COX-2/5-LOX dual inhibition was subjected to RP-HPLC to purify COX-2/5-LOX dual inhibitor(s). These studies resulted in isolation of four active molecules with potent inhibition of COXs and/or 5-LOX enzymes, out of which one is characterized as 6-hydroxy salvinolone (6-HS).

2.2 MATERIALS AND METHODS

2.2.1 Reagents and chemicals

Phosphate buffered saline (PBS), Culture medium DMEM, fetal bovine serum (FBS), penicillin, streptomycin and trypsin-EDTA were purchased from GIBCO, Ltd. (BRL Life Technologies, Inc., Grand Island, NY). TMPD (N,N,N,N-tetramethyl-p-phenylenediamine), Hematin, MTT (3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide), lipopolysaccharide (LPS) and Griess reagent, NDGA (Nordihydroguaiaretic acid) were purchased from Sigma–Aldrich, USA. Arachodonic acid (AA) was procured from Nu-Chek-Prep (MN, USA). Enzyme immunoassay kit for IL-1β, IL-6, IL-2 and PGE_2 were purchased from R&D systems, Inc. (MN, USA). Anti-COX-2 antibody and anti 5-LOX antibodies were obtained from Santa Cruz Biotechnology Inc. (Texas, USA). Anti-iNOS antibody was purchased from Thermo Fisher Scientific (MA, USA). Anti-TNF-α and anti IL-1β antibodies were purchased from R&D system, Inc. (MN, USA). Solvents for chromatography were of HPLC grade. All other chemicals were of analytical grade.

2.2.2 Collection of *Premna integrifolia* roots

Plant material was collected from Tirupati region of Andhra Pradesh, India. The plant was identified as *Premna integrifolia* L. belonging to Verbenaceae by Late Prof. Kottapalli Seshagirirao, Department of Plant Sciences, the University of Hyderabad on 4th April 2016. The root voucher specimen number was assigned as Rajaram-R2022 and voucher specimen was deposited at the University of Hyderabad Herbarium (World Acronym: UH).

2.2.3 Preparation of *Premna integrifolia* roots extracts

The roots of *Premna integrifolia* were sun dried for 15 days, powdered and successively extracted using soxhlet apparatus with petroleum ether, ethyl acetate, methanol and water in the increasing polarity index. These extracts were dried using a rotatory evaporator followed by lyophilization. Petroleum ether, ethyl acetate, and methanol extracts were dissolved in dimethyl sulphoxide (DMSO) while the water extract was dissolved in double distilled water. These extracts were screened for inhibition of 5-LOX, COX-1and COX-2 enzymes.

2.2.4 Fractionation of petroleum ether extract (PEE) through silica column chromatography

PEE of *Premna integrifolia*, the most potent extract, was partially purified through open silica column and nine different fractions were collected. These fractions were dried through a rotary vacuum evaporator followed by lyophilization and further screened for 5-LOX, COX-1, and COX-2 inhibitory activities. Out of nine fractions, the eighth fraction showed COX-2/5-LOX dual inhibition (Table 2).

2.2.5 RP-HPLC column chromatography

The lyophilized eighth fraction obtained above was subjected to reverse phase HPLC (RP-HPLC) by employing C^{18} column (shim-pack column with particle size 5 µm and dimension 250X4.6 mm) with a flow rate of 1 mL/min and the eluants were monitored at 235 nm. The mobile phase consisted of a gradient of solvent A (water: acetic acid-1000:1) and solvent B (methanol: acetic acid-1000:1). Gradient profile was: 0-5 min, 95% of A, 5% of B; 5-15 min, 95-

70% of A, 5-30% of B; 15-35 min, 70-40% of A, 30-60% of B; 35-55 min, 40-5% of A, 60-95% of B; 55-60 min, 5-95% of A, 95-5% of B.

Isolated peaks were assayed for COX-1, COX-2 and 5-LOX inhibition. Preparative RP-HPLC (shim-pack PREP-ODS column with dimensions500X46 mm and particle size 5 µm) was employed to purify COX-2/5-LOX inhibitory peaks at large scale using the gradient described above at flow rate of 6 mL/min.

2.2.6 Extraction and isolation of COX-1 from Ram seminal vesicles

Ram seminal vesicles were collected from slaughter house under ice pack, and all the process of extraction and isolation of microsomes were carried out below 6 °C. Preparation of microsomes was carried out, according to the method of Hemler et al [85] with some modifications. Ram seminal vesicles were cut into small pieces and homogenized with a blender in buffer containing 5 mM EDTA disodium salt, 0.05 M Tris–HCl (pH 8), 0.01% sodium azide and 5 mM diethyl dithiocarbamate. The homogenate was centrifuged at 15,000 × g for 15 min at 4 °C. The supernatant was filtered through cheese cloth. The supernatant was further centrifuged at 33,000 rpm for 1 h 15 min at 4 °C, using ultracentrifuge (Himac, CP-100α HITACHI) to obtain a microsomal pellet. The microsomal pellet was dissolved in a solubilizing buffer containing 0.1 mM EDTA disodium salt, 0.05 M Tris–HCl (pH 8), 0.01% sodium azide and 0.1 mM diethyl dithiocarbamate. These solubilized microsome fractions were stored in small aliquots at −80 °C and used for further studies as the COX-1 enzyme source.

2.2.7 Extraction and isolation of the COX-2 enzyme

2.2.7.1 Expression and isolation of COX-2

COX-2 was prepared according to the method of Reddy et al [86] with some modifications. Human recombinant COX-2 expression was carried out in *Spodoptera frugiperda* (Sf9) cell line. Sf9 cells were maintained at 28 °C in Grace's insect culture medium. At 70% confluence, the Sf9 cells were infected with recombinant baculovirus containing human COX-2. After 68-72 h of infection, the cells were collected by centrifugation at 2500 × g for 5 min at 4 °C. The pellet was resuspended in a minimum volume of Tris–HCl buffer (50 mM, pH 7.2) containing 300 mM sucrose, 5 mM EDTA, 1 mM phenol, 5 mM diethyl-dithiocarbamate and sonicated for 3 min. The cell lysate was centrifuged at 100,000 × g for 1 h 15 min at 4 °C by using ultracentrifuge (Himac, CP-100α HITACHI), and the microsomal pellet obtained was resuspended in Tris–HCl buffer (2.5 mM, pH 7.2) containing 0.5% glycerol, Tween 20, 0.8% and 1 mM phenol. This solubilized microsomal fraction was stored in small aliquots at −80 °C and used as a COX-2 enzyme source.

2.2.7.2 Cyclooxygenase (COX-1 and COX-2) assay

COX-1 and COX-2 enzyme activities were measured according to the method of Copeland et al [87] with slight modifications using a chromogenic assay based on the oxidation of N,N,N,N-tetramethyl-p-phenylenediamine (TMPD), during the reduction of PGG2 to PGH2 [13,88]. The reaction mixture contained Tris–HCl buffer (5 mM, pH 8.0), EDTA (5 mM), hematin (5 mM), enzyme (COX-1 or COX-2) and the test compound. The assay mixture was pre-incubated at 25 °C for 5 min, and the reaction was initiated by addition of arachidonic acid and TMPD. The enzyme activity was determined by estimating the rate of TMPD oxidation at

610nm, for the first 60s of the reaction. Non-enzymatic oxidation at a low rate that occurs during the reaction in the absence of COX-1 and COX-2 was subtracted from the actual experimental value while calculating the activity. The activity of COX-1/COX-2 enzymes was expressed as µmoles of product formed/ (min x mL).

2.2.8 Purification and assay of 5-LOX

Purification of 5-LOX from potato tubers was carried out and assayed as described earlier according to Reddanna et al [89]. Enzyme activity was measured using the polarographic method with Clark's oxygen electrode on Strathkelvin Instruments (model 782, RC-300). The 3 mL reaction mixture contained 100 µL of the enzyme, 10 µL of 40 mM arachidonic acid, 2 mL of 100 mM potassium phosphate buffer of pH 6.3, 30 µl of test compound and 860µl of double distilled water. Since 5-LOX add oxygen to the substrate, the rate of decrease in oxygen concentration in the reaction mixture was measured as enzyme activity. Enzyme activity was expressed as µmoles of product formed/ (min x mL).

2.2.9 *In vitro* assays on RAW 264.7 cell line

RAW 264.7 cell line was maintained in a humidified atmosphere with 5% CO_2 at 37 °C in DMEM medium, 10% heat activated fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin. The cultured cells were passaged at 70-80% confluence. Before the treatment, the cells were washed with PBS. The cells were pre-treated in presence or absence of different concentrations (1 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL) of PEE and stimulated with 100ng/mL lipopolysaccharide for 6 h, 18 h and 24 h for cytokine analysis, nitrite estimation and for protein expression studies respectively.

2.2.9.1 Cytotoxicity Assay

Cytotoxicity of PEE on RAW 264.7 cells was assayed by MTT assay. RAW 264.7 cells were seeded in 96 wells plate at a density of 5X 10^3 cells/well. After overnight incubation, the cells were washed with cold PBS to remove unattached cells and then pre-treated in the presence and absence with different concentrations (1 μg/mL, 10 μg/mL, 25 μg/mL and 50 μg/mL) of PEE for 1 h and then stimulated with 100ng/mL lipopolysaccharide for 24 h. Cell viability was measured calorimetrically by MTT assay as described by Mosmann [90].

2.2.9.2 Nitrite Estimation

RAW 264.7 cells were pre-treated in the presence or absence with different concentrations (1 μg/mL, 10 μg/mL, 25 μg/mL and 50 μg/mL) of PEE for 1 h then stimulated with or without LPS (100 ng/mL) for 18 h. After LPS stimulation for 18 h, nitrite level in the culture supernatant was measured by Griess reagent according to the manufacturer's protocol and determined by using the standard curve of sodium nitrite.

2.2.9.3 Analysis of prostaglandin E_2, pro and anti-inflammatory cytokines

Analysis of prostaglandin PGE_2 and pro and anti-inflammatory cytokines (IL-1β, IL-6, and IL-2) were carried out on LPS stimulated RAW 264.7 cells by ELISA using commercially available kits (R&D system, MN, USA). In a 12 well plate, $1X10^5$ cells were seeded for overnight culturing. After cold PBS wash, cells were pre-treated in presence or absence with different concentrations (1 μg/mL, 10 μg/mL, 25 μg/mL and 50 μg/mL) of PEE for 1 h then stimulated with or without LPS (100 ng/mL) for 6 h. After LPS stimulation, the cytokines level in the culture supernatant were determined by ELISA as per the manufacturer's protocol.

2.2.9.4 Preparation of whole cell lysates for protein expression studies

RAW 264.7 cells were seeded in 6-well plates (2 x 10^5 cells/well) and at its 70-80% confluence, pre-treated with the presence or absence of PEE for 3 h, followed by treatment with and without LPS (100 ng/mL) for 24 h. The whole cell lysates were prepared for measuring the protein levels for Western blotting. Briefly, the cells were washed twice with ice-cold PBS and lysed in 80 µL of RIPA buffer with protease inhibitor followed by 30 min incubation on ice. The tubes were vigorously rocked for 5 min, and the homogenates were centrifuged for 30 min at 14,000 rpm at 4 °C. The supernatants were collected and stored at -80 °C. The protein concentrations were determined before protein expression studies according to the Bradford method. An equal amount of protein was loaded and separated on 10–15% SDS-PAGE and transferred to nitrocellulose membrane. Then membranes were blocked with 5% blotting-grade blocker (BIO-RAD) in TBS for 1 h at room temperature followed by washing three times with TBST. Membranes were incubated with primary antibodies (0.5–1.0 µg/mL) for overnight at 4 °C with gentle shaking on rocker, then thrice washing with TBST. The membranes were then incubated with respective secondary antibodies conjugated with HRP. Signals were then detected with SuperSignal® West Femto Maximum Sensitivity Substrate from Thermo Scientific.

2.2.9.5 Lymphocyte proliferation assay (LPA)

Lymphocytes were isolated from fresh blood donated by human health volunteers by Ficoll Histopaque (Sigma) method [91]. Isolated lymphocytes were washed twice with PBS and resuspended in complete DMEM media containing β-mercaptoethanol (50 mM). Cells were seeded at the density of 1 X 10^4 cells in each well in 96 well plates and grown for 16 h at 37 °C and 5% CO_2. Cells were treated with the *Premna integrifolia* roots extracts at four different

concentrations (50 µg/mL, 25µg/mL, 10 µg/mL and 1 µg/ mL) and incubated for 24 h. Cells treated with Concanavalin A (Con-A) (5 µg/mL) served as positive control and without Con-A served as negative control. After 24 h, 20 µl of MTT (5 mg/mL in PBS) was added in each well and incubated for an additional 3 h at 37 °C. After incubation, 50 µL of DMSO was added in each well to dissolve the formazan crystals. Absorbance was measured at 570nm after 30 min addition of DMSO in BioTek Synergy Mx multimode reader. Lymphocytes proliferation was measured as percentage growth keeping control as a reference.

2.2.9.6 *In vivo* assay-carrageenan-induced paw edema animal model

Male BALB/c mice weighing 20-25 grams of 6-8 week age were purchased from the National Institute of Nutrition (NIN), Hyderabad, India. Mice were housed in University of Hyderabad animal house at constant room temperature 23±1 °C and allowed to water and food freely in 12 h dark/light cycle. The mice were kept at least a week in the animal house before experimentation. The mice used in this study were handled carefully and according to the *Guide lines for the care and use of Laboratory animals* published by NIH (National Institute of Health). The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC), University of Hyderabad, India.

PEE the most promising extract with COX-2/5-LOX dual inhibition was further studied for acute inflammation in a mouse model. Briefly, the mice were randomly grouped (n=6) and were intraperitoneally injected with 100 µL DMSO or with PEE (25mg/Kg bw/50mg/Kg bw) or Dexamethasone (20mg/Kg bw). After 3 h of intraperitoneal injection, paw edema was induced through subcutaneous injection of 20µL of carrageenan (1%) into left paw or an equal volume of 0.9% saline into the left hind paw [92]. Prior to carrageenan dose, the thickness of the left hind

paw was measured using a digital caliper, as a baseline reference [93]. Edema calculation was made as the average difference in paw thickness and compared with the data for the saline group and the corresponding baseline reference.

2.2.10 Statistical analysis

All the data were analyzed using Sigma Plot 10.0 and GraphPad Prism version 6.01 software. Numerical data for all experiments were presented as mean ± S.D. of three independent experiments. *p*-value was determined using one way ANOVA + Tukey's post hoc test. The p-value of <0.05 was considered as statistically significant.

2.3 RESULTS

2.3.1 *In vitro* anti-inflammatory effects.

2.3.1.1 Effects of *Premna integrifolia* extracts on COX-1, COX-2 and 5-LOX activities

To study the effects of *Premna integrifolia* on COX-1, COX-2, and 5-LOX activities, the young roots (age 1 year) of *Premna integrifolia* were powdered and extracted with four different solvent systems, petroleum ether, ethyl acetate, methanol and aqueous, as described in the methodology. These four solvent extracts were screened for their anti-inflammatory effects by the inhibition studies of COX-1, COX-2, and 5-LOX, the key mediators of inflammation (Figure 2-1). Of all the extracts tested, petroleum ether extract (PEE) showed the least IC_{50} value for COX-2 (6.15 µg/mL) and 5-LOX (11.33 µg/mL), followed by ethyl acetate extract, COX-2 (37.77 µg/mL) and 5-LOX (16.5 µg/mL). Methanolic and aqueous extracts showed less than 50% inhibition of above enzymes at the highest concentration employed (100 µg/mL) (Table 2-

1). As the PEE showed maximum inhibition with least IC_{50} values for COX-2 and 5-LOX, further studies on anti-inflammatory activity and isolation of active principles were taken up only with PEE.

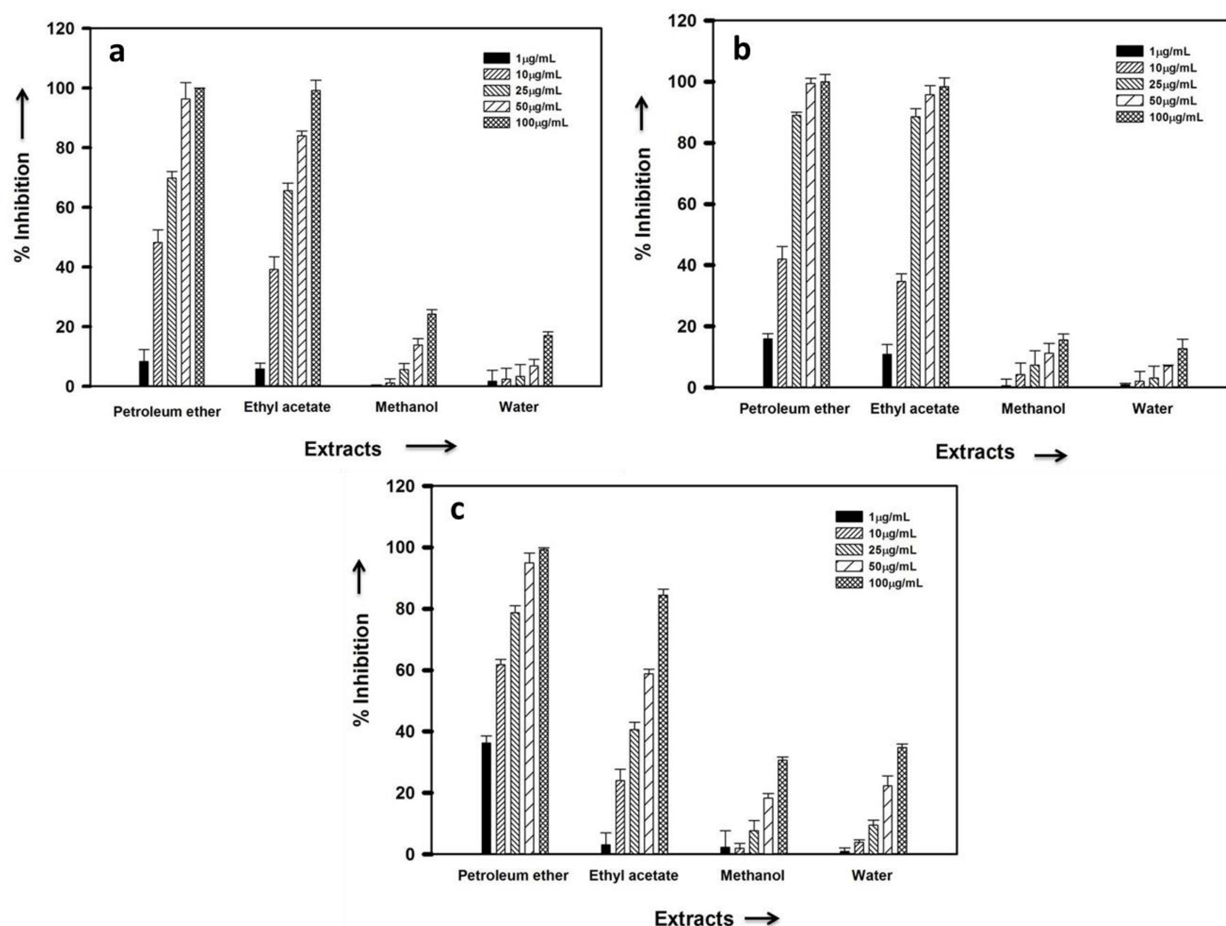


Figure 2-1: Anti-inflammatory effects of *Premna integrifolia* roots extracts on COX-1, COX-2 and 5-LOX. (a) Effect of *Premna integrifolia* extracts on 5-LOX enzyme activity. The inhibitory properties of extracts were checked using polarography method with a Clark's oxygen electrode. The enzyme was incubated for five minutes at 25^0C with different concentrations of extracts and activity was examined by the rate of decrease in oxygen in presence of arachidonic acid as the substrate. (b) Effect of *Premna integrifolia* extracts on COX-1 enzyme activity. The inhibitory properties of extracts were checked through spectrophotometric method. The enzyme was incubated for five minutes at 25 ^{0}C with different concentrations of extracts and COX-1

activity was measured as the oxygenation of arachidonic acid as the substrate. (c) Effect of *Premna integrifolia* extracts on COX-2 enzyme activity. The inhibitory properties of extracts were checked through spectrophotometric method. The enzyme was incubated for five minutes at 25 ^{0}C with different concentrations of extracts and activity was measured as the oxygenation of arachidonic acid as substrate.

Table 2-1: Data showing IC_{50} values (µg/mL) of extracts of *Premna integrifolia*, petroleum ether, ethyl acetate, methanol and water on 5-LOX, COX-1 and COX-2 enzymes. NDGA, Indomethacin and Celecoxib served as the standard inhibitors of 5-LOX, COX-1 and COX-2 respectively.

Plant extracts	IC_{50} value (µg/mL)			
	Petroleum ether	Ethyl acetate	Methanol	Water
5-LOX	11.33	16.5	>100	>100
COX-1	12.47	13.65	>100	>100
COX-2	6.15	37.77	>100	>100

- COX-1 activity level : 0.687 µ mol product formed/(min x mL)
- Std. Inhibitor Indomethacin (COX-1) IC_{50} Value: 15.8 µM
- 5-LOX activity level : 10.14 µ mol product formed/(min x mL)
- Std. Inhibitor NDGA IC_{50} Value: 10.6 µM
- COX-2 activity level : 0.108 µ mol product formed/(min x mL)
- Std. Inhibitor Celecoxib (COX-2) IC_{50} Value: 0.136 µM

2.3.1.2 *In vitro* anti-inflammatory effects of various extracts of *Premna integrifolia* roots on mouse macrophage cell line, RAW 264.7

2.3.1.2.1 *Premna integrifolia* extracts showed very little or no cytotoxicity on RAW 264.7 cells

Four solvent root extracts of *Premna integrifolia* were tested for their anti-inflammatory effects *in vitro* on RAW 264.7 cell line stimulated with LPS. There was no cell death for aqueous, methanolic and ethyl acetate extracts at all ranges of concentrations used. Cell viability

was significantly reduced for PEE at 100 μg/mL, but no cell death was recorded at and below 50 μg/mL (Figure 2-2.A).

As PEE of *Premna intigrefolia* has shown most potent COX-2/5-LOX dual inhibition, we have selected different concentrations (1 μg/mL, 10 μg/mL, 25 μg/mL and 50 μg/mL) of PEE at which there was no cell death, was used for further anti-inflammatory studies.

2.3.1.2.2 Effects of PEE on LPS-induced nitric oxide levels in RAW 264.7 cells

The effects of PEE on LPS induced inflammatory marker, nitric oxide (NO) in RAW 264.7 cells were evaluated. The LPS stimulated RAW 264.7 cells were treated with different concentrations of PEE. Upon LPS stimulation, there was a significant elevation of NO production in the cells and this elevated NO level was significantly reduced by PEE in a dose-dependent manner (Figure 2-2.B). This significant reduction of the pro-inflammatory marker (NO) suggests anti-inflammatory properties of PEE.

2.3.1.2.3 Effects of PEE on PGE_2 and pro-inflammatory cytokines in RAW 264.7 cells

PEE was further evaluated for its effect on the level of PGE_2, and pro-inflammatory cytokines (IL-1β, IL-6) in LPS challenged RAW 264.7 cell line. Levels of PGE_2, IL-1β, and IL-6 were significantly elevated by LPS. PEE showed dose-dependent decrease in PGE_2 level (Figure 2-2.C) in LPS stimulated cells at 1 μg/mL, 10 μg/mL, 25 μg/mL and 50 μg/mL. LPS induced levels of IL-1β (Figure 2-2.D) and IL-6 (Figure 2-2.E) were significantly reduced by PEE at 10 μg/mL, 25 μg/mL, and 50 μg/mL. The significant reduction of these pro-inflammatory markers further supports the anti-inflammatory effects of PEE.

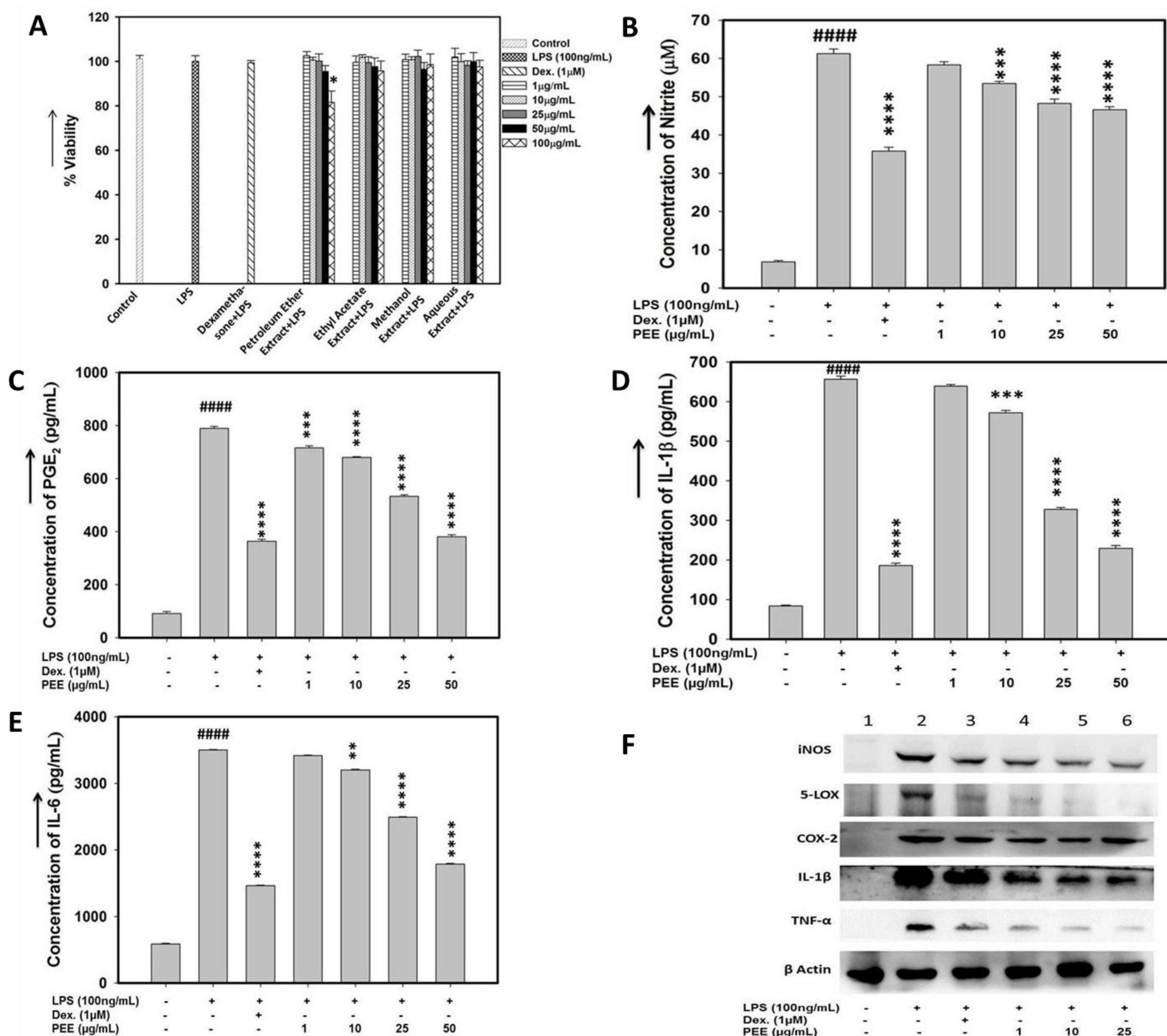


Figure 2-2: Anti-inflammatory effect of *Premna integrifolia* root extracts on LPS challenged mouse macrophage cell line, RAW 264.7 cells. (A) Effect of *Premna integrifolia* extracts on RAW 264.7 cell line. Cells were pre-treated with various concentrations of extracts/compounds for 1 h (as shown in graph) and then challenged with LPS for 24 h. The cell viability was examined by MTT assay. **(B)** Effect of PEE on LPS induced NO production in RAW 264.7cell line. Cells were pretreated with indicated concentrations for 1 h and then challenged with LPS (100 ng/mL). After 18 h of incubation, the culture supernatants were isolated and analyzed for nitrite levels. **(C), (D)** and **(E)** Effect of PEE on LPS induced PGE_2, IL-1β and IL-6 production in RAW 264.7cell line. Cells were pretreated with indicated concentrations for 1 h and then challenged with LPS (100 ng/mL). After 6 h of incubation, the

culture supernatants were collected and analyzed for PGE_2, IL-1β and IL-6 levels respectively. The values represent mean ± S.D. of three independent experiments. $^{\#\#\#\#}p<0.0001$ vs. control; ****p<0.0001, ***p<0.001; **p<0.01; *p<0.05 vs. LPS alone. Dex.: Dexamethasone, PEE: Petroleum ether extract, LPS: Lipopolysaccharides. **(F)** Immunoblot analysis of PEE showed anti-inflammatory activities against LPS challenged RAW 264.7 cells. Cells were pre-incubated with PEE (1 µg/mL, 10 µg/mL and 25 µg/mL) for 3 h, and challenged with or without LPS(100 ng/mL) for additional 24 h. β actin was used as internal control and dexamethasone was used as positive control.

2.3.1.2.4 Effects of PEE on the expression of iNOS, COX-2, 5-LOX, IL-1β, and TNF-α in LPS challenged RAW 264.7 cells

The protein expression of iNOS, COX-2, 5-LOX, IL-1β, and TNF-α in RAW 264.7 cells challenged with LPS in the presence and/or absence of PEE at different concentrations was studied. Untreated cells showed no or little expression of these pro-inflammatory markers while LPS alone treated cells showed induced expression. However, the expression of these pro-inflammatory markers was significantly reduced in a dose-dependent manner in RAW 264.7 cells that were pre-treated with PEE and then challenged with LPS (Figure 2-2.F).

2.3.1.3 *In vitro* immunomodulatory and anti-inflammatory effects of PEE of *Premna integrifolia*

2.3.1.3.1 Immunomodulatory effects of *Premna integrifolia* extracts on lymphocytes

All four solvent extracts of *Premna integrifolia* roots were tested for immunomodulation. Con A treated lymphocytes significantly induced proliferation by nearly two folds as measured by MTT assay. Among four extracts, methanol extract at 50 µg/mL only showed significant

proliferation of lymphocytes, whereas PEE showed lymphocytes proliferation significantly at 10 μg/mL, 25 μg/mL and 50 μg/mL (Figure 2-3.A).

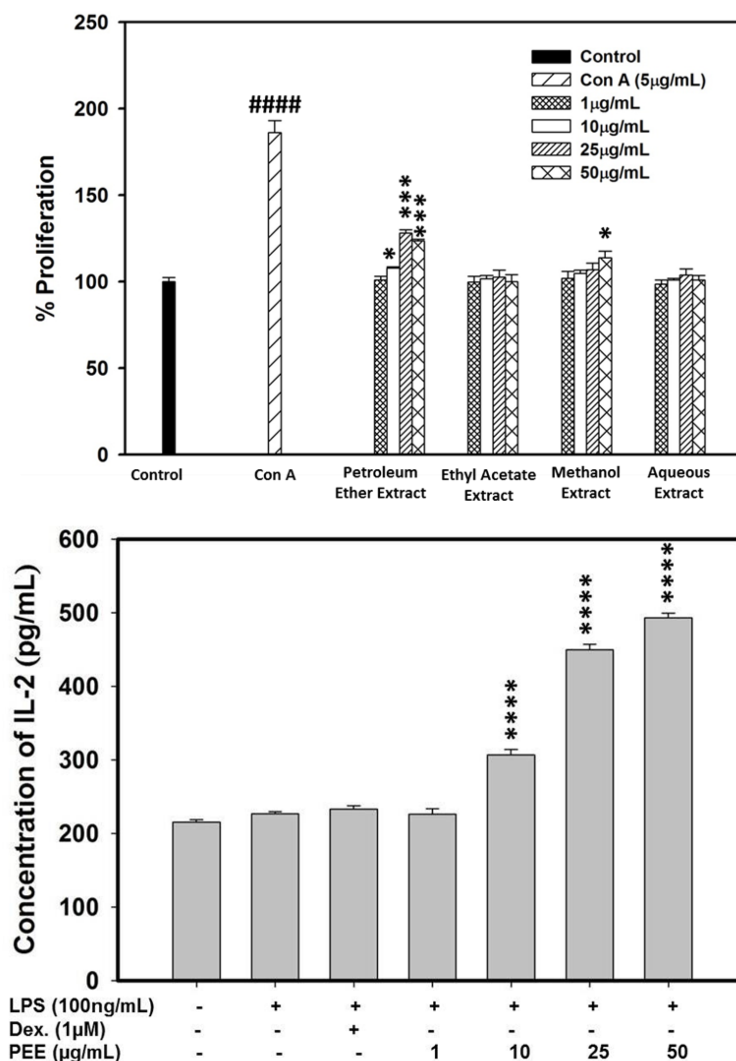


Figure 2-3: Immunomodulatory and anti-inflammatory effects of *Premna integrifolia* extracts. (A) Effect of different solvent extracts of roots of *Premna integrifolia* on human lymphocytes proliferation. The cells were incubated with different concentrations as shown in graph for 24 h. Con A represents positive control. **(B)** Effect of PEE on LPS induced IL-2 production in RAW 264.7cells. Cells were pretreated with indicated concentrations for 1h and then challenged with LPS (100 ng/mL). After 6 h of incubation, the culture supernatants were collected and analyzed for IL-2 levels. Data are mean ±S.D. of three independent experiments.

****$p<0.0001$; ***$p<0.001$; *$p<0.05$ vs. control. Dex: Dexamethasone, PEE: Petroleum ether extract, LPS: Lipopolysaccharides.

2.3.1.3.2 Effects of PEE on IL-2 production in RAW 264.7 cell line

PEE was further evaluated for its effect on anti-inflammatory and immunomodulatory cytokine, IL-2 produced by LPS challenged RAW 264.7 cells. PEE showed a dose-dependent increase in the level of IL-2 production from 10 µg/mL onwards (Figure 2-3.B). This significant induction of IL-2 by these extracts indicates the immunomodulatory and ant-inflammatory properties of PEE.

2.3.1.4 *In vivo* effects of PEE on Carrageenan-induced paw edema in BALB/c mice

The *in vivo* anti-inflammatory effects of PEE were evaluated by intraplantar injection of 20 µL of 1% carrageenan and by measuring paw thickness in millimeter (mm). These studies revealed an increase in the thickness of the mouse paw in response to carrageenan treatment. The paw edema peaked at 3 hours after carrageenan induction, and the thickness was 1.63 ± 0.06 mm in the carrageenan group and 0.71 ± 0.03 mm in the dexamethasone group. In the PEE treatment groups (25 mg/kg body weight and 50 mg/kg body weight), the mean peak thicknesses were 1.07 ± 0.04 mm and 0.98 ± 0.09 mm, respectively, this showed PEE significantly attenuated paw edema compared to the carrageenan group (Figure 2- 4).

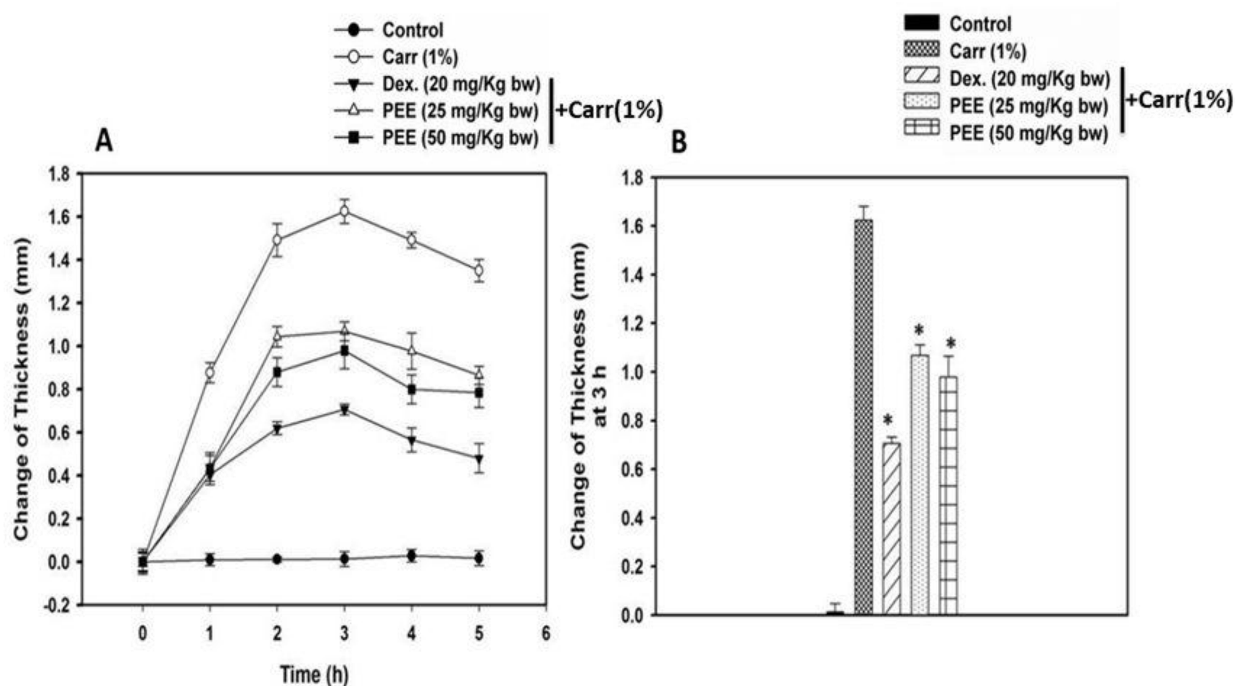


Figure 2-4: Effect of *Premna integrifolia* PEE on carrageenan induced paw edema in BALB/c mice. Animals in the group were injected with PEE [50 mg/kg body weight and 25 mg/kg body weight], dexamethasone (20 mg/kg body weight) or equal volume of the vehicle (100 µL, 1% DMSO) intraperitoneally. One hour later, paw inflammation was induced by injecting 20 µL of 1% solution of carrageenan in 0.9% saline subcutaneously into plantar region of the left hind paw. The thickness of the paw was measured by digital vernier caliper after injecting carrageenan in the dorsal plantar axis at the metatarsal level at the indicated times and data presented as line diagram **(A)**. The thickness of the paw edema at 3 h after induction was shown in the form of bar diagram **(B)**. All data are expressed as mean ± S.D. *$p < 0.05$ compared with the Carr (1%).

2.3.2 Isolation and characterization of COX-2/5-LOX dual inhibitor(s) from PEE of *Premna integrifolia*

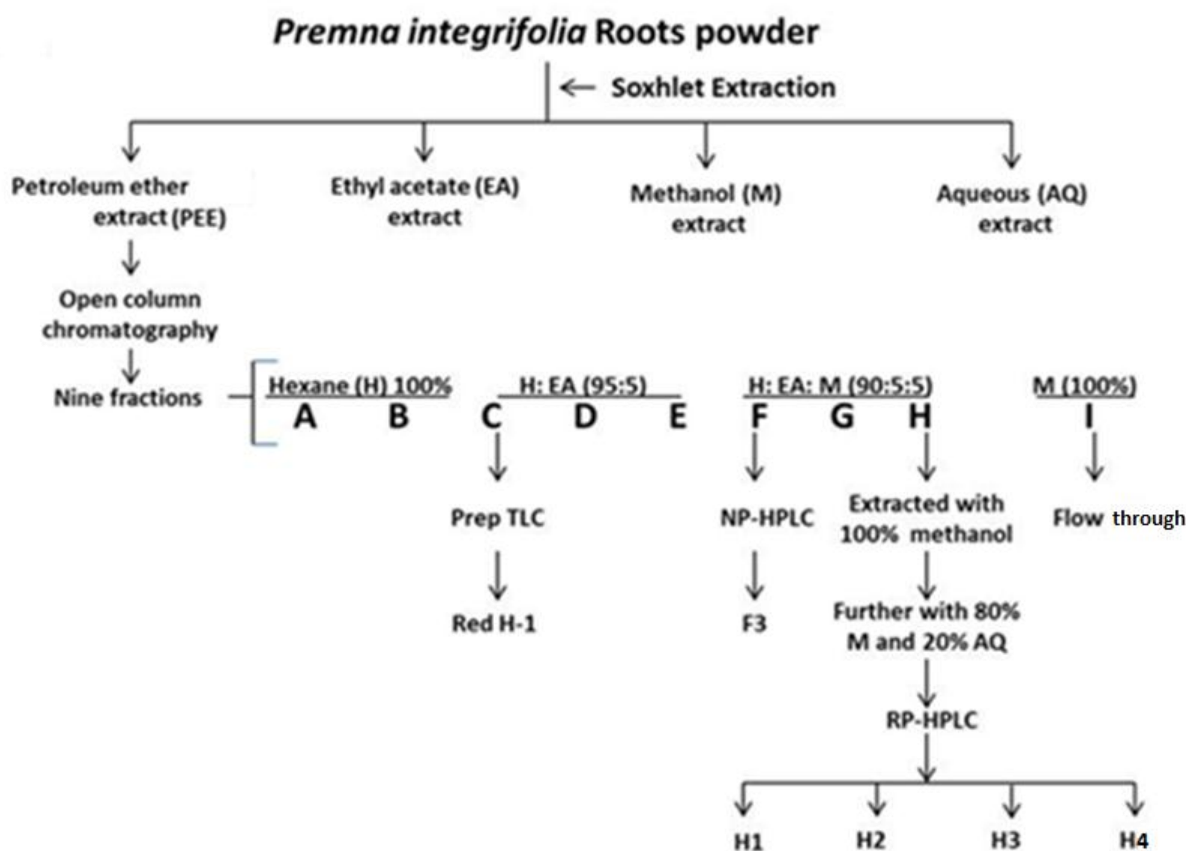


Figure 2-5A: Isolation of active principle(s) from *Premna integrifolia*. Schematic presentation of the process involved in the preparation of various extracts and purification of COX-2/5-LOX dual inhibitor from PEE of *Premna integrifolia*.

Table 2-2: Effect of different fractions of petroleum ether extracts of *Premna integrifolia* on open silica column on 5-LOX, COX-1 and COX-2 enzymes. NDGA, Indomethacin and Celecoxib are standard inhibitors of 5-LOX, COX-1 and COX-2.

S.No.	Petroleum ether extract fractions of *Premna integrifolia*	5-LOX		COX-1		COX-2	
		% Inhibition at 100 µg/mL	IC_{50} µg/mL	% Inhibition at 100 µg/mL	IC_{50} µg/mL	% Inhibition at 100 µg/mL	IC_{50} µg/mL
1.	Fraction 1(A)	NIL	NIL	NIL	NIL	05.21	>100
2.	Fraction 2 (B)	6.43	>100	NIL	NIL	NIL	NIL
3.	Fraction 3 (C)	83.12	27.78	NIL	NIL	NIL	NIL
4.	Fraction 4 (D)	14.8	>100	5.44	>100	15.87	>100
5.	Fraction 5 (E)	2.51	>100	27.62	>100	48.94	>100
6.	Fraction 6 (F)	1.89	>100	56.51	41.84	92.66	20.41
7.	Fraction 7 (G)	47.85	>100	64.22	39.17	50.22	46.21
8.	Fraction 8 (H)	96.27	2.18	70.42	36.84	53.97	68.19
9.	Fraction 9 (I)	47.11	>100	88.13	27.84	46.77	>100

- COX-1 activity level : 0.625 µ mol product formed/(min x mL)
- Std. Inhibitor Indomethacin (COX-1) IC_{50} Value: 16 µM
- 5-LOX activity level : 11.11 µ mol product formed/ (min x mL)
- Std. Inhibitor NDGA IC_{50} Value: 11 µM
- COX-2 activity level : 0.099 µ mol product formed/(min x mL)
- Std. Inhibitor Celecoxib (COX-2) IC_{50} Value: 0.12 µM
- NIL: No inhibition/Not determined

PEE which showed potent inhibition of COX-2 and 5-LOX was subjected to open column chromatography (Figure 2-5A) using silica gel and eluting with hexane, ethyl acetate and methanol in sequence and eluants were collected in various fractions. Nine fractions were collected and tested for COXs, 5-LOX assays (Table 2-2). A highly non-polar fraction (# third) with only 5-LOX inhibition (IC_{50} value, 27.78 µg/mL) was purified by preparatory TLC (data

not shown). Another fraction (# eighth) with maximum COX-2/5-LOX dual inhibition was selected for isolating COX-2/5-LOX, dual inhibitor. This fraction (# eight) was initially fractionated in 100% methanol followed by 20% water and 80% methanol. The supernatant obtained after centrifugation was subjected to RP-HPLC. Four peaks H1, H2, H3, and H4, were obtained (Figure 2-5B). These peaks were tested for COXs/5-LOX assay and for the IC_{50} values (Table 2-3).

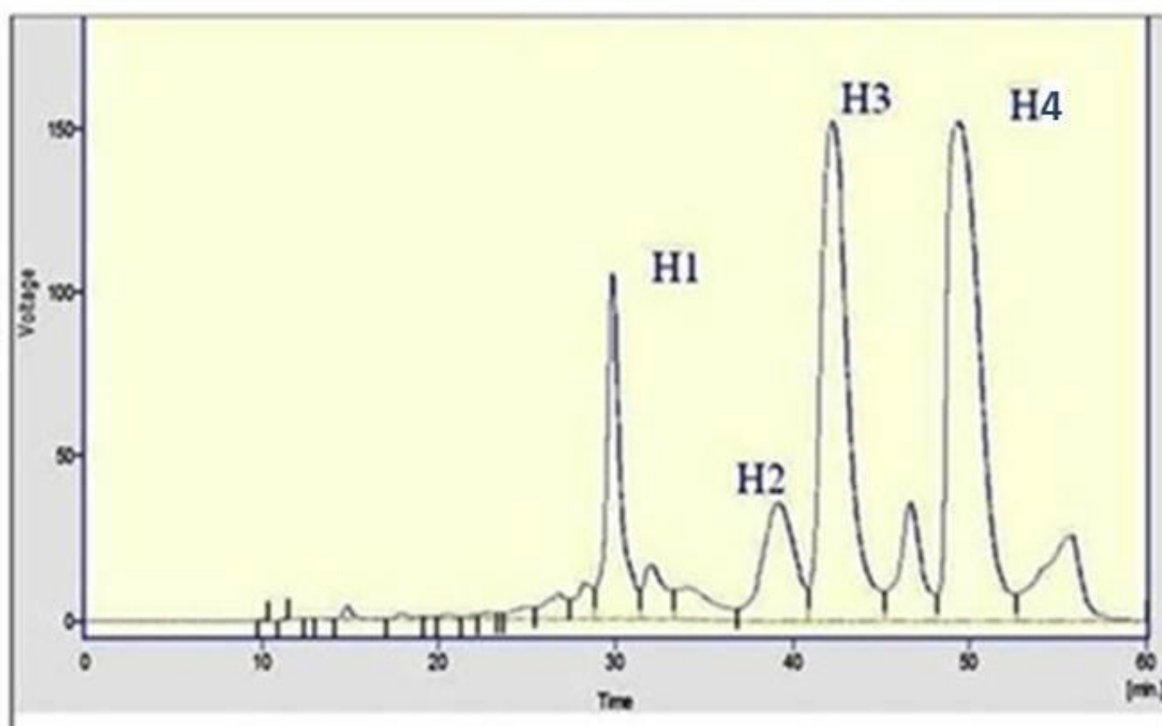
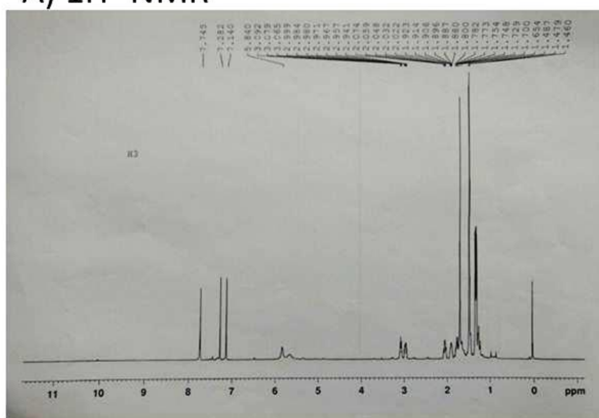


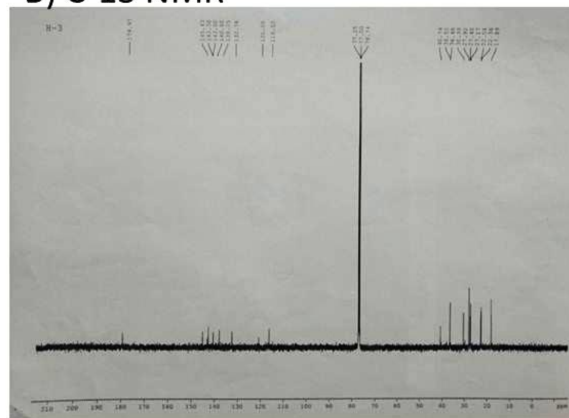
Figure 2-5B: Isolation of active principle(s) from *Premna integrifolia*. RP-HPLC Chromatograph of fraction H, obtained from open column chromatography of petroleum ether extract, on C^{18} column.

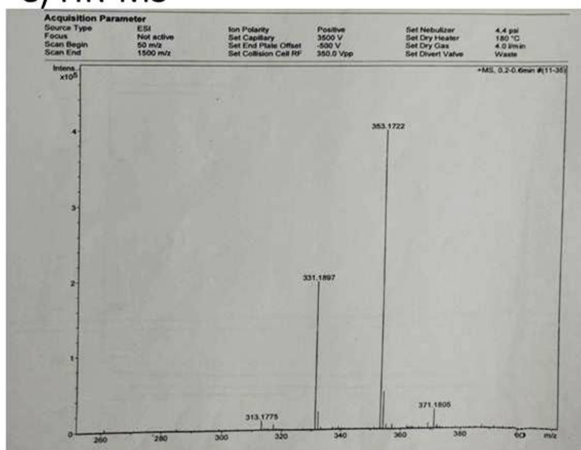
Table 2-3: IC_{50} values for 5-LOX, COX-1 and COX-2 by different peaks-H1, H2, H3 and H4 obtained on RP-HPLC (Figure 2-5B) of the fraction H from open column chromatography, as shown in Figure 2-5A. IC_{50} values for peaks H1, H2 and H4 are given in µg/mL and for H3 (6-HS) in µg/mL and µM.

Peak No.	**5-LOX** [IC_{50} (µg/mL)]	**COX-1** [IC_{50} (µg/mL)]	**COX-2** [IC_{50} (µg/mL)]
H1 (RP-HPLC)	19.94	26.95	5.33
H2 (RP-HPLC)	36.83	29.28	49.13
H3 (RP-HPLC)/ **6-HS**	5.76 **(17 µM)**	54.17 **(164 µM)**	71.03 **(215 µM)**
H4 (RP-HPLC)	60.82	31.01	66.01

Among these, H3 peak showed potent inhibition of 5-LOX (IC_{50} value 17 µM). H3 also inhibited COX-1 and COX-2 with IC_{50} values 164 µM and 215 µM respectively (Figure 2-7.C). H1 showed very potent inhibition of COX-2 with IC_{50} value of 5.33 µg/mL, along with inhibition of 5-LOX (19.94 µg/mL) and COX-1 (26.95 µg/mL). The other peaks H2 and H4 also showed comparable inhibition of COX-1, COX-2 and 5-LOX. H3, one of the major peaks on RP-HPLC inhibiting both COX-2 and 5-LOX, was taken up for further studies to identify the structure by NMR, IR, HR-MS (Figure 2-6) and single X-ray diffraction and its unit cell (Figure 2-7.A & 2-7.B).







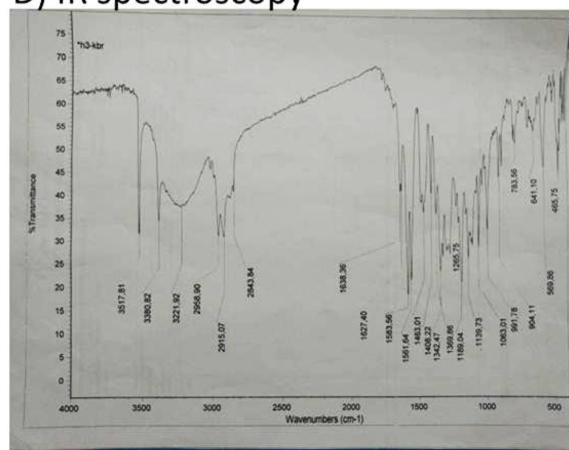


Figure 2-6: Details of (A) 1H-NMR (Proton Nuclear Magnetic Resonance); (B) C-13-NMR (Carbon-13 Nuclear Magnetic Resonance); (C) HR-MS (High Resolution Mass Spectrometry) and (D) Infrared Spectroscopy of peak H3 from RP-HPLC.

- **A.** ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 7.74 (s), 5.84 (s), 5.75 (s), 3.10-3.05 (m), 2.99-2.94 (m), 2.08-2.02 (m), 1.92-1.87 (m), 1.82-1.77 (m), 1.69 (s), 1.48 (d), 1.27-1.34 (m).
- **B.** ^{13}C NMR: (400 MHz, $CDCl_3$, δ ppm) 17.89, 22.36, 22.54, 27.17, 27.40, 27.92, 30.39, 36.48, 36.52, 40.74, 116.53, 121.05, 132.76, 138.25, 140.88, 143.00, 143.38, 145.43, 179.97.
- **C.** HR-ESI-MS: Experimental 331.1897 (M+1), calculated for $C_{20}H_{26}O_4$, 330.1831.
- **D.** FT-IR (KBr, cm^{-1}): 3517 (-OH), 3380 (-OH), 1638 (>C=O), 1583, 1561, 1408, 1189, 1063.

From these studies, the peak is identified as 6-hydroxy salvinolone (6-HS) with a molecular weight (MW) of 330.41 which is similar to earlier findings [94]. Further characterization of other peaks, H1, H2 and H4 are in progress.

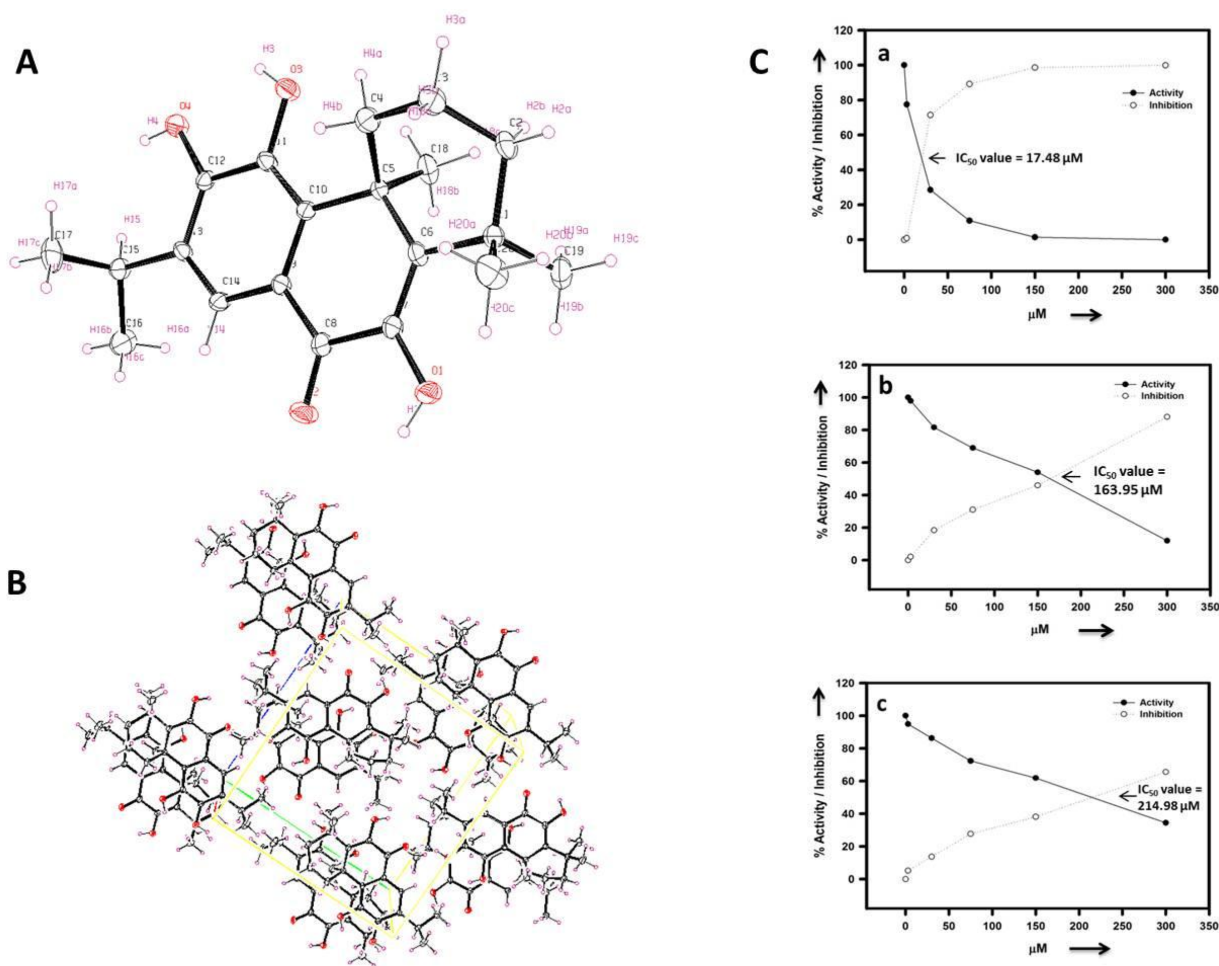


Figure 2-7: Characterization of COX-2/5-LOX dual inhibitor, 6-hydroxy salvinolone (6-HS), and its effects on COX-1, COX-2 and 5-LOX. **(A)** ORTEP (Oak Ridge Thermal Ellipsoid Plot diagram – Drawn at *20*% probability) of 6-hydroxy salvinolone {systematic name: 5,6,10-trihydroxy-7-isopropyl-1,1,4a-trimethyl-2,3,4,4a-tetrahydrophenanthren-9(1*H*)-one}. **(B)** Unit Cell of 6-Hydroxy salvinolone. **(C)** Effect of 6-hydroxy salvinolone (6-HS) isolated from PEE of *Premna integrifolia* on (a) 5- Lipoxygenase, (b) Cyclooxygenase-1 and (c) Cyclooxygenase-2 enzyme activities. IC_{50} values are shown in the above graph.

2.4. DISCUSSION

Premna integrifolia is one of the medicinal plants from Brihatpunchmoola that is widely used in traditional medicine especially in Ayurvedic formulations. Its formulation is used for treatment of many diseases such as allergy, inflammation, cough & cold, asthma, bronchitis, pain, wound healing, arthritis and diabetes. The known pharmacological activities of *Premna integrifolia* are anti-inflammatory, analgesic, antibacterial, anti-arthritic, antimicrobial [95], antioxidant, anti-obesity/hypolipidemic, antiparasitic, cardiac stimulant/cardio protective, antiulcer/gastro protective, central nervous system (CNS) depressant, hypoglycemic, hepatoprotective, immunomodulatory, neuroprotective, longevity-promoting, etc. [96,97].

Earlier work done on the plant was mainly on their therapeutic applications, isolation of several components and their pharmacological properties. There have been no studies carried out that focus on the molecular target and the specific compounds involved. In the present study, we have focused on the evaluation of anti-inflammatory and immunomodulatory properties of *Premna integrifolia* through *in vitro* and *in vivo* assays, followed by assay-guided isolation of active principle(s) with COX-2/5-LOX inhibition.

Eicosanoids are the key mediators of inflammation that are formed by COXs and 5-LOX specifically the prostaglandins and leukotrienes respectively. Non-selective COXs inhibitors are known for gastric and renal side effects. After the discovery of COX-2 as the main mediator of inflammation, several COX-2 specific inhibitors were developed such as celecoxib, rofecoxib and valdecoxib. These specific COX-2 inhibitors were without or less gastric side effects, but have shown cardiac side effects on long use in cases such as arthritis [98]. In the light of the

above information, search for new anti-inflammatory drugs without gastric and cardiac side effects were intensified. Due to this reason, next generation anti-inflammatory drugs, COXs-LOX dual inhibitors have gained importance, because of their superior anti-inflammatory properties with no gastric or cardiac side effects [66]. It is in this connection that the present study was taken up to isolate and identify specific natural products with anti-inflammatory properties from *Premna integrifolia*, a well-known medicinal plant widely being used in Ayurvedic formulations for treatment of inflammatory disorders. These studies revealed potent anti-inflammatory and immunomodulatory effects of the petroleum ether extracts (PEE) of young roots of *Premna integrifolia*, both *in vitro* and *in vivo*.

Immunomodulatory effect of traditional medicinal plants can be an alternative to the conventional therapy for a variety of diseases [99]. In certain disease conditions for examples immune deficiency, autoimmunity or in cancer, it regulates immune response in order to maintain a disease-free state. Our studies, examined immunomodulatory effects of *Premna integrifolia* extracts on human peripheral blood mononuclear cells where Con A was used as a mitogen. The Con A, one of the most effective and widely used mitogen, is being used for studying proliferation and differentiation of leukocytes [100]. PEE and methanolic extract of *Premna integrifolia* have shown potent immunomodulatory effect at nontoxic doses, suggesting their possible application in the treatment of immune compromised diseases, including cancer. The recent Nobel Prize in medicine awarded for cancer immunotherapy [101,102], highlights the importance of immune-modulators in the treatment of various diseases.

In addition to major roles of COX-2 and 5-LOX in inflammation, several pro-inflammatory cytokines are associated in promoting inflammation. Cytokines exist in “cascades” where interruption of one cytokine leads to interruption of the whole cascade that might be sufficient to

control the inflammatory diseases [103–105]. IL-2, the anti-inflammatory cytokine was shown to play an important role in controlling immune homeostasis by the spontaneous lethal autoimmunity in mice lacking IL-2 expression [106]. In our study, *in vitro* anti-inflammatory activities of PEE of *Premna integrifolia* were analyzed on LPS challenged RAW 264.7 cell line and found no cytotoxicity up to 50 µg/mL. PEE showed decrease in nitrite level induced by LPS in RAW 264.7 cell line and these effects were comparable to dexamethasone, a known steroidal anti-inflammatory drug. PEE also showed down-regulation of pro-inflammatory (IL-1β and IL-6) and induction of anti-inflammatory (IL-2) cytokines. Further, PEE down-regulated the expression of LPS -induced pro-inflammatory markers (iNOS, 5-LOX, COX-2, IL-1β and TNF-α) at protein level in Raw 264.7 cell line. These finding demonstrate the anti-inflammatory effects of PEE of *Premna integrifolia in vitro* on mouse macrophage cell line, RAW 264.7.

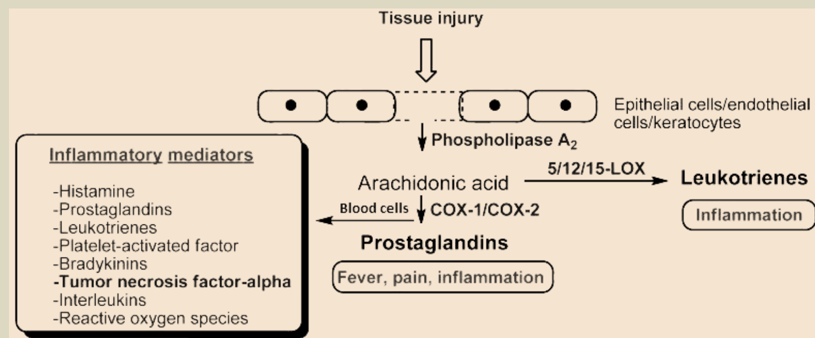
The carrageenan-induced acute inflammatory model is one of the widely used methods to test the anti-inflammatory effects of various agents, *in vivo*. It has been reported that the methanolic extract of roots of *Premna integrifolia* significant inhibits carrageenan induced rat paw edema [76]. This animal model has biphasic inflammatory condition, First phase that is from 1-2 h, is mainly mediated by histamine and serotonin. PEE being an inhibitor of 5-LOX that inhibits leukotriene formation may inhibits acute phase of edema formation. PEE contains terpenoids (diterpenoid) that are known to impair histamine release from mast cells and therefore exert anti-inflammatory effects [107]. The second phase is from 3-5 h onwards and is due to the release of newly synthesized prostaglandins, arachidonic acid metabolites and other neutrophil-derived mediators [108]. Our studies clearly demonstrate the potent inhibition of paw edema by PEE, may be through inhibition of COX/LOX pathway in addition to modulation of cytokines.

Further attempts at isolating the active principle(s) involved in these anti-inflammatory effects of PEE resulted in the isolation and characterization of 6-HS, which is inhibiting both COX-2 and 5-LOX. These studies indicate that 6-HS, being a COX-2/5-LOX dual inhibitor, may form a potential anti-inflammatory drug candidate without gastric and cardiac side effects. As 6-HS is highly potent in inhibiting 5-LOX (IC_{50} value 17 μM), it may have greater potential for the treatment of allergy and asthmatic conditions. Further in-depth studies, however, are required to evaluate its efficacy and safety in other cell lines and animal models. The other compounds, with equal potency in inhibiting the COXs/5-LOX pathway, are being characterized.

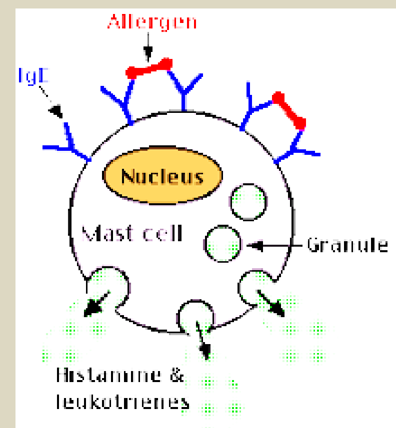
In summary, the results of the present study provides evidence that the petroleum ether extract of *Premna integrifolia* exhibits potent anti-inflammatory and immunomodulatory properties *in vitro* and *in vivo*. Further the studies also identify COX/LOX pathway as the target in mediating the anti-inflammatory properties of *Premna integrifolia*. Another key finding of the present study is the isolation of four peaks with potent inhibition of COXs/5-LOX pathways, out of which one is identified as 6-hydroxy salvinolone, which may be one of the factors contributing to the anti-inflammatory properties of *Premna integrifolia*.

Chapter 3

(A) EVALUATION OF ANTI-INFLAMMATORY EFFECTS OF 6-HYDROXY SALVINOLONE (6-HS), A COX-2/5- LOX DUAL INHIBITOR, *IN VITRO* ON MOUSE PERITONEAL MACROPHAGE/RAW 264.7 CELL LINE AND *IN VIVO* ON PAW EDEMA AND AIR POUCH MODELS OF INFLAMMATION



(B) EVALUATION OF ANTI-ALLERGIC EFFECTS OF 6-HS ON RBL-2H3 CELL LINE *IN VITRO* AND *IN VIVO* ON PASSIVE SYSTEMIC ANAPHYLAXIS MOUSE MODEL



3.1 Introduction:

In the last chapter we have isolated and identified a COX-2/5-LOX dual inhibitor, 6-hydroxy salvinolone, from the petroleum ether extract of a medicinal plant, *Premna integrifolia*. In view of the gastric side effects associated with the conventional NSAIDs and cardiac adverse effects of specific COX-2 inhibitor, there is unmet need and active search for anti-inflammatory drugs without gastric and cardiac side effects. In this scenario, the COX-2/5-LOX dual inhibitors are emerging as promising molecules. Licofelone, one such COX-2/5-LOX dual inhibitor (CLOXIB) has been approved recently. In the light of the above, the 6-HS identified as COX-2/5-LOX dual inhibitor assumes importance. 6-HS, being a natural product isolated from a medicinal plant, *Premna integrifolia*, has a greater chance of becoming a potential anti-inflammatory drug candidate without side effects. However, in-depth studies on its anti-inflammatory properties *in vitro* and *in vivo* are required to evaluate its efficacy for anti-inflammatory drug candidate. Hence this study is taken for to investigate anti-inflammatory effects of 6-HS on LPS challenged RAW 264.7 cell line *in vitro* and *in vivo* on carrageenan induced paw edema and air pouch animal models. Further, its anti-allergic effects were studied on stimulation with A23187/IgE mediated response on RBL-2H3 cell line *in vitro* and *in vivo* on passive systemic anaphylaxis in Balb/c mice.

3.2 Materials and methods

3.2.2 Cell culture

Mouse macrophage cells, RAW 264.7 cell line was obtained from NCCS, Pune. It was maintained in a humidified atmosphere with 5% CO_2 at 37 °C in DMEM medium, 10% heat inactivated fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin. The cultured cells were passaged at 70-80% confluence.

3.2.3 Isolation of Mouse Peritoneal Macrophages

BALB/c male mice were source for isolation of peritoneal macrophages and were elicited with thioglycolate [109]. In Brief, mice were injected with 2% thioglycolate broth (2 mL) through intra-peritoneal route. After 48 hours, mice were euthanized by cervical dislocation. Ice cold DMEM, 5 mL was carefully injected in to the peritoneal cavity, followed by finger tapping of abdomen for 4-5 times in order to loosen attached macrophages from peritoneum. Peritoneal fluid was then carefully taken out into sterile syringe and collected in sterile 15 mL centrifuge tubes on ice. Centrifugation of the tubes were carried out at 800 rpm for 10 min, pellets containing cells were carefully, re-suspended in cold DMEM and cells were counted. Further, seeding and maintenance were done as per earlier stated for RAW 264.7 cell line.

3.2.4 Cytotoxicity Assay of 6-HS on mouse peritoneal macrophages and RAW 264.7 cells

Cytotoxicity of 6-HS for both RAW 264.7 cells and primary mouse peritoneal macrophages were assayed by MTT assay. Both the cells were seeded seperately in 96 wells plate at a density of 5 X 10^3 cells/well. After overnight incubation, the cells were washed with cold PBS to remove unattached cells and then pre-treated in presence and absence with different concentrations (30 µM, 15 µM, 3 µM and 0.3 µM) of 6-HS for 1 h and then stimulated with 100 ng/mL lipopolysaccharide for 24 h. Cell viability was measured colorimetrically by MTT assay as described by Mosmann [90] at 570 nm using microtitre plate reader.

3.2.5 Nitrite Estimation

Mouse peritoneal macrophages were seeded in 12 well plates for overnight. After overnight, wells were washed with cold PBS and pre-treated in presence or absence of 6-HS (30 µM, 15 µM and 3 µM) for 1 h then stimulated with or without LPS(100ng.mL) for 18h. After 18 h of LPS stimulation, nitrite level in the culture supernatant were measured by Griess reagent according to manufacturer's protocol and determined by using standard curve of sodium nitrite.

3.2.6 Analysis of IL-1β.

Analysis of IL-1β was carried out using LPS stimulated mouse peritoneal macrophages by ELISA using commercially available kits (R&D system, MN, USA). In a 12 well plates, 1X10^5 mouse peritoneal macrophages were seeded for overnight. After cold PBS wash, cells were pre-treated in presence or absence with different concentrations of 6-HS (30 µM, 15 µM and 3 µM) for 1 h then stimulated with or without LPS (100ng/mL) for 6h. After 6 h of LPS

stimulation, cytokine level in the culture supernatant was determined by ELISA as per manufacturer's protocol

3.2.7 Analysis of the NF-κB translocation through confocal microscopy

RAW 264.7 cells were seeded on sterile coverslips inside 6 well plate and at its 70-80% confluence, pre-incubated with 6-HS (5 μM) or licofelone (1 μM) for 3 h, then stimulated with LPS (100ng/mL) for 30 min. After end point of treatment (30 min), cells were fixed in ice cold 4% paraformaldehyde for 5 min at 4°C. Cells after fixation were washed with PBST. Cells permeabilization were carried out using ice-cold methanol:acetone (3:1) for 15 min at room temperature and again washed in PBST. The non-specific binding was avoided by blocking with 5% FBS in PBST for the period of 1 h at room temperature, then washed with PBST twice, followed by incubation with primary antibody at dilution of 1:100 in 2% BSA in PBST, overnight at 4°C and then washed three times with PBST for 10 min each. Then cells were further incubated with secondary antibodies at room temperature for 1 h, where dilution of secondary antibody was 1:500 that are fluorescent conjugated.After secondary incubation, washed with PBST and a drop of DAPI (Prolong Gold Antifade regent, Invitrigen, USA) was laid over slide and cells containing coverslip was mounted over slide. Image analysis was taken on a confocal microscope, Zeiss LSM700, USA.

3.2.8 mRNA expression studies in LPS challenged RAW 264.7 cells : Real time-Polymerase Chain Reaction

RAW 264.7 cells was grown in 6 well plate and on 70-80% confluence, these cells were pre-treated with 6-HS (15 μM, 3 μM and 0.3 μM) or dexamethasone (1 μM) for the period of 1 h

and then challenged with LPS (10 ng/mL) for 12 h. TRIzol reagent protocol was used to extract total RNA. RNA quantification was carried out by nanodrop instrument. cDNA for each sample was prepared by using 1 µg of RNA with the help of cDNA synthesis kit. Synthesized cDNA were diluted 10 fold with molecular grade water. Analysis by real time PCR was carried out on synthesized cDNA and to detect iNOS, COX-2, 5-LOX, IL-1β, TNF-α and HPRT1 with 10µl reaction volume.

Table 3: List of Primers used for Real Time PCR

List of Primers used for Real Time PCR	
mRNA	**Sequences of Primers**
iNOS	**Forward Primer 5’ TCCTACACCACACCAAAC 3’,** **Reverse Primer 5’ CTCCAATCTCTGCCTATCC 3’,**
LO-5	**Forward Primer 5’ CGGCTTCCCTTTGAGTATTGATGC 3’,** **Reverse Primer 5’ CAGGAACTGGTAGCCAAACATGAG 3’,**
COX- 2	**Forward Primer 5’ GAAGTCTTTGGTCTGGTGCCTG 3’,** **Reverse Primer 5’ GTCTGCTGGTTTGGAATAGTTGC 3’,**
IL- 1β	**Forward Primer 5’ GCAACTGTTCCTGAACTCAACT 3’,** **Reverse Primer 5’ ATCTTTTGGGGTCCGTCAACT 3’,**
TNF- α	**Forward Primer 5’ AGCACAGAAAGCATGATCCG 3’,** **Reverse Primer 5’ CTGATGAGAGGGAGGCCATT 3’,**
HPRT1	**Forward Primer 5’ TGGATACAGGCCAGACTTTGT 3’,** **Reverse Primer 5’ TTTTACTGGCAACATCAACAGGAC 3’,**

3.2.9 Preparation of whole cell lysate for immunoblots analysis

RAW 264.7 cells (3 x 10^5 cells/well) were seeded in 60 mm disc and at its 70-80% confluence, pre-treated in the presence or absence of 6-HS (15 µM, 3 µM and 0.3 µM) for 1 h, followed by treatment with and without LPS (10 ng/mL) for 24 h. The whole cell lysates were

prepared for measuring the protein levels for Western blotting. Briefly, the cells were washed twice with ice-cold PBS and lysed in 100 µL of RIPA buffer with protease inhibitor followed by 30 min incubation on ice. The tubes were vigorously rocked for 5 min, and the homogenates were centrifuged for 30 min at 14,000 rpm at 4 °C. The supernatants were collected and stored at -80 °C. The protein concentrations were determined before protein expression studies according to the Bradford method. An equal amount of protein was loaded and separated on 10–15% SDS-PAGE and transferred to nitrocellulose membrane. Then membranes were blocked with 5% blotting-grade blocker (BIO-RAD) in TBS for 1 h at room temperature followed by washing three times with TBST. Membranes were incubated with primary antibodies (0.5–1.0 µg/mL) for overnight at 4 °C with gentle shaking on rocker, then thrice washing with TBST. The membranes were then incubated with respective secondary antibodies conjugated with HRP. Signals were then detected with SuperSignal® West Femto Maximum Sensitivity Substrate from Thermo Scientific.

3.2.10 *In vitro* anti-allergic studies on RBL-2H3 cell line

Rat basophilic leukaemia cell line, (RBL-2H3), were cultured in DMEM as described for RAW 264.7 cell line. Two different stimuli: immunological stimulus [IgE anti-DNP (100 ng/mL) followed by DNP-HSA (100 ng/mL)] that is called IgE/antigen and 1µM calcium ionophore (A23187), were used for anti-allergic studies. In case of A23187 stimulus, cells were pretreated with 6-HS for 15 min, followed by incubation with stimulus A23187 for 30 min (Figure 3-1A). Supernatant was collected and analyzed for *in vitro* studies. For immunological stimulus, cells were incubated with IgE anti-DNP for 16 h along with 6-HS. Then, cells were washed with PBS twice and then stimulated for 1 h with DNP-HSA along with 6-HS (Figure 3-1B). After treatment, cells supernatant was collected and analyzed for anti-allergic studies (Figure 3-1).

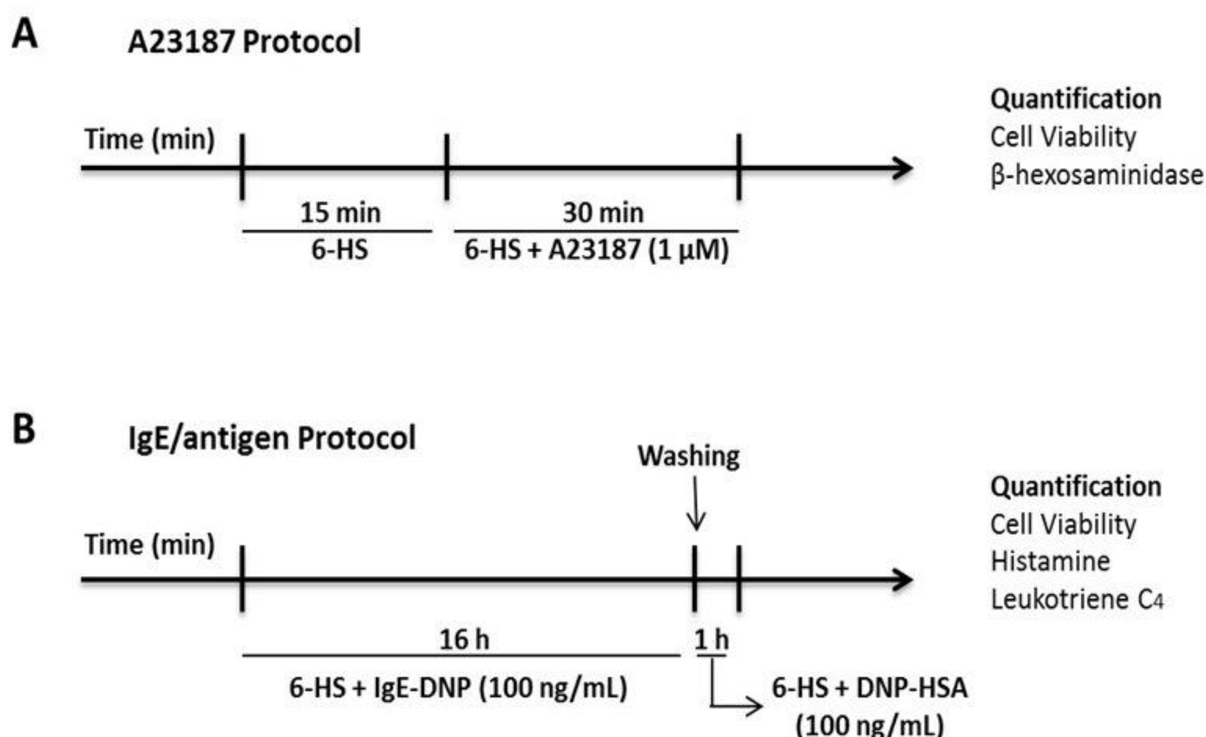


Figure 3-1: Schematic representation of RBL-2H3 basophils' degranulation assay protocols using calcium ionophore (A23187) (A) or IgE/antigen as stimuli (B).

3.2.11 Cytotoxicity assay in presence of 6-HS and two degranulating stimuli on RBL-2H3 cell line

Rat basophilic leukemia cell line, (RBL-2H3) was maintained as reported earlier and its cells viability was checked in presence and absence of 6-HS and two degranulating stimuli were employed using MTT assay. Cells were challenged with degranulating stimulus (A23187) and with 6-HS (30 µM, 15 µM and 3 µM) for 30 min and another stimulus (DNP-HSA) with 6-HS (30 µM, 15 µM and 3 µM) for 1 h, then the cell viability was assessed as described earlier.

3.2.12 *In vitro β*-hexosaminidase Quantification

RBL-2H3 cell line was stimulated with calcium ionopore (A23187) in presence and / or absence of 6-HS (30 μM, 15 μM and 3 μM)/Zileuton (1 μM) and then β-hexosaminidase released in mediawas assayed as described [110]. In brief, 96 well plate was taken containing 30 μL of cell supernatant and 50 μL of substrate solution, *p*-nitrophenyl N-acetyl-D-glucosamine in citrate buffer, pH 4.5 were added, followed by incubation for 1h at 37°C. Reaction was stopped with addition of 80 μL of 0.5M NaOH. *P*-nitrophenolate , the product of reaction was meseared at 405 nm at BioTek Synergy Mx, multimode reader.

3.2.13 *In vitro* histamine quantification

Supernatant collected after stimulating the RBL-2H3 cells with above mention specified antigen in present or absence of 6-HS (30 μM, 15 μM and 3 μM)/Zileuton (1 μM) and were used for quantification of histamine release via histamine ELISA kit from Abcam as per their protocol.

3.2.14 *In vitro* Leukotriene C_4 quantification

Supernatant collected after stimulating RBL-2H3 cells with above mention specified antigen in present or absence of 6-HS (30 μM, 15 μM and 3 μM)/Zileuton (1 μM) were used for quantification of LTC_4 using LTC_4 ELISA kit from Abcam as per their protocol.

3.2.15 Animal Experiments

Male BALB/c mice weighing 20-25 grams of 6-8 weeks age were used for all the studies and were purchased from the National Institute of Nutrition (NIN), Hyderabad, India. Mice were housed in University of Hyderabad animal house at constant room temperature 23±1 °C and

allowed to water and food freely in 12 h dark/light cycle. The mice were kept at least a week in the animal house before experimentation. The mice used in this study were handled carefully and according to the *Guide lines for the care and use of Laboratory animals* published by NIH (National Institute of Health). The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC), University of Hyderabad, India.

3.2.16 *Paw edema model* for acute inflammation

6-HS, a COX-2/5-LOX dual inhibitor was further studied for acute inflammation in a mouse model. Briefly, the mice were randomly grouped, each with 6 animals and were intraperitoneally injected with 100 μL DMSO or with 6-HS (20 mg/Kg bw and 10 mg/Kg bw) or Celecoxib (20 mg/Kg bw). After 1 h of intraperitoneal injection, paw edema was induced through subcutaneous injection of 20μL of carrageenan (1%) into left paw or an equal volume of 0.9% saline into the left hind paw [92]. Prior to carrageenan dose, the thickness of the left hind paw was measured using a digital caliper, as a baseline reference [93]. Edema calculation was made as the average difference in paw thickness and compared with the data for the saline group and the corresponding baseline reference.

3.2.17 Mouse air pouch model of inflammation

Carrageenan induced inflammatory mouse air pouch model was developed as previously described [111]. In brief, mice were randomly grouped, each with 6 animals. Air cavities in mice were produced with sterile 5 mL air injected subcutaneously at dorsal side in the inte-capsular area. On third day, again sterile air of 3 mL was injected in the air pouch that was developed. On day seven, 1 mL 1% carrageenan was injected into the air pouch to induce inflammatory responses. Control group of animal were given 1 mL of saline. After time point, pouch tissues

were carefully opened and inflammatory exudate was collected for analyses of various inflammatory parameters. The pouch lining was carefully washed in saline and then taken for processing further.

3.2.18 Administration of 6-HS

In mouse paw edema model, 6-HS or celecoxib were given intra peritoneally whereas carrageenan was injected, one hour later into the paw directly. In the case of mouse air purch model all the treatment are together (Carrageenan and 6-HS) into air pouch cavity. Animals were randomly divided into 5 different groups each with 6 animals for paw edema as follows: Saline treated; Carrageenan treated (1 mL of carrageenan 1% (w/v) in saline); celecoxib + carrageenan treated (20 mg/kg body weight); carrageenan + 6-HS treated (10 mg/kg body weight); Carrageenan + 6-HS treated (20 mg/kg body weight). For air pouch model, animals were divided into 4 different groups each with 6 animals as follows: Saline treated; Carrageenan treated (1 mL of carrageenan 1% (w/v) in saline); Carrageenan + celecoxib treated (20 mg/kg body weight); Carrageenan + 6-HS treated (20 mg/Kg body weight).

3.2.19 Histopathology of mouse air pouch tissue

Air pouch tissue from each experiment animal group were collected and washed twice in PBS. The washed air pouch tissues were fixed in Bouin's fixative overnight and then washed thorough using double distilled water. Pouch tissue obtained above were dehydrated with alcohol 70%, 80% 90% and 100% sequentially for the duration of 10 min each. Then, tissues were processed further through alcohol and benzene in ratio of 3:1 and then 1:1 for 10 min each, followed by paraffin and benzene in ratio of 1:1 for 10 min to embed in paraffin wax. Further,

tissues were placed in molten wax for 2-3 h so that paraffin get infiltrated in to the tissues and on cooling get harden. Thin sections of 10 µm were made on microtome and mounted on poly lysine coated slides. Using xylene, each sections were de-paraffinised and then sequentially rehydrated in decreasing concentration as shown above of alcohol for 10 min each. The section were observed at 400x magnification under contrast light microscope and photograph were taken.

3.2.20 Immunoblots analysis for expression of various pro-inflammatory markers in air pouch Tissue

Pouch tissue lining was homogenized in 100 mM Tris-HCl (pH 8.0) buffer containing 0.3 M mannitol, 1 mM EDTA, 1 mM DTT, 4 mM K_2HPO_4, 1 mM Sodium orthovanadate, 2 mM PMSF, 0.1% SDS, and 20 µl/mL of protease inhibitor solution. Homogenates were centrifuged at 12,000 rpm for 20 min at 4° C and resultant supernatants were utilized for protein expression studies. Protein expression studies were carried out for the detection of iNOS, 5-LOX, COX- 2, IL-1β, TNF-α and β actin by the same procedure mention earlier.

3.2.21 Mouse model for induction of passive systemic anaphylaxis.

Passive systemic anaphylaxis was done as previously described [112,113]. In brief, the antibody IgE that reconizes the hapten DNP (anti-DNP-IgE) was injected through intraperitoneal injections of 0.1 mL in sterile saline. Twelve hours after injection of the anti-DNP-IgE, the allergen DNP-Human Serum Albumin (DNP-HSA) was injected by intravenous injection of 0.1 mL in sterile saline. Immediately following injection of the DNP-HSA, the temperature of the

animals were recorded, using a rectal thermometer, every 5 minutes for the first 20 minutes, then every 20 minutes until 60 minutes post-injection. Control animals received 0.1 mL of saline only. Animals after end point of experiment were sacrificed that is at 60 minutes time point

All treatments given 1 hour prior to the icednjection of DNP-HSA via intraperitoneal injection. Animals were divided into 3 different groups each with 5 animals. They were: anti-DNP-IgE (100 ng in 0.1mL of saline) + Saline (0.1mL); anti-DNP-IgE + DNP-HSA (100 ng in 0.1mL of saline); anti-DNP-IgE +DNP-HSA + 6-HS (2.5 mg/Kg body weight).

3.2.22 Determination of histamine release *in vivo* animal model

Analysis of *in vivo* histamine release was determined as previously described [114]. Treatment regime and animal grouping were similar as above (passive systemic anaphylaxis model). In brief, mice were injected with 100ng anti-DNP IgE intraperitoneally, 12 h later, 100ng DNP-HSA was injected intravenously. Three minutes later, animals were sacrificed and serum was collected and analyzed for histamine content using ELISA kit (Abcam as per their protocol).

3.2.23 Statistical analysis

All the data were analyzed using Sigma Plot 10.0 and GraphPad Prism version 6.01 software. Numerical data for all experiments were presented as mean ± S.D. *P* value was determined using one way ANOVA + Tukey's post hoc test. The *P* value <0.05 was considered as statistically significant.

3.3 Results

3.3.1 Evaluation of anti-inflammatory effects of 6-HS *in vitro* on mouse peritoneal macrophages/RAW 264.7 cell line and RBL-2H3 cells.

3.3.1.1 Effect of 6-HS on mouse peritoneal macrophages/RAW 264.7 cell viability

The cytotoxic effects of 6-HS on mouse peritoneal macrophages and RAW 264.7 cell line were evaluated by MTT assay. 6-HS showed no cytotoxicity on both type of cells up to 30 µM (Figure 3-2). Therefore, based on this result, other cell based experiments were carried at/or below 30 µM of 6-HS to analyze its anti-inflammatory activity.

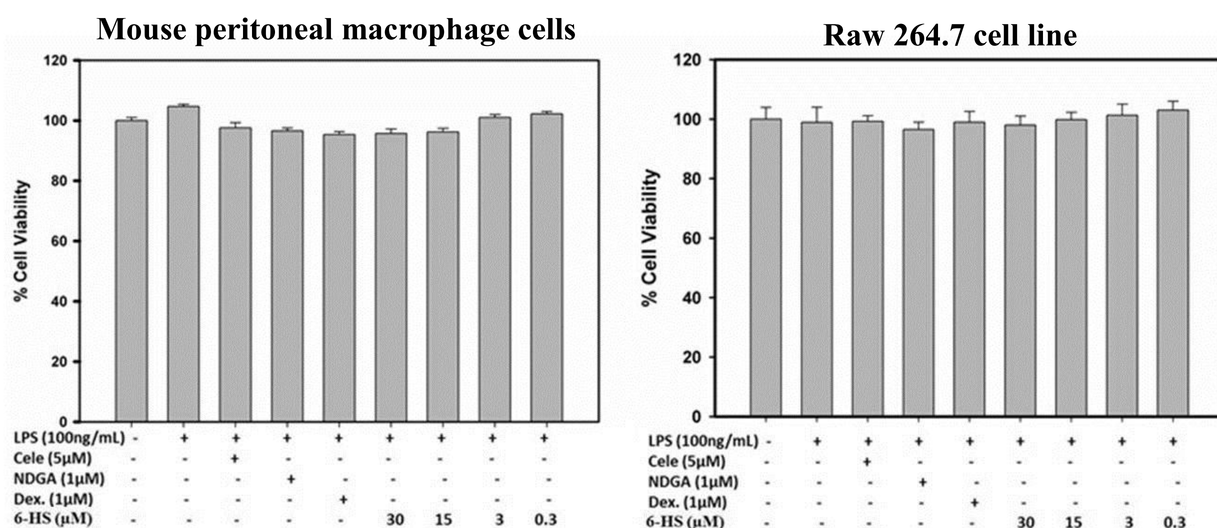


Figure 3-2: Effect of 6-HS on cell viability. Mouse peritoneal macrophages and RAW 264.7 cells were pre-incubated with or without Celecoxib, NDGA, Dexamethasone and 6-HS for 1h and then challenged with 100 ng/mL of LPS.

3.3.1.2 6-HS reduced NO and IL-1β secretion in LPS stimulated mouse peritoneal macrophages

Mouse peritoneal macrophages were cultured as described in the methods. These cells were given pre-treatment with dexamethasone or 6-HS (as described in the methods) for 1 h

followed by LPS treatment. After 6 h and 18 h of LPS treatment, supernatants were collected and tested for IL-1β and nitrite levels respectively. These studies revealed dose dependent reduction of NO and IL-1β level on 6-HS treatment, while dexamethasone used for positive control (Figure 3-3).

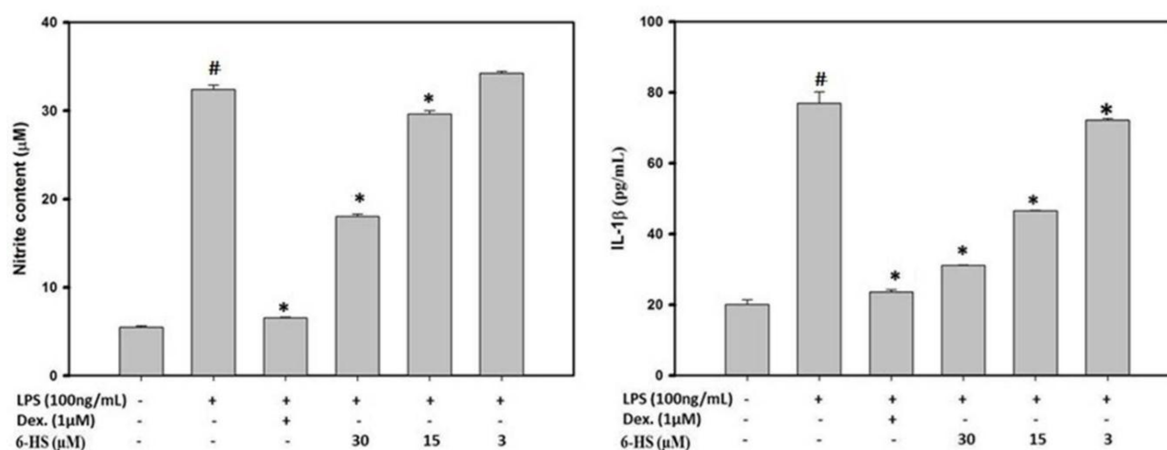


Figure 3-3: Effect of 6-HS on LPS induced nitric oxide and IL-1β released from mouse peritoneal macrophages. Cells were pretreated with 3, 15 and 30 μM of 6-HS for 1h and then challenged with LPS (100 ng/mL). After 6h and 18 h of incubation, the culture supernatants were isolated and analyzed for IL-1β and nitrite levels.

3.3.1.3 Effect of 6-HS on LPS induced NF-kB nuclear translocation in RAW 264.7 cell line

Translocation of NF-kB from cytoplasm to nucleus is critical for activation of pro-inflammatory markers (COX-2, 5-LOX, iNOS, IL-1β, etc.). LPS challenged Raw 264.7 cells showed increase in translocation of NF-kB into the nucleus. RAW 264.7 cells were pre-treated with 6-HS (5 μM) or licofelone (1 μM) for 3 h, followed by LPS treatment showed significant reduction in NF-kB translocation into nucleus (Figure 3-4). Licofelone, a dual inhibitor of COX-2 and 5-LOX was taken as the positive control.

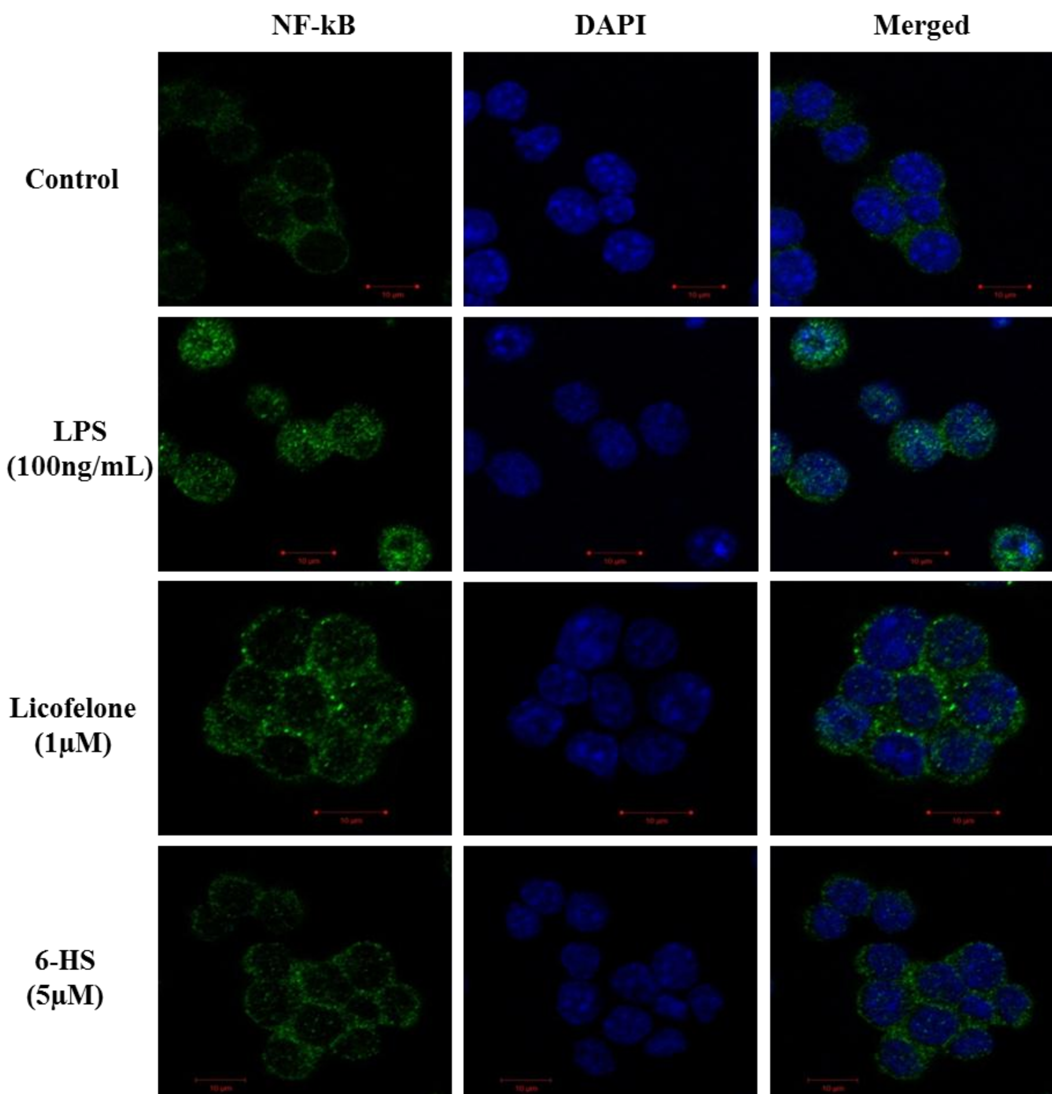


Figure 3-4: 6-HS inhibits translocation of NF-κB in LPS stimulated RAW 264.7 cell line. RAW 264.7 cells were pre-treated with or without 6-HS (5 µM) for 3 h and then challenged with LPS for 30 min. Cells were then immunostained for NF-κB (Green) and DAPI (Blue). Bar Scale: 1cm=10 µm.

3.3.1.4 Effect of 6-HS on expression of pro-inflammatory markers at mRNA and protein levels in LPS stimulated RAW 264.7 cells

6-HS showed inhibition of both NO and translocation of NF-kB to nucleus, therefore, pro-inflammatory markers mRNA expression studies were taken up, followed by protein

expression studies. These studies showed significant up-regulation of pro-inflammatory markers in response to LPS alone (Figure 3-5) and 6-HS inhibited these pro-inflammatory markers significantly in dose dependent manner.

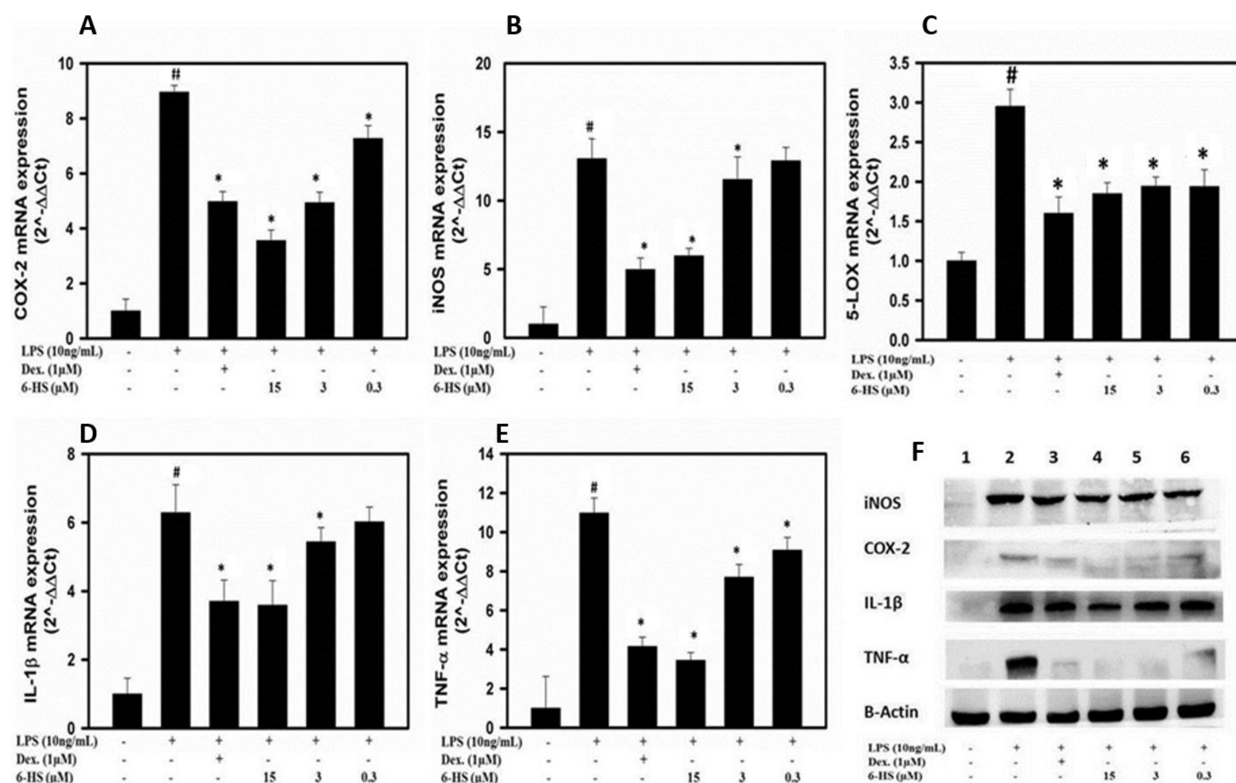


Figure 3-5: Effect of 6-HS on expression of mRNA of (A) COX-2, (B) iNOS, (C) 5-LOX, (D) IL-1β and (E)TNF-α and protein expression (F) in LPS challenged RAW 264.7 cells. RAW 264.7 cells were pre-treated with different concentrations as shown above and challenged with LPS for 12 h. cDNA was prepared from total RNA for Real time PCR for pro-inflammatory markers gene expression analysis and protein expression studies were carried out at 24 h after LPS challenge.

3.3.2 Evaluation of anti-inflammatory effects of 6-HS *in vivo* on paw edema and air pouch inflammatory mouse models.

3.3.2.1 *In vivo* effects of 6-HS on Carrageenan-induced paw edema in BALB/c mice

The *in vivo* anti-inflammatory effects of 6-HS were evaluated by intraplantar injection of 25 µL of 1% carrageenan and measuring paw thickness in millimeter (mm). Thickness of edema developed in paw was taken for calculation as per their average difference in thickness (mm) of the paw edema in the treated groups with the carrageenan group and are normalized by control group (0.1% DMSO in saline). Injection at intraplantar with 25µL, 1% carrageenan increased the thickness of paw edema to maximum peak at 3h, 1.48±0.40mm. In celecoxib (20mg/Kg bw), the mean peak of thickness was 0.725mm±0.36. In 6-HS treated group (20mg/Kg bw, 10mg/Kg bw), the mean peak thickness are 1.19 mm±0.71 and 1.30±0.90 respectively at 3 h after carrageenan treatment. Treated groups thus have shown significant attenuation of paw edema induced by carrageenan (Figure 3-6).

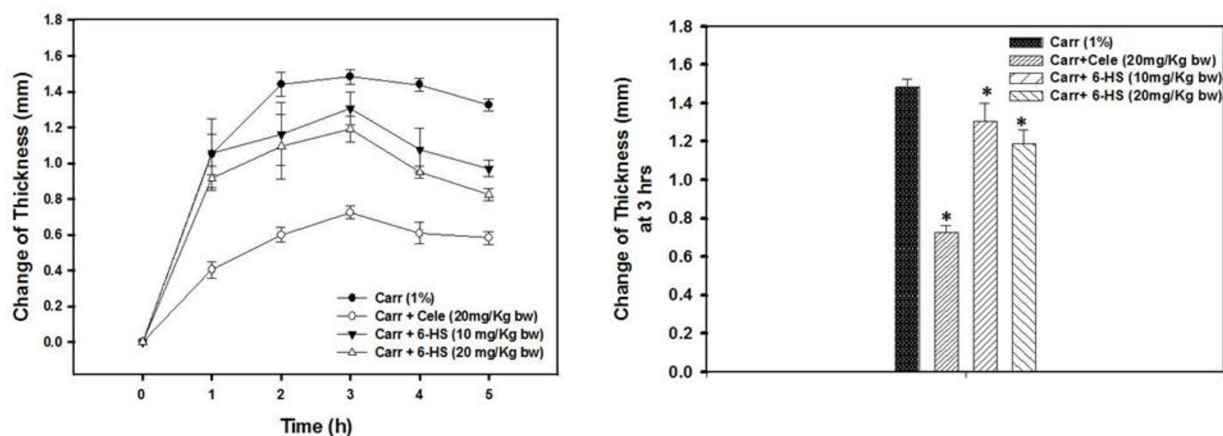


Figure 3-6: Effect of 6-HS on carrageenan induced paw edema in Balb/c mice. Animals were pre-treated with 100 µL of saline containing 6-HS (20, 10 mg/Kg bw), Celecoxib (20 mg/Kg bw) and saline (0.1% DMSO in saline) intraperitoneally and after 1 h, paw inflammation was induced with carrageenan.

3.3.2.2 Evaluation of anti-inflammatory effects of 6-HS *in vivo* on mouse air pouch model

3.3.2.2.1 Inflammatory reaction

Air pouch inflammatory mouse model was established as described in the methods. Animals were treated as follows: Saline treated; Carrageenan treated; Carrageenan + celecoxib treated; Carrageenan + 6-HS treated. Redness and swelling are important parameters for acute inflammation. In our studies, we have observed at 24 h carrageenan treatment, redness was maximum seen in animals treated with carrageenan alone and redness was decreased in celecoxib and 6-HS treated animals groups in comparison to animals treated with carrageenan alone (Figure 3-7).

3.3.2.2.2 Leukocytes infiltration into the air pouch tissue

Cells that get infiltrated into air pouch tissue were collected by gavaging with 5 mL of saline repeatedly to ensure complete recovery of cells from air pouch tissue. Collected cells were centrifuged, washed in DMEM and re-suspended in saline for its counting on hemocytometer. As shown (Figure 3-8), carrageenan alone (1%) group showed increase in cell infiltration from 6 h to 24 h. Infiltration of cells in 6-HS and celecoxib treated animal groups showed significant reduction in cells number in both time points. These values were highly significant when compare to carrageenan alone treated group.

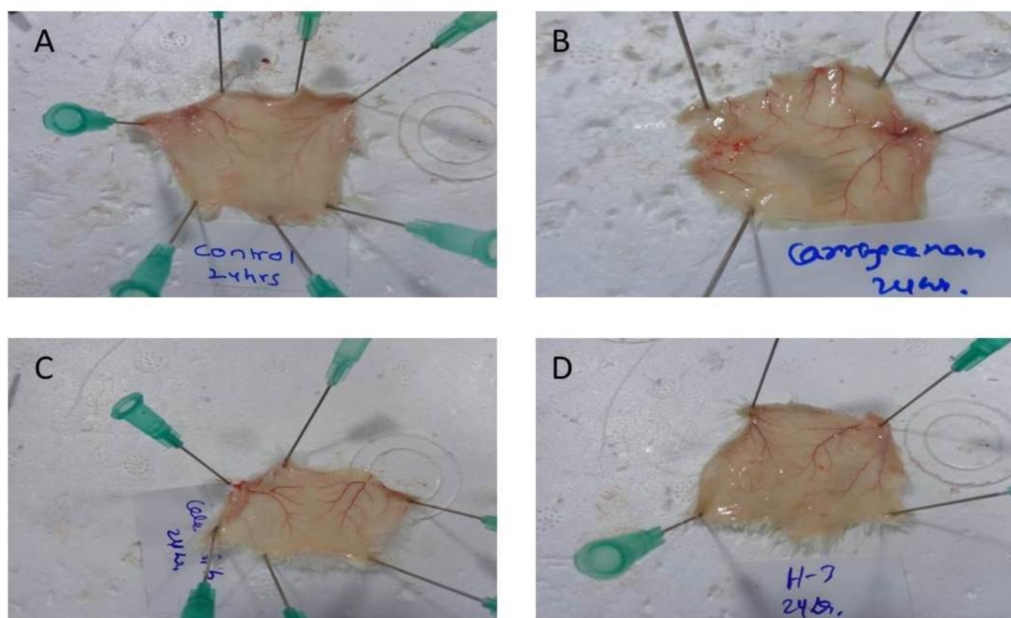


Figure 3-7: The photographs of air pouch tissue after 24 h treatment with saline (A), Carrageenan alone (B), Celecoxib + Carrageenan (C) and 6-HS + Carrageenan (D). As shown in the figure, carrageenan induced extensive vascularization as evidenced by redness (B), which was significantly reduced by 6-HS (D) treatment. The effect of 6-HS was comparable to that of celecoxib (C).

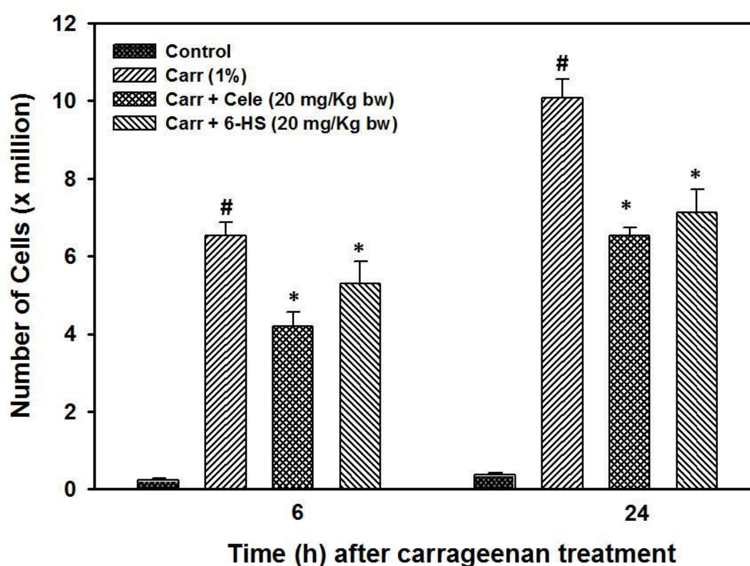


Figure 3-8: Effect of 6-HS on number of cells infiltrated into the pouch tissue of either carrageenan or carrageenan + 6-HS treated mice. Animals were sacrificed at 6 h and 24 h

time points after treatments. $^{\#}p<0.05$ vs control; $^{*}p<0.05$ when compared with the carrageenan and with the treated group.

3.3.2.2.3 Histological observation of neutrophils infiltration in air pouch tissue

Infiltration of cells during inflammation in air pouch tissue lead to formation of granulomatous tissue, also called pouch lining. The section of these linings showed increased infiltration of neutrophils. Carrageenan alone treated animal group showed maximum infiltration of neutrophils in pouch lining (Figure 3-9B), which demonstrates the induction of inflammation in air pouch tissue. The 6-HS and celecoxib treated animal groups, on the other hand, reduced infiltration of neutrophils, suggesting the anti-inflammatory property of 6-HS and celecoxib.

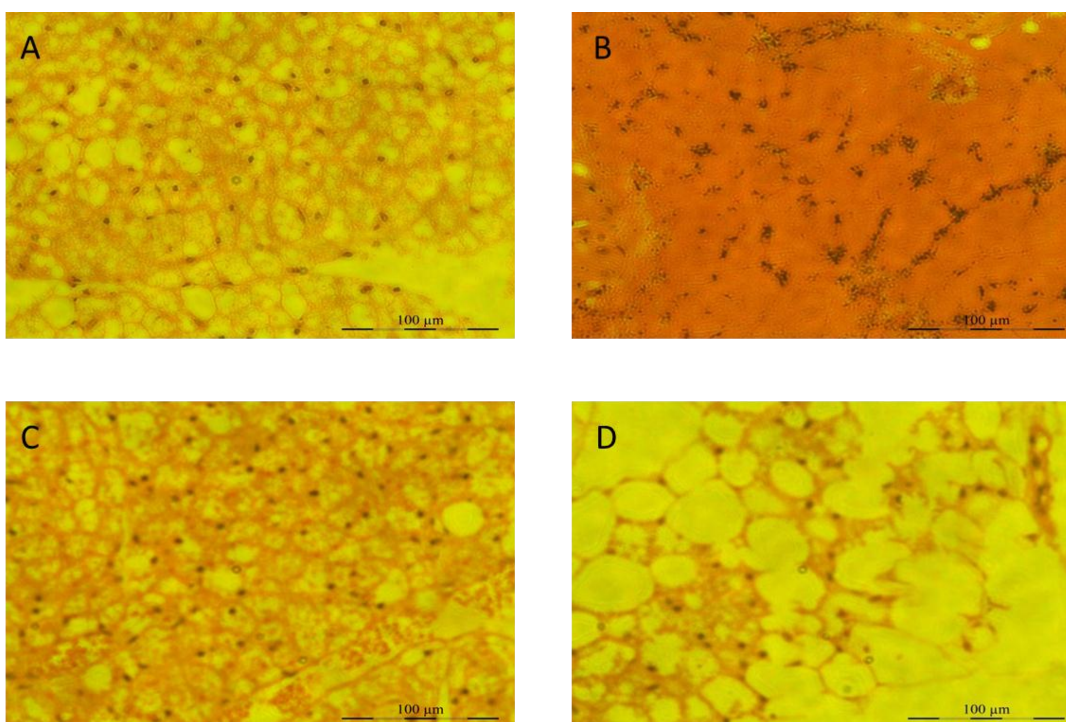


Figure 3-9: The photomicrographs (20x) of histological sections of the air pouch tissue after 24 h treatment with saline group (A), Carrageenan alone (B), Celecoxib + Carrageenan group (C) and 6-HS + Carrageenan (D). The air pouch tissue was fixed in formalin buffer and section was stained with hematoxylin and eosin.

3.3.2.2.4 Effect of 6-HS on expression of pro-inflammatory proteins (COX-2, iNOS, 5-LOX, TNF-α and IL-1β) in air pouch tissue.

Earlier it is shown that 6-HS reduced inflammatory conditions in mouse air pouch model of inflammation. In order to evaluate the mechanism behind these anti-inflammatory property, further studies on protein expression was carried out to check whether 6-HS has any effect on the expression of pro-inflammatory proteins, COX-2, iNOS, 5-LOX, TNF-α and IL-1β. From these studies, it is clear that pro-inflammatory proteins were significantly up-regulated in response to carrageenan alone and 6-HS potently reduced pro-inflammatory protein level. This down regulation of proteins showed strong anti-inflammatory effects of 6-HS in the present study (Figure 3-10).

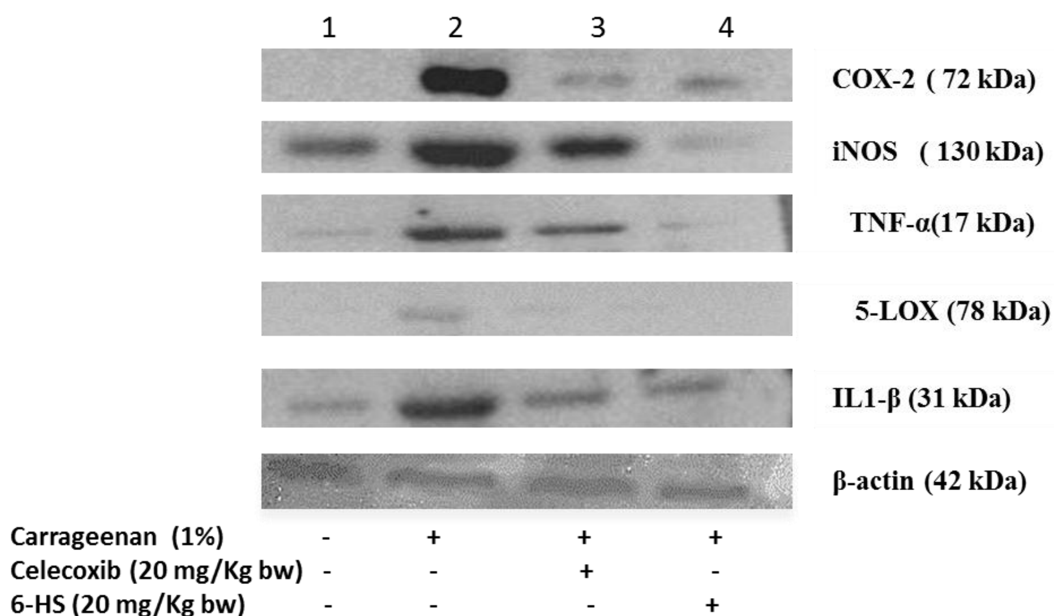


Figure 3-10: Effect of 6-HS on carrageenan induced protein expression in iNOS, COX-2, 5-LOX, TNF-α and IL-1β in air pouch tissue of mouse model. Immunoblots showed expression of COX-2, iNOS, 5-LOX, TNF-α and IL-1β protein in air pouch tissue of mice. Lane 1 is Control (0.1% DMSO in saline) as vehicle control, Lane 2 is carrageenan 1%, Lane 3 is celecoxib (20 mg/Kg wt) and lane 4 is 6-HS (20 mg/Kg wt).

3.3.3 Evaluation of anti-allergic effects of 6-HS on RBL-2H3 cell line *in vitro* and *in vivo* on passive systemic anaphylaxis mouse model.

3.3.3.1 Effect of 6-HS on cell viability of calcium ionophore (A23187) or IgE/antigen challenged RBL-2H3 cells

The cytotoxic effect of 6-HS on the cell viability of calcium ionophore (A23187) or IgE/antigen challenged on RBL-2H3 cell line were evaluated by MTT assay. 6-HS along with stimulus, calcium ionophore or IgE/antigen showed no cytotoxicity on the cells up to 30 µM (Figure 3-11). Therefore, based on this result, other cell based experiments were carried at or below 30 uM of 6-HS to analyze its anti-allergic activity.

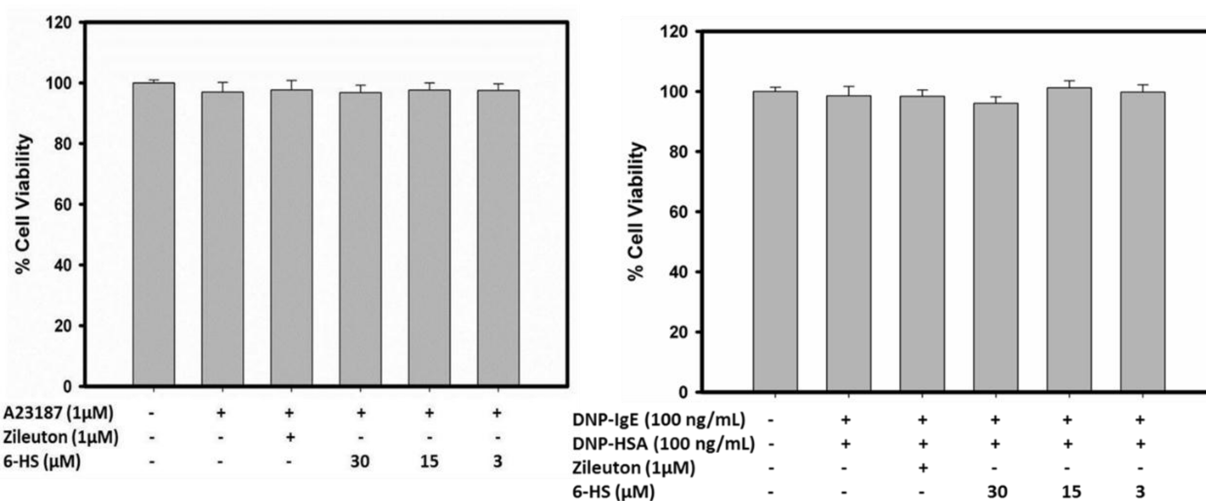


Figure 3-11: Effect of 6-HS on cell viability of calcium ionophore or IgE/antigen challenged in RBL-2H3 cells. RBL-2H3 cells were pre-incubated and or stimulated as per protocols mentioned in methodology.

3.3.3.2 Effect of 6-HS on release of β-hexosaminidase, histamine and leukotriene C_4 (LTC_4)

The anti-allergic property of 6-HS was also evaluated by ability to inhibit RBL-2H3 basophils' degranulation by two different stimuli: calcium ionophore and IgE/antigen (1µg/mL and 100 ng/mL respectively). In both the treatments, the calcium ionophore (A23187) and IgE/antigen assay, there were no significant cell death upto 30µM of 6-HS. In A23187, 6-HS showed significant dose dependent (3, 15, and 30 µM) reduction of *β*-hexosaminidase activity. 6-HS also showed dose dependent decrease in histamine and LTC_4 content when stimulated with IgE/antigen. In all these assays, zileuton (1 µM) served as the positive control.

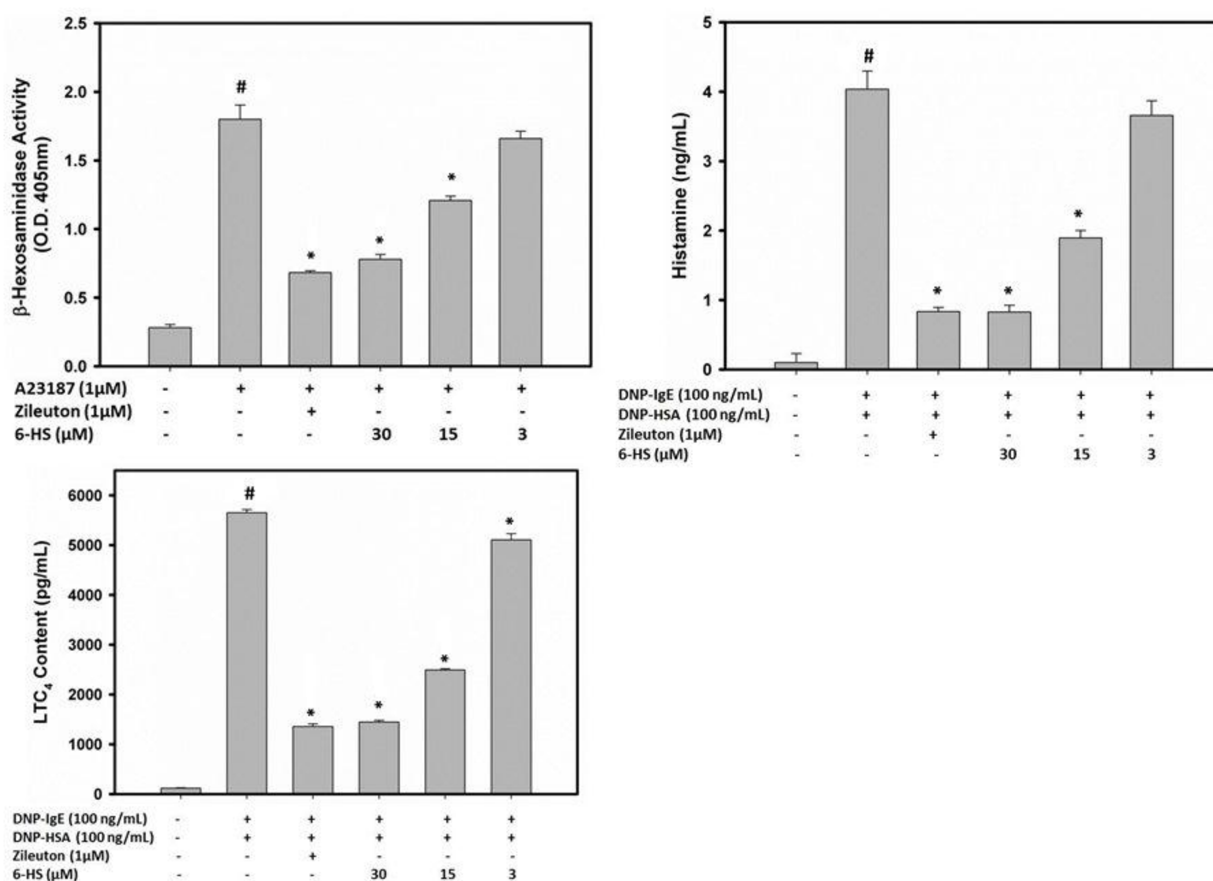


Figure 3-12: Effect of 6-HS on release of β-hexosaminidase, histamine and LTC_4. RBL-2H3 cells were pre-incubated and or stimulated as per protocols mentioned in the methodology.

3.3.3.3 Effect of 6-Hydroxy salvinolone on change in body temperature after passive systemic anaphylaxis reaction

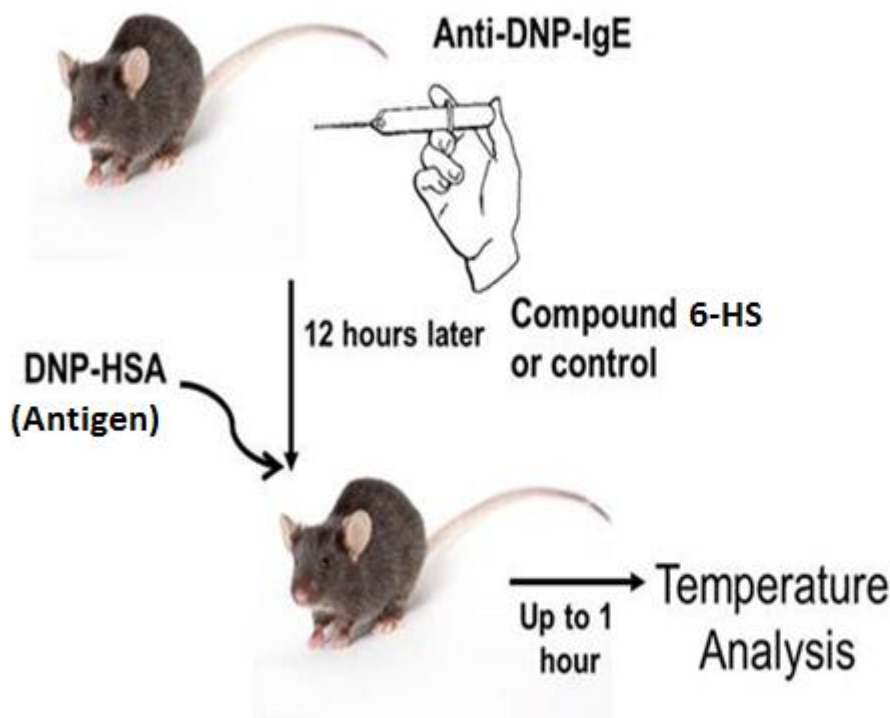


Figure 3-13: Schematic representation of passive systemic anaphylaxis using IgE/antigen as stimuli.

Balb/C mice were injected with anti-DNP specific IgE intraperitoneally, 12 hour later mice were pre-treated with 6-HS and one hour later antigen DNP-HSA was given intravenously (Figure 3-13). Body temperature was recorded for one hour just after antigen challenge. The results showed a decrease in body temperature and maximum drop in body temperature was recorded at 20 minutes with $2.8^0 \pm 0.3^0$C drop in antigen group. This decrease in body temperature was transient as it returned to its normal level within 60 minutes. 6-HS group also decreased body temperature by only $1.5^0 \pm 0.2^0$C with faster recovery to initial level within 25

min. These results indicate a role for 6-HS in exerting resistance to hypothermia during passive systemic anaphylaxis.

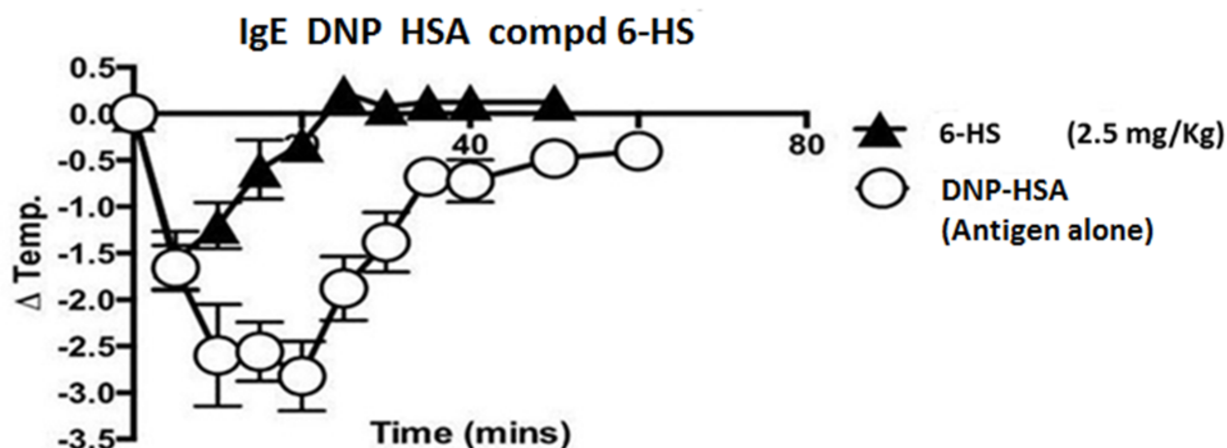


Figure 3-14: Effect of 6-HS on change in body temperature of BALB/c mice after the passive systemic anaphylaxis reaction. Body temperature was measured after intravenous injection of DNP-HSA for 60 min.

3.3.3.4 Effect of 6-HS on change in plasma histamine concentration after passive systemic anaphylaxis reaction

Passive systemic anaphylaxis was indued in BALB/c mice with antigen challenged as described in the methodology and the plasma histamine concentration was analyzed just before and after 3 minutes of antigen challenged. These studies showed a sharp increase in plasma

histamine levels from 0.14 ± 0.02 ng/mL to 7.76 ± 2.08 ng/mL in antigen challenged group and was significantly reduced (2.76 ± 1.51 ng/mL) in 6-HS group (Figure: 3-15).

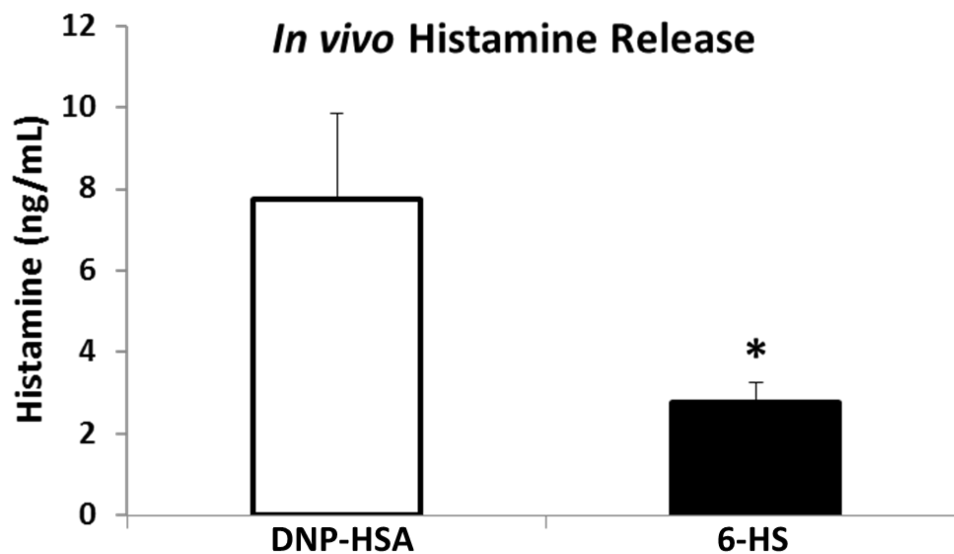


Figure 3-15: 6-HS inhibits IgE mediated histamine release in passive systemic anaphylaxis in murine model. Serum histamine release was measured 3 min after intravenous injection of DNP-HSA. *P<0.05.

3.4 Discussion:

Inflammation has a key role in host's defense against pathogens invasion. Inflammatory response is generally protective and aims in the removal of tissues of both the cause and consequences of tissue injury. Uncontrolled inflammation is associated with several disorders which includes respiratory, cardiovascular, neurological and many life style diseases. Anti-inflammatory drugs presently available in the market which includes NSAIDs, COXIBs, immune-suppressants, glucocorticoids etc are either less effective or with several side effects. Hence there is search for anti-inflammatory drugs which are safe and efficacious. In this scenario, there is greater demand for natural products with anti-inflammatory properties [115,116]. Earlier *Premna integrifolia* extracts were shown to have anti-bacterial, anti-arthritic, anti-tumor, anti-parasitic, anti-microbial, anti-inflammatory, cardiprotective, hepatoprotective, hypoglycemic, immunomodulatory, longevity promoting, and neuroprotective activities [96,97]. However, the specific molecule(s) involved in mediating these effects and the mechanism of action is largely unknown.

In the present study, 6-hydroxy salvinolone (6-HS), isolated from *Premna integrifolia* belonging to Brihatpunchmoola group of species, showed inhibition of both COX-2 and 5-LOX and also anti-inflammatory effects *in vitro* on RAW 164.7 cells and *in vivo* on paw edema and air pouch model of inflammation. Further 6-HS was shown to exert anti-allergic effects *in vitro* on RBL-2H3 cell line and *in vivo* on mouse model of passive systemic anaphylaxis. The present study also revealed inhibition of pro-inflammatory cytokine IL-1β, at nontoxic dose of 6-HS in LPS challenged mouse peritoneal macrophages *in vitro*. These studies suggest a role for 6-HS in interrupting whole cytokines cascade responsible for eliciting inflammatory responses [103–105]. The p^{50}/p65 heterodimers of NF-κB are held in the cytoplasm by an inhibitory molecule,

IκBα in most cells. Stimulatory signals such as LPS and TNF-α cause degradation of IκBα, leading to translocation of p50/p65 to the nucleus, leading to the induction of target genes. Our studies *in vitro* on RAW 264.7 cells, monitored through DAPI staining, showed inhibition of the translocation of NF-κB into nucleus by 6-HS. This inactivation of NF-kB may be responsible for inhibition of several pro-inflammatory genes and mediators observed in the present study. Nitric oxide (NO), one of the pro-inflammatory mediators produced by inducible nitric oxide synthase (iNOS) during inflammation, modulates cytokine production. NO is known to increase vascular permeability and thus increasing exudation by acting on endothelium [117]. Mouse Paw edema and air pouch models are considered as straightforward model systems to investigate inflammatory responses *in vivo*. The air pouch model has been proven to be an ideal model system for studies on acute and chronic inflammation [118], where inflammatory parameters like infiltration of cells to the pouch could be analyzed. In the present study a decrease in paw edema was observed on treatment with different doses of 6-HS after induction with carrageenan. Air pouch model was exploited to evaluate the effect of 6-HS on carrageenan induced infiltration of inflammatory cells. At a dose of 20 mg/kg body weight 6-HS reduced carrageenan-induced infiltration of leukocytes. These along with histopathological observations it is clearly demonstrated that 6-HS is involved in the reduction of inflammation. Further in this study, 6-HS treatment caused significant reduction in the levels of pro-inflammatory mediators (iNOS, COX-2, 5-LOX, Il-1β and TNF-α) *in vitro* and *in vivo* model as evidenced by mRNA expression and Western blot analysis. COX-2 is known to exist in two isoforms, COX-1 and COX-2. COX-2 is known to be induced under inflammatory conditions and contributes to inflammation through production of pro-inflammatory signaling molecules. In the present study 6-HS not only

inhibited COX-2 but also decreased the LPS/carrageenan induced expression of COX-2 *in vitro* as well as *in vivo* respectively.

Allergy mediated disorders are known to affect individuals throughout the world. The allergic response is quantified by inflammatory mediators that are released such as pro-inflammatory cytokines, histamines, leukotrienes [119] from activated mast cells by its degranulation by substances like IgE [120]. Many anti-histaminic drugs, steroids, inhibitors or suppressants of inflammatory mediators are known to be anti-allergic agents. Many of these are potent but known to have adverse side effects. Therefore, need for development of naturally occurring substances with anti-allergic properties that can modulates allergy is most needed [121]. Cysteinyl leukotrienes (LTC_4, LTD_4 and LTE4) act as inflammatory mediators in several inflammatory diseases [122]. LTC_4, a glutathione conjugated product of LTA_4, was known to increase in nasal secretions of allergic rhinitis patients after nasal antigen exposure. It was found to be 1000 times more potent than histamine in mediating allergic responses [123]. Increased levels of LTC_4 was reported in the serum as well as skin lesions of AD (Anti-diuretic) patients [124]. In the present study, LTC_4 content was decreased dose dependently on treatment with 6-HS in Rat basophilic leukemia (RBL) cells. It is known that upon allergen exposure, cross-linkage of IgE bound to its receptor triggers complex reactions which includes calcium influx, leading to degranulation. 6-HS showed dose dependent decrease in β-Hexosaminidase and histamine release during RBL-2H3 cells stimulated with Calcium Ionophore (A23187) /IgE antigen. These effects of 6-HS, observed in the present study, may be mediated through the inhibition of 5-LOX, the key enzyme in the formation of leukotrienes.

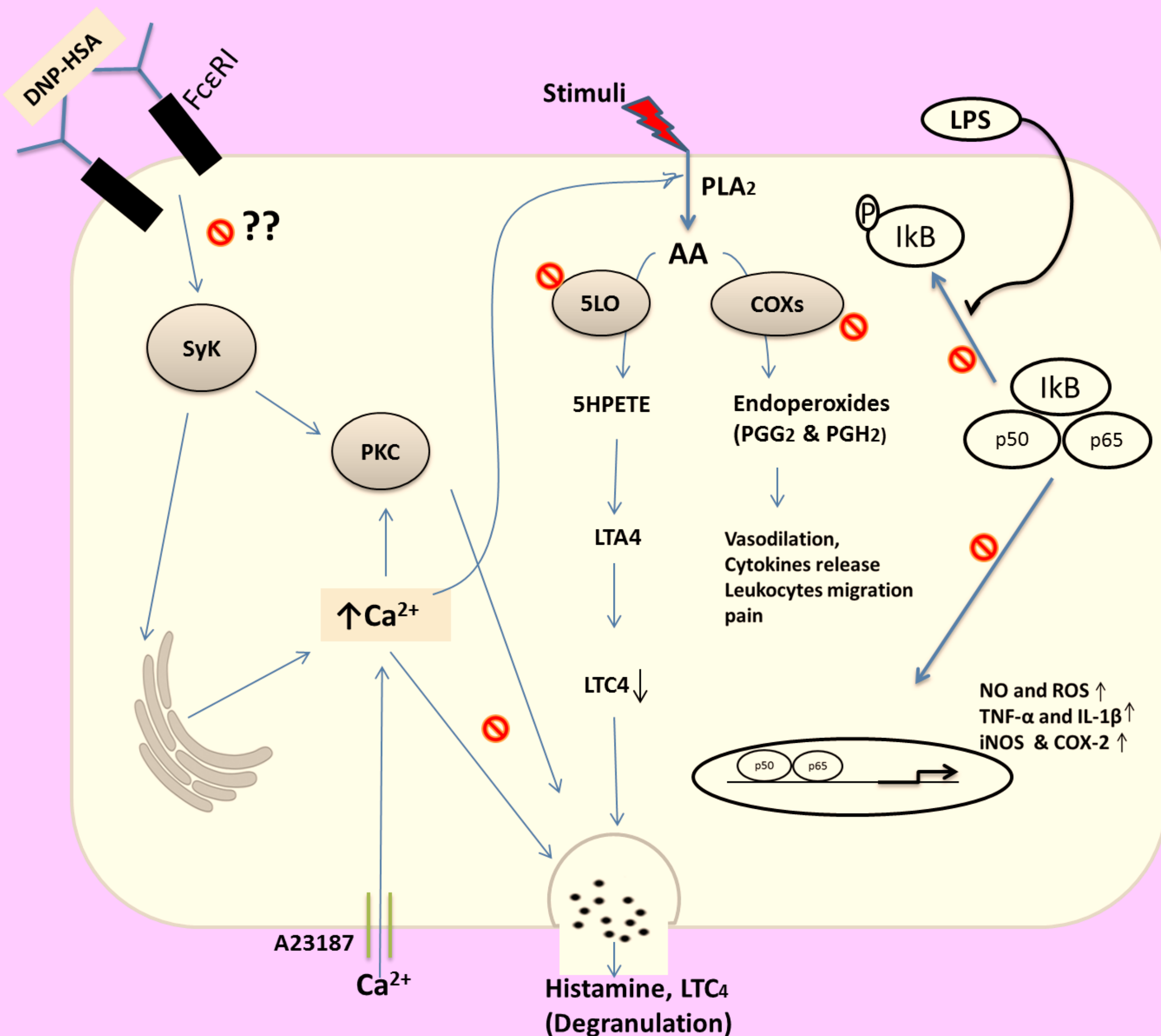
In the present study, we induced the systemic anaphylaxis reaction [113] [125] in Balb/C mice by passive immunization of the mice with anti-DNP-specific IgE and then by triggering an

allergic reaction with DNP-HSA. In these animals, hypothermia, one of the symptoms of systemic anaphylaxis, was assessed. 6-HS along with DNP-HSA, when injected into mice, showed lesser drop in body temperature and histamine release than DNP-HSA alone challenged group. These studies suggest that 6-HS could be a potential candidate for treating allergic disorders. Further-in-depth studies on safety and efficacy in other animal models, however, are required to develop the molecule into a potential clinical candidate for treatment of allergic disorders.

In conclusion, the present study reveals that 6-HS, a COX-2/5- LOX dual inhibitor isolated from the medicinal plant, *Premna integrifolia*, acts as a potent anti-inflammatory agent by down regulating the expression of pro-inflammatory markers and also blocking the release of histamine *in vitro* and *in vivo*, suggesting that it could be a potential pre-clinical candidate for treatment of inflammatory and allergic disorders. Further, pre-clinical studies on the safety and efficacy, however, are required before taking up clinical studies.

Chapter 4

SUMMARY AND CONCLUSIONS



Premna integrifolia, one of the medicinal plants from Brihatpunchmoola, is widely used in traditional medicine especially in Ayurvedic formulations. Its formulation is used for treatment of many diseases such as allergy, inflammation, cough & cold, asthma, bronchitis, pain, wound healing, arthritis and diabetes. The known pharmacological activities of *Premna integrifolia* are anti-inflammatory, analgesic, antibacterial, anti-arthritic, antimicrobial [95], antioxidant, anti-obesity/hypolipidemic, anti-parasitic, cardiac stimulant/cardio protective, antiulcer/gastro protective, central nervous system (CNS) depressant, hypoglycemic, hepatoprotective, immunomodulatory, neuroprotective, longevity-promoting, etc. [96,97].

4.1 Discovery and Development of anti-inflammatory drugs: Eicosanoids as the targets.

Eicosanoids are metabolites of eicosapolyenoic acid like arachidonic acid that are formed by oxidation of these fatty acids via COXs and LOXs pathways, specifically the prostaglandins and leukotrienes form the key mediators of allergy, asthma and other inflammatory disorders. The conventional NSAIDs like ibuprofen and aspirin and indomethacin etc. that inhibits both COX-1 and COX-2 non-specifically and thus, prostaglandin biosynthesis. However, these are not free from side effects like gastric and renal complication. After COX-2 discovery, that reveals that it is mainly induced during inflammation, leads to several COX-2 specific inhibitor development such as valdecoxib, celecoxib and rofecoxib and many more. The specific inhibitors of COX-2 inhibitors, though were without gastric side effects, but were found to have cardiac side effects on long term use in cases such as arthritis [98]. In the light of the above, search for new anti-inflammatory drugs without gastric and cardiac side effects were intensified. In this search for next generation anti-inflammatory drugs, COXs-LOX dual inhibitors have

gained importance, because of their superior anti-inflammatory properties with no gastric or cardiac side effects [66]. It is in this connection the present study was taken up to isolate and identify specific natural products with anti-inflammatory properties from *Premna integrifolia*.

4.2 Petroleum ether extract (PEE) of *Premna integrifolia* showed anti-inflammatory and immunomodulatory properties

Earlier work done on *Premna integrifolia* was mainly on their therapeutic applications, isolation of several components and their pharmacological properties. There have been no studies that focus on the molecular target and the specific compounds involved. In the present study, we have focused on the evaluation of anti-inflammatory and immunomodulatory properties of petroleum ether extract of *Premna integrifolia*, followed by assay-guided isolation and characterization of active principle(s) with COX-2/5-LOX dual inhibition. These systematic studies have led to the isolation, identification and characterization of 6-hydroxy salvinolone (6-HS) from the petroleum ether extract (PEE) of young roots of *Premna integrifolia* as the COX-2/5-LOX dual inhibitor. Further, 6-HS was found to be anti-allergic and anti-inflammatory as evaluated by *in vitro* studies on cell line and *in vivo* studies on animal models.

Immunomodulatory effect of traditional medicinal plants can be an alternative to the conventional therapy for a variety of diseases [99]. In certain disease conditions for examples immune deficiency, autoimmunity or in cancer, immunomodulators play an important role to maintain a disease-free state. Our studies, examined immunomodulatory effects of *Premna integrifolia* extracts on human peripheral blood mononuclear cells where Con A was used as the mitogen. PEE and methanolic extracts of young roots of *Premna integrifolia* showed potent

immunomodulatory effect at nontoxic doses, suggesting their possible application in the treatment of various diseases, including cancer. The recent Nobel Prize in medicine awarded for cancer immunotherapy [101,102] highlights the importance of immune-modulators in the treatment of various diseases.

In addition to major role of COX-2 and 5-LOX in inflammation, several pro-inflammatory cytokines are associated in promoting inflammation. Cytokines exist in “cascades” where interruption of one cytokine leads to interruption of the whole cascade that might be sufficient to control the inflammatory diseases [103–105]. IL-2, the anti-inflammatory cytokine was shown to play an important role in controlling immune homeostasis by the spontaneous lethal autoimmunity in mice lacking IL-2 expression [106]. In the present study, *in vitro* anti-inflammatory activities of PEE of *Premna integrifolia* were analyzed on LPS challenged RAW 264.7 cell line. PEE showed induction of anti-inflammatory (IL-2) cytokines. PEE also showed dose dependent decrease in nitrite level and pro-inflammatory cytokines (IL-1β and IL-6) and these effects were comparable to dexamethasone. Further, PEE down-regulated the expression of LPS -induced pro-inflammatory markers (iNOS, 5-LOX, COX-2, IL-1β and TNF-α) at protein level in RAW 264.7 cell line. These findings demonstrate the anti-inflammatory effects of PEE of *Premna integrifolia in vitro* on mouse macrophage cell line, RAW 264.7.

The carrageenan-induced acute inflammatory model is one of the widely used methods to test the anti-inflammatory effects of various agents *in vivo*. It has been reported that the methanolic extract of roots of *Premna integrifolia* inhibits carrageenan induced rat paw edema [76]. This animal model has biphasic inflammatory conditions. First phase that is from 1-2 h is mainly mediated by histamine and serotonin. PEE being an inhibitor of 5-LOX that inhibits leukotriene formation may inhibit this phase of edema formation [126]. PEE contains terpenoids

(diterpenoid) that are known to impair histamine release from mast cells and therefore, exert anti-inflammatory effects [107]. The second phase is from 3-5 h and is due to the release of newly synthesized prostaglandins, arachidonic acid metabolites formed via cyclooxygenase pathway [108]. Our studies clearly demonstrate the potent inhibition of paw edema by PEE, may be through inhibition of COX/LOX pathway in addition to modulation of cytokines.

4.3 Assay guided purification of 6-hydroxy salvinolone (6-HS) from PEE of *Premna integrifolia*

Further attempts at isolating the active principle(s) involved in these anti-inflammatory effects of PEE resulted in the isolation and characterization of 6-hydroxy salvinolone (6-HS) with a molecular weight (MW) of 330.41, which is inhibiting both COX-2 and 5-LOX. These studies indicate that 6-HS, being a COX-2/5-LOX dual inhibitor, may form a potential anti-inflammatory drug candidate without gastric and cardiac side effects. As 6-HS is highly potent in inhibiting 5-LOX (IC_{50} value, 17.5 μM), it may have greater potential for the treatment of allergy and asthmatic conditions.

4.4 6-hydroxy salvinolone (6-HS), a COX-2/5-LOX dual inhibitor is anti-inflammatory agent

Inflammation is an immune response being regulated by series of cytokines, prostaglandins, NO and growth factors that are mainly produced by activated macrophages. Therefore, the molecules or agents that reduce the expression of these inflammatory genes such as iNOS, COX-2, 5-LOX, IL-1β and TNF-α show therapeutic potency towards anti-inflammatory disorders treatment. This present study showed, 6-HS inhibit production of IL-1β and NO at concentration dependent manner and partly this may be due to COX-2 inhibition by 6-

HS in LPS challenged mouse peritoneal macrophage cells. To understand further mechanism of inhibition of 6-HS, we studied the mRNA expression levels of iNOS, COX-2, 5-LOX, IL-1β and TNF-α and further validated by protein expression studies of iNOS, COX-2, IL-1β and TNF-α. The inhibition in COX-2 and iNOS genes and protein expressions by 6-HS in dose dependent manner, was evidenced by reduction in their mRNA and protein levels. Also, the observed decrease in NO production with increase in 6-HS concentration may be due to down regulation of expression in the iNOS at mRNA and protein levels. Mammalian transcription factor NF-kB, which controls cell survival genes expression and is activated by LPS, is also responsible for induction of pro-inflammatory genes expression, such as COX-2, iNOS, IL-1β and TNF-α. In our studies, we found that 6-HS inhibits the LPS-induced translocation of NF-κB. Therefore, this result tells that pro-inflammatory markers studied in cells are down regulated in LPS challenged cells, due to blockage of the activation of NF-κB by the COXs/5-LOX dual inhibitor, 6-HS.

Animal experiments are extremely important to understand the entire inflammatory process because it demonstrates a complete length of events, right from the point of time of the stimulus given and till the reaction get naturally resolved [127]. In this study, we have chosen, air pouch mouse model to study the progression of inflammatory process. Air pouch model in rodents are very similar to condition of the arthritis in humans because both the air pouch fluid and the synovial fluid are found to be very similar [128].

In the present study, the inflammatory response in mouse air pouch was defined as acute, as with increasing time of exposure of inflammatory stimulus leads to more migration of leukocytes into pouch. These cells are mainly responsible for inflammatory response in pouch lining. The inflammatory response can be quantified by degree of redness, number of infiltrated leukocytes into the pouch and infiltration of neutrophils. The quantification of these parameters

is of significance in assessing the efficacy of anti-inflammatory agents. In this study, 6-HS showed a decrease in these pro-inflammatory parameters suggesting its anti-inflammatory property. In addition, the air pouch model was employed to further evaluate the efficacy of 6-HS, as anti-inflammatory agent, and to understand the molecular mechanisms involved in its action. In carrageenan induced inflammatory model, 6-HS showed down regulation of pro-inflammatory markers COX-2, iNOS, 5LOX, IL-1β and TNF-α at protein level. These studies indicate that 6-HS acts as an anti-inflammatory agent, which may be mediated by both the mechanisms – inhibition of COX-2 and 5-LOX enzymes and down regulation of expression of COX-2, iNOS and 5-LOX as observed in the present study. These *in vitro* and *in vivo* studies further strengthen the anti-inflammatory potential of 6-HS and could form an ideal anti-inflammatory drug candidate.

4.5 6-HS showed anti-allergic activity *in vitro* and *in vivo*

Basophils and mast cells play a central role in immediate allergic and inflammatory reaction. Histamine one of the important potent mediators of allergy and/or inflammation. Majority of histamine in the body is stored as pre-formed amine in mast cells or basophils. Leukotriene C_4 (LTC_4), another important mediator of allergy, is thousand times more potent than histamine [123]. To explore the anti-allergic properties of 6-HS, we have estimated the β-hexosaminidase, histamine and Leukotriene C_4 (LTC_4) concentration in 6-HS pretreated RBL-2H3 cells induced with A23187 and/ or DNP-HSA. Further, anti-allergic property of 6-HS was validated in passive systemic anaphylaxis mouse model. Manifestation of systemic anaphylaxis includes hypothermia, airway obstruction and hypotension and mainly mediated by histamine [129]. The systemic anaphylaxis reaction was induced in mice with the help of anti-DNP-specific

IgE and was triggered vis DNP-antigen. Hypothermia, one of symptoms of systemic anaphylaxis, was assessed in these animals. 6-HS group of mice showed lesser drop in body temperature and histamine release than DNP antigen alone challenged mice. These studies suggest that 6-HS could be a potential candidate for further evaluation in the treatment of allergic disorders.

In conclusion, the present work involving systematic evaluation through *in vitro* as well as *in vivo* studies reveals that 6-HS, a COX-2/5- LOX dual inhibitor isolated from medicinal plant *Premna integrifolia*, acts as a potent anti-inflammatory agent by down regulating the expression of pro-inflammatory markers and by inducing anti-inflammatory markers. Further *in vitro* and *in vivo* studies also demonstrate that 6-HS inhibits the release of histamine and leukotriene C_4 in response to antigen challenge, suggesting it as a potential candidate for treatment of allergic disorders. The present study thus provides a clear scientific evidence for several of the claims in the Ayurvedic literature on the anti-inflammatory and anti-allergic properties of the medicinal plant, *Premna integrifolia*. The study not only identifies 6-HS as a potential candidate but also provides molecular mechanism behind these anti-inflammatory and anti-allergic properties. A schematic presentation showing the possible mechanisms behind the anti-inflammatory and anti-allergic properties of 6-HS is presented in figure 4.

Further in-depth pre-clinical studies on efficacy and safety in other animal models, however, are required to develop 6-HS as a clinical candidate for the treatment of inflammatory and allergic disorders.

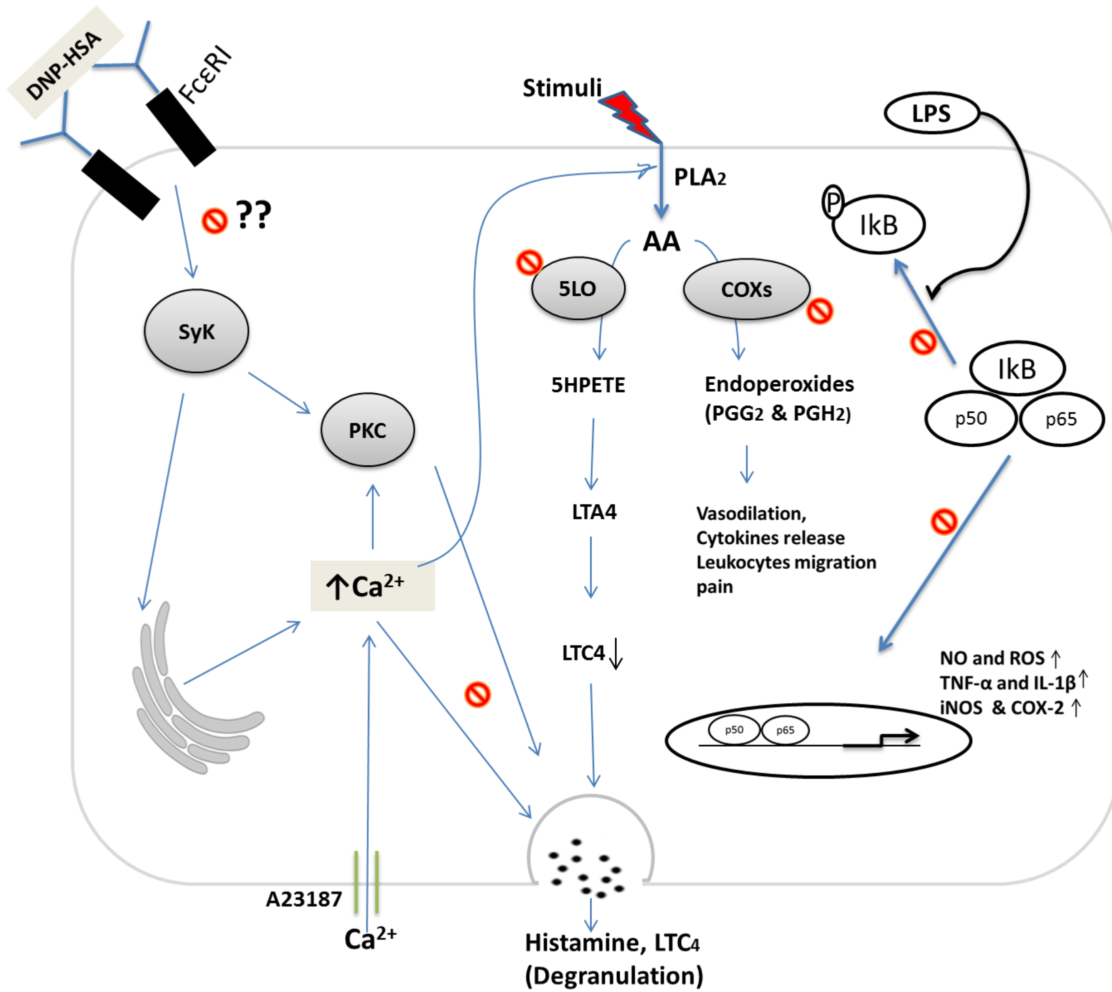


Figure 4: Schematic presentation showing the possible mechanisms behind the anti-inflammatory and anti-allergic properties of 6-hydroxy salvinolone (6-HS), isolated from the young roots of *Premna integrifolia*. The potential sites of action of 6-HS are indicated as "🚫" in the scheme.

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INDEX

Air pouch model of inflammation, 60

Allergy, 12

Cyclooxygenases, 8

Eicosanoids, 7

Inflammation, 1

Leukotriene C_4, 59

Lymphocyte proliferation assay, 31

Mast cells, 15

Mediators of inflammation, 4

Mouse peritoneal macrophages, 53

Passive systemic anaphylaxis, 62

Paw edema animal model, 32

Petroleum ether extract (PEE), 26

***Premna integrifolia*, 23**

Prostaglandin E_2 , 30

Rat basophilic leukemia Cells, 17

RAW 264.7 cell line, 29

RBL-2H3 cell line, 57

Real Time-PCR, 56

RP-HPLC column chromatography, 23

β-hexosaminidase , 59

5-Lipoxygenase, 9

6-hydroxy salvinolone, 47

List of Publications and Manuscripts

1. **Azad R**, Babu NK, Gupta AD, Reddanna P. "Evaluation of anti-inflammatory and immunomodulatory effects of Premna integrifolia extracts and assay-guided isolation of a COX-2/5-LOX dual inhibitor." Fitoterapia. 2018 Nov; 131:189-199.

2. **Rajaram A**, Vanaja GR, Vyakaranam P, Rachamallu A, Reddy GV, Anilkumar K, Arunasree KM, Dhyani A, Prasad NK, Sharma S, Chandra Joshi M, Kimothi GP, Brindavanam NB, Reddanna P. "Anti-inflammatory profile of Aegle marmelos (L) Correa (Bilva) with special reference to young roots grown in different parts of India". J Ayurveda Integr Med. 2018 Apr - Jun; 9(2):90-98.

3. Anilkumar K, Reddy GV, **Azad R**, Yarla NS, Dharmapuri G, Srivastava A, Kamal MA, Pallu R. "Evaluation of Anti-Inflammatory Properties of Isoorientin Isolated from Tubers of *Pueraria tuberosa"*. Oxid Med Cell Longev. 2017; 2017:5498054.

4. Lokwani D, **Azad R**, Sarkate A, Reddanna P, Shinde D. "Structure Based Library Design (SBLD) for new 1,4-dihydropyrimidine scaffold as simultaneous COX-1/COX-2 and 5-LOX inhibitors". Bioorg Med Chem. 2015 Aug 1; 23(15):4533-43.

5. Yarla NS, **Azad R**, Basha M, Rajack A, Kaladhar DS, Allam BK, Pragada RR, Singh KN, K SK, Pallu R, Parimi U, Bishayee A, Duddukuri GR. "5-Lipoxygenase and cyclooxygenase inhibitory dammarane triterpenoid 1 from Borassus flabellifer seed coat inhibits tumor necrosis factor-α secretion in LPSInduced THP-1 human monocytes and induces apoptosis in MIA PaCa-2 pancreatic cancer cells". Anticancer Agents Med Chem. 2015; 15(8):1066-77.

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7. Anti-Inflammatory and Anti-allergic effects of 6-hydroxy salvinolone, a COX-2/5-LOX Dual Inhibitor Isolated from the roots of *Premna integrifolia* (manuscript in preparation).

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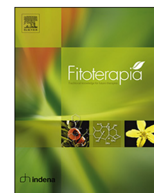
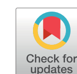


Evaluation of anti-inflammatory and immunomodulatory effects of *Premna integrifolia* extracts and assay-guided isolation of a COX-2/5-LOX dual inhibitor



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ABSTRACT

Premna integrifolia (Agnimantha brihat) is a traditional medicinal plant with a prominent place in Ayurveda, Siddha and Unani systems of medicine. In this study we have evaluated the anti-inflammatory and immunomodulatory properties of the *Premna integrifolia* root extracts employing cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and 5-lipoxygenase (5-LOX) enzyme-based assays, lymphocyte proliferation assay, pro- and anti-inflammatory cytokines measurement. Petroleum ether extract (PEE) of *Premna integrifolia* showed potent inhibition of COX-2 and 5-LOX with IC_{50} values of 6.15 μg/mL and 11.33 μg/mL respectively. In *in vitro* studies on RAW 264.7 cell line, PEE showed inhibition in the formation of nitric oxide (NO), pro-inflammatory cytokines (IL-1β, IL-6), prostaglandin E_2 (PGE_2) production, induction of anti-inflammatory cytokine (IL-2) and down-regulation of expression of COX-2, 5-LOX, TNF-α, IL-1β and iNOS. PEE also significantly reduced carrageenan-induced paw edema in mouse model of inflammation. Further, attempts in isolating the active principle (s) involved in these anti-inflammatory effects of PEE by separation on RP-HPLC resulted in the isolation of four active peaks, H1, H2, H3 and H5, inhibiting COX-1, COX-2 and 5-LOX, out of which H3 was identified as 6-hydroxy salvinolone (6-HS). Present findings reveal that PEE of roots of *Premna integrifolia* exhibits potent anti-inflammatory and immunomodulatory activities, which could form a potential source for development of anti-inflammatory drugs. 6-HS, a COX-2/5-LOX dual inhibitor along with other lead molecules isolated from PEE of *Premna integrifolia* may form lead molecules for the development of COX-LOX dual inhibitors.

1. Introduction

Premna integrifolia belongs to the family Verbenaceae commonly known as Arani or Agnimantha [1]. Roots of *Premna integrifolia* are one of the essential constituents of Ayurvedic formulation, “Dasmula” [2] used in the treatment of various ailments in traditional systems of medicine [3]. *Premna integrifolia* is a large shrub or a small tree widely distributed throughout the dry parts of India, Bangladesh, Sri Lanka, Myanmar and south-east Asia. All parts of this plant, *i.e.*, leaves, stems, barks have medicinal value. The roots are widely used in the treatment of diabetes, inflammation, swellings, bronchitis, dyspepsia, liver diseases, fever, constipation, piles *etc.* [4,5]. Also this plant is widely used by the traditional practitioners as cardiotonic, antibiotic, carminative, hepatoprotective and antitumor [6]. The pharmacological properties reported in the literature include its antidiabetic [7], anti-atherosclerotic [8], hypolipidemic, analgesic and antimicrobial [9] and antioxidant [10] activities. Phytochemical investigations of *Premna integrifolia* revealed the presence of flavonoid luteolin [11], sterols, triterpenes, alkaloids premnine, ganikarine and premnazole.

The non-steroidal anti-inflammatory drugs (NSAIDs), which block the formation of pro-inflammatory prostaglandin E_2, are commonly used to treat pain and inflammatory disorders. NSAIDs inhibit non-selectively both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), enzymes involved in the production of prostaglandins [12]. However, the use of NSAIDs is associated with several side effects that include gastrointestinal complications, bleeding, perforation of the esophagus, stomach, duodenum and mucosal lesions, platelet dysfunction, rare kidney [13,14] and liver problems, headaches, allergic reactions, *etc.* These side effects were attributed to the inhibition of COX-1, which is cytoprotective, in addition to COX-2 which is involved in all inflammation disorders. To overcome these problems, the selective COX-2 inhibitors (COXIBs) were developed. Though COXIBs are

Abbreviations: LPS, lipopolysaccharides; Con A, Concanavalin A; PEE, petroleum ether extract; COX, Cyclooxygenase; 5-LOX, 5-Lipoxygenase

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without gastric side effects, they were found to enhance cardiac-related complications on long term use [15]. These cardiac side effects of COXIBs have been attributed to be due to activation of 5-lipoxygenase (5-LOX), that mediates allergy and asthma. With this background the COX-2/5-LOX dual inhibitors (CLOXIBs) are emerging as the front runners of the next generation anti-inflammatory drugs. COX-2 and 5-LOX involved in arachidonic acid metabolic pathways are associated with various inflammatory and allergic disorders [16]. The COX-2/5-LOX dual inhibitors showed least side effect than NSAIDs and COXIBs [17,18]. In this scenario, the use of natural plant extracts/compounds with COX-2/5-LOX dual inhibition may have an added safety benefit.

In the present study, the roots of *Premna integrifolia* were extracted with four solvent systems, out of which PEE showed the most potent inhibition for both COX-2 and 5-LOX enzymes and also showed immunomodulatory effect on human lymphocytes. PEE also showed potent anti-inflammatory effects *in vitro* on RAW 264.7 cells as well as *in vivo* on mouse paw edema model of inflammation. In the quest to isolate COX-2/5-LOX dual inhibitor(s), the PEE extract was subjected to partial purification on an open silica column and most active fraction towards COX-2/5-LOX dual inhibition was subjected to RP-HPLC to purify COX-2/5-LOX dual inhibitor(s). These studies resulted in isolation of four active molecules with potent inhibition of COX/LOX enzymes, out of which one is characterized as 6-hydroxy salvinolone (6HS).

2. Materials and methods

2.1. Reagents and chemicals

Phosphate buffered saline (PBS), Culture medium DMEM, fetal bovine serum (FBS), penicillin, streptomycin and trypsin-EDTA were purchased from Gibco. TMPD (*N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine), Hematin, MTT (3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide), lipopolysaccharide (LPS) and Griess reagent were purchased from Sigma–Aldrich, USA. Arachodonic acid (AA) was procured from Nu-Chek-Prep (MN, USA). Enzyme immunoassay kit for IL-1β, IL-6, IL-2 and PGE_2 were purchased from R&D system, Inc. (MN, USA). Anti-COX-2 antibody and anti 5-LOX antibodies were obtained from Santa Cruz Biotechnology Inc. (Texas, USA). Anti-iNOS antibody was purchased from Thermo Fisher Scientific (MA, USA). Anti-TNF-α and anti IL-1β antibodies were purchased from R&D system, Inc. (MN, USA). Solvents for chromatography were of HPLC grade. All other chemicals were of analytical grade.

2.2. Collection of Premna integrifolia roots

Plant material was collected from Tirupati region of Andhra Pradesh, India. The plant was identified as *Premna integrifolia* L. belonging to Verbenaceae by Late Prof. Kottapalli Seshagirirao, Department of Plant Sciences, the University of Hyderabad on 4th April 2016. The root voucher specimen number was assigned as Rajaram-R2022 and voucher specimen was deposited at the University of Hyderabad Herbarium (World Acronym: UH).

2.3. Preparation of Premna integrifolia roots extracts

The roots of *Premna integrifolia* were sun dried for 15 days, powdered and successively extracted with soxhlet apparatus with petroleum ether, ethyl acetate, methanol and water in the increasing polarity index. These extracts were dried using a rotatory evaporator followed by lyophilization. Petroleum ether, ethyl acetate, and methanol extracts were dissolved in dimethyl sulphoxide (DMSO) while the water extract was dissolved in double distilled water. These extracts were screened for inhibition of 5-LOX, COX-1and COX-2 enzymes.

2.4. Fractionation of petroleum ether extract (PEE) through silica column chromatography

PEE of *Premna integrifolia*, the most potent extract, was partially purified through open silica column and nine different fractions were collected. These fractions were dried through a rotary vacuum evaporator followed by lyophilization and further screened for 5-LOX, COX-1, and COX-2 inhibitory activities. Out of nine fractions, the eighth fraction showed 5-LOX/COX-2 dual inhibition at least IC_{50} value (Table 2).

2.5. RP-HPLC column chromatography

The lyophilized eighth fraction obtained above was subjected to reverse phase HPLC (RP-HPLC) by employing C^{18} column (shim-pack column with particle size 5 μm and dimension 250 × 4.6 mm) with a flow rate of 1 mL/min, and the eluants were monitored at 235 nm. The mobile phase consisted of a gradient of solvent A (water: acetic acid-1000:1) and solvent B (methanol: acetic acid-1000:1). Gradient profile was: 0–5 min, 95% of A, 5% of B; 5–15 min, 95–70% of A, 5–30% of B; 15–35 min, 70–40% of A, 30–60% of B; 35–55 min, 40–5% of A, 60–95% of B; 55–60 min, 5–95% of A, 95–5% of B.

Isolated peaks were assayed for COX-1, COX-2 and 5-LOX inhibition. Preparative RP-HPLC (shim-pack PREP-ODS column with dimensions500X46 mm and particle size 5 μm) was employed to purify COX-2/5-LOX inhibitory peaks at large scale using the gradient described above at flow rate of 6 mL/min.

2.6. Extraction and isolation of COX-1 from Ram seminal vesicles

Ram seminal vesicles were collected from slaughter house under ice pack, and all the process of extraction and isolation of microsomes were carried out below 6 °C. Preparation of microsome was carried out, according to the method of Hemler et al. [19] with some modifications. Ram seminal vesicles were cut into small pieces and homogenized with a blender in buffer containing 5 mM EDTA disodium salt, 0.05 M Tris–HCl (pH 8), 0.01% sodium azide and 5 mM diethyl dithiocarbamate. The homogenate was centrifuged at 15,000 ×*g* for 15 min at 4 °C. The supernatant was filtered through cheese cloth. The supernatant was further centrifuged at 33,000 rpm for 1 h 15 min at 4 °C, using ultracentrifuge (Himac, CP-100α HITACHI) to obtain a microsomal pellet. The microsomal pellet was dissolved in a solubilizing buffer containing 0.1 mM EDTA disodium salt, 0.05 M Tris–HCl (pH 8), 0.01% sodium azide and 0.1 mM diethyl dithiocarbamate. These solubilized microsome fractions were stored in small aliquots at −80 °C and used for further studies as the COX-1 enzyme source.

2.7. Extraction and isolation of the COX-2 enzyme

2.7.1. Expression and isolation of COX-2

COX-2 was prepared according to the method of Reddy et al. [20] with some modifications. Human recombinant COX-2 expression was carried out in *Spodoptera frugiperda* (Sf9) cell line. Sf9 cells were maintained at 28 °C in Grace's insect culture medium. At 70% confluency, the Sf9 cells were infected with recombinant baculovirus containing human COX-2. After 68–72 h of infection, the cells were collected by centrifugation at 2500 ×*g* for 5 min at 4 °C. The pellet was resuspended in a minimum volume of Tris–HCl buffer (50 mM, pH 7.2) containing 300 mM sucrose, 5 mM EDTA, 1 mM phenol, 5 mM diethyl thiocarbamate, and sonicated for 3 min. The cell lysate was centrifuged at 100,000 ×*g* for 1 h 15 min at 4 °C by using ultracentrifuge (Himac, CP-100α HITACHI), and the microsomal pellet obtained was resuspended in Tris–HCl buffer (2.5 mM, pH 7.2) containing 0.5% glycerol, Tween 20, 0.8% and 1 mM phenol. This solubilized microsomal fraction was stored in small aliquots at −80 °C and used as a COX-2 enzyme source.

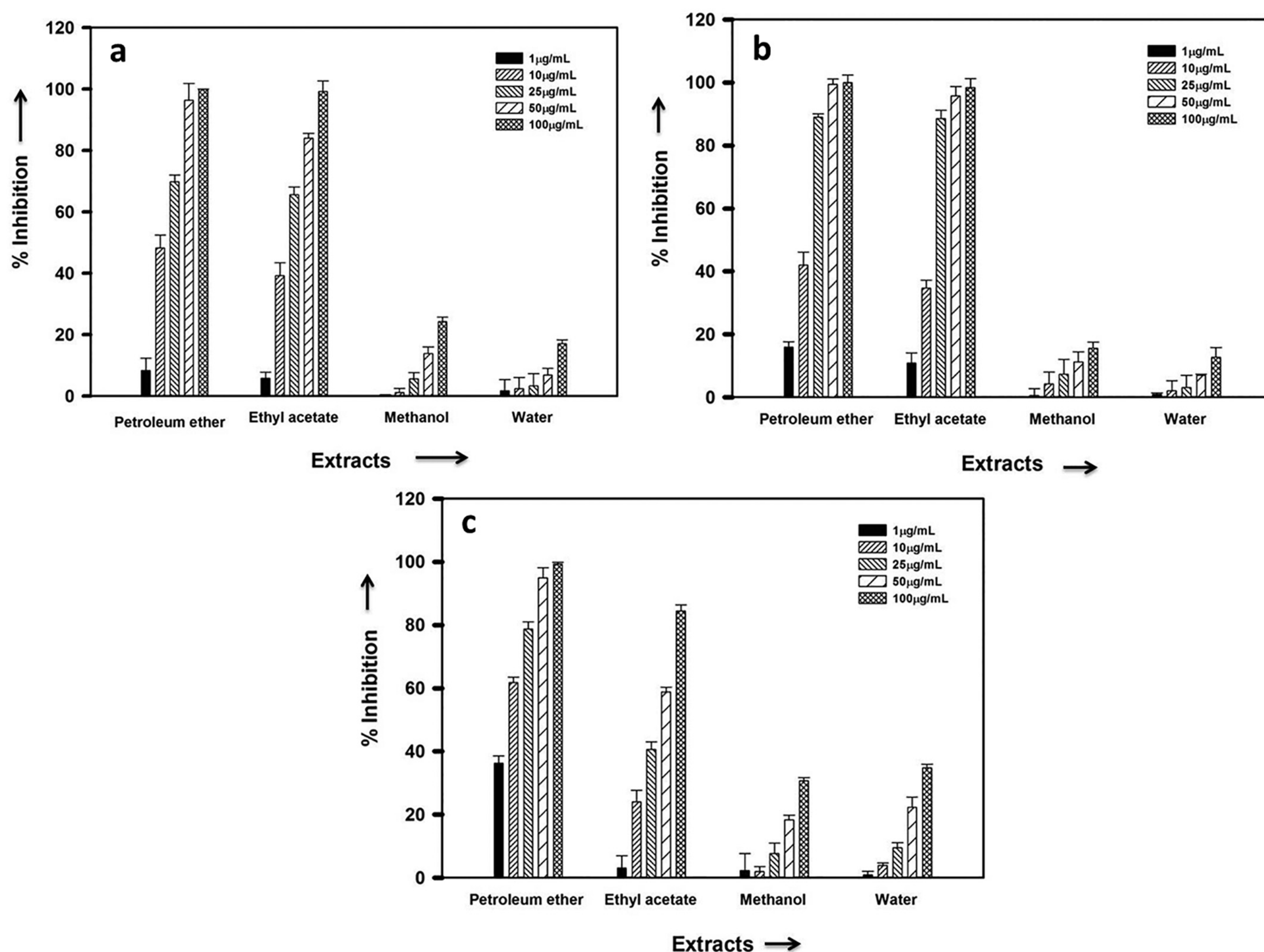

Fig. 1. Anti-inflammatory effects of *Premna integrifolia* roots extracts on COX-1, COX-2 and 5-LOX. (a) Effect of *Premna integrifolia* extracts on 5-LOX enzyme activity. The inhibitory properties of extracts were checked using polarography method with a Clark's oxygen electrode. The enzyme was incubated for five minutes at 25 °C with different concentrations of extracts (as shown in graph) and activity was examined by the rate of decrease in oxygen in presence of arachidonic acid as the substrate. (b) Effect of *Premna integrifolia* extracts on COX-1 enzyme activity. The inhibitory properties of extracts were checked through spectrophotometric method. The enzyme was incubated for five minutes at 25 °C with different concentrations of extracts (as shown in graph) and COX-1 activity was measured as the oxygenation of arachidonic acid as the substrate. (c) Effect of *Premna integrifolia* extracts on COX-2 enzyme activity. The inhibitory properties of extracts were checked through spectrophotometric method. The enzyme was incubated for five minutes at 25 °C with different concentrations of extracts (as shown in graph) and activity was measured as the oxygenation of arachidonic acid as substrate.

Table 1
Data showing IC_{50} values (μg/mL) of extracts of *Premna integrifolia*, petroleum ether, ethyl acetate, methanol and water on 5-LOX, COX-1 and COX-2 enzymes. NDGA, Indomethacin and Celecoxib served as the standard inhibitors of 5-LOX, COX-1 and COX-2 respectively.

Plant extracts	IC_{50} value (μg/mL)			
	Petroleum ether	Ethyl acetate	Methanol	Water
5-LOX	11.33	16.5	>100	>100
COX-1	12.47	13.65	>100	>100
COX-2	6.15	37.77	>100	>100

COX-1 activity level: 0.687 μ mol product formed/(min x mL)
Std. Inhibitor Indomethacin (COX-1) IC_{50}: 15.8 μM
5-LOX activity level: 10.14 μ mol product formed/(min x mL)
Std. Inhibitor NDGA IC_{50}: 10.6 μM
COX-2 activity level: 0.108 μ mol product formed/(min x mL)
Std. Inhibitor Celecoxib (COX-2) IC_{50}: 0.136 μM

2.7.2. Cyclooxygenase (COX-1 and COX-2) assay

COX-1 and COX-2 enzyme activities were measured according to the method of Copeland et al. [21] with slight modifications using a chromogenic assay based on the oxidation of *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine (TMPD), during the reduction of PGG2 to PGH2 [22,23]. The reaction mixture contained Tris–HCl buffer (5 mM, pH 8.0), EDTA (5 mM), hematin (5 mM), enzyme (COX-1 or COX-2) and the test compound. The assay mixture was pre-incubated at 25 °C for 5 min, and the reaction was initiated by addition of arachidonic acid and TMPD. The enzyme activity was determined by estimating the rate of TMPD oxidation at 610 nm, for the first 60 s of the reaction. Non-enzymatic oxidation at a low rate that occurs during the reaction in the absence of COX-1 and COX-2 was subtracted from the actual experimental value while calculating the percent inhibition.

2.8. Purification and assay of 5-LOX

Purification of 5-LOX from potato tubers were carried out and assayed as described earlier according to Reddanna et al. [24]. Enzyme activity was measured using the polarographic method with Clark's

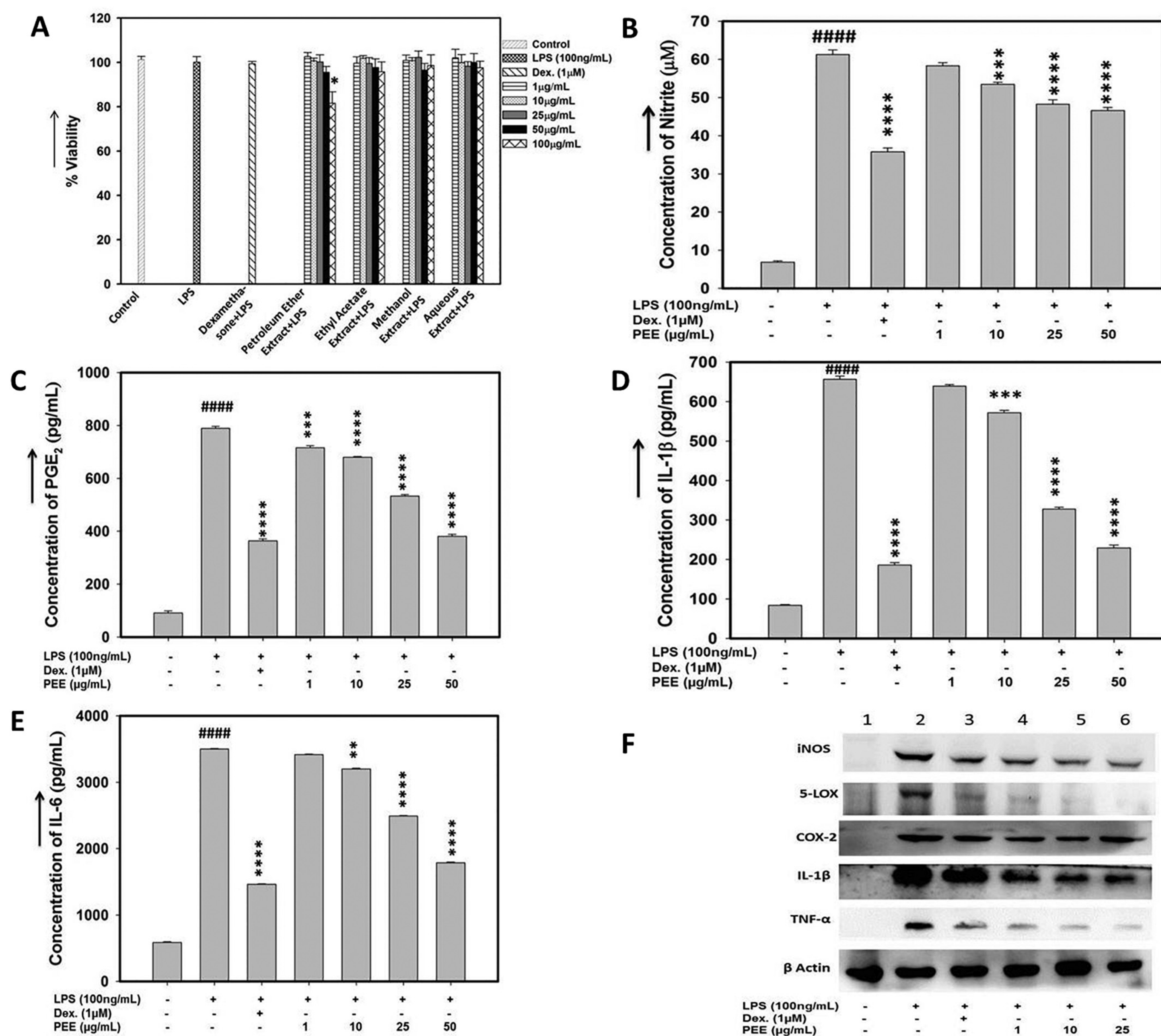

Fig. 2. Anti-inflammatory effect of *Premna integrifolia* root extracts on LPS challenged mouse macrophage cell line, RAW 264.7 cells. (A) Effect of *Premna integrifolia* extracts on RAW 264.7 cell line. Cells were pre-treated with various concentrations of extracts/compounds for 1 h (as shown in graph) and then challenged with LPS for 24 h. The cell viability was examined by MTT assay. (B) Effect of PEE on LPS induced nitrite production in RAW 264.7cell line. Cells were pretreated with indicated concentrations for 1 h and then challenged with LPS (100 ng/mL). After 18 h of incubation, the culture supernatants were isolated and analyzed for nitrite levels. (C), (D) and (E) Effect of PEE on LPS induced PGE_2, IL-1β and IL-6 production in RAW 264.7cell line. Cells were pretreated with indicated concentrations for 1 h and then challenged with LPS (100 ng/mL). After 6 h of incubation, the culture supernatants were collected and analyzed for PGE_2, IL-1β and IL-6 levels respectively. The values represent mean ± SD of three independent experiments. $^{\#\#\#\#}p < 0.0001$ *vs.* control; $^{****}p < 0.0001$ $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < .05$ *vs.* LPS alone. Dex.: Dexamethasone, PEE: Petroleum ether extract, LPS: Lipopolysaccharides. (F) Immunoblot analysis of PEE showed anti-inflammatory activities against LPS challenged RAW 264.7 cells. Cells were pre-incubated with PEE (1 μg/mL, 10 μg/mL and 25 μg/mL) for 3 h, and challenged with or without LPS (100 ng/mL) for additional 24 h. β actin was used as internal control and dexamethasone was used as positive control.

oxygen electrode on Strathkelvin Instruments (model 782, RC-300). The 3 mL reaction mixture contained 100 μL of the enzyme, 10 μL of 40 mM arachidonic acid, 2 mL of 100 mM potassium phosphate buffer of pH 6.3, 30 μL of test compound and 860 μL of double distilled water. Since 5-LOX add oxygen to the substrate, so the rate of decrease of oxygen in the reaction mixture was measured as enzyme activity.

2.9. In vitro assays on RAW 264.7 cell line

RAW 264.7 cell line was maintained in a humidified atmosphere with 5% CO_2 at 37 °C in DMEM medium, 10% heat activated fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin. The cultured cells were passaged at 70–80% confluency. Before the cell treatment, the cells were washed with PBS. The cells were pre-treated in presence or absence of different concentrations (1 μg/mL, 10 μg/mL, 25 μg/mL, 50 μg/mL and 100 μg/mL) of PEE and stimulated with 100 ng/mL lipopolysaccharide for 6 h, 18 h and 24 h for cytokine analysis, nitrite estimation and for protein expression studies respectively.

2.9.1. Cytotoxicity assay

Cytotoxicity of PEE on RAW 264.7 cells was assayed by MTT assay. RAW 264.7 cells were seeded in 96 wells plate at a density of 5×10^3

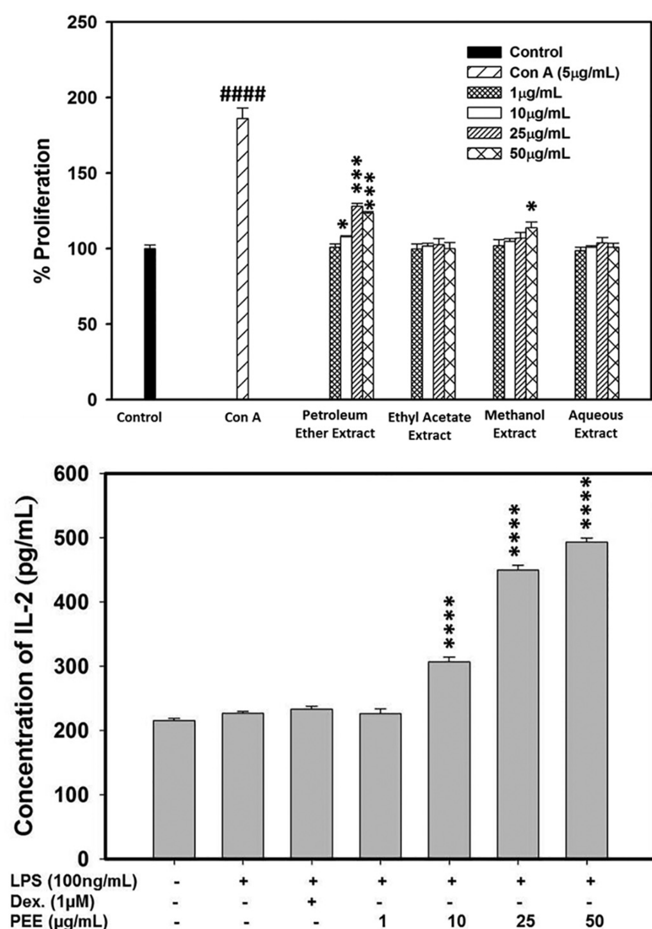

Fig. 3. Immunomodulatory and anti-inflammatory effect of *Premna integrifolia* extracts. (A) Effect of different solvent extracts of roots of *Premna integrifolia* on human lymphocytes proliferation. The cells were incubated with different concentrations as shown in graph for 24 h. Con A represents positive control. (B) Effect of PEE on LPS induced IL-2 production in RAW 264.7cells. Cells were pretreated with indicated concentrations for 1 h and then challenged with LPS (100 ng/mL). After 6 h of incubation, the culture supernatants were collected and analyzed for IL-2 levels. Data are mean ± SD of three independent experiments. ****p < 0.0001; ***p < 0.001; *p < 0.05 *vs.* control. Dex: Dexamethasone, PEE: Petroleum ether extract, LPS: Lipopolysaccharides.

cells/well. After overnight incubation, the cells were washed with cold PBS to remove unattached cells and then pre-treated in presence and absence with different concentrations (1 μg/mL, 10 μg/mL, 25 μg/mL and 50 μg/mL) of PEE for 1 h and then stimulated with 100 ng/ml lipopolysaccharide for 24 h. Cell viability was measured colorimetrically by MTT as described by Mosmann [25].

2.9.2. Nitrite estimation

RAW 264.7 cells were pre-treated in presence or absence with different concentrations (1 μg/mL, 10 μg/mL, 25 μg/mL and 50 μg/mL) of PEE for 1 h then stimulated with or without LPS (100 ng/mL) for 18 h. After LPS stimulation, nitrite level in the culture supernatant was measured by Griess reagent according to the manufacturer's protocol and determined by using the standard curve of sodium nitrite.

2.9.3. Analysis of prostaglandin E_2, pro and anti-inflammatory cytokines

Analysis of prostaglandin PGE_2 and pro and anti-inflammatory cytokines (IL-1β, IL-6, and IL-2) were carried out on LPS stimulated RAW 264.7 cells by ELISA using commercially available kits (R&D system, MN, USA). In a 12 well plate, 1×10^5 cells were seeded for overnight culturing. After cold PBS wash, cells were pre-treated in presence or absence with different concentration (1 μg/mL, 10 μg/mL, 25 μg/mL and 50 μg/mL) of PEE for 1 h then stimulated with or without LPS (100 ng/mL) for 6 h. After LPS stimulation, the cytokines level in the culture supernatant were determined by ELISA as per the manufacturer's protocol.

2.9.4. Preparation of whole cell lysates for protein expression studies

RAW 264.7 cells were seeded in 6-well plates (2×10^5 cells/well) and at its 70–80% confluence, pre-treated with the presence or absence of PEE for 3 h, followed by treatment with and without LPS (100 ng/mL) for 24 h. The whole cell lysates were prepared for measuring the protein levels for western blotting. Briefly, the cells were washed twice with ice-cold PBS and lysed in 80 μL of RIPA buffer with protease inhibitor followed by 30 min incubation on ice. The tubes were vigorously rocked for 5 min, and the homogenates were centrifuged for 30 min at 14,000 rpm at 4 °C. The supernatants were collected and stored at −80 °C. The protein concentrations were determined before protein expression studies according to the Bradford method. An equal amount of protein was loaded and separated on 10–15% SDS-PAGE and transferred to nitrocellulose membrane. Then membranes were blocked with 5% blotting-grade blocker (BIO-RAD) in TBS for 1 h at room temperature followed by washing three times with TBST. Membranes were incubated with primary antibodies (0.5–1.0 μg/mL) for overnight at 4 °C with gentle shaking on rocker, then thrice washing with TBST. The membranes were then incubated with respective secondary antibodies conjugated with HRP. Signals were then detected with west femto maximum sensitivity substrate from Thermo Scientific.

2.9.5. Lymphocyte proliferation assay (LPA)

Lymphocytes were isolated from fresh blood donated by human health volunteers by Ficoll Histopaque (Sigma) method [26]. Isolated lymphocytes were washed twice with PBS and resuspended in complete DMEM media containing β-mercaptoethanol (50 mM). Cells were seeded at the density of 1×10^4 cells in each well in 96 well plates and grown for 16 h at 37 °C and 5% CO_2. Cells were treated with the *Premna integrifolia* roots extracts at four different concentrations (50 μg/mL, 25 μg/mL, 10 μg/mL and 1 μg/ mL) and incubated for 24 h. Cells treated with Concanavalin A (Con-A) (5 μg/mL) served as positive control and without Con-A served as negative control. After 24 h, 20 μl of MTT (5 mg/mL in PBS) was added in each well and incubated for an additional 3 h at 37 °C. After incubation, 50 μL of DMSO was added in each well to dissolve the formazan crystals. Absorbance was measured at 570 nm after 30 min addition of DMSO in BioTek Synergy Mx multimode reader. Lymphocytes proliferation was measured as percentage growth keeping control as a reference.

2.9.6. In vivo assay-carrageenan-induced paw edema animal model

Male BALB/c mice weighing 20–22 g of 5–6 week age were purchased from the National Institute of Nutrition (NIN), Hyderabad, India. Mice were housed in University of Hyderabad animal house at constant room temperature 23 ± 1 °C and allowed to water and food freely in 12 h dark/light cycle. The mice were kept at least a week in the animal house before experimentation. The mice used in this study were handled carefully and according to the *Guide lines for the care and use of Laboratory animals* published by NIH (National Institute of Health). The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC), University of Hyderabad, India.

PEE the most promising COX-2/5-LOX dual inhibitor was further studied for acute inflammation in a mouse model. Briefly, the mice were randomly grouped ($n = 6$) and were intraperitoneally injected with 100 μL DMSO or with PEE (25 mg/Kg bw/50 mg/Kg bw) or Dexamethasone (20 mg/Kg bw). After 3 h of intraperitoneal injection, paw edema was induced through subcutaneous injection of 20 μL of carrageenan (1%) into left paw or an equal volume of 0.9% saline into the left hind paw [27]. Prior to carrageenan dose, the thickness of the left hind paw was measured using a digital caliper, as a baseline

Fitoterapia 131 (2018) 189–199

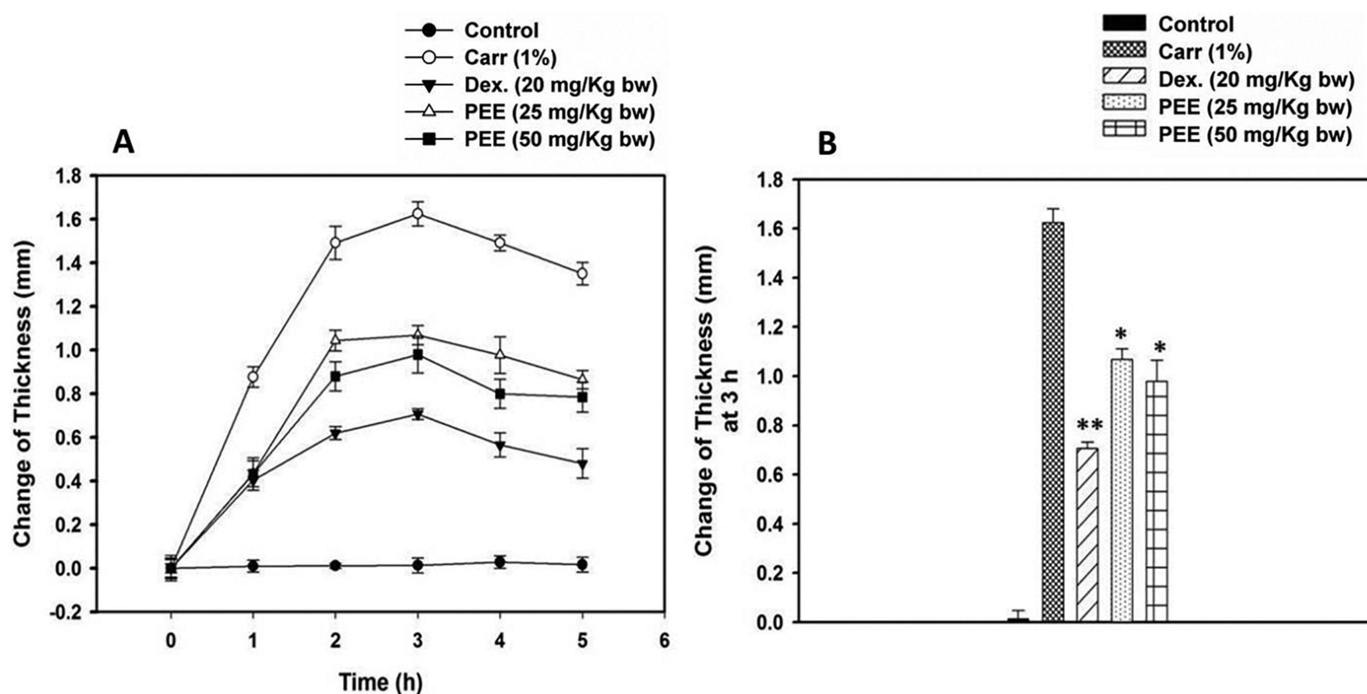

Fig. 4. Effect of *Premna integrifolia* PEE on carrageenan induced paw edema in BALB/c mice. Animals in the group were injected with PEE [50 mg/kg body weight and 25 mg/kg body weight], dexamethasone (20 mg/kg body weight) or equal volume of the vehicle (100 μL, 1% DMSO) intraperitoneally. One hour later, paw inflammation was induced by injecting 20 μL of 1% solution of carrageenan in 0.9% saline subcutaneously into plantar region of the left hind paw. The thickness of the paw was measured by digital vernier caliper after injecting carrageenan in the dorsal plantar axis at the metatarsal level at the indicated times and data presented as line diagram **(A)**. The thickness of the paw edema at 3 h after induction was shown in the form of bar diagram **(B)**. All data are expressed as mean ± S.D. *p < 0.05; **p < 0.01 compared with the Carr (1%).

reference [28]. Edema calculation was made as the average difference in paw thickness and compared with the data for the saline group and the corresponding baseline reference.

2.10. Statistical analysis

All the data were analyzed using Sigma Plot 10.0 and GraphPad Prism version 6.01 software. Numerical data for all experiments were presented as mean ± S.D. of three independent experiments. *p*-value was determined using one way ANOVA + Tukey's *post hoc* test. The p-value < .05 was considered as statistically significant.

3. Results

3.1. In vitro anti-inflammatory effects

3.1.1. Effects of Premna integrifolia extracts on COX-1, COX-2 and 5-LOX activities

To study the effects on COX-1, COX-2, and 5-LOX, the roots powder of *Premna integrifolia* was extracted with four different solvent systems, petroleum ether, ethyl acetate, methanol and aqueous. These four solvent extracts were screened for their anti-inflammatory effects by the inhibition studies of COX-1, COX-2, and 5-LOX, the key mediators of inflammation (Fig. 1). Of all the extracts tested, PEE showed the least IC_{50} value for COX-2 (6.15 μg/mL) and 5-LOX (11.33 μg/mL), followed by ethyl acetate COX-2 (37.77 μg/mL) and 5-LOX (16.5 μg/mL). Methanolic and aqueous extracts showed < 50% inhibition of above enzymes at highest concentration employed (100 μg/mL) (Table 1). As the PEE showed maximum inhibition with least IC_{50} values for COX-2 and 5-LOX, further studies on anti-inflammatory activity and isolation of active principles were taken up only with PEE.

3.1.2. In vitro anti-inflammatory effects of various extracts of Premna integrifolia roots on mouse macrophage cell line, RAW 264.7

3.1.2.1. Premna integrifolia extracts showed very little or no cytotoxicity on RAW 264.7 cells. Four solvent root extracts of *Premna integrifolia* were tested for their anti-inflammatory effects *in vitro* on RAW 264.7 cell line stimulated with LPS. There was no cell death for aqueous, methanolic and ethyl acetate extracts at all range of concentrations used. Cell viability was significantly reduced for PEE at 100 μg/mL, but no cell death was recorded at and below 50 μg/mL (Fig. 2.A).

As PEE of *Premna intigrefolia* has shown most potent COX-2/5-LOX dual inhibition, we have selected different concentrations (1 μg/mL, 10 μg/mL, 25 μg/mL and 50 μg/mL) of PEE at which there was no cell death. The above concentrations were used for further anti-inflammatory studies.

3.1.2.2. Effects of PEE on nitric oxide levels in RAW 264.7 cells. The effects of PEE on LPS induced inflammatory marker, nitric oxide (NO) in RAW 264.7 cells were evaluated. The LPS stimulated RAW 264.7 cells were treated with different concentrations of PEE. Upon LPS stimulation, there was a significant elevation of NO production in the cells and this elevated NO level was significantly reduced by PEE in a dose-dependent manner (Fig. 2.B). This significant reduction of the pro-inflammatory marker (NO) suggests anti-inflammatory properties of PEE.

3.1.2.3. Effects of PEE on PGE_2 and pro-inflammatory cytokines in RAW 264.7 cells. PEE was further evaluated for its effect on the level of PGE_2 and pro-inflammatory cytokines (IL-1β, IL-6) in LPS challenged RAW 264.7 cell line. Levels of PGE_2, IL-1β, and IL-6 were significantly elevated by LPS. PEE showed dose-dependent decrease in PGE_2 level (Fig. 2.C) in LPS stimulated cells at 1 μg/mL, 10 μg/mL, 25 μg/mL and 50 μg/mL. LPS induced levels of IL-1β (Fig. 2.D) and IL-6 (Fig. 2E) were significantly reduced by PEE at 10 μg/mL, 25 μg/mL, and 50 μg/mL.

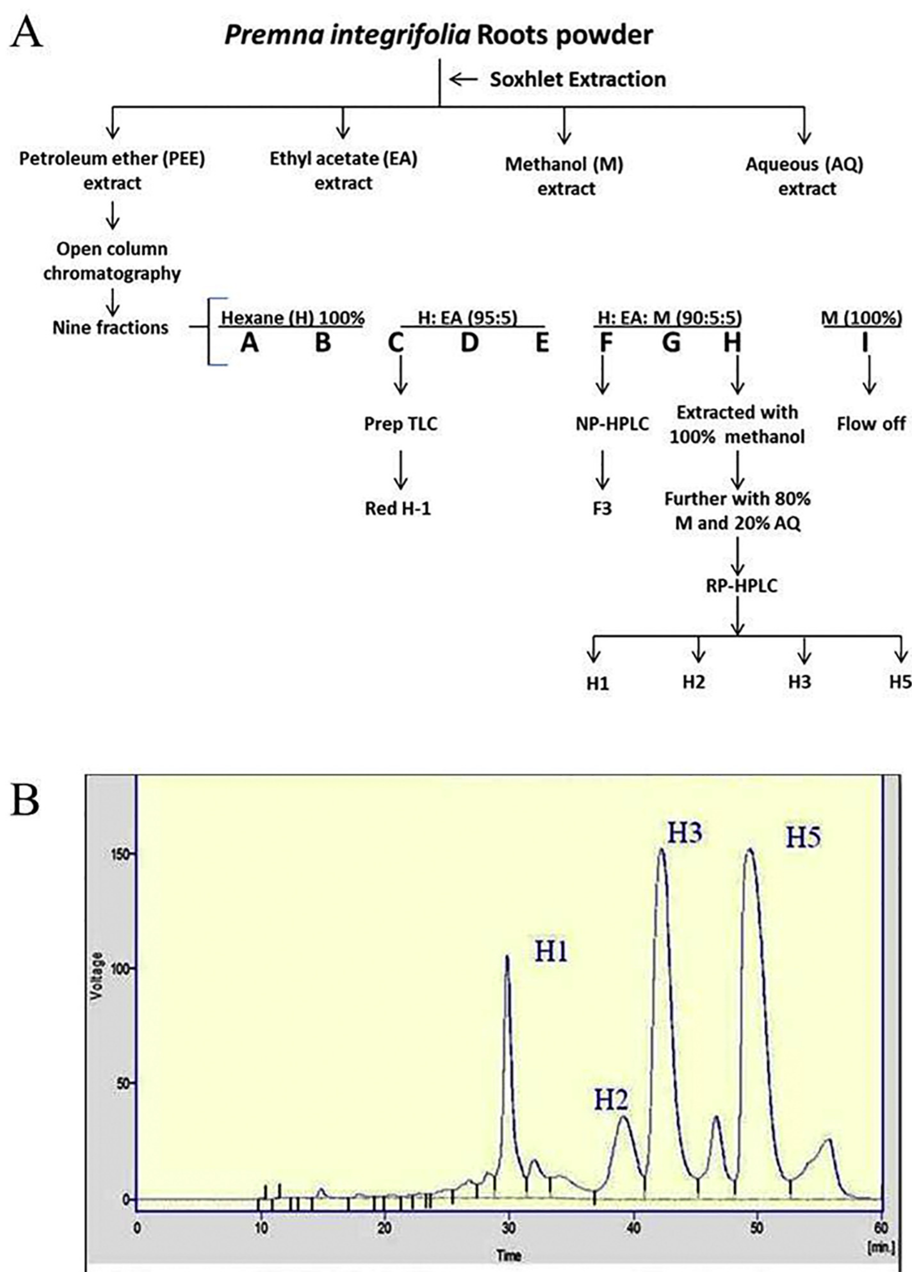

Fig. 5. Isolation of active principle(s) from *Premna integrifolia*. (A) Schematic presentation of the process involved in the preparation of various extracts and purification of COX-2/5-LOX dual inhibitor from PEE of *Premna integrifolia*. (B) RP-HPLC Chromatograph of H fraction obtained from open column chromatography of petroleum ether extract on C^{18} column.

The significant reduction of these pro-inflammatory markers further supports the anti-inflammatory effects of PEE.

3.1.2.4. Effects of PEE on the expression of iNOS, COX-2, 5-LOX, IL-1β, and TNF-α in LPS challenged RAW 264.7 cells. The protein expression of iNOS, COX-2, 5-LOX, IL-1β, and TNF-α in RAW 264.7 cells challenged with LPS in the presence and/or absence of PEE at different concentrations was studied. Untreated wells showed no or little expression of these pro-inflammatory markers while LPS alone treated cells showed induced expression. However, the expression of these pro-inflammatory markers was significantly reduced in a dose-dependent manner in RAW 264.7 cells that were pre-treated with PEE and then challenged with LPS (Fig. 2.F).

3.1.3. In vitro immunomodulatory and anti-inflammatory effect of PEE of Premna integrifolia

3.1.3.1. Immunomodulatory effects of Premna integrifolia extracts on lymphocytes. All four solvent root extracts of *Premna integrifolia* were tested for immunomodulation. Con A treated lymphocytes significantly induced proliferation by nearly two folds as measured by MTT assay. Among four extracts, methanol extract at 50 μg/mL only showed significant proliferation of lymphocytes, whereas PEE showed lymphocytes proliferation significantly at 10 μg/mL, 25 μg/mL and 50 μg/mL (Fig. 3.A). These findings were additionally validated by counting of cells (Supplementary Fig.2).

3.1.3.2. Effects of PEE on IL-2 production in RAW 264.7 cell line. PEE was further evaluated for its effect on anti-inflammatory and immunomodulatory cytokine, IL-2 produced by LPS challenged RAW

Table 2
Effect of different fractions of petroleum ether extracts of *Premna integrifolia* on open silica column on 5-LOX, COX-1 and COX-2 enzymes. NDGA, Indomethacin and Celecoxib are standard inhibitors of 5-LOX, COX-1 and COX-2.

S.No.	Petroleum ether extract fractions of *Premna integrifolia*	5-LOX		COX-1		COX-2	
		% Inhibition at 100 µg/mL	IC_{50} µg/mL	% Inhibition at 100 µg/mL	IC_{50} µg/mL	% Inhibition at 100 µg/mL	IC_{50} µg/mL
1.	Fraction 1(A)	NIL	NIL	NIL	NIL	05.21	>100
2.	Fraction 2 (B)	6.43	>100	NIL	NIL	NIL	NIL
3.	Fraction 3 (C)	83.12	27.78	NIL	NIL	NIL	NIL
4.	Fraction 4 (D)	14.8	>100	5.44	>100	15.87	>100
5.	Fraction 5 (E)	2.51	>100	27.62	>100	48.94	>100
6.	Fraction 6 (F)	1.89	>100	56.51	41.84	92.66	20.41
7.	Fraction 7 (G)	47.85	>100	64.22	39.17	50.22	46.21
8.	Fraction 8 (H)	96.27	2.18	70.42	36.84	53.97	68.19
9.	Fraction 9 (I)	47.11	>100	88.13	27.84	46.77	>100

COX-1 activity level: 0.625 µ mol product formed/(min x mL)
Std. Inhibitor Indomethacin (COX-1) IC_{50}: 16 µM
5-LOX activity level: 11.11 µ mol product formed/(min x mL)
Std. Inhibitor NDGA IC_{50}: 11 µM
COX-2 activity level: 0.099 µ mol product formed/(min x mL)
Std. Inhibitor Celecoxib (COX-2) IC_{50}: 0.12 µM
NIL: No inhibition/Not determined

264.7 cells. PEE showed a dose-dependent increase in the level of IL-2 production from 10 µg/mL onwards as shown in Fig. 3.B. This significant induction of IL-2 by these extracts indicates the immunomodulatory and ant-inflammatory properties of PEE.

3.1.4. In vivo effects of PEE on Carrageenan-induced paw edema in BALB/c mice

The *in vivo* anti-inflammatory effects of PEE were evaluated by intraplantar injection of 20 µL of 1% carrageenan and measuring paw thickness in millimeter (mm). These studies revealed an increase in the thickness of the mouse paw. The paw edema peaked at 3 h after induction, and the thickness was 1.63 ± 0.06 mm in the carrageenan group and 0.71 ± 0.03 mm in the dexamethasone group. In the PEE treatment groups (25 mg/kg body weight and 50 mg/kg body weight), the mean peak thicknesses were 1.07 ± 0.04 mm and 0.98 ± 0.09 mm, respectively, this showed PEE significantly attenuated paw edema compared to the carrageenan group (Fig. 4).

3.2. Isolation and characterization of COX-2/5-LOX dual inhibitor(s) from PEE of Premna integrifolia

PEE which showed potent inhibition of COX-2 and 5-LOX was subjected to open column chromatography (Fig. 5.A) using silica gel and eluting with hexane, ethyl acetate and methanol in sequence and eluents were collected in various fractions. Nine fractions were collected and tested for COXs, 5-LOX assays (Table 2). A highly non-polar fraction (# third) with only 5-LOX inhibition (IC_{50} value, 35.73 µg/mL) was purified by preparatory TLC (data not shown). Another fraction (# eighth) with maximum COX-2/5-LOX dual inhibition was selected for isolating COX-2/5-LOX, dual inhibitor. This fraction (# eight) was initially fractionated in 100% methanol followed by 20% water and 80% methanol. The supernatant obtained after centrifugation was subjected to RP-HPLC. Four peaks H1, H2, H3, and H5, were obtained as shown in Fig. 5.B. These peaks were tested for COX/5LOX assay and the IC_{50} values are shown in supplementary table-1. Among these, H3 peak showed potent inhibition of 5-LOX (IC_{50} value 17.48 µM). H3 also inhibited COX-1 and COX-2 with IC_{50} values 163.95 µM and 214.98 µM respectively (Fig. 6.C). H1 showed very potent inhibition of COX-2 with IC_{50} value of 5.33 µg/mL, along with inhibition of 5-LOX (19.94 µg/mL) and COX-1 (26.95 µg/mL). The other peaks H2 and H5 also showed comparable inhibition of COX-1, COX-2 and 5-LOX. H3, one of the major peaks on RP-HPLC inhibiting both COX-2 and 5-LOX, was taken up for further studies to identify the structure of H3 by NMR, IR, HR-MS (shown in supplementary Fig.1) and single X-ray diffraction and its unit cell (Fig. 6.A & 6.B). From these studies, the peak is identified as 6-hydroxy salvinolone (6-HS) with a molecular weight (MW) of 330.41 which is similar to earlier findings [29]. Further characterization of other peaks, H1, H2 and H5 are in progress.

4. Discussion

Premna integrifolia belongs to Brihatpunchmoola group of medicinal plants widely used in traditional medicine especially in Ayurvedic formulations. Its formulation is used in treating various diseases such as inflammation, allergy, cough & cold, bronchitis, asthma, pain, wound healing, diabetes, and arthritis. The pharmacological activities of *Premna integrifolia* are analgesic and antibacterial, anti-arthritic, anti-inflammatory, antimicrobial [30], anti-obesity/hypolipidemic, anti-oxidant, antiparasitic, antiulcer/gastro protective, cardiac stimulant/cardio protective, central nervous system (CNS) depressant, hepato-protective, hypoglycemic, immunomodulatory, longevity-promoting, neuroprotective *etc.* [31,32].

Earlier work on this plant has been mostly on their therapeutic applications, chemical composition, isolation of several components and their pharmacological properties with no studies focusing on the molecular target and the specific compounds involved. In the present study, we have focused on the evaluation of anti-inflammatory and immunomodulatory properties of *Premna integrifolia* through *in vitro* and *in vivo* assays, followed by assay-guided isolation of active principle(s) with COX-2/5-LOX inhibition.

Eicosanoids form one of the key mediators of inflammation, specifically the prostaglandins and leukotrienes formed by COX-2 and 5-LOX respectively. Many NSAIDs with known gastric and renal side effects inhibit both COX-1 and COX-2 non-selectively. With the discovery of COX-2 as the main mediator of inflammation, the COX-2 specific inhibitors such as celecoxib, rofecoxib and valdecoxib were developed. However, these are known to have cardiac side effects on long-term use in cases such as arthritis [33]. In the light of the above, there is intense search for the development of anti-inflammatory drugs without gastric and cardiac side effects. Among these next generation anti-inflammatory drugs, COX-LOX dual inhibitors have gained importance, because of their superior anti-inflammatory properties with no gastric or cardiac side effects [34]. It is in this connection that the present study was taken up to isolate and identify specific natural products with anti-inflammatory properties from *Premna integrifolia*. These studies revealed potent anti-inflammatory and immunomodulatory effects of

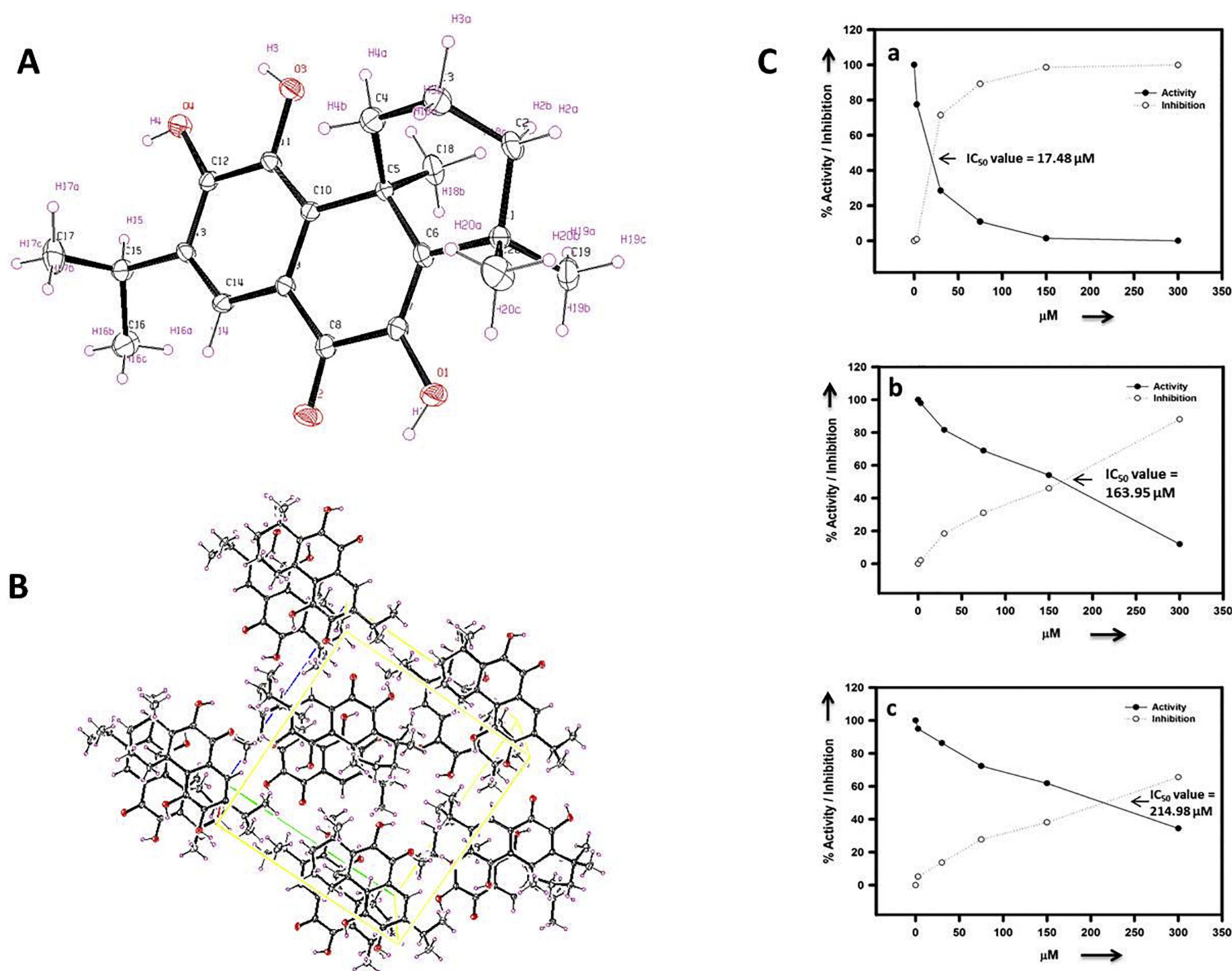

Fig. 6. Characterization of COX-2/5-LOX dual inhibitor, 6-hydroxy salvinolone (6-HS), and its effects on COX-1, COX-2 and 5-LOX. (A) ORTEP (Oak Ridge Thermal Ellipsoid Plot diagram – Drawn at *20*% probability) of 6-Hydroxysalvinolone {systematic name: 5,6,10-trihydroxy-7-isopropyl-1,1,4a-trimethyl-2,3,4,4a-tetra-hydrophenanthren-9(1*H*)-one}. (B) Unit Cell of 6-Hydroxy salvinolone. (C) Effect of 6-hydroxy salvinolone (6-HS) isolated from PEE of *Premna integrifolia* on (a) 5-Lipoxygenase, (b) Cyclooxygenase-1 and (c) Cyclooxygenase-2 enzyme activities. IC_{50} values are shown in the above graph.

the petroleum ether extracts (PEE) of *Premna integrifolia*, both *in vitro* and *in vivo*.

Immunomodulatory effect of medicinal plants can provide an alternative to the conventional therapy for a variety of diseases [35]. Under certain disease conditions such as autoimmunity, immune deficiency or in cancer, it regulates immune response and maintains a disease-free state. In our studies, we examined immunomodulatory effects of *Premna integrifolia* extracts on human peripheral blood mononuclear cells where Con A was used as a mitogen. The Con A is one of the most potent mitogens being used for leukocytes proliferation and differentiation [36]. PEE and methanolic extract of *Premna integrifolia* showed potent immunomodulatory effect at nontoxic doses, suggesting their possible application in the treatment of various diseases, including cancer. The recent Nobel Prize in medicine or physiology awarded to the scientists, who have come up with cancer immunotherapy [37,38], highlights the importance of immunomodulators in the treatment of various diseases.

In addition to COX-2 and 5-LOX, several cytokines are associated with promoting inflammation. Blocking one of the cytokines might be sufficient to control the inflammatory diseases as cytokines exist in “cascades” and interruption of one cytokine leads to interruption of the whole cascade [39–41]. IL-2, the anti-inflammatory cytokine was shown to play an important role in controlling immune homeostasis by the spontaneous lethal autoimmunity in mice lacking IL-2 expression [42]. In our study, evaluation of *in vitro* anti-inflammatory activities of PEE of *Premna integrifolia* on LPS induced RAW 264.7 cell line were analyzed with no cytotoxicity up to 50 µg/mL. PEE showed a reduction in nitrite level induced by LPS and these effects are comparable to dexamethasone. It also showed down-regulation of pro-inflammatory (IL-1β and IL-6) and induction of anti-inflammatory (IL-2) cytokines. Further, PEE down-regulated the expression of LPS -induced pro-inflammatory markers (iNOS, COX-2, 5-LOX, IL-1β and TNF-α) at protein level in Raw 264.7 cell line. These studies demonstrate the anti-inflammatory effects of PEE of *Premna integrifolia in vitro* on mouse macrophage cell line, RAW 264.7.

The carrageenan-induced acute inflammatory model is a sensitive method to test the anti-inflammatory potential of various agents. Earlier it was reported that the methanolic extract of roots of *Premna integrifolia* produced significant inhibition on carrageenan induced rat paw edema [10]. This animal model is known for biphasic inflammatory condition, First phase $(1-2h)$ is mediated mainly by histamine and serotonin. PEE being an inhibitor of 5-LOX that inhibits

leukotriene formation may impede this phase of edema. PEE contains terpenoids (diterpenoid) known to impair histamine release from mast cells and exert anti-inflammatory effects [43]. The second phase (3–5 h) of edema is due to the release of prostaglandins, arachidonic acid metabolites and other neutrophil-derived mediators [44]. Our studies clearly demonstrate the potent inhibition of paw edema by PEE, may be through inhibition of COX/LOX pathway in addition to modulation of cytokines.

Further attempts at isolating the active principle(s) involved in these anti-inflammatory effects of PEE resulted in the isolation and characterization of 6-HS, which is inhibiting both COX-2 and 5-LOX. These studies indicate that 6-HS, being a COX-2/5-LOX dual inhibitor, may form a potential anti-inflammatory drug candidate without gastric and cardiac side effects. As 6-HS is highly potent in inhibiting 5-LOX (IC_{50} value, 17.48 μM),it may have greater potential for the treatment of allergy and asthmatic conditions. Further in-depth studies, however, are needed to evaluate its efficacy and safety in cell lines and animal models. The other compounds, with equal potency in inhibiting the COX/LOX pathway, are being characterized.

In summary, the results of the present study provides evidence that the petroleum ether extract of *Premna integrifolia* exhibits potent anti-inflammatory and immunomodulatory properties *in vitro* and *in vivo*. Further the studies also identify COX/LOX pathway as the target in mediating the anti-inflammatory properties of *Premna integrifolia*. Another key finding of the present study is the isolation of four peaks with potent inhibition of COX/LOX pathways, out of which one is identified as 6-hydroxy salvinolone, which may be one of the factors contributing to the anti-inflammatory properties of *Premna integrifolia*.

Conflict of interest

Authors do not have conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2018.10.016.

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Original Research Article (Experimental)

Anti-inflammatory profile of *Aegle marmelos* (L) *Correa* (*Bilva*) with special reference to young roots grown in different parts of India

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A B S T R A C T

Background: *Aegle marmelos* (*Bilva*) is being used in Ayurveda for the treatment of several inflammatory disorders. The plant is a member of a fixed dose combination of Dashamoola in Ayurveda. However, the usage of roots/root bark or stems is associated with sustainability concerns.
Objectives: The present study is aimed to compare the anti-inflammatory properties of different extracts of young roots (year wise) and mature parts of *Bilva* plants collected from different geographical locations in India, so as to identify a sustainable source for Ayurvedic formulation.
Materials and methods: A total of 191 extracts (petroleum ether, ethyl acetate, ethanol and aqueous) of roots, stems and leaves of *A. marmelos* (collected from Gujarat, Maharashtra, Odisha, Chhattisgarh, Karnataka and Andhra Pradesh region) were tested for anti-inflammatory effects *in vitro* on isolated target enzymes cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX), lymphocyte proliferation assay (LPA), cytokine profiling in LPS induced mouse macrophage (RAW 264.7) cell line and *in vivo* carrageenan induced paw edema in mice.
Results: Of 191 extracts, 44 extracts showed COX-2 inhibition and 38 extracts showed COX-1 inhibition, while none showed 5-LOX inhibition. Cytokine analysis of the 44 extracts showing inhibition of COX-2 suggested that only 17 extracts modulated the cytokines by increasing the anti-inflammatory cytokine IL-2 and reducing the pro-inflammatory cytokines like IL-1β, MIP1-α and IL-6. The young (2 and 3 years) roots of *Bilva* plants from Gujarat and young (1 yr) roots from Odisha showed the most potent anti-inflammatory activity by suppressing the pro-inflammatory cytokines and inducing anti-inflammatory cytokines. These three extracts have also shown *in vivo* anti-inflammatory activity comparable to that in adult stem and root barks.
Conclusion: The present study reveals that young roots of *Bilva* plants from Gujarat and Odisha region could form a sustainable source for use in Ayurvedic formulations with anti-inflammatory activities. The present study also indicates that the region in which the plants are grown and the age of the plants play an important role in exhibiting the anti-inflammatory effect.
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1. Introduction

Aegle marmelos (L.) Correa is a member of *Dashamoola* (10 root drugs) group. This combination is widely used in generic Ayurvedic formulations such as *Dasmularishta*, *Dasamoola Kashayam*, and *Dasamulakatutrayadi Kashayam*. The plant grows wild in dry forest in outer Himalayas and Shivaliks. *Bilva* is a medium to large sized deciduous glabrous armed tree with axillary and 2.5 cm long

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Research Article

Evaluation of Anti-Inflammatory Properties of Isoorientin Isolated from Tubers of *Pueraria tuberosa*

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Inflammation is the major causative factor of different diseases such as cardiovascular disease, diabetes, obesity, osteoporosis, rheumatoid arthritis, inflammatory bowel disease, and cancer. Anti-inflammatory drugs are often the first step of treatment in many of these diseases. The present study is aimed at evaluating the anti-inflammatory properties of isoorientin, a selective cyclooxygenase-2 (COX-2) inhibitor isolated from the tubers of *Pueraria tuberosa*, in vitro on mouse macrophage cell line (RAW 264.7) and in vivo on mouse paw edema and air pouch models of inflammation. Isoorientin reduced inflammation in RAW 264.7 cell line in vitro and carrageenan induced inflammatory animal model systems in vivo. Cellular infiltration into pouch tissue was reduced in isoorientin treated mice compared to carrageenan treated mice. Isoorientin treated RAW 264.7 cells and animals showed reduced expression of inflammatory proteins like COX-2, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), 5-lipoxygenase (5-LOX), and interleukin 1-β (IL-1-β) both in vitro and in vivo. The antioxidant enzyme levels of catalase and GST were markedly increased in isoorientin treated mice compared to carrageenan treated mice. These results suggest that isoorientin, a selective inhibitor of COX-2, not only exerts anti-inflammatory effects in LPS induced RAW cells and carrageenan induced inflammatory model systems but also exhibits potent antioxidant properties.

1. Introduction

Inflammation is a cellular, immune, and metabolic response to injury/infection. It is a normal protective vascular connective tissue response to eliminate the cause of injury and clean up the dead and dying cells but when it occurs in uncontrolled or inappropriate manner it results in pathogenesis of several disorders which include cardiovascular, respiratory, neurological, and many lifestyle diseases. Inflammation is a complex interplay of cellular and particulate mediators, which include chemokines, plasma enzymes, lipids, and cytokines. Among these the lipid mediators such as eicosanoids, the oxygenated metabolites of arachidonic acid formed via the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, play a predominant role in mediating the inflammatory disorders. The anti-inflammatory drugs are being targeted against COX pathway. These include the conventional nonsteroidal anti-inflammatory drugs (NSAIDs) that target both COX-1 and COX-2 and the selective COX-2 inhibitors (COXIBs). Although these drugs are effective in controlling signs of inflammation, number of adverse effects encountered is the biggest limitation to their use [1, 2]. These include the gastric

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Structure Based Library Design (SBLD) for new 1,4-dihydropyrimidine scaffold as simultaneous COX-1/COX-2 and 5-LOX inhibitors

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ABSTRACT

The various scaffolds containing 1,4-dihydropyrimidine ring were designed by considering the environment of the active site of COX-1/COX-2 and 5-LOX enzymes. The structure-based library design approach, including the focused library design (Virtual Combinatorial Library Design) and virtual screening was used to select the 1,4-dihydropyrimidine scaffold for simultaneous inhibition of both enzyme pathways (COX-1/COX-2 and 5-LOX). The virtual library on each 1,4-dihydropyrimidine scaffold was enumerated in two alternative ways. In first way, the chemical reagents at R groups were filtered by docking of scaffold with single position substitution, that is, only at R_1, or R_2, or R_3, ... R_n on COX-2 enzyme using Glide XP docking mode. The structures that do not dock well were removed and the library was enumerated with filtered chemical reagents. In second alternative way, the single position docking stage was bypassed, and the entire library was enumerated using all chemical reagents by docking on the COX-2 enzyme. The entire library of approximately 15,629 compounds obtained from both ways after screening for drug like properties, were further screened for their binding affinity against COX-1 and 5-LOX enzymes using Virtual Screening Workflow. Finally, 142 hits were obtained and divided into two groups based on their binding affinity for COX-1/COX-2 and for both enzyme pathways (COX-1/COX-2 and 5-LOX). The ten molecules were selected, synthesized and evaluated for their COX-1, COX-2 and 5-LOX inhibiting activity.
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1. Introduction

Non-Steroidal Anti-Inflammatory Drugs (NSAID's) are the most popular and well recognized drug for the treatment against inflammatory diseases.[1] These drugs prevent the metabolism of arachidonic acid to prostaglandins (PGs) by binding and inhibiting cyclooxygenase (COX) enzymes and hence exert their therapeutic effects.[2] The two distinct isoforms (COX-1 and COX-2) of COX enzyme discovered in 1990, which led to the discovery of a new class of NSAID's, selective for COX-2, name coxibs.[3] COX-1 is constitutive and responsible for the biosynthesis of PGs, which protects the stomach lining from the secreted acid and maintains blood flow in gastric mucosa. The other isoform, COX-2 is triggered only in proinflammatory conditions. Thus the long term use of traditional NSAID's cause adverse gastrointestinal effects, which are overcome by the use of selective COX-2 inhibitors.[4] Unfortunately, the withdrawal of rofecoxib from the market in the fall of the year 2004 after its clinical report of its linkage to increased risk of cardiovascular toxicity has widely affected the safety of coxib.[5,6] Studies suggest that selective COX-2 Inhibitors also cause GI injury like conventional NSAIDs and increased systemic blood pressure and thus have severe side effects on the cardiovascular system and promote thrombogenesis.[7]

Arachidonic acid, but also be metabolized through lipoxygenase (LOX) pathway, leading to the production of leukotrienes (LTs), which are potent inflammatory mediators. Therefore, it has been shown that selective COX-2 inhibitors, beside of preventing side effect of NSAIDs associated with inhibition of COX-1, lead to upregulation of arachidonic acid metabolism by the 5-LOX enzyme.[8,9] This result in the increased levels of LTB_4 in the gastric mucosa of NSAIDs and selective COX-2 inhibitors treated patients. The high level of LTs may also contribute to the development of atherosclerosis, and myocardial infarction. It is revealed from literature that simultaneous inhibition of both enzymatic pathways (COX-1/COX-2 and 5-LOX enzymes) would possibly exhibit enhanced anti-inflammatory potency along with reduced GI tract damage and other inflammatory side effects.[10–12] Thus licofelone, a competitive inhibitor of COX-1, COX-2 and 5-LOX, is currently in clinical development for the treatment of osteoarthritis and is

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Isolation and Characterization of COX-2/5-LOX Dual Inhibitors from Premna integrifolia: Potential Drug Candidates for Treatment of Inflammatory disorders.

by Raja Ram Azad

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