EVALUATION OF ANTI-INFLAMMATORY ACTIVITY OF
OPUNTIA DILLENII VIA INHIBITION OF
INFLAMMATORY MEDIATORS

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DEDICATED TO
MY LOVING PARENTS
AND
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1. INTRODUCTION

1.1 Inflammation and homeostasis

Inflammare a Latin word meaning to set on fire was first defined in 1st century AD by Cornelius Celsus on the basis of signs of inflammation such as 1) redness and 2) swelling with 3) heat and 4) pain due to wounds and infections in his book treatise De medicina. Later, Augustus Waller (1846) and Julius Cohnheim (1867) explained migration of leukocyte from blood vessel and vascular changes in acute inflammation. He was the first to observe microscopically the vasodilatation, leakage of plasma exudates and migration of leukocytes to the surrounding inflamed tissue. Rudolph Virchow (1858) explained 5th sign of inflammation and its cellular basis in his book Cellular pathology. Another milestone in the field of inflammation was the discovery of phagocytosis by Elie Metchnikoff and his proposed theory on cellular immunity in 1892 pointing the role of macrophages and neutrophils in host defense mechanism and in regulation of tissue homeostasis. Finally in late 19th century Robert Koch and Louis Pasteur proposed germ theory of disease explaining the microbial agents contribution in acute inflammation (Medzhitov, 2010).

Apparently inflammation is normal physiological response of a body against foreign pathogens or tissue injuries. It is characterized by cardinal signs of redness (rubor), pain (dolor), swelling (tumor/tugor) and loss of function (functio laesa). After tissue injury, the cellular components of immunological system participate to eliminate the foreign particles from body (Cruvinel et al., 2010). Thus process of inflammation is essential for protection against pathogens by destroying infectious agents, healing of wound and repairing damaged part of the tissues (Iismaa et al., 2009). The tissue repair process begins by the release of various growth factors (epidermal- and fibroblast growth factors) from serum of injured blood vessels and activating degranulated platelets (Muller et al., 2009).

Homeostasis was first introduced by Claude Bernard (1865) who explained it as a state of free and independent life. Later, Metchnikoff (1892) proposed "physiological inflammation" explaining the role of phagocytes in the maintenance of homeostasis (Medzhitov, 2010). Termination of inflammatory mediators and transition to the homeostatic state is highly controlled process which includes switch from pro-inflammatory mediators to anti-inflammatory mediators, resolution-inducing various lipoxins and the monocytes replacing the neutrophils which results in the clearance of cell debris and finally initiating the process of tissue repair. However, if homeostatic control
mechanism fails to clear the stimulus or the initiation of resolution phase might induce the chronic phase (Xu et al., 2014).

Inflammation involves series of events and consists of two parts i.e. acute and chronic inflammation.

1.2 Acute inflammation

This phase is short lived (hours or days) depending upon the magnitude of insults (Borges et al., 2014). In case of sterile tissue injury, acute inflammation promotes tissue repair and prevents the growth of opportunistic microorganisms in the damaged area. It is self-limiting process, and subsides by the removal of stimulus ultimately the damaged tissue is repaired (Rotelli et al., 2003). Anti-inflammatory mediators such as interleukin-10 (IL-10), tumor growth factor (TGF-β) and glucocorticoid act at the site of inflammation itself to repair the tissue damages (Siqueira-junior et al., 2003).

In acute inflammation after encounter with stimuli vascular and cellular responses come into play (Medzhitov, 2008). The vascular phase is transient phase involving vasodilatation of arterioles, venules and capillaries as a result of an increase in blood flow (redness) to the site of inflammation and heat which is followed by an increase in vessels permeability leading to swelling and extravasation of plasma protein and fluids, referred as edema (Nathan, 2002). It is mediated by pro-inflammatory mediators such as histamine, serotonin, bradykinin (Geppetti, 1993), prostaglandins (PGE₂, PGl₂), leukotrienes (LTB₄, LTC₄, LTD₄) and cytokines (TNF-α and IL-1β) (Nathan, 2002).

The cellular response is mediated by the activation of resident macrophages, dendrite cells, granulocytes and mast cells to induce the inflammatory responses and initiate the repair process (Basbaum et al., 2009). These cells recognize foreign particle and rapidly release vasoactive amines (histamine, serotonin), peptides (bradykinin), lipid mediators (PGE₂, LTB₄), platelets activating factor and cytokines (TNF-α) (Li et al., 2013).

Neutrophils or polymorphonuclear leukocytes (PMN) a type of white blood cells, their normal range in human is 2.0 - 7.5 x 10⁹ mm³, 40-60% of white blood cells. Under normal condition they exist in the resting state, with average life span of 1 or 5 days (Pillay et al., 2010) in circulation followed by the process of apoptosis for spontaneous death (Geering and Simon 2011). Its main role is to phagocytose the foreign antigen (Cunha et al., 2008). During inflammation, these are earliest cells appear at the site of insult reaching within 30-60 minutes (Asako et al., 1992). The infiltration of neutrophils occurs by multiple processes in which first they adhere with endothelium of capillary
venules followed by capture, rolling and adhesion to the vascular endothelium, migrated through the vessel wall and finally enters in inflamed tissue (Muller, 2002). These steps are mediated by selectins (E and P) and enhance the release of PGE₂, LTB₄, nitric oxide (NO) (Albelda et al., 1994), TNF-α, IL-1 and 8 (Silva et al., 2014) and generation of reactive oxygen species (ROS) (Fantone and Ward 1982). During inflammation life span of neutrophils increased to kill the foreign stimuli (Wright et al., 2010). After their programmed cell death they appear in the form of pus at the site of damaged area. Parallel to the inflammatory responses, resolution phase of inflammation begins which is further preceded by engulfment of dead neutrophils by macrophages (Kebir and Filep 2010). It’s over expression involved in the several diseases such as atherogenesis, rheumatoid arthritis (Tanaka et al., 2006), asthma and psoriasis (Corrigan and Kay 1992).

### 1.3 Chronic inflammation

If the inflammatory process fails to clear the stimulus and acute inflammation persists or resolution phase is not initiated due to unrepaired tissue damages, persistent allergic agents, undigestable foreign particles or endogenous crystals (monosodium urate) provides favorable conditions for development of chronic phase (Medzhitov, 2010).

Chronic inflammation is characterized by infiltration of mononucleated cells like macrophages and lymphocytes, proliferation of fibroblasts and collagen fibers and synthesis of new connective tissues forming 0.5-2.0 mm large size granuloma (swelling appears like tumor). It is mainly mediated by ROS and proteases formed from infiltrated inflammatory cells (Suleyman et al., 2004). These ROS are mutagenic and interact with DNA in proliferation of epithelium which may leads to permanent changes in genes e.g. point mutations, deletions or rearrangements (Maeda and Akaike 1998). The p53 mutations have been associated with chronic inflammatory diseases such as inflammatory bowel diseases (IBW) and rheumatoid arthritis, thereby facilitating the development of cancer (Coussens and Werb 2002). Chronic inflammation is basically silent killer causing tissue or organ damages by various inflammatory mediators (Calder, 2006) and might lead to other chronic inflammatory diseases such as diabetes (Wellen and Hotamisligil 2005), psoriasis, Alzheimer’s (Kim et al., 2012) and asthma (Sevenoaks and Stockley 2006).

### 1.4 Eicosanoids as mediators of inflammation

Phospholipase A₂ located in cell membrane responsible for degranulation of mast cell and release of histamine and serotonin (Cirino et al., 1989), activates neutrophils and increase
chemotactic properties of endothelial cells (Rizzo et al., 2000) and precursor of eicosanoids synthesis. Eicosanoids first discovered in 1936 by Ulf Von Euler, these are located in phospholipid membrane, participates in the process of cell activation and pathways of signal transduction. Upon hydrolytic cleavage of ester bond at Sn-2 position of cell membrane free arachidonic acid (AA, all-cis-5-8-11-14-eicosatetraenoic acid) is released which is polyunsaturated acid (Venable et al., 1993) and one of the most important precursor for eicosanoids synthesis. The eicosanoids include the products of cyclooxygenases (prostaglandins and thromboxanes) and 5-lipoxygenase (leukotrienes and 5-hydroxyeicosatetraenoic acid (HETE), 12-lipoxygenase (12-hydroxyeicosatetraenoic acid) and lipoxins (Lewis et al., 1990) as described in Figure-1. Both prostaglandins and leukotrienes play an important role in inflammation (Bhattacheryee, 1989) among them PGE2 and LTB4 are most studied and important mediators (James et al., 2000).

1.4.1 Prostaglandins

Arachidonic acid is converted into unstable prostaglandin PGG2 which is peroxidized to PGH2 in the presence of prostaglandin H synthase. Prostaglandin H (PGH) synthase is also termed as cyclooxygenase (COX). An intermediate PGH2 serve as a precursor for the synthesis of further different prostaglandins such as PGD2, PGE2, PGF2, by their corresponding synthases enzyme as well as thromboxanes. After their production, prostanoids leave the cell through prostaglandin transporters and immediately converted into inactive form of metabolites; therefore, exert less systemic effects and produce their effect on neighboring cells or from the cells they were generated. Their actions mediated via specific G-protein coupled receptors (GPCR) present in most of tissues and predominantly cells of immune system (Rocha and Carvalho 2005).

The COX was first isolated from ram seminal vesicles (Hemler and Lands 1976) and is membrane bound enzyme, existing in two isoforms COX-1 and COX-2. COX-1 is constitutive present in all nucleated cell and responsible for production of prostanoids which are involved in normal physiology of body, regulation of tissue homeostasis, cytoprotection, platelet aggregation, renal blood flow and maintenance of gastric mucosa (Vane and Warmer 2000). While, COX-2 is inducible, is only constitutive in neurons and gastric mucosa (O’Neil and Ford-Hutchinson 1993) and over expressed in the condition of inflammation consequently enhance the production of prostanoids in inflammatory diseases such as arthritis and glomerulonephritis. The COX-3 mainly residing in brain and spinal cord, its byproduct PGD2 is involved in fever and pain which is inactivated by
acetoaminophen. However, there are controversies regarding its separate entity and proposed to be a COX-1 variant (Nicolaou, 2013).

The PGE$_2$ is generated by resident cells at the site of inflammation and stimulates erythema, increase blood flow, promotes edematous response (Willliams and Peck 1977), vascular permeability; stimulate plasma exudation and causes immunosuppression (Wang et al., 2013). It is also involved in fever by acting on neurons of thermoregulatory mechanism of hypothalamus and increase body temperature (Egg, 1984). It also triggers the release of other inflammatory mediators such as histamine, bradykinin, recruitment of neutrophils (Plummer et al., 1999) and increase metalloproteinase production in other cells (Clancy et al., 1998) thus amplifying inflammatory process. PGE$_2$ promotes inflammatory pain by sensitizing the afferent nerve terminals present on the peripheral endings of sensory neurons (Rhodes et al., 2009). At the site of inflammation the biosynthesis of PGE$_2$ in large quantities within short duration is enhanced due to up-regulation of COX-2 enzyme (Smith et al., 1998) and involved in several inflammatory diseases such as rheumatoid arthritis, atopic dermatitis and colon cancer (Sheng et al., 1998).

1.4.2 Leukotrienes

Leukotrienes (LTs) are lipid mediators, synthesized in many cells by 5-lipoxygenase (5-LOX) which is the major enzyme in the formation of LTs pathways. The 5-LOX is activated by FLAP, and converts AA to 5(S)-hydroperoxy-6-trans-8, 11, 14-cis-eicosatetraenoic acid (5-HPETE) which is unstable and is converted into LTA$_4$ and also reduced to 5-HETE. The LTA$_4$ is parent molecule in leukotriene (LT) family either it is converted to LTB$_4$ by an enzyme LTA$_4$ hydrolase or is conjugated with glutathione to form cysteinyl leukotriene (Borgeat and Samuelsson 1979).

The 5-LOX enzyme was discovered in 1976 from glycogen induced rabbit neutrophils (Borgeat et al., 1976). It is protein resides mainly in neutrophils, eosinophils, monocytes, macrophages and lymphocytes (Borgeat and Samuelsson 1979).

This enzyme act differently from the members of its lipoxygenase family of arachidonic acid pathway as it requires interaction with 5-lipoxygenase–activating protein (FLAP) a specific activating protein found at the nuclear envelop. Presence of cPLA$_2$, 5-LOX and FLAP at nuclear envelop are required, to activate the process of 5-lipoxygenation of AA in cells for LT synthesis (Pouliot et al., 1996). There are various 5-LOX activation and leukotrienes synthesis stimuli including Ca$^{2+}$ mobilizing agents such as calcium ionophore A23187 facilitates the translocation of enzyme into the nuclear
membrane (Brock et al., 2001), phagocytic agents like zymosan and cytokine like IL-8 (Schroder, 1989).

LTB₄ is synthesized by monocytes, macrophages and neutrophils (Nicosia et al., 2001). It is the potent chemotactic factor for mobilization and recruitment of neutrophils (Casale et al., 1992) and prolongs its existence at the site of inflammation; stimulate neutrophil accumulation in skin. It enhances endothelial leakage (Rosengren et al., 1991), synergistic effect in combination with PGs and thus increases vasodilatation and vascular permeability, also trigger the release of lysosomal enzyme (Feinmark et al., 1981) and cause hyperalgesia (Levine et al., 1984). It stimulates the production of superoxide consequently increases the inflammatory signals (Damtew et al., 1993). It also acts upon other cells of defense system such as eosinophils, monocytes and lymphocytes (Rola-Plezczynski et al., 1987). It is over expressed in gastrointestinal cancer (Shimakura and Boland, 1992), asthma, pulmonary fibroblast (Wilborn et al., 1996) and skin diseases (Brain et al., 1984). According to Shindo et al. (1995) normal levels of LTB₄ in humans were 360 ng/mL which increases and reached to 560 and 810 ng/mL in asthmatic and chronic obstructive pulmonary diseased (COPD) patients.

1.5 Other mediators of inflammation

It includes vasoactive amines, peptides, nitric oxide, cytokines, matrix metalloproteinases and C-reactive proteins.

Vasoactive amine i.e. histamine [2-(4-imidazole) ethylamine] is formed in Golgi apparatus by decarboxylation of histidine via L-histidine decarboxylase from where it is translocated to the granules of mast cells and basophils and stored there until release (He, 2004). They are present in all tissues, upon activation mast cells degranulate to release histamine (Marone et al., 1997) secrete in the quantity of 2-5 pg/cell (Church and Caulfield 1993). It is potent inflammatory mediator plays an important role in the anaphylactic and allergic reaction, increases the vascular permeability, involved in nociception (Marone et al., 1997), release LTs and platelet activating factors (Ammon, 2010). The antagonists such as cryproheptadine, pyrilamine and chlorpheneramine suppress its production.

Serotonin [5-hydroxytryptamine (5-HT)] another vasoactive amine synthesized by decarboxylation of tryptophan, stored in human platelets and granules of mast cells in rodents. It increases the vascular permeability, dilates capillaries and the elevated levels of serotonin is considered as a marker of irritable bowel syndrome (Barnes et al., 1998).

Bradykinin is a nanopeptide formed from system of plasma Kallikrein- Kininogen
(Bhoola et al., 1992). It is also potent vasodilator involved in allergic reactions and enhances the synthesis of prostaglandins and pain production, intensify algesic action by the excitation of sensory neurons and thus provokes the release of neuropeptides (substance P, calcitonin gene related peptide (CGRP) and neurokinin A (Geppetti, 1993).

Nitric oxide (NO) is also potent inflammatory mediator. It is synthesized via L-arginine oxidation by nitric oxide synthases (NOS) present at macrophages, endothelial cells, hepatocytes and certain neurons. Constitutively NO synthases produce picomole-nanomole amounts of NO for short period provide nonspecific immunity as a first-line defense against invading pathogens as well as regulating vessels tone. During inflammation the inducible NO generates in larger and sustained amounts promotes vasodilatation and increases the volume of exudates to cause edema (Kumari et al., 2014), stimulates tumor necrosis factor, COX pathway, reacts with free radicals to form toxic peroxynitrite and contribute in tissue injury and progression of various diseases such as arthritis, osteoarthritis, multiple sclerosis, ulcerative colitis, psoriasis and diabetes mellitus (Clancy et al., 1998).

The first cytokine was recognized by Beeson in 1948 as a pyrogenic agent extracted from neutrophils, later referred as IL-1β. Afterwards, various other cytokines identified they are small, low molecular weight glycoproteins, exert broad spectrum of actions and act as mediators between cells (Ozaki and Leonard 2002). These are multifunctional molecules having synergistic as well as antagonistic interactions (Feghali and Wright 1997) and act as intercellular signaling mediators. The process of inflammation is mediated by a number of cytokines by binding to target cells through specific high affinity cell-surface receptors which initiate a series of intracellular signal transduction pathways. Various cytokines are involved in inflammation include tumor necrosis factor-α (TNF-α), members of interleukin (IL) i.e. IL-1, -6, -11 and -8 among them most potent is TNF-α and IL-1 (α and β) (Padi and Kulkarni 2008).

TNF-α and β produced by activated macrophages/monocytes, fibroblasts, mast cells, endothelial cells, natural killer (NK) cells, cardiac myocytes, adipose tissue and neurons (Walsh et al., 1991). Its biological roles in the host defense against bacteria, virus and parasitic infections however, inappropriate or excessive production of TNF-α can be harmful, its local increase in concentration associated with cardinal signs of inflammation by increase expression of COX-2 resulting in increased blood flow and vascular permeability through endothelial cells (Aggarwall et al., 2012), involved in the induction of acute phase reactant protein production by the liver. Its production also stimulates the release of IL-1β and IL-6, consequently production of prostaglandins, sympathomimetic
amines and nitric oxide (NO) which are associated with nociceptors sensitization (Cunha et al., 1992). It induces fever, either directly via stimulation of PGE₂ synthesis by the vascular endothelium of the hypothalamus, or indirectly by inducing release of IL-1.

TNF-α and IL-1 further exert secondary inflammatory effects by stimulating IL-6 synthesis in several cell types. IL-6 then mediates its own effects and those of TNF-α and IL-1 in inducing fever and the acute phase responses (Padi and Kulkarni 2008), thereby intensify the inflammatory response through a cascade of cytokines with overlapping properties. High concentrations induce shock like symptoms and implicated in variety of diseases including Alzheimer’s disease (Gaur and Aggarwal 2003), inflammatory bowel disease (Carswel et al., 1975), cancer, depression and psoriasis (Aggarwal, 2003).

Various TNF-α antagonist (infliximab, etanercept, and adalimumab) are indicated against arthritis, psoriasis, ulcerative colitis, and Crohn’s disease. According to the sale report of TNF-α blocker in 2010 exceeded US $20 billion. But these drugs are associated with serious adverse effects such as immuno-suppression and carry black-box warning labels regarding the increased risk of infections (such as tuberculosis), CNS demyelinating disorder, also cause liver injury, hepatitis B, and drug-induced lupus. Besides safety issues, most of the TNF-α blockers are very expensive i.e. ~ US15–18000$/patient/annum in USA and approximately 10,000£ in Europe (Kuek et al., 2007).

Thus, alternatives that are safer and more affordable, yet effective, are needed (Aggarwall et al., 2012).

Balance between pro-inflammatory and anti-inflammatory mediators is necessary for regulating immune system against pathogen. The pro-inflammatory response is essential for fighting the pathogen. Conversely, it is necessary to limit and resolve the inflammatory process to avoid damaging the host itself. An excessive inflammatory reaction can be stopped by various anti-inflammatory cytokines (such as IL-10 and tumor growth factor (TGF) (Asadullah et al., 2003). Interleukin-IL-10 is an 18 kDa, mainly synthesized via granulocytes, natural killer cells (NK cells) or lymphocytes, but may also be produced by non-immune cells such as epithelial cells, keratinocytes and fibroblasts. It is also referred as anti-inflammatory or anticytokine by inhibiting cytokine production and inhibits pro-inflammatory agent IFNγ (Savan et al., 2009) thus turning off the inflammatory processes (Ozaki and Leonard, 2002).

Matrix metalloproteinases (MMPs) are member of zinc and calcium dependent endoproteinases produced by epithelial, endothelial, fibroblast cells and neutrophils. It consist of MMP1-28 members, regulate extracellular matrix and basement membrane during inflammation they involved in the cleavage of chemokine precursors (CXCL-1
and CXCL-8) (Fournier and Parkos 2012), recruit at inflammatory site and contributes in inflammatory diseases such as arthritis, atherosclerosis (Cawley and Matrisian 2001) and tumor growth (Nagase, 1997).

C-reactive protein (CRP) belongs to the family of pentraxin plasma protein synthesized by liver. Its physiological role is to activate the complement system and contribute in the metabolic, scavenging and host defense mechanism promoting phagocytosis by macrophages thus clears necrotic or apoptotic cells and bacteria (Pepys and Hirschfield 2003). In healthy human its concentration is 0.8 mg/L, following an acute-phase stimulus values may increase by 10,000 folds (Licastro et al., 2005). Its elevated concentrations in blood is cytokine mediated response (TNF-α) found in most forms of inflammation and infection and widely use as diagnostic tool for several diseases such as Alzheimer’s, coronary heart disease, especially myocardial infarction (MI) (Thompson et al., 1995).

1.6 Skin and eicosanoids

Rodents (rats, mice and guinea pig) skin has similarities in histologically and biochemically to human (Simon and Maibach 1998). However, human dermis has more elastic fibers than rodents (Montagna and Parakkal 1974). Skin is the body’s first and largest defensive barrier against exogenous (chemical shock, heat, injury or pathogenic microbes) and endogenous stimuli and to repair the damage tissue and regulates the homeostasis mechanism of body. The skin histological study provides information about skin layers, epidermis and dermis, former being the outermost layer is thick and consists of stratified squamous epithelium, stratum corneum appears as mass of black cells in toluidine blue dye whereas remaining part of epidermis and dermis stains light blue. Therefore black stain quantification provides accurate estimation of stratum corneum thickness. Epidermis constituent about 95% of keratinocytes, with small population of lymphocytes and Langerhans cells (immune cells) (15 cells/ mm of epidermis section), merkel cells (sense of touch), melanocytes (regulate melanin pigment of skin) and had small and large intracellular vacoules (~10 μm) which have been damaged during inflammation with the sign of spongiosis and decrease in the number of Langherns cells (Montagna et al., 1989), over production of vacuolated basal keratinocytes, melanocytes, and polarity of cell reduced with dead and dying cells. Additionally, epidermis also regulate amount of water in the body. Both epidermis and dermis are segregated by basement membrane which is composed of thin sheet of fibers. It controls the trafficking
of cells between them and also serves for the binding of cytokines and growth factors for
the physiological remodelling and repair processes.

The dermis, inner layer of the skin composed of connective tissues, elastic fibers, dermal fibroblast and provides elasticity to the skin. It consists of three less well-defined layers: papillary, intermediate and reticular dermis. The papillary dermis composed of very small (~1 µm) and small (~8-10 µm) collagenous fiber bundles, the thickness of papillary layer is equal to epidermis thickness. The upper portion of intermediate layer consist of medium sized fiber bundles with blood vessels plexues in its deeper parts, it is lack of elastic fiber and rich of fibroblasts. The lower part of intermediate dermis contains medium and large fiber bundles (> 20 µm in diameter). The innermost layer reticular dermis contains tightly bound large, packed fiber bundles (Smith et al., 1982). It also contains nerve endings for the sense of touch and heat, hair follicles, various glands (sweat, sebaceous and apocrine glands), fibroblasts, mast cells, endothelial cells, macrophages, lymphatics and blood vessels (Proksch et al., 2008) as well as mast cells and infiltrating leukocytes (Kupper and Fuhlbrigge 2004). The undamaged dermis contains fibroblast below the dermoepidermal junction which is smaller in size compared to middle layer of intermediate dermis. While in case of severe inflammation the elastic fibers were overgrown in the lower part of intermediate dermis with granular and foamy masses and fibers become fragmented, normal skin contains smaller amount of large size melanophages at the epidermis where as in inflammation numerous large melanophages in the papillary dermis were observed and necrosis of papilary dermis with marked increase in inflammatory cell population with moderate increase in lymphocytes, histocytes and mast cells (Montagna et al., 1989).

The prostaglandins (PGE₁, PGE₂, PGE₃, PGD₂, PGF₂α, PGI₂, and TXB₂) are present on cutaneous cells express by COX-1 and -2 (Nicolaou, 2013). Among them PGE₂ is predominant eicosanoids in skin synthesized by keratinocytes of epidermis and fibroblasts of dermis (Rhodes et al., 2009) involved in keratinocyte proliferation and differentiation, these actions are mediated by G-protein coupled receptors subtypes present in all cells of the skin (Konger et al., 1998). Involvement of 5-LOX in skin is less and is found in epidermal keratinocytes and infiltrating leukocytes its activity is increased after keratinocytes differentiated, however 12-LOX is abundantly present in skin cells (Nicolaou, 2013). Inappropriate or failure of immune system involve in the pathogenesis of variety of cutaneous inflammatory diseases although mortality rates for these skin diseases are relatively low (Njoronge and Bussmann 2007) but persistent chronically and difficult to treat such as atopic dermatitis and psoriasis (Maldini et al., 2009) which are
characterized by increased vascular permeability, accumulation of plasma exudates and infiltration of inflammatory cells mediated by TNF-α, IL-1β, PGs, LTs and platelet-activating factor (Camussi and Brentjens 1987).

For skin and tissue histopathological studies, hematoxylin and eosin (H&E) are most widely used dye (Avwioro, 2011). Most of the cells are transparent therefore; histological sections need to be stained to make the cell visible (cytoplasm, nuclear, and extracellular matrix). Hematoxylin is derived from logwood, *Hematoxylin campechianum* (Willis, 1951), it do not have staining properties unless oxidized to hematin with deep blue in color, basic in nature, positively charged and stains chromatin material of nucleic acids which is negatively charged whereas; eosin is pink in color, acidic in nature, negatively charged and bind to positively charge proteins and connective tissue (cathionic in nature) in cytoplasm. H&E is important for recognizing various types of tissue and the morphologic changes for the diagnosis of various disease states. However, it is incompatable with immunofluorescence (Fischer et al., 2008).

### 1.7 Animal models of inflammation

The cellular function in humans and animals are remarkably similar and the pathological process of various inflammatory diseases can be observed and studied in animal models, followed by further *in vitro* studies (Balcombe et al., 2004). Various acute inflammatory animal models have been developed, because single animal model is not sufficient to detect whole inflammatory process. There are many animal models for evaluating anti-inflammatory activity to determine the possible mode of action (Patel et al., 2012) some of them are as follows:

#### 1.7.1 Acute animal models of inflammation

A) **Ear edema** model offers particular advantages as it is quick, simple and reproducible (Garrido et al., 2006). Various irritant agents such as arachidonic acid (AA) (Young et al., 1984), 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), xylene (Saraiva et al., 2011), croton oil (Tonelli et al., 1965), phorbol myristate acetate (Kuehl et al., 1977), capsaicin (Gabor and Razga 1992), ethyl phenylpropiolate (Brattsand et al., 1982), mustard oil (Inoue et al., 1997) or histamine applied topically on the ear have been popularly used to induce edema in rodents. The weight of ear punches or diameter of ear was measured using vernier caliper provides a reliable tool for screening of anti-inflammatory agents with possible mode of action (Gabor, 2000).
(i) **Croton oil or TPA-induced ear edema in mice**

Croton oil contains 12-0-tetradecanoyl-phorbol-13-acetate (TPA) and other phorbol esters. Its local application on ear induces vasodilatation, slow and long lasting edema, vascular permeability, polymorphonuclear leukocytes, epidermal hyperplasia (Clark et al., 1985), increase influx of neutrophils, releases hydrogen peroxide (Wei and Frenkel 1993) which is responsible for further damage and may form skin tumors. However, repetitive application induces chronic inflammation (Stanley et al., 1991). The TPA activates protein kinase C (PKC) and thus initiates series of events (such as mitogen activated protein kinases (MAPK), and phospholipase A2 which causes the release of platelet activating factor and AA pathway for synthesis of eicosanoids (Ferrandiz et al., 1996) with predominant formation of PGE₂ (Inoue et al., 1988) and LTB₄ (Ferrandiz et al., 1996). PGE₂ is major mediator of inflammation predominantly involved in the redness while leukotrienes induce edematous response (Young et al., 1984). In ear edema LTB₄ is synthesized for longer period than PGE₂ and it may be due to eicosanoids cellular source (Ford-Hutchinson et al., 1980).

TPA also stimulate over expression of other inflammatory mediators such as inducible nitric oxide synthase (iNOS), COX-2, nuclear factor kappa B (NFκB) (Ban et al., 2009), TNF-α, IL-1β and IL-6 (Song et al., 2008). In this animal model COX, LOX inhibitors (Murakawa et al., 2006), dual inhibitor of COX and LOX and corticosteroid showed good anti-inflammatory activity (Inoue et al., 1988).

(ii) **Arachidonic acid (AA)-induced ear edema in mice**

Topical application of AA has rapid onset of vasodilatation and erythema within five minutes that last for 60-90 min and produce short-lived edema, accompanied by protein and leukocyte extravasation (Young et al., 1984). Its multiple applications cause keratinocyte proliferation (Doherty et al., 1988). It mediates the release of arachidonate metabolites with markedly increased levels of prostaglandins (PGE₂, 6-keto PGF₁α and PGF₂α), thromboxane B₂ (TxB₂) and leukotrienes (LTB₄, LTC₄, LTD₄ in small amounts) via COX-1 and -2 (Gabor, 2000) and 5-LOX (Carlson et al., 1985) pathways.

These metabolites also cause the release of histamine by mast cell degranulation (Young et al., 1984) and increased myeloperoxidase activity (Ban et al., 2009). The COX, LOX and histamine inhibitors cause reduction in AA-induced ear edema in mice however corticosteroids has no or little effect compare to NSAIDs (Carlson et al., 1985; Gabor 2000).
(iii) Other irritants

Ear edema can also be induced by topical administration of mustard oil that induces acute inflammatory response such as plasma extravasation and edema formation and its maximum effect lasts for 30 min (Patel et al., 2009). Xylene and capsaicin induced ear edema related to neurogenic response by the release of substance P from sensory neurons of spinal cord and excites the pain pathway peripherally causing vasodilatation and extravasation (Hernandez-Ceruelos et al., 2002; Inoue et al., 1993), while phenol applied topically causes rupture of keratinocyte membrane and release of pro-inflammatory cytokines (TNF-α, IL-1 and -8) and also activate the release of metabolites of AA and generation of ROS (Muray et al., 2007).

B) Paw edema Several phlogistic agents have been used to induce paw edema. Some of them are as follows:

(i) Carrageenan

It is derived from the Irish Sea moss from the species of red algae *Chondrus crispus* and discovered by Stanford in 1862. It is mucopolysaccharide, with repeated galactose monomers and classified into 3 types as kappa, lambda and iota having different gel characteristics and need thermal induction except lambda which does not form gel even at room temperature (Patel et al., 2012). Upon its administration in animal it causes redness, increase the permeability of blood vessels, edema, neutrophil recruitment and hyperalgesia (Oka et al., 2007) by activating the release of vasoactive amines (bradykinin, histamine and serotonin), eicosanoids (PGs and leukotrienes) and cytokines (TNF-α) producing edema and the levels of PGs and TNF-α increased by 4-5 folds (Smith et al., 1998).

The carrageenan (1%, 50 μL)-induced paw edema was introduced by Winter et al. (1962) that elicit above mention inflammatory signs such as developed immediately after its administration subcutaneously in to the paw of rat. The paw edema volume was measured by plethysmometer in which the displacement of volume by immersing the animal paw during various time intervals was noted. It consists of biphasic events first phase last for 2 h and second phase last for 4-5 h. In the first phase vasoactive amines are released followed by the second phase in which Prostaglandins, lysosome and protease are released. This animal model provides a suitable parameter for assessment of anti-inflammatory agents particularly NSAIDs (COX inhibitors) are useful in this animal model (Patel et al., 2012).
(ii) **Phospholipase A\(_2\) (PLA\(_2\))**

In this method inflammation is induced by subcutaneous administration of PLA\(_2\) (2 unit/25 \(\mu\)L) into the sub plantar region of mice paw. The increase in weight of paw edema can be measured using plethysmometer at different interval. The principle involved in this model is PLA\(_2\) activates the release of arachidonate metabolites. It also stimulates the release of histamine and serotonin. PLA\(_2\), COX, LOX, histamine and serotonin antagonist cyproheptadine are useful in this animal model (Giner-Larza et al., 2001).

(iii) **Histamine and Egg-albumin**

Subcutaneous injection of histamine (1\%) (Amann et al., 1995) or egg albumin (0.1 mL) to the right hind paw of rodent induces swelling and vascular permeability. Increase in paw edema was measured by plethysmometer. Edema formation by egg albumin is biphasic event. Early phase begins immediately after the administration and last up to 2 h during which histamine and serotonin are released producing edema, plasma extravasation and neutrophil extravasation. In the later phase that last for 3-5 h bradykinin, protease, PGs, lysosome are released (Yankanchi and Koli 2010).

C) **Peritonitis** one of the central features of inflammation is vascular permeability induced by \(i.p\) administration of various chemical agents in rodent’s as a result acute inflammation and painful reaction emere in the peritoneal area and stimulation of peritoneal nociceptors and endogenous substances release (Gyires and Torna 1984).

The intraperitoneal cavity is membrane bound and fluid filled cavity of mammals and contains variety of immune cells including macrophages, B and T cells. The presence of large number of macrophages in peritoneal cavity makes it preferred site as they are responsible to produce inflammatory mediators (Zhang et al., 2008).

i) **Glycogen**

Glycogen (1 g/kg) has been used for induction of sterile inflammatory exudates with influx of leukocytes predominantly polymorphonuclear neutrophils (PMNs) and plasma proteins which increase the circulating neutrophils by 4 fold and accumulation of 14-23 million neutrophils in the peritoneal cavity was evident. NSAIDs and glucocorticoid are effective in this model (Yamashita et al., 1982). Furthermore these neutrophils can also use for cytotoxicity assay to evaluate the substance ability to kill the cells (Goldberg and Frazier 1989). Trypan blue exclusion assay is widely used to determine neutrophil viability. It is blue color diazodye and based on the principle that cell membrane is the basic component to distinguish between viable and non viable cells (Kroemer et al.,
2009). Cell membrane of live cells exclude various dyes to penetrate in it and thus remained unstained whereas, trypan blue absorb into the cytoplasm of dead cell because of loss of cell membrane selectivity and thereby dead cells appear as blue in color under the microscope (Song et al., 2012).

ii) Carrageenan
Carrageenan (1%, 0.1 mL) used to induce peritonitis in mice causing dilation of the blood vessels and increase the permeability of blood vessels, recruitment of neutrophils thereby produced edema mediated by release of inflammatory mediators such as vasoactive amines, prostaglandins, leukotrienes and cytokines (Kolaczkowska et al., 2002) acting on their corresponding receptors localized on vascular endothelium in the peritoneal cavity (van Hinsberg and van Amerongen 2002). The inflammatory cell population attributed in the pores or gaps formation between the cells of endothelium and magnitude of vascular permeability can be represented as amount of extravasation of Evans blue dye leaks into the peritoneal cavity which is measured by means of absorbance optical density (OD). Evans blue (EB) dye is non toxic (Matsuda et al., 1995) and used as diagnostic tool to measure the vascular and protein leakage in vivo and cellular permeability by in vitro study (Ferrero, 2004). This dye can easily be visualized when injected directly into the blood stream of animal (Reeve et al., 1965) and have high binding affinity for albumin and used as diagnostic marker (Rawson, 1943).

iii) Acetic acid
Acetic acid (0.7%, 10 mL/kg, i.p) increases the dilation of arterioles and venules, enhance vascular permeability and hyperalgesia. All these inflammatory features mediated by histamine, serotonin, PGs and LTs in peritoneal cavity (Park et al., 2007).

In sub-acute animal model of inflammation air pouch was formed by injecting air sub-cutaneously on the back skin of rat skin followed by the injection of carrageenan (2%). After 8 days exudates is collected, weighed and white blood cells are counted. This model is useful for the study of extravasation, migration of leukocyte, prostaglandins, leukotrienes and interleukins (IL). Synthesis of nitric oxide, release of kinin, collagen formation and angiogenesis are associated with granuloma formation. Angiogenesis that is involved in chronic phase of inflammatory state facilitates the recruitment of inflammatory cells, supplies nutrition and oxygen to the granulation tissue. Therefore, attenuation of angiogenesis in granulation tissue is important to target the chronic inflammation (Selye, 1953).
1.7.2 Chronic animal models of inflammation

The proliferative phase of proliferation can be evaluated by following methods:

Cotton pellet and glass rod-induced granuloma in rat sterile piece of cotton (Goldstein et al., 1976) or glass rod (6 mm) (Vogel, 1996) was implanted sub-cutaneously into the axila of rat. After 20 or 40 days cotton pellet or glass rod was removed surgically, weighed and newly formed undifferentiated connective tissues and histologically giant cells were studied.

In these models cotton or glass rod act as foreign body, associated with proliferative stage of inflammation in which neutrophils, macrophages, fibroblasts accumulate around foreign particles together with epitheloid and giant cells derived from macrophages and collagen synthesis initiates granuloma formation (to form a ball of cell). The undifferentiated connective tissues, macrophages and lymphocytes are considered as indicative of severity of chronic inflammation (Paschapur et al., 2009).

1.8 Routes of administration

The route of administration is the selection of path for the entry of drug into the body. It is depend on the properties of test agent (molecular size, ionization state and lipophilicity). These physicochemical properties are independent of test species (DeSesso and Jacobson 2001). Route of administration classified into systemic and local route. The systemic route is further classify into enteral (oral, sublingual and rectum) and parenteral (intravascular (i.v), intramuscular (i.m), subcutaneous sc and inhalation) route (Verma et al., 2010).

Oral route is most common and convenient route in which drug is swallowed by placing in mouth followed by its absorption via hepatic metabolism of test agents in gastrointestinal tract (Abe et al., 2009). However, oral route has some limitation such as slow onset of action compared to parenteral route, first pass effect by liver, lack of absorption of drug due to chemical polarity, degradation of substance by digestive acid and enzymes, unconscious patient (Becker, 2006). Rectal route (enema or suppository) is an alternate route of oral administration comprise of rich blood supply. However, this route is not recommended for animals.

Parenteral route includes entry of drug directly into the circulation without passing through GI tract (Becker, 2006) and inhalation route includes volatile gases for anesthesia or aerosols preparations. Particles (3-5 µm) distribute throughout the lung while particles (<3 µm) penetrate into the alveoli. Lung has large surface area consist of adequate capillary network which provide rapid absorption (Verma et al., 2010).
When test agents applied topically it absorb through the skin and enters to the systemic circulation. Absorption of test agent in the epidermis occurs through para and transcellular mechanisms into layers of epidermis i.e. stratum corneum to the stratum spinosum followed by basal layers of skin finally entry in to the dermis as well as into the space subcutaneous via hair follicles and other accessory glands (Gonzalez-Mariscal et al., 2005). Topical application includes direct absorption of test agent into the surface of skin. For percutaneous absorption the extent of absorption of test agent via skin into the blood depend upon the area large surface area of the site of application, skin thickness, substance contact duration with skin, degree of hydrated skin (absorption is more faster in hydrated skin than dry skin hydrated skin well) length of time conc. of substance applied and lipid solubility of test agent (Ngo and Maibach 2010).

Test agent administration in laboratory animal is critical component of designing of experiment. Drug can be administered in animals by mouth (oral) or directly delivered into stomach (intragastric), delivered direct into blood stream (i.v) or administered into muscle (i.m) or instilled into eye (intraocular) or into brain (intracerebral) into the space surrounds dura mater (epidural) or into the peritoneal cavity (i.p), inhaled into the lungs (inhalation) or applying topical (Gerwin et al., 2006).

Placing the test agent directly into the mouth or mix it with feed or intragastric route is most common routes in lab animals. The humans and rodents share similar anatomy and physiological features of hepatic system except rat lack of gallbladder (Rogers and Fox 2004).

Administration of substance into the peritoneal cavity is also one of most common route in laboratory animals but rarely used in humans. After i.p administration drug absorb via mesenteric vessels, which drain into liver’s portal vein (Lukas et al., 1971). Thus substance enters through parenteral route followed by hepatic metabolism and finally reached into the systemic circulation. In addition small amount may directly cross the diaphragm through small lacunae and enter into the thoracic lymph (Abu-Hijleh et al., 1995).

1.9 Anti-inflammatory drugs

The discovery of anti-inflammatory drugs originated from the concept of use of Willow bark (Salix alba) and Filipendula ulmaria extract for the treatment of inflammatory conditions such as fever and pain (Rainsford, 2004). During the period of 17th century because of popularity of Willow bark the clinical trials were evident by Reverend Edward Stone for the treatment of fever. In 19th century with the advancement in the field of
chemistry principle active ingredient salicylate from the Willow bark was isolated followed by commercialization of this product in 1899 by Bayer as Aspirin over 100 years ago. Later several other agents of same class were also identified (Prescott, 2001).

1.9.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

Based on the selectivity of cyclooxygenases NSAIDs can be classified as non-selective and selective COX-2 inhibitors. The non-steroidal anti-inflammatory drugs (NSAIDs) are most commonly prescribed as anti-inflammatory, analgesic and antipyretic agents, with more than 98 million prescriptions in 2012 in the U.S, according to IMS Health report, 2012 (Consumers report, 2013).

The non-selective NSAIDs consist of following classes: carboxylic acid includes salicylate derivatives (aspirin); heterocyclic and carboxylic acid derivatives (indomethacin); derivatives of propionic acid (ibuprofen, flurbiprofen, ketoprofen, and naproxen), phenyl acetic acid derivatives (diclofenac) and derivatives of fenamic acid includes mefenamic acid. The enolic acid consists of oxicams (piroxicam and meloxicam) and pyrazoles (phenylbutazone) (Derle et al., 2006).

They are also indicated for chronic inflammatory conditions such as rheumatoid and osteoarthritis, sports related injuries, gouty tendonitis, muscle strain, postoperative and post injury inflammation, dysmenorrhea, thrombophlebitis and vasculitis also indicated in the cancer and cardiovascular diseases (Pulichino et al., 2006). NSAIDs inhibits the prostaglandin synthesis by inhibiting both COX-1 and inducible COX-2 enzymes involved in the pathway of arachidonate metabolism. However COX-1 involved in the regulation of homeostasis and protects the lining of gastrointestinal tract (GI). On long term use of NSAIDs and continuous blockade of the physiological effect of prostacyclin (PGI₂), PGE₂ and thromboxane A₂ cause erosion of stomach lining which lead to bleeding and stomach and duodenal ulcers. It has been reported that asymptomatic damage of mucosa in 80% of patients after NSAIDs therapy (Ehsanullah et al., 1988). Other adverse effects are hemorrhage, bronchospasm, kidney and liver dysfunction (Lin et al., 2006). After the blockage of the COX pathway the inflammatory response shifts to LOX pathway leading to bronchoconstriction and gastro damaging (Knights, 2006). It is estimated by IMS Health report, in 2012 undesired effects due to NSAIDs causes more than 100,000 patients admitted in hospitals and more than 16,000 deaths in the U.S. every yearly (Consumers report, 2013).

Considering the unwanted effects of classical NSAIDs as mentioned above and discovery of COX-2 in 1991 by Daniel L. Simmons and meloxicam (more selectivity
towards COX-2 inhibitor) was the first step for the development of true COX-2 inhibitors. NSAIDs new coxib class introduced in 1999 included celecoxib, valdecoxib, rofecoxib, paracoxib and etoricoxib having selectivity for COX-2 pathway without disturbing the COX-1 pathway (Hawkey, 1999) and indicated for arthritis. The COX monomer consists of three domains: an N-terminal, membrane binding domain (48 amino acid) and C-terminal have COX active site where the substrate or inhibitors bind. Its active site consists of long hydrophobic channel from membrane binding domain and extended to the C-domain for binding of arachidonate and NSAIDs (Picot et al., 1994). The arachidonate bind on the upper part of channel from Arg-120 to Tyr-385 while NSAIDs bind to Ser-530 in the middle of channel. The major difference for selectivity between COX-1 and -2 is presence of 3 amino acids which provide larger (20%) and more access to COX-2. The isoleucine at 523 position in COX-1 which is replaced by valine in COX-2 causes structural changes. These changes allows access to the additional COX-2 side pocket which is specific for COX-2 binding also replacement of isoleucine-434 for valine in COX-2 allowing neighbour residue such as phenylalanine-518 to out of the pocket thus further increase the access of side pocket. Placement of arginine in the side pocket of COX-2 instead of histidine-513 in COX-1 increases its interactions with polar moieties. These differences in amino acids sequence allow selectivity for COX enzyme (Charlier and Michaux 2003).

Clinical studies demonstrated that coxibs have similar efficacy to diclofenac and naproxen with lower incidence of gastrointestinal tract adverse effects on prolonged use (Alvaro-Gracia, 2004). Rofecoxib was one of the most widely used coxib but its clinical studies evident that selective COX-2 inhibitors increase the risk of cardiovascular diseases such as myocardial infarction and stroke in patients with rheumatoid arthritis (Bresalier et al., 2005). This cardiac risk is mainly due to attenuation of prostacyclin (PGI2) which in turn increased the levels of prothrombotic thromboxane A2 and decrease vasodilatory prostacyclin levels in the endothelium, enhance platelet aggregation and vasoconstriction. Thus rofecoxib was baned in 2004 because ~ 140,000 heart attacks were associated with it and only prescribed under strict conditions (Lin et al., 2006). One approach to prevent cardiac risk associated with COX-2 inhibitors is use of thromboxane A2 receptors antagonist (Rovati et al., 2010).

In cyclooxygenase inhibiting nitric oxide donors (CINODs) coupling of nitric oxide (NO) to NSAIDs in the form of acidic ester reduce the gastrointestinal or cardiovascular disturbance. Naproxinod is one of the members of CINODs in phase-III clinical trials (Schnitzer et al., 2005). After absorption these drugs are cleaved into
original NSAIDs and nitric oxide moiety (Wallace and Del Soldato 2003). NO reduced the gastrointestinal and cardiovascular related adverse effects as it protect mucosa from damaging effect of NSAIDs and posses relaxant effect on vascular smooth muscle thereby, reducing vasoconstriction (Ignarro, 2002).

5-LOX inhibitor classified in two major groups i) leukotriene biosynthesis inhibitors ii) leukotriene receptor antagonists.

The leukotriene biosynthesis inhibitors can be further subdivided into three groups: redox active inhibitors, non-redox competitive 5-LOX inhibitors and FLAP inhibitors.

(i) Redox active inhibitors

These are non-selective antioxidants; participate in uncoupling of catalytic cycle of the 5-Lipoxygenase. They act by reducing the active site iron of 5-LOX or interfere in the oxidation of Fe$^{+2}$ to Fe$^{+3}$ (Eleni and Dimitra 2003). Variety of compounds containing catechol groups such as nordihydroguaiaretic acid (NDGA) extracted from leaves of Larrea tridentata (Arteaga et al., 2005), α-pyrene containing coumarins and flavonoids (quercetin) inhibit 5-LOX activity (Eleni and Dimitra 2003).

Non-redox competitive 5-LOX inhibitors

They compete with AA for binding to 5-LOX. Compounds such as dioabicyclooctanyl naphthalene, methoxyalkylthiazoles and methoxytetrahydropyrans were reported for in vitro inhibition (McMillan et al., 1990). However, poor solubility, short half life was less potent in animals (Steinhilber, 1999).

(ii) FLAP inhibitors

The leukotriene synthesis is inhibited by antagonizing the FLAP function without directly interfering with 5-LOX enzyme. The indole derivative (MK-886, MK-5911) has poor bioavailability as a crystalline sodium salt and led to maculopapular rash incidences in humans. The quinoline derivatives (DG0312, formerly known as BAYX1005) are weak FLAP inhibitors that have progressed to clinical trials in human with good clinical safety profiles with no hepatotoxicity (Evans et al., 2007).

The leukotriene receptor antagonists exert their action by binding and activating their receptors (BLT, Cys LT-1 and -2). Montelukast and zafirlukast block these receptors and are effective against chronic asthma although it is popularly used especially in paeds but associated with adverse effect such as fever and upper respiratory tract infection (Knorr et al., 2001). Zileuton inhibits the production of both LTB$_4$ and the Cys LTs thus
efficacious in asthma but is not widely prescribed due to poor pharmacokinetic parameters and hepatotoxic incidences (Berger et al., 2007).

The COX/5-LOX dual inhibitors, licofelone ([2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,3-dihydro-1H-pyrrolizine-5-yl]-acetic acid) is the only drug which inhibit both (COX-1, COX-2) and 5-LOX enzymes (Singh et al., 2006) and has cleared clinical trials phase-III for the treatment of osteoarthritis but not marketed yet. Additionally, it suppresses leukocyte rolling and adhesion to the endothelium and prevents cellular influx during acute inflammatory state (Ulbrich et al., 2005). Its safety profiles on GI tract and cardiovascular system are encouraging (Tries et al., 2002).

1.9.2 Steroidal anti-inflammatory drugs

Glucocorticoid hormone was developed by Hench and colleagues in 1940s and received Nobel Prize in 1949 for evaluating its effect in rheumatoid arthritis followed by variety of glucocorticoids was synthesized (Hench et al., 1950). It belongs to the class of steroid hormones; ability to bind cortisol receptor and also known as corticosteroids. This cortisol-glucocorticoid complex translocate to nucleus where it binds to the DNA sequences thus resulting protein complex act as co activator or corepressor and modify the structure of chromatin thus facilitating or inhibiting the transcriptional factors (Rhen and Cidlowski 2005).

They act via multiple mechanisms in suppression of inflammation including interaction with NF-κB, attenuate the transcriptional activity of inflammatory genes which mediate cytokines, chemokines and complement factors (Barnes, 1999). Suppress the activation of cPLA2 mediated by GPCRs, sPLA2, and COX-2, thereby inhibiting arachidonate metabolites, also inhibiting mast cell degranulation thus reduced the capillary permeability. They are prescribed for both acute and chronic inflammatory conditions such as rheumatic arthritis, asthma, Grave’s disease, systemic lupus erythmatosus, psoriasis, vasculitis, Wegener’s granulomatosis, multiple sclerosis, dermatoses and eczema (Rhen and Cidlowski 2005). Chronic use is associated with large variety of adverse effects atherosclerosis, hypertension, visceral obesity, type-2 diabetes mellitus, glaucoma, muscle wasting, osteoporosis, Cushing’s syndrome and glucocorticoid-induced psychoses (Goppelt-Struebe, 1997).

1.10 Complementary and alternative therapies

Deviation from conventional therapy is referred as complementary and alternative medicine (CAM). It consists of vast range of diagnostic and therapeutic procedures
include herbal medicine, traditional Chinese medicine, Ayurvedic medicine, homeopathy, acupuncture aromatherapy, color therapy and reflexology (Langmead and Rampton 2005). These therapies are not regulated by the Food and Drug Administration (FDA) as conventional drugs and their products are available to consumers as over the counter items in various dosages forms (Abebe, 2002).

Since thousands of years plants used for food and to treat the different ailments. In 15th century knowledge about plant containing medical information were compiled in the form of herbal books and became most popular selling books at that time (Adams et al., 2009). People used plants prophylactically or for the treatment of diseases. Herbal preparations make up an important trend toward alternative medicine because of their biological active compounds (Andrade et al., 2007), diversity in chemical structures, pharmacological properties, safety profiles and less side effects (Yang et al., 2013). Plant based medicinal active constituents and their phytochemicals are investigated continuously for the discovery of new therapeutic agents (Balunas and Kinghorn 2005) and available as standardized plant extracts form from plants or in purified form. Almost six thousand species of plants are used as medicines.

According to WHO ~ 60% of the world population rely on traditional herbal remedies (Mondal et al, 2012) which is reflected by its worth sell in 1997 according to World market annual sale report of medicinal plant earn $10 billion (Rates, 2001) which reached up to US$ 60 billion in 2008 (Tilburt and Kaptchuk 2008). and it is estimated by the end of 2015 it will reach to $93 billion about 9.3 times increase from the 1997-2001 (San Jose, 2011).

However, some undesirable and toxic effects are also associated with herbal medication because of contaminants such as microbial, pesticides, fumigants, toxic heavy metals and adultrants mostly steroids lead to different types of allergic reactions (Kunle et al, 2012). Some commercially available herbal products such as anti-inflammatory agent (Aconitum carmichaeli) containing alkaloids of aconite reported for cardio and neurotoxicity (Singhuber et al., 2009), weight reducing products Arislolochia fangchi containing aristolochic acid is associated with cardiac and nephrotoxicity (Nortier et al., 2000) and Pteleopsis hylodendron stem barks extract are hepato and nephrotoxic (Nana et al., 2011). Camellia sinensis contain epigallocatechin-3-gallate and St. John’s Wort containing hypericin used as weight reducing (Grove and Lambert 2010) and anti-depressant associated with hepatotoxicity (Licata et al., 2013). Thus toxicological evaluations of plants are important for their safety point of view. As liver is the first major organ to received ingested toxins, play important role in metabolism of exogenous
chemicals such as drugs, pesticides and metals by portal blood supply during the first pass effect resulting in the formation of metabolites which is less toxic hence provides increased hepatic injury while protection to other organs (Cieslinski and Humes 1994). Liver is located at the upper right side of abdomen consist of two main lobes; right lobe is six fold larger in size than left one. Liver cells (hepatocytes), portal canals and hepatic vein together forms the lobules of liver. The human and pig liver lobules are similar having distinct fibrous septa while in rodents there is no separate fibrous septa (Fraser et al., 1995).

Hepatocytes are varying in size and shape with dense cytoplasm and round nucleus. Hepatic portal vein connected to the GI tract to received 75% of blood containing dissolved foods while aorta supplied oxygenated blood to the hepatic artery finally blood drains into the vena cava from their reaches to the right atrium of heart (Wynne et al., 1989). The portal vein and hepatic artery are branched and unite to form portal canals which linked to the bile duct. From the branched structure of portal canal, both arterial and portal blood moves into the spaces between cords of liver cells called sinusoids. Sinusoids provide passage for blood into central hepatic vein of liver (Boyer, 2013).

The hepatic portal system is similar in human and rodent which consist of blood flow from small intestine capillaries to the hepatic portal vein and to the liver sinusoids (DeSesso and Jacobson 2001).

Liver function test are helpful in assessment of toxic effect of natural plants on liver these tests include determination of alanine aminotransferase (ALT/SGPT) responsible to transform metabolites across the cell membrane, aspartate aminotransferase (AST/SGOT) involved in amino acid catabolism and gamma glutamyltransferaseare (γGT) regulates the γ-glutamyl transfer from peptides to different amino acids, these enzymes present at hepatocytes, biliary cells of epithelium and renal tubules (Rosalki et al., 1971). Elevated levels of total bilirubin results from reduced uptake and bilirubin conjugation with liver due to dysfunctioning of liver cells while raised levels of direct/conjugated bilirubin due to its less secretion from liver or blockage of bile ducts (Anosike et al., 2008). The dysbalance of these enzymes are indicative of hepatodamage effects (Tilkian, 1979). In case of hepatotoxicity the cell membrane of liver is damaged and varieties of enzymes which are normally located in the cytosol are released in the circulation (Ncibi et al., 2008). In 1999 thirty four cases were reported on consumption of green tea based commercially available supplements to induce hepatotoxicity associated with rise in serum transaminase and serum albumin. Histological profiles also revealed
inflammatory and necrotic liver damage due to epigallocatechin gallate or its metabolites which affects patient’s metabolism mechanism (Mazzanti et al., 2009).

Kidney is located in the retroperitoneal in the abdominal cavity responsible for removal of waste from the body in the form of urine. Its basic unit is nephron comprise of glomerulus responsible to filter the solutes and well defined loop of Henle (Moffat, 1981). The renal medulla which is the innermost part comprise of nephrons structures and responsible for regulation of water and salt balance. The renal capsule provides protection to the kidney it is composed of tough layers of fibrous and adipose tissue. Kidney is important for efficient ultra filtration of blood to remove toxic wastes. Thus xenobiotics effects on liver and kidney should be studied (Cieslinski and Humes 1994).

Around 200 traditional plants have been reported for anti-inflammatory activity (Aggarwarl et al., 2011). In Pakistan about 75% of population used traditional medicines for the management of diseases (Qureshi et al., 2007). Medicinal plants widely used for the treatment of inflammatory diseases and pain such as Rosemary (Mengoni et al., 2011), boswellic acid (Ammon, 2010), turmeric (Garg et al., 2008), garlic (Sengupta et al., 2004), Willow bark, basil (Sing et al., 1996) and ginger (Park et al., 1998) but there is little knowledge regarding the mode of action of various herbal medicines which are extensively used in traditional medicines for the treatment of several diseases all over the world. WHO has expressed high interest in traditionally used medicinal plants thus it is important to evaluate these scientifically. Different industries has also invested hundreds of millions of US$ for search of promising medicinal herbs and novel compounds (Choudhari et al., 2013). Table-1 explained some plant derived active constituents against inflammation.

Drug interactions is a serious problem such as NSAIDs particularly aspirin interact with herbal supplements that possess antiplatelet activity (ginger, ginkgo, garlic, ginseng, bilberry, dong quai, feverfew, meadowsweet, turmeric, and willow bark), coumarin containing herbs (horse chestnut, fenugreek chamomile, red clover and motherworth) enhanced the risk of bleeding. Acetaminophen also interacts with ginkgo and with some of the above herbs to increase bleeding risk. Furthermore, hepat- and nephrotoxicity incidences were reported by concomitant use of acetaminophen with Echinacea and kava herbs (potential to cause hepatotoxicity) and with salicylate containing herbs (willow, meadowsweet). The opioid analgesics when used concomitantly with herbal supplements having sedative effects (kava, valerian and chamomile) increase central nervous system (CNS) depression. Ginseng inhibited the analgesic effect of opioids (Abebe, 2002).
Diet plays important part in promoting or suppressing the process of inflammation. Foods such as hydrogenated vegetable oil or corn, soy and safflower oils containing eicosatetraenoic acid and linoleic acid which converts into omega-6 (belongs to family of fatty acids) can aggravate inflammation by triggering the release of AA and its metabolites (PGs and LTs). On other side, diet rich in alpha-linolenic acid found in green leafy vegetables, fish oils (abundant in n-3 PUFA (polyunsaturated fatty acid) converted into omega-3 which includes eicosapentaenoic acid reduce the production of LTB4, inhibits neutrophil chemotaxis and beneficial in reducing the risk of cardiovascular diseases, asthma and psoriasis (Das et al., 1992). Other dietary components such as vegetables: olives, broccoli, rosemary; peppers, cloves, mints; fruits: berries, black currants, pineapple and lemon. Nuts: walnuts, almonds, sunflower seeds and hazelnuts can reduce inflammation if eaten regularly.

Other therapies for inflammation includes heating pads used in the treatment of chronic inflammatory diseases to stimulate the blood flow to the inflamed area while cold therapy (ice packs applied externally to the damage area for 10-20 min) is widely used in the management of pain and acute musculoskeletal injuries causing vasoconstriction, prevents edema, immediate release of endorphins (the body’s natural opiates system), decrease central nervous system transmission to the pain fibers, raises the pain threshold and also decreases free nerve endings excitability.

Aromatherapy is a form of alternative medicine in which aromatic plant oils (essential oils) used for prevention or treatment of diseases such as arthritis, mood altering, relaxation, pain, hair loss prevention, eczema and relaxation. These essential oils act on limbic system through olfactory system or by pharmacological actions. Several essential oil possess anti-inflammatory effects such as essential oil from leaves of *Cyperus giganteus* inhibit 5-LOX pathway and possess antioxidant property (Alitonou et al., 2006).

Another form of alternative therapy is color therapy also referred as chemotherapy, visible range of spectrum (colors) is used to treat diseases. The color produces electrical impulses and various magnetic fields which stimulates the biochemical and hormonal mechanisms of body (Samina and Raza 2005) that different colors are used for different diseases such as red light use for wound healing and for the treatment of cancer while blue light used for neonatal jaundice, rheumatoid arthritis and act as antioxidants (Ebbesen et al., 2003).
1.11 Reactive oxygen species (ROS) and inflammation

Under normal physiological condition ROS responsible to kill or oxidize harmful pathogens, polymorphonuclear leukocytes (neutrophils, eosinophils), macrophage and lymphocytes produce larger amount of ROS to destroy pathogens however, its imbalance causing tissue damage and worsen the inflammatory conditions and cell exposed to oxidative stress resulting cell death (Halliwell, 1989). Variety of cellular enzymes also involved in its synthesis including xanthine oxidoreductase (XOR), NADPH oxidases (Nox), aldehyde oxidase (AO), mitochondrial electron transport proteins, dysfunctional (uncoupled) nitric oxide synthases (NOS) (Kim et al., 2010) and cyclooxygenases and lipoxygenases (Machlin and Bendich 1987). Free radicals superoxides anions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl free radical (·OH) and hypochlorite (HOCl) are highly reactive, toxic and can damage the tissue. The ROS increases the vascular permeability (Salvemini et al., 2001) and act as second messengers that triggers the production of pro-inflammatory mediators (Maxwell, 1995) such as TNF-α release from mast cells which stimulate PLA$_2$ and consequently release COX and LOX pathway (Gossart et al., 1996), enhance the release of cytokines (IL-1 and IL-8) (Nardi et al., 2007) and ultimately leading to variety of diseases including cancer (Waris and Ahsan, 2006) such as in colon cancer the levels of O$_2^-$ and H$_2$O$_2$ raised by 2 and 20x (Aykin-Burns et al., 2009) atherosclerosis, neurodegenerative disorders (Manton et al., 2004), rheumatoid arthritis and aging (Thomas and Kalyanaraman, 1997). ROS also amplify pain responses and involved in inflammatory and neuropathic pain (Kumar, 2011).

One of the methods to determine ROS generation is ROS sensitive dye 2', 7´-dichlorodihydrofluoresceindiacetate (DCFH-DA) from skin homogenate (Driver et al., 2000). It is a stable, non-fluorescent that easily passes from cell membrane and is hydrolyzed via intracellular esterases by cleavage of diacetate to non-fluorescent 2',7´-dichlorofluorescin (DCFH). DCFH during incubation intracellularly trapped with ROS which induced the cleavage of diacetate by esterases and immediately oxidized in the presence of oxygen reactive species to fluorescent 2´,7´-dichlorofluorescein DCF (Bass et al., 1983) that can be measured directly (Scott et al., 1988).

ROS production controlled by endogenous natural antioxidant, vitamins and antioxidant enzymes which protect cells against the damages of ROS (Kim et al., 2010) such as, superoxide dismutase (SOD), glutathione peroxidase (GSH) and catalase (Cat) (Mates, 2000). SOD catalyzes the conversion of superoxide to generate hydrogen peroxide which later converted to water and oxygen by glutathione peroxidase and
catalase (Halliwell and Gutteridge 1990). But high levels of glutathione peroxidase (31%) may also involve in cancerous state such as colon cancer (Kanbagli et al., 2000).

There is close association of anti-oxidant, anti-inflammatory and analgesic activities thus targeting the ROS production minimize the causes associated with inflammatory disorders and provides evaluation of anti-inflammatory potential of compounds (Iwalewa et al., 2007).

Anti-oxidant prevents oxidations of oxidizable substrate including endogenous substances (Halliwell and Gutteridge 1985). Nutrients which directly scavenge free radicals and commercially available as antioxidants are: vitamin E (α-tocopherol) present at cellular membrane and protects against lipid peroxidation. It acts directly against peroxy radical (ROO.) and HO. (Machlin, 1980). Vitamin C (ascorbic acid, water soluble), quench singlet oxygen and free radicals (Hemila et al., 1985). β-carotene (precursor of vitamin A) found in plants also efficiently quench the singlet oxygen (Burton and Ingold 1984). In addition minerals such as copper, manganeese, zinc and selenium act as antioxidant (Machlin and Bendich 1987). Around 150,000 research papers have been published regarding antioxidant property such as O. ficus (Gentile et al., 2004), Nepeta sibthorpii (Micelia et al., 2005), Scutelleria baicalensis (Huang et al., 2006), Forsythiae fructus (Kang et al., 2008), Aegiceras corniculatum (Roome et al., 2008a), Allanblackia monticola (Nguemfo et al., 2009) and Areca catechu (Khan et al., 2011).

1.12 Pain

In the beginning of 20th century, Sherrington was the first to coin the term nociception referring pain. It is a Latin word meaning harm. Nociceptive system plays an important role in control mechanism of body i.e. homeostasis. Pain is the sensation which is produced in response to injury. It is evoked by various noxious stimuli either physical (heat, cold and pressure) or chemical (acetic acid, formalin) resulting in the activation of high-threshold nociceptor primary sensory neurons (Bars et al., 2001). Injury of tissue generates noxious stimuli which are sensed by nociceptors. They are small-diameter unmyelinated C-fiber or medium-diameter thinly myelinated Aδ-fiber sensory neurons which carry pain signals from peripheral tissues to the central nervous system (CNS). The cell bodies of these pseudo-unipolar nociceptors exhibited in the dorsal root ganglia (DRG) and trigeminal ganglia (TRG) and extend axons to the periphery and viscera as well as centrally to the dorsal horn of the spinal cord. The first synapse occurs in the region of dorsal horn either with interneurons or with supraspinally projecting neurons,
followed by transmit pain information to higher levels of brain such as the thalamus and cortex (Miller et al., 2014).

Pain is categorized in to acute and chronic. Acute pain may cause by burns, cuts, broken bones, labor and childbirth and surgeries etc (IASP, 2011). It is associated with sympathetic nervous system (increase tachycardia, respiratory rate and blood pressure, diaphoresis and dilation of pupils) (Farshchi et al., 2009). It may be mild or severe and persist for few moments or weeks or months; however continuous presence of acute pain stimulus may lead to chronic pain (IASP, 2011). Chronic pain may present even after healing of injury because afferent pain signals remain active in the nervous system for months or years (Cherkin et al., 2001). It does not involve sympathetic hyperactivity but may be associated with fatigue, changes in appetite, lipid (Farshchi et al., 2009), anger, anxiety, depressed mood and fear of re-injury. Chronic pain complaints are associated with pathological conditions such as headaches, arthritis, cancers, neurogenic pain, psychogenic pain (Cherkin et al., 2001).

Inflammation is the most common cause of pain resulting from tissue injury. It synthesized mixture of mediators which act to sensitize high-threshold nociceptors, thereby, producing peripheral sensitization. Peripheral inflammation generates pain due to the increase neuronal excitability in the spinal cord (central sensitization). COX-2 involved in the CNS pain responses by regulating central prostanoid production. Prostaglandins particularly PGE$_2$ take part in the inflammatory and nociceptive processes by increasing neuronal activity at nociceptive nerve fibers. It increased cAMP levels hence produce hyperalgesia at sensory nerve endings (Zakaria et al., 2010). Other pro-inflammatory mediators which are also involved in pain production are: nitric oxides, leukotrienes, serotonin, histamine, neurotrophic factors, neurokinin, neuropeptides (substance P, CGRP) and cytokines sensitized the peripheral nociceptors and amplify nociceptive responses (Coulaux et al., 2005). In addition NF-κB capable of modulating the expression of a wide range of genes involved in inflammatory and pain responses and aggravates the activation of intracellular pain signaling pathway (Wang et al., 2011). NSAIDs reduce the pain and inflammation by inhibiting the synthesis of prostaglandins (Ballou et al., 2000).

Several inflammatory cytokines such as TNF-α, IL-1β, -6 and -17 at the site of inflammation are involved in the pain pathway and act directly to excite dorsal root ganglia (DRG) neurons to regulate conductance by voltage gated sodium (VGS) and transient receptor potential (TRP) channels. TNF-α is predominant signaling molecule in the pain either directly increase the neuronal excitability or by expression of downstream
cytokines (Jin and Gereau 2006). It enhances the firing of A and C-fibers and increase the release of calcitonin gene-related peptide (CGRP) from the nociceptors of peripheral terminals (Opree and Kress 2000). It has been also reported that elevated levels of TNF-α occurring in hippocampus, locus coeruleus and red nucleus part of brain in neuropathic pain through glial systems thus mediates central mechanisms of pain (Covey et al., 2000). It is also involved in the pathogenesis of osteoarthritis. The cytokine IL-1β produced both fast and slow pain effects on DRG neurons and increase the response of G protein coupled receptors on DRG neurons (von Banchet et al., 2011). Upon exposure of heat stimuli IL-1β sensitized the DRG neurons (Obreja et al., 2002) and release neuropeptide (CGRP). IL-10 an anti-inflammatory and analgesic cytokine expressed at DRG neurons. It reduces the Na channel expression induced by TNF-α (Shen et al., 2013). It has been reported that intrathecal administration of IL-10 for 3 days suppressed the mechanical allodynia in chronic injuries (Milligan et al., 2005).

1.13 Animal models of pain

Pain cannot be monitored directly because it is the perception and absence of verbal communication is an hurdle for the evaluation of pain but it can be estimating by the pain related behavior of animal to various noxious stimuli such as chemical, thermal, electrical and mechanical (Bars et al., 2001).

A) Chemical stimuli

(i) Acetic acid-induced wthithes in mice

This method was introduced by Koster et al., (1959). The writhing induced by intraperitoneal injection of algogenic chemical agent acetic acid (0.8%) which act as nociceptive stimulus and irritates the serous membrane thus provokes the stereotyped response in mouse which is characterized by movement of hind paws, abdominal contractions, abdominal cramps, twisting of abdominal muscles, reduced motor activity and motor co-ordination. This test is also known as stretching test or abdominal contortion test. These behaviors are evidence of peritoneovisceral pain (Vyklicky, 1979). Writhing test is non-specific (Chen et al., 2008), simple, reliable, quick and used for rapid evaluation of peripheral analgesic test (Singh and Majumdar 1995). This analgesic response is considered to be mediated by peripheral receptors located at the surface of the cell lining of peritoneal cavity, peritoneal macrophages, mast cells (Ribeiro et al., 2000) and acid sensing ion channels (Voilley, 2004). Acetic acid also causes the release of free arachidonic acid from phospholipids membrane thus releases prostaglandin. High levels
of PGE₂ (Alam et al., 2008) and leukotrienes (Hajare et al., 2000) were also evident from the peritoneal exudates. Several other inflammatory mediators such as histamine, serotonin, bradykinin (Ribeiro et al., 2000), acetyl choline, substance P (Vogel and Vogel 1997), TNF-α, IL-1 and IL-8 have been reported to be associated with the activation and sensitization of nociceptive terminals against acetic acid in mice. However the disadvantage of this model is duration of action of analgesic agent cannot be evaluated because of spontaneous reduction in the frequency of abdominal cramps with time (Michael-Titus and Costentin 1988). This test resembles to human clinical pain conditions (Bars et al., 2001).

(ii) Formalin-induced paw licking response in mice

This method was introduced by Dubuisson and Dennis (1977) in which injection of plogistic agent formalin (1%) into the dorsal surface of mice paw provokes painful behavior characterized by injected paw being licked or shaken or nibbled and the time spent on licking or biting on injected paw is to be measured (Dubuisson and Dennis 1977). Formalin-induced biphasic response and discriminate the pain into two phases on the basis of time. The first or early transient phase (0-5 min) is neurogenic phase starts immediately after injection associated with direct activation of nociceptors presents on afferent C and in part Aδ fibers thus generates pain by the activation of glutamate, release of substance P, bradykinin, histamine and serotonin (Hunskaar and Hole 1987). Then, there is an interval of about 10 min of minimum pain response of mice (Prabhu et al., 2011). The second or late phase began at 15th min and persists for 15-30 min reflects integration between peripheral and central (spinal/supraspinal) signaling (Dallel et al., 1995) this phase is associated with inflammatory pain caused by local tissue inflammation and functional alteration in the dorsal horn of the spinal cord. Several pro-inflammatory mediators are involved in this phase such as histamine, serotonin, bradykinin, prostaglandins (Shibata et al., 1989). Formalin-induced peripheral pain is typically resembles to moderate and chronic human clinical pain (Khan et al., 2010).

B) Thermal stimuli

(i) Hot plate-induced jumping response in mice

Centrally mediated analgesic hot plate assay was introduced by Eddy and Lambach (1953). Animal is introduced into cylinder which is open from upper end and floor is consist of metallic plate which is heated by a thermodoe it elevates pain threshold of mice; it is non-inflammatory response and works through chemical processes. This heated plate
produced two types of behavior in mice which are measured in terms of reaction time and are characterized by paw licking and jumping. Both these behaviors are considered as supraspinal response and associated with central neurotransmission when the heat activates nociceptors (Aδ and C fibers) by transmit the impulse of the dorsal horn of the spinal cord and eventually to cortical centers (Chapman et al., 1985). Particularly the paw licking behavior is affected only by opioids and jumping reaction time is affected by less potent analgesic agents such as NSAIDs (acetylsalicylic acid and paracetamol) (Ankier, 1974). Centrally acting agents activate the release of endogenous peptide by periaqueductal gray matter (PAG), which are transmit to the spinal cord to attenuate the pain muscle transmission within the region of dorsal horn thus opioid analgesics increase the latency time of the animals on the hot plate (Freitas et al., 2009).

(ii) Cold plate test in mice

It is related to acute, allodynia neuropathic pain model in animal. However use of this animal model is very rare (Pizziketti et al., 1985).

(iii) Tail-flick test in mice

It consist of two variants 1) tail-flick test using radiant heat method was developed by Hardy et al., (1940) in this thermal radiation was applied to the tail of the rodent. The lengthening of reaction time of withdrawal of tail was recorded and referred as tail flick latency (D’Amour and Smith 1941). 2) Tail-flick test using immersion of tail into the hot water results in the abrupt tail movement or turning of the body (Ben-Bassat et al., 1959).

From the mechanistic point of view tail flick test is central analgesic activity and related to spinal reflex control by supraspinal region of central nervous system (CNS) (Mitchell and Hellon 1977).

C) Electrical and mechanical stimuli

In this test slowly increasing intensities of trains (current last for some hundreds of milliseconds) was delivered into the tail of animal by placing subcutaneous electrodes or by applying the electrical stimuli on the paw through the floor of the cage (Evans, 1961). The reflex movement and vocalization during the time of trains and after the discharge can be noted. These responses depend on the nociceptive signals in the CNS which include spinal cord, brain stem and thalamus (Borszcz, 1995).

The preferred area for applying mechanical nociceptive stimuli is the hind paw and the tail. In this test constant pressure applied using analgesia meter to the area of punctiform of hind paw or less commonly on the tail a pressure of increasing intensity
When increased pressure (weight in grams) applied on the paw, the reflex withdrawal of the paw or a vocal reaction was noted. These reactions are associated with supraspinal response (Bars et al., 2001).

1.14 Analgesic drugs

The currently available therapeutic agents such as steroidal (SAID) and non-steroidal anti-inflammatory drugs (NSAIDs) indicated for the treatment of painful and inflammatory conditions. Opioid agonist the second most popular medicine after NSAIDs are used to treat moderate to severe cancer related pains (Clark, 2002). NSAIDs act peripherally via blocking of COX enzyme (Alam et al., 2008) and opiates acts centrally by excitation of opioid receptors (meo (μ), kappa (κ) and delta (δ) (Almeida et al., 2001). Opioid agonist directly acts on spinal cord by inhibiting the transmission of pain regulated by dorsal horn. However long term use of NSAIDs may lead to undesirable side effects such as lesions in gastrointestinal tract or liver and renal failure (Rao and Knaus 2008) while opioids may cause dependency, nausea, drowsiness, respiratory depression, decrease movement of gastrointestinal tract and various changes in endocrine and autonomic nervous system (Almeida et al., 2001).

The limited effectiveness or therapeutic failure in some conditions of neuropathic pain and even refractory to opioids (Portenoy and Hagen 1990) and adverse effect associated on chronic use of available therapeutic agents has become one of major challenge for the scientist. For this reason search for new therapeutic alternatives is arising and it is need to recognize the importance of herbal preparations for better treatment of pain (Table-2). Over the years, natural products have been shown source of drug discovery especially in pain treatment (Shu, 1998) such as salicylic acid (Salix alba) and morphine (Opium poppy) is one of its examples. Therefore, scientific research on the plants that have been traditionally used as pain relievers is logical and fruitful research strategy for the search of new analgesic agents (Elisabetsky et al., 1995).

1.15 Opuntia dillenii

The genus Opuntia is succulent shrubs belongs to the family Cactaceae and consists of about 300 species. It is native of New York, Mexico and widely grown in warmer areas of the world. Its 7-8 species were introduced into Indo-Pak subcontinent, but only 2-3 species have been naturalized such as O. dillenii, O. ficus. Its fruit is edible and thus commonly known as prickly Pears (Mizrahi et al., 1997) also known as cactus. It is cultivated cultivated naturally by simply fall of its pod on ground,
rapid growth and good adaptation even in poor soil condition and required less water to grow (Pintado et al., 2001). It has soft, flattened stem also known as cladodes or phylloclades and rich source of mucilage, pectin, vitamin A and C, minerals and sugars. Its young stem is referred as “nopalito” and used as vegetable while mature cladode used as animal fodder (Mizrahi et al., 1997). Its leaves are small and spiny. Seeds are hard, pale, discoid or angular and good source of oil production (Sastri, 1969). Flowers are large, red or yellowish in color. Fruit of cactus used world wide contains nutrients, minerals, amino acids and rich in ascorbic acid and betalins. Its ripe fruit consume by human and unripe for cattle feed, also use in preparation of syrups, jams and dairy products (Mizrahi et al., 1997), tea, soft candies, vinegar, alcoholic beverages, juices, jam, natural colorants, sweeteners and jellies and ice-cream (Chang et al., 2008). This plant also used as ornamental and for cosmetic purposes (Ahmed et al., 2005). From medical point of view species of Opuntia are widely used traditionally. Early European travelers use it as a vegetable to prevent scurvy disease during their long journeys (Sastri, 1969). People of Trongta tribe used juice of cactus for treatment of dysentery. Europeans and Africans utilized its leaves for treatment of sours, boils, ulcers and for painful conditions. South Africans and Australians used the decoction of the cladode for the treatment of diabetes. In the South Europe and West Indies it was employed for the treatment of arthritis and gout (Pintado et al., 2001). Its cladode was effective against insect bites, burns, rash, wounds, hemorrhoids, asthma and in ear aches (DerMarderosian and Beutler 2002). Plant juice used as anthelmintic and purgative (Pintado et al., 2001).

O. dillenii Haw commonly known as Nagphana (Loro et al., 1999) grows widely in arid and semi-arid regions including Pakistan (Saleem et al., 2005) abundant at Himalayas mountains. Its modified leaves in the form of spines were used as protection of town during the era of Tippu Sultan, ruler of Mysore, India (Sastri, 1969).

1.15.1 Chemical constituents and Pharmacological activities

Chemical constituents of O. dillenii are mention in (Table-3). Traditionally it is popularly used worldwide including Pakistan (Mahmood et al., 2011) and China (Jiang et al., 2006) for cure of ailments. Cladode used for the treatment of inflammation, snake bites, ulcers, eye and heart diseases. Its fruit used for remedy of cough, asthma, hepatic congestion, increase the secretion of bile and useful in the treatment of gonorrhoea (Sastri, 1969).

It exhibited anti-inflammatory and analgesic activity as previously reported that lyophilized aqueous extract of fruit used against carrageenan-induced paw edema and hot plate test (Loro et al., 1999), methanol extract of spines (Ahmed et al., 2004), the alcohol
extracts of stems, flowers and fruits using carrageenan-induced paw edema and electric stimulus in rats. (Ahmed et al., 2005), aqueous ethanol extract of cladode against xylene-induced ear edema in mice (Qiu et al., 2007) and wound healing property (Teixeira, et al., 2000) has also been reported. *O. dillenii* in combination with Radix et caulis and herba taraxaci reported for the treatment of pelvic inflammatory disease (Liu et al., 2003). It has been also described for treatment of skin pruritis when used in combination of Chinese medicinal plant (Huang, 2006).

The cladode possesses radical scavenging activity (Qiu et al., 2002; Cho et al., 2006). Its fruit extract showed low density lipid peroxidation activity (Chang et al., 2008). Extracts of seed, seed oil, peel and pulp also displayed antioxidant activity against DPPH assay and β-carotene bleaching tests (Liu et al., 2009). Polysaccharides (xylose, arabinose, glucose, fructose, rhamnose and galacturonic acid) of *O. dillenii* possess hypoglycemic activity against diabetes induced in mice (Zhao et al., 2011). According to some studies its derived tablets use for the management of type-2 diabetes mellitus and it is under clinical trials (Yang et al., 2008). These polysaccharides also used in the treatment of HIV and against Herpes simplex virus infection (Liu et al., 2002), neuroprotective activity and antihyperlipidaemic effects (Huang et al., 2009).

Methanol extract of cladode (1g/kg) has been described for non-toxic effects and hypotensive property (Saleem et al., 2005). Polysaccharides derived from this plant showed protective role against carbon tetrachloride-induced injury of liver in mice and also attenuated the increase levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Yang et al., 2009).

Its phylocladode extract identified for antispermatogenic effect in male rats (Gupta et al., 2002). *O. dillenii* in combination with other plants exhibited property for management of hepatitis, cirrhosis, hepatic cancer and for various types of tumor (Cho et al., 2006), its medicine in combination with plant material use for the treatment lung cancer (Song, 2007) and blood lipid lowering activity (Trombetta et al., 2006).

*O. dillenii* has synergistic effect with alumen for treatment of common cold, tonsillitis, pharyngitis and pneumonia (Lou et al., 2003). An ointment prepared from *O. dillenii* extract for the treatment of acne, sore and furuncle (Wang and Wang 2006). Isolated compounds opuntiol (6-hydroxymethyl-4-methoxy-2H-pyran-2-one, C7H8O4, mol wt: 156.13) and opuntioside (a glycoside of opuntiol) (6-β-D-glucopyanosyloxymethyl-4-methox-H-pyran-2-one, C9H18O9, mol wt: 318) are signature compounds of *O. dillenii* belong to α-pyrones (six member ring containing one oxygen atom and a ketone functional group, a lactone). In opuntioside glucose moiety is attached at 7th position on
opuntiol ring structure. Opuntioside (10 mg/kg) has been reported for hypotensive activity causing 44% reduction in blood pressure (Saleem et al., 2005). Opuntiol and opuntioside showed radical scavenging effect (Qiu et al., 2002).

1.16 Objective of the study

The edible *O. dillenii* cladode is being used in traditional medicine against various ailments including inflammation. Therefore, its methanol extract, fractions and pure compounds (opuntiol and opuntioside) were evaluated for anti-inflammatory and analgesic properties to justify its use and to elucidate their possible mode of action as described below:

1) Induction of edema in mice ear and paw by AA, TPA and PLA₂ and its reduction by monitoring their weight and volume, respectively.

2) Vascular permeability was measured in peritoneal cavity induced by carrageenan in mice using Evans blue dye.

3) Levels of inflammatory mediators i.e. LTB₄ in rat neutrophils (HPLC), PGE₂, TNF-α (ELISA kit) and ROS (2´, 7´-dichlorodihydrofluorescein diacetate (DCFH-DA) dye) derived from mice edematous ear as well as anti-inflammatory mediator, cytokine IL-10 (ELISA kit) from carrageenan-induced peritonitis.

4) Histological changes in the inflamed ear punches and its diminution using hematoxylin and eosin (H&E) staining.

5) Analgesic effects were monitored by chemical (acetic acid-induced writhes and formalin-induced paw licking response in mice) and thermal stimuli (hot plate-induced jumping response in mice) against peripheral and centrally mediated pain.

6) Safety of plant extracts was assessed in mice (acute toxicity, biochemical parameters of liver and kidney and histology of liver and kidneys) and cytotoxicity using rat neutrophils by typan blue exclusion test.
2. MATERIALS AND METHODS

2.1 Animals

The handling and the use of laboratory animals for the present studies were performed in accordance with guidelines provided by Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), USA and after clearance of the Institutional Animal Use Committee (protocol #: 2014-0003), International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan. The animals were maintained on a 12 h light/dark cycle with temperature (22 ± 2 ºC) and humidity (50 ± 10%) and were fed with standard diet and water ad libitum. The Naval Medical Research Institute (NMRI) mice of both sexes (22-29 gm) and Sprague-Dawley male rats (350-400 gm) from animal house of ICCBS were used throughout the study.

2.2 Chemicals, consumables and equipments

i) Commonly used chemicals in various experiments: Acetic acid, acetone, ammonium chloride (NH₄Cl), β-sitosterol (Sigma-Aldrich, Germany); acetonitrile (J.T. Baker, USA); acetylsalicylic acid, α-D-glucose, celecoxib, dexamethasone, diclofenac sodium, dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), ethylenediaminetetraacetic acid disodium salt (Na₂EDTA), hydrochloric acid (HCl), ibuprofen, indomethacin, lithium carbonate, nordihydroguaiaretic acid (NDGA), picric acid, phenidone, potassium bicarbonate (KHCO₃), potassium chloride (KCl), potassium phosphate monobasic and dibasic, quercetin, sodium carbonate, sodium phosphate dibasic heptahydrate (Na₂HPO₄.7H₂O), trifluoroacetic acid (TFA) (Sigma Co, St. Louis, Mo, USA); chromium potassium sulfate (Merck, Germany); diethyl ether (Lab-scan, Ireland); DL-Dithiothreitol (DTT) (GoldBio, USA); eosin 0.5%, formaldehyde 37%, gelatin, mounted media, (Carl Roth, Germany); ethanol (Tedia, USA); fraction-1 (Fisher Scientific, UK), glycogen (type ix from bovine liver) (Molekula, UK); hematoxylin Gill 2, paraffin, xylene (Scharlau, Spain); isopropyl alcohol (Tedia, USA); methanol (VWR, USA); morphine sulphate (brand name: magnus MR, AGP (Pvt) Pakistan), naloxone hydrochloride (Sam Chun Dang Pharm Co Ltd, South Korea), octyl phenoxypolyethoxylethanol-40 (NP40), sodium orthovanadate (Na₃VO₄) (Biobasic, Canada); paracetamol (brand name: panadol, GlaxoSmithKline Ltd Pakistan); sodium fluoride (NaF) (BDH, U.K); sodium thiopental (brand name: pentothal sodium, Abbott,
Pakistan); tris base (hydroxymethyl aminomethane) (Boehringer Mannheim) and trypan blue (Sigma Co, St. Louis, Mo, USA).

ii) Specific chemicals used in following experiments

a) Histology of liver, kidney and ear punches: Chromium potassium sulfate (Merck, Germany); eosin 0.5%, formaldehyde 10% and 37%, gelatin, mounted media, (Carl Roth, Germany); hematoxylin Gill 2, paraffin, xylene (Scharlau, Spain); isopropyl alcohol (Tedia, USA) and lithium carbonate, picric acid (Sigma Co, St. Louis, Mo, USA).

b) Anti-inflammatory in vivo and in vitro assays

Ear and paw edema and peritonitis in mice: Arachidonic acid (AA), λ-carrageenan, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma-Aldrich, Germany); cyproheptadine, phospholipase-A2 (PLA2, honey bee venom, Apis mellifera) (Sigma-Aldrich, Germany) and Evans blue (BDH, USA).

Prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) levels: The enzyme immuno assay (EIA) kit-monoclonal (Cayman Chemical Co., Arbor, MI, USA) contained: EIA buffer concentrate, Ellman’s reagent, goat anti-mouse IgG coated plate, PGE2-AChE tracer, polysorbate 20, PGE2 monoclonal antibody, PGE2 EIA standard, wash buffer concentrate, 96 well cover sheet. Ultra pure water (Cayman Chemical Co., USA). Glycogen (type IX from bovine liver) (Molekula, UK) Leukotriene B4 and prostaglandin B2 (Sigma-Aldrich, Germany), PGE2 (TCI, Japan).

Tumor necrosis factor (TNF-α) Interleukin 10 (IL-10) ROS levels: TNF-α mouse Enzyme linked immunosorbant assay (ELISA) kit (Invitrogen Co, Carlsbad, CA, USA) containing: TNF-α standard, standard diluent buffer, antibody coated wells, TNF-α biotin conjugate, streptavidin-horse radish peroxidase (HRP) and its diluent, wash buffer concentrate, stabilized chromogen, stop solution and plate covers.

Mouse IL-10 ELISA kit (Millipore, USA) containing: IL-10 standards and its detection antibody, Avidin HRP D, assay buffer A, wash buffer, substrate and stop solution. In ROS 2´, 7´-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, Germany) were used.

iii) Consumables

Blotting paper (local market Karachi); centrifuge tubes (Vivantis, USA); nitrile gloves powder-free (VWR, Malaysia); microscopic slides (HECOS, China) and cover slips (24 x 50 mm) (Carl Roth, Germany); pipette tips (1-200 µL, 200-1000 µL) (Biologix, USA); syringes of 1 mL (29 G), 5 mL (23 G), and 10 mL (21½ G) (BD, USA, USA); syringe
driven filter unit 0.22 µm (Millipore, Ireland); 96-well clear (Falcon, USA) and black flat-bottom plates (Corning, USA).

iv) Equipments

Analytical balance (Sartorius, Germany); centrifuge (Himac, Japan) and (Heraeus biofuge Stratos, USA); deionizer (Millipore, USA); High performance liquid chromatography (HPLC) system (Prominence 20A, Shimadzu, Japan) with pump (LC-20A), auto sampler (SIL-20A), diode array detector (SPD-M20A) communication bus module (LC-CBM-20A), helium degasser (Shimadzu DGU-1A), reverse phase nucleosil column (C18, 250/4.6 mm, 100-5), nucleosil guard column (C18, CC8/4 mm, 100-5), solid phase extraction column (Chromabond C18, 45 µm, 1 mL with 100 mg matrix, Macherey Nagel, USA), HPLC vial (Shimadzu, Japan); hot plate (model-DS 37, Ugo Basile, 25x25 cm, Italy); microscope (model: TE 2000-E; 90 I, Nikon eclipse, Japan); magnetic stirrer (I sopad, UK); multi-well microplate reader, sonicator (Fisher Bioblock Scientific, France); micro plate shaker (PMS-1000, Grant-bio instrument Ltd, England); water bath (Grant Instruments, England), Neubauer’s cell counting chamber (0.1 mm and 0.0025mm²) (HBG, Germany); oven (Lab tech, USA); pH meter (Corning, USA); pressure pump (Neuberger 78 Friburg VDE 0530, USA); simplicity 185 deionizer (Millipore, USA); tissue homogenizer (Ultra-Turrax T25, Janke and Kunkel IKA-Labortechnik, Germany); tissue sectioning and embedding system: Microtome (Yidi, Japan), slide cooling plate, tissue conserving table and embedding center (Kedee, China), strengthener (Medite, Germany); spectrophotometer (SpectraMax M2, molecular Devices CA, USA); uni thermo shaker (Eyela, Japan) and vortex mixer (Yamato, Japan).

v) Solubility and storage of chemicals and plant extracts

Calcium ionophore A23187, calcium chloride, tris and trypan blue were solubilized in water. Acetic acid, carrageenan, cyproheptadine, dexamethasone, diclofenac sodium, Evans blue, formalin (1%), naloxone, phospholipase A₂ (PLA₂) and O. dillenii methanol extract, fraction-2, opuntioside were prepared in physiological saline (0.9% NaCl) whereas, phosphate buffer saline (PBS) was used for glycogen. Acetylsalicylic acid, β-sitosterol, celecoxib, ibuprofen, NDGA, paracetamol, quercetin as well as fraction-1 fraction and opuntiol were dissolved in DMSO (10%). Arachidonic acid (AA), leukotriene B₄ (LTB₄), morphine sulphate, prostaglandin E₂ (PGE₂), 13-O-tetradecanoyl-phorbol-13-acetate (TPA) were solubilized in ethanol while methanol was used for dye
2’, 7’-dichlorodihydrofluorescein diacetate (DCFH-DA). Sodium carbonate (1%) was used to solubilize indomethacin.

The aliquots of AA, LTB₄, PGE₂ and TPA were stored in micro-centrifuge tube (1.5 mL), covered with tin foil and after flushing with argon gas stored at -20°C, while DCFH-DA was placed at 2-8 °C.

2.3 Extraction and isolation of pure compounds from *O. dillenii*

The cladodes of *O. dillenii* were collected in October 2001 from the gardens of ICCBS and identified by taxonomist Prof. Dr. Surraya Khatoon, Department of Botany, University of Karachi. A voucher specimen (KUH GH No.68218) was deposited in the herbarium of the same department.

The extraction and isolation of pure compounds from *O. dillenii* was performed by Professor Shaheen Faizi’s group, chemist at ICCBS. Initially, the green cladodes (45 Kg) were cut into one inch pieces and percolated twice with methanol. The thickish methanol extract residue (200 g), obtained on removal of the solvent *in vacuo* was subjected to vacuum liquid chromatography with various solvents and their combinations [VLC, silica gel, pet ether (PE), fraction-1 (EA) and methanol (MeOH)], furnishing 31 fractions including EA and MeOH fractions. In VLC fractions 10 and 11 (PE:EA 1:9), crystals deposited which were filtered and identified through thin layer chromatography (TLC) and spectral studies identified it as pure opuntiol. The VLC fraction 15 (EA: MeOH 6:4) showing a major spot on TLC on further purification through preparative thin layer chromatography (PTLC) (silica gel, chloroform CHCl₃: 7:3 MeOH) identified through spectral studies as opuntioside. Fractions 6-12 mainly contained opuntiol (F6-12) while fractions 14-17 (F14-17) were rich in opuntioside (Figure-2).

2.4 Acute toxicity in mice and cellular toxicity in rat neutrophils

The methanol extract was evaluated for its toxic effects if any *in vivo* in mice and *in vitro* in rat neutrophils; however, due to limited quantities of opuntiol and opuntioside only cytotoxicity test was performed.

Mice (22-30 g, n=10 /group) of either sex were gavaged with *O. dillenii* methanol extract (1 and 5 g/kg) or with vehicle (0.9% saline) for a continuous period of 7 days (11.00 am). During this period the animals were kept under close observation for pharmacotoxic signs such as restlessness, agitation, sedation, diarrhea, sensitivity to touch and sound, motor activity, gasping, arching and piloerection as described earlier (Lorke, 1983) with slight modification i.e. instead of single dose the test agents were administered
daily. Body weights of animals were also noted at the start and end of the experiment and mortality, if any was also noted. On the 7th day of experiment the animals were sacrificed via cervical dislocation, blood was collected by cardiac puncture for biochemical tests: liver enzymes: total bilirubin, direct bilirubin, alanine transaminase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (γGT) and kidney end product (creatinine) were assayed from Diagnostic Lab and Clinical Research Facility of ICCBS and liver and kidney were excised for histopathological studies.

2.4.1 Rat neutrophils collection, concentration and viability

Male rats were fasted overnight with free access to water; next day they were anaesthetized by placing them in glass chamber containing cotton soaked with diethyl ether (5 mins) followed by intraperitoneal administration (i.p) of warm glycogen solution (1 g/kg in 10 mL of PBS). After 4 h, rats were sacrificed and PBS (20 mL) was administered, massaged at the injected site and the animal was rolled gently to dislodge leukocytes from walls of blood vessels facilitating their entry into peritoneal fluid. It was aspirated and further PBS (20 mL) twice was used to collect cell suspension (total PBS = 60 mL) and centrifuged (15 min, 5000 rpm) as described earlier (Ammon et al., 1991; Roome et al., 2008b). The neutrophils pellet was re-suspended in PBS (2 mL), washed twice with it and finally suspended in incubation buffer (1 mL) prior to conduct experiments (Appendix-I). If needed, lysis buffer (1 mL) was used to remove blood from neutrophils cell pellet and centrifuged followed by washing with PBS as described above.

The neutrophil cell suspension (15 μL) was mixed with equal volume of trypan blue solution (0.4 %) and viable (unstained transparent granular) and non-viable cells (stained blue) were counted in 5 squares within 3 min using Neubauer cell counting chamber microscopically at 20x magnification (Rosengard and Cochrane 1983). The following formula was employed to calculate cell concentration and percent cell viability:

\[
\text{Cell concentration} = \frac{\text{Total cell count} \times 2 \times 10^4 \times \text{total volume of cell suspension}}{\text{Cell concentration}}
\]

\[
\text{Percent cell viability} = \frac{\text{Number of viable cells}}{\text{Number of viable cells + non viable cells}} \times 100
\]

2.4.2 Neutrophils toxicity in the presence and absence of test agents

The rat peritoneal neutrophils cell suspension (5 x 10^6 cells/mL) having more than 95% viability were incubated with 5 μL of methanol extract (10,100, 200, 500 and 1000


\( \mu g/mL \), fraction-1 and fraction-2 (10, 100 and 200 \( \mu g/mL \)), opuntiol (1, 10 and 100 \( \mu g/mL \)), opuntioside (1, 10, 50 and 100 \( \mu g/mL \)), nordihydroguaiaretic acid (NDGA) and quercetin (0.1, 1 and 10.0 \( \mu M \)) in shaking water bath (37 °C) for 0, 0.5, 1, 2 and 3 h. Three independent experiments were performed in duplicate and the percent viability was calculated using following formula:

\[
\text{Percent cell viability} = \frac{\text{Number of viable cells in the presence of test agent}}{\text{Number of viable cells in control}} \times 100
\]

2.5 Histology of liver, kidney and ear punches

2.5.1 Fixation, dehydration and embedding

The liver and kidney samples were fixed in Bouin’s fixative for 2-4 h and transferred in deionized water for 5 mins, washed and repeated 3x. The samples were dehydrated by sequential exposure with isopropyl alcohol (IPA 70% and 90%) containing lithium carbonate solution (2-3 drops) and kept overnight in IPA (70%). Next day, the process of dehydration was continued with sequential changes (3x) with IPA (70%, 90%, and 100%) for a period of 1 h in each solution, finally exposed to xylene for 1 h and repeated it 3x.

The tissues were dipped in xylene: paraffin (1:1) solution in glass vials and kept in oven (66 °C). After 30 min. tissues were transferred to new glass vials containing pure paraffin and kept in oven (66 °C) overnight. The next day, each tissue was immersed in melted paraffin in moulds (Paraffin embedding system, Slee, Germany) and after slight solidification tissue cassettes were fixed onto individual mould followed by placing them on cooling plate (1 °C) for 30 min. and used for tissue sectioning.

2.5.2 Sectioning, deparaffinization and rehydration of tissues

The aforementioned paraffin blocks were fixed in microtome (Semi-automated microtome, Yidi, China) to obtain 6 \( \mu m \) sized sections (Microtome blade, C.I. Sturkey Inc. USA). Individual sections were transferred in a glass container with deionized water and kept in water bath (42 °C) (LabTech, Korea). After 2-3 mins, the stretched sections were carefully captured on to the gelatin coated glass slides, air dried (2-3 mins) and placed on slide strengthener (Medite, Germany) at 42 °C for overnight (Appendix-I).

After 10-12 h, gelatin coated slides containing sections were de-paraffinization by placing them in xylene (15 min) followed by sequential rehydration in IPA (100%, 90% and 70%) for 3 min in each solution. After the process of rehydration, the slides were immersed in deionized water (10 min) and subjected to staining.
2.5.3 Hematoxylin/Eosin staining (H&E) and microscopy

The liver and kidney sections (6 µm) on the gelatin coated slides were stained with few drops of hematoxylin (5 min). After washing with de-ionized water these were stained with eosin solution (0.5%) for 2 min and washed again with de-ionized water. After sequential dehydration with IPA (70%, 90% and 100%) the slides were transferred in xylene for 5 min. Finally, the slides were mounted in two drops of mounting media, covered with cover slips, dried and stored at room temperature until microscopic observations (Abbas, 2011).

In the case of AA and TPA-induced mice ear edema punches all the aforementioned steps were followed except after collection they were fixed in formalin (10%) and the size of the sections were 5 µm.

The control and treated liver, kidney and ear punches specimen slides were observed at 20x magnification (Nikon Eclipse TE2000-E and Nickon Eclipse 90i). Any anatomical changes in the architecture of liver and kidney and morphology of hepatic and renal cells (Awodele et al., 2012) were analyzed and interpreted accordingly. In ear punches edema and inflammation intensity, vasodilatation and leukocyte infiltration were noted (Saraiva et al., 2011). The photographs were captured (NIS element 3.0 AR software) while images were processed using photoshop CS2.

2.6 Inflammatory animal models

Inflammatory animal models included AA and TPA-induced ear edema, PLA₂-induced hind paw edema and carrageenan-induced peritonitis. For analgesic assays acetic acid-writhing assay, formalin and hot plate test were used. The *O. dillenii* methanol extract, fraction-1, fraction-2 and pure compounds opuntiol and opuntioside derived from it were screened as described below:

2.6.1 AA and TPA-induced ear edema in mice

Concentration and duration of AA and TPA-induced ear edema in mice

Mice (n=10) received 10 µL of different concentrations of either AA (1, 2 and 3 mg/20 µL/ear) or TPA (2.5 or 4 µg/20 µL/ear) on both anterior and posterior surfaces (20 µL) of the right ear whereas, left ear anterior and posterior surfaces served as control receiving similar amount of ethanol using micropipette. The edema was noted at different time intervals for AA (0 min, 30, 60 and 90 min) and TPA (0 min, 0.5, 2, 4, 6, 8 and 24 h) as described earlier (Young et al., 1984; Saraiva et al., 2011 and Xian et al., 2011). Animals were sacrificed by cervical dislocation, right and left ear punches (6 mm diameter, using
cork borer) were collected weighed, dipped in liquid nitrogen and stored at -80 °C until further use. Increase in weight of ear punch (mg) was expressed as edema weight. The percent of edema formation was determined as shown below:

\[
\text{Ear edema formation (\%)} = \frac{\text{Weight of right ear punch} \times 100}{\text{Weight of left ear punch}} - 100
\]

After selection of most suitable concentration and time for maximum edema formation (Figure 5A and B) for AA (2 mg/20 µL/ear) and TPA (2.5 µg/20 µL/ear) were used to induce ear edema for 1 h or 6 h respectively.

**Topical, oral and intraperitoneal routes for test agents**

Test agents were administered by three different routes (topical, oral and i.p) prior to the screening of anti-inflammatory activity and selection of most suitable and effective route of their administration was determined.

i) Left ear topically received acetone (20 µL, vehicle control) while right ear received test agents including methanol extract, fraction-1, fraction-2 (1, 10 and 20 mg/ear), opuntiol and opuntioside (0.1, 1.0 and 10 mg/ear) and reference drugs diclofenac sodium (0.5, 1 and 1.5 mg/ear), indomethacin (1, 2 and 3 mg/ear) and dexamethasone (0.05 and 0.08 mg/ear) five minutes prior to topical application of AA (2 mg/20/µL/ear) (Young et al., 1984; Saraiva et al., 2011) or TPA (2.5 µg/20/ µL/ear) (Xian et al., 2011). After 1 and 6 h of topical application of irritants, animals were sacrificed by cervical dislocation ear punches were collected and weighed as described above and percent edema inhibition was calculated as described above.

ii) Oral administration (10mL/ kg) of methanol extract (1-300 mg/kg), fraction-1 (1-250 mg/kg), fraction-2 (1- 400 mg/kg mg/kg), opuntiol (1-100 mg/kg), opuntioside (0.5-100 mg/kg mg/kg), acetylsalicylic acid (10-200 mg/kg), diclofenac sodium (0.5-25 mg/kg), ibuprofen (1-150 mg/kg), indomethacin (1- 25 mg/kg), celecoxib (10-100 mg/kg), NDGA (1-75 mg/kg) and dexamethasone (1-50 mg/kg) to mice of both sex 30 mins. prior to topical application of either AA (2 mg/ 20 µL/ear) or TPA (2.5 µg/ 20 µL/ear) on the right ear and left ear received ethanol (20 µL, vehicle control). Likewise, the control group received saline (0.9%) or Na₂CO₃ (1%) or DMSO (10%). The ear punches from mice were collected and percent edema inhibition was calculated as described above.

iii) The highest dose of test agents employed in oral route of administration after AA or TPA induced ear edema as described above were used for their anti-inflammatory
effect using i.p. route. Thus the methanol extract (200 or 300 mg/kg), fraction-1 (200 or 250 mg/kg), fraction-2 (150 or 400 mg/kg), opuntiol (100 mg/kg), opuntioside (20 or 100 mg/kg), indomethacin (15 or 25 mg/kg) and dexamethasone (20 or 50 mg/kg). Likewise, the control group received saline (0.9%) or Na₂CO₃ (1%) or DMSO (10%) and ear punches were collected and percent ear edema inhibition was calculated.

\[
\text{Ear edema inhibition (\%)} = \frac{\text{Ear edema control} - \text{Ear edema treated}}{\text{Ear edema control}} \times 100
\]

### 2.6.2 PLA₂-induced paw edema in mice

Mice (23-30 g) of either sex (n=3/dose) received orally either saline or Na₂CO₃ (1%) or DMSO (10%) (vehicle control) or IC₅₀ values of test agents methanol extract (158 or 269 mg/kg), fraction-1 (203 mg/kg), fraction-2 (113 or 342 mg/kg), opuntiol (85 mg/kg) and opuntioside (15 or 99 mg/kg) reducing AA or TPA-induced ear edema (Table-8) were used in PLA₂-induced hind-paw edema in mice (Neves et al., 1993). The positive control included cyproheptadine, indomethacin and dexamethasone (1, 5 and 10 mg/kg). Initially right hind paw of all mice were marked at ankle joint and paw volume was measured in triplicate by means of plethysmometer (Ugo Basile 7150, Italy) representing initial paw volumes (\(V₀\)). After 30 min of administration of test agents except cyproheptadine (60 min), PLA₂ (2 units in 25 µL saline) was injected into the plantar region of right hind paw of mice. The paw volumes were measured at 30, 60 and 90 min, representing final paw volumes (\(V_f\)). The percent inhibition of paw edema volume was calculated at different time intervals as follows:

\[
\text{Paw edema inhibition (\%)} = \frac{[(V_f-V₀) \text{ control} - (V_f-V₀) \text{ treated}]}{(V_f-V₀) \text{ control}} \times 100
\]

### 2.6.3 Carrageenan-induced peritonitis in mice

Mice (20-24 g) of either sex (n=3/dose) received either saline or Na₂CO₃ (1%) or DMSO (10%) (control), or IC₅₀ values of test agents methanol extract (158 or 269 mg/kg), fraction-1 (203 mg/kg), fraction-2 (113 or 342 mg/kg), opuntiol (85 mg/kg) and opuntioside (15 or 99 mg/kg) reducing AA or TPA-induced ear edema (Table-8) or dexamethasone (1, 2 and 3 mg/kg) and indomethacin (1, 5 and 10 mg/kg) as positive control were introduced orally 30 mins prior to carrageenan induced peritonitis (Montanher et al., 2007; Longhi-Balbinot et al., 2012). Immediately, mice were
immobilized in restrainer and its tail was immersed in hot water (65 °C) so to the tail vein becomes prominent and Evans blue dye solution (0.6%, 10 mg/kg) was administered intravenously in lateral tail vein. After 30 min, carrageenan (1%, 0.1 mL) was injected i.p to induce peritonitis and mice were sacrificed after 4 h by cervical dislocation. The animals were dissected and 1 mL of cold saline was introduced in the peritoneal cavity which was collected and centrifuged at 3000 rpm for 5 min. The supernatant sample (200 µL) was transferred in 96-well plate and absorbance was noted at 620 nm using spectrophotometer.

Vascular permeability inhibition (%) = \frac{\text{OD of control} - \text{OD of treated}}{\text{OD of control}} \times 100

For standard curve different concentrations Evans blue (2, 4, 6, 8, 10, 15, 20, 25, 30 µg/mL) were transferred in 96 well plate and absorbance of 200 µL samples was measured at 620 nm. The graph between concentrations (X-axis) vs absorbance (Y-axis) was plotted. The best fit line generated was use to obtain the concentration of Evans blue in the presence or absence of test agents and expressed as µg/mL dye.

2.7 Determination of inflammatory mediators

The levels of inflammatory mediators such as PGE$_2$, ROS and TNF-α were determined from AA and/or TPA-induced ear edema using ELISA and DCFH-DA dye. The LTB$_4$ release was stimulated from rat neutrophils and detected by HPLC. The anti-inflammatory mediator IL-10 was also determined by ELISA from carrageenan-induced peritonitis.

a) Optimization of solvent system for PGE$_2$ and LTB$_4$ levels

Separation and quantification of PGE$_2$ and LTB$_4$ from ear punches were conducted by HPLC followed by UV detection. During the entire analytical procedure exposure of light and temperature above 25 °C was avoided.

PGE$_2$ (10 and 50 ng) and LTB$_4$ (0.1 and 10 ng) prepared in ethanol were subjected to HPLC (Prominence 20A, Shimadzu, Japan) on a reversed phase nucleosil column (C18, 250/4.6 mm, 100-5) two different mobile phases in various combinations $i$) methanol: water (74:26 and 80:20) containing trifluoroacetic acid TFA (0.007%) and $ii$) acetonitrile: water (35:65, 40:60 and 45:55) containing TFA (0.001 %) at a flow rate of 1 mL/min. The sample (20 µL) was injected with the duration of run was 30 min and the
absorbance was recorded using UV detector (SPD-M20A) at 214 and 280 nm for PGE\textsubscript{2} and LTB\textsubscript{4}, respectively.

b) Extraction of PGE\textsubscript{2} and LTB\textsubscript{4} from mice ear punch

Mice ear punches from AA (2 mg/20 µL/ear) or TPA (2.5 µg/20 µL/ear) induced edema was used for the extraction of eicosanoid metabolites (PGE\textsubscript{2} and LTB\textsubscript{4}) as described by Lloret and Moreno, (1995). Ear punches (n = 10) were collected and homogenized in methanol (1 mL) containing HCl (1 M, 1%) followed by addition of deionized water (2 mL) and kept on ice for 30 min, followed by centrifugation (30,000 g) for 20 min at 4 ºC (Heraeus Biofuge Stratos, Germany). The eicosanoids were extracted with fraction-1 (6 mL) and centrifuged (1000 rpm) for 3 min at 4 ºC resulting in two separate layers. The aqueous layer was discarded and the organic layer was evaporated in a stream of nitrogen. The residue obtained was dissolved in mobile phase (acetonitrile 45:55, 200 µL), filtered through syringe driven filter unit (0.22 µm, Millipore, Ireland) and 20 µL sample was injected in C-18 column for HPLC. Since separation of eicosanoids was not satisfactory therefore concentrations of irritants {AA (3 mg/20 µL/ear) or TPA (4 µg/20 µL/ear)} and the number of ear punches (20) were increased while the volumes of methanol (500 µL) and deionized water were reduced (1 mL) and further steps were performed as described earlier, however, no success was obtained. Because of failure of detection of PGE\textsubscript{2} and LTB\textsubscript{4} by HPLC methods alternative methods were used as described below.

2.7.1 Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) levels

The levels of PGE\textsubscript{2} were detected successfully in AA (2 mg/ 20 µL/ear) or TPA (2.5 µg/20 µL/ear) sensitized ear punches as described by Xian et al. (2011), therefore further experiments were performed using IC\textsubscript{50} values of methanol extract (158 or 269 mg/kg), fraction-1 (200 or 203 mg/kg), fraction-2 (112 or 342 mg/kg), opuntiol (77 or 85 mg/kg), opuntioside (15 or 99 mg/kg) and reference drugs celecoxib (42 or 95 mg/kg), indomethacin (18 mg/kg), dexamethasone (13 mg/kg) obtained using corresponding irritants (Table-8). The respective ear punches were homogenized (Ultra-turrax T25, Janke and Kunkel IKA-Labortechnik, Germany) in potassium phosphate buffer (500 µL, 0.1 M, pH 7.4) (Appendix-I) and kept on ice for 15 min followed by centrifugation (10,000 g, 30 min, 4 ºC). The levels of PGE\textsubscript{2} in supernatants were determined using commercially available PGE\textsubscript{2} EIA kit (Cayman chemical Co., USA) according to manufacturer guidelines wells were labeled as blank (Ellman’s reagent only), NSB (non-specific binging), B\textsubscript{0} (maximum binding), PGE\textsubscript{2} (standard) and various samples (vehicle
control or test agents or reference drugs). The enzyme immunoassay buffer (EIA) was added in NSB (100 µL) and also to B₀, PGE₂ and sample wells (50 µL). Immediately, 50 µL of samples were added in appropriate wells. The tracer (acetyl cholinesterase linked to PGE₂, 50 µL) was added to all the wells except blank wells followed by addition of PGE₂ antibody (50 µL) to B₀, PGE₂ and samples wells. After 18 h of incubation at 4 °C the 96 well plates were rinsed 5x with wash buffer (200 µL), followed by addition of Ellman’s reagent (containing acetyl cholinesterase substrate) to all wells (Appendix-I). The plastic film was used to cover the plate and placed on plate shaker (20 rpm, 70 min) in dark. The absorbance of yellow color developed was measured at 412 nm using spectrophotometer (SpectraMax M2, Molecular Devices CA, USA). Each absorbance value after subtraction from blank values was used for calculation of corrected value:

\[
\text{Corrected value} = B₀ - \text{NSB}
\]

Where B₀ is maximum tracer bound in the absence of sample and NSB is non-immunological binding of tracer in the absence of PGE₂ antibody.

The PGE₂ bound to tracer (B) in the samples and its maximum binding with tracer in the absence of sample (B₀) is represented as percent B/ B₀ and calculated as:

\[
\text{Percent } B/ B₀ = \frac{\text{Subtracted values} - \text{NSB} \times 100}{\text{Corrected value}}
\]

Various concentrations of PGE₂ (50 µL) (7.8, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 pg/mL) was added to the appropriate wells of 96 well plate and the same procedure as described above was performed. The graph between PGE₂ concentrations (X-axis) vs %B/ B₀ values (Y-axis) was plotted and best fit line generated was used to obtain the concentration of PGE₂ in the presence or absence of test agents.

2.7.2 Leukotriene B₄ (LTB₄) levels

The rat neutrophils as described above in the cellular toxicity assay section were used to release LTB₄ from them followed by its isolation and measurement as shown below:

Rat neutrophils (5 x 10⁶ cells/ mL) in incubation buffer (975 µL) were pre-incubated (5 min, 37°C) with shaking (20-22 rpm) on Uni Thermo shaker (Eyela, Japan). The control tubes contained 5 µL of either incubation buffer, DMSO (0.5%), or saline (0.9%), positive control contained NDGA (1, 2 and 3 µg/mL) quercetin (1, 4, 8 and 10 µM) whereas, the treated group with either methanol extract, fraction-1 (10, 20, and 50 µg/mL), fraction-2 (20, 50 and 100 µg/mL), opuntiol (1, 10 and 20 µg/mL) and
opunsioside (1, 10 and 30 µg/ mL) were incubated for 5 min at 37°C. After incubation calcium ionophore (10 µL, 1.9 µM) was introduced and incubated for further 2 min followed by the addition of calcium chloride (10 µL, 1.8 mM) and incubated for 5 mins. The reaction was terminated by 1 mL of acidified ice cold methanol: 1N HCl (97: 3, pH 3). All the reaction tubes (2 mL) were stored at -20 °C until further analysis within 2-3 days (Appendix-I).

The above reaction tubes (2 mL) were brought to room temperature and PGB₂ (50 ng, 10 µL) was introduced in all the tubes as an internal standard. The contents were thoroughly mixed, centrifuged (10 min, 5000 rpm) and the supernatant was subjected to solid phase extraction (SPE) (Chromabond C18, 45 µm, 1 mL and 100 mg matrix) under air pressure using SPE column pre-equilibrated with methanol (100%, 1 mL) with flow rate (100 µL/sec). It was followed by the addition of deionized water (1 mL) with flow rate (200 µL/sec). Each reaction tube containing supernatant (2 mL) as mentioned earlier was gently applied to SPE column (flow rate: 5 µL/sec) followed by sequential addition of deionized water (1 mL, flow rate: 8.5 µL/sec) and methanol (25%, 1 mL, flow rate: 7.0 µL/sec). Finally, LTB₄ was eluted in methanol (100%, 300 µL) at 3 µL/sec flow rate and collected in an argon flushed aluminum foil wrapped micro centrifuge tube (1.5 mL). The eluted methanol samples were analyzed on HPLC as reported by (Steinhilber et al., 1989; Sala et al., 2003).

The eluted methanol samples (300 µL) were filtered through syringe driven filter unit (0.22 µm, Millipore, Ireland) and subjected to HPLC (Prominence 20A, Shimadzu, Japan) on a reversed phase nucleosil column (C18, 250/4.6 mm, 100-5). The sample (20 µL) was injected and isocratic elution was performed in methanol: water (74: 26) solvent containing TFA (0.007 %, pH 4.8) at a flow rate of 1.0 mL/min for 30 min using UV detector (SPD- M20A) at 280 nm for both LTB₄ and PGB₂. These were identified on the basis of their retention time using LTB₄ and PGB₂ standards and the concentration of LTB₄ was determined by generating its standard curve (Ammon et al., 1991; Roome et al., 2008b).

The standard curve was plotted with various concentrations of LTB₄ (0.05, 0.1, 0.3, 0.5, 0.7 and 1.0 ng) in triplicates. In graph, the best fit line between concentration (X-axis) vs area under the peak (Y-axis) was calculated by using software (LC solution).

2.7.3 Reactive oxygen species (ROS) levels

Mice (20-28 g) of either sex (n=3/dose) received orally either saline (0.9%) and DMSO (10%) (Vehicle control) or treated with IC₅₀ values of methanol extract (269 mg/kg),
fraction-1 (203 mg/kg), fraction-2 (113 and 342 mg/kg), opuntiol (85 mg/kg) and opuntioside (15 and 99 mg/kg) or NDGA (55 mg/kg) from TPA-induced ear edema (Table-8). Ear punches (1/10 w/v) were homogenized in phosphate buffer (pH 7.4) (Appendix-I) followed by centrifugation (14000 g, 5 min). Supernatant (50 µL) and 2’-7’-dichlorodihydrofluorescein diacetate (DCFH-DA) (50 µL, 20 µM) were incubated in 96 black wells plate (30 min, 37 ºC) in dark. In the presence of ROS, the DCFH-DA dye is converted into highly fluorescent 2’,7’-dichlorodihydrofluorescein (DCF) and was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm by fluorescence microplate reader (SpectraMax M2, Molecular Devices CA, USA) according to the method described by Best et al., (1999); Xian et al., (2011).

2.7.4 Tumor necrosis factor (TNF-α) levels

Mice (20-24 g) of either sex (n=3/dose) received orally either saline or IC50 values of methanol extract methanol extract (269 mg/kg), fraction-1 (203 mg/kg), fraction-2 (342 mg/kg), opuntiol (85 mg/kg) and opuntioside (99 mg/kg) or dexamethasone (13 mg/kg) from TPA-induced ear edema (Table-8). The ear punches were collected and homogenized in lysis buffer (500 µL) (Appendix-I) and incubated in ice for 15 mins. followed by centrifugation (10,000 g, 4 ºC) for 30 min. The levels of TNF-α in the supernatant was measured as described earlier (Xian et al., 2011) using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s guidelines (Invitrogen USA). To 100 µL of each sample, diluent buffer (50 µL), biotinylated MS TNF-α Biotin conjugate (secondary antibody) (50 µL) were added except to chromogen blank wells followed by incubation at room temperature. After 90 min all the wells were washed (4 times) with wash buffer (300 µL) and streptavidin-HRP (streptavidin-peroxidase enzyme, 100 µL) solution was added and incubated at 25 ºC for 30 min. Each well was washed again with wash buffer and stabilized chromogen (substrate of streptavidin-peroxidase enzyme, 100 µL) was added to blank, standard and sample wells. The solution begins to turn blue and immediately incubated in dark at room temperature for 20 min and the reaction was stopped by the addition of stop solution (sulphuric acid 2N, 100 µL). The yellow color was developed and measured at 450 nm using spectrophotometer. The absorbance of chromogen blank was subtracted from all the value and used to calculate TNF-α value.

Different concentrations of TNF-α (100 µL) (0, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 pg/mL) were added in wells of 96 well plate and the same procedure as described above was performed. The graph between TNF-α concentrations (X-axis) vs
absorbance (Y-axis) was plotted and best fit line generated was used to obtain the concentration of TNF-α in the presence or absence of test agents.

2.8 Determination of anti-inflammatory mediator

The levels of anti-inflammatory mediator IL-10 was also determined by ELISA from carrageenan-induced peritonitis in mice.

2.8.1 Interleukin-10 (IL-10) levels

Mice (20-28 g) of either sex (n=3/dose) received orally either saline (0.9%) (vehicle control) or IC50 values of methanol extract (269 mg/kg), fraction-1 (203 mg/kg), fraction-2 (113 and 342 mg/kg), opuntiol (85 mg/kg) and opuntioside (15 and 99 mg/kg) (Table-8) or dexamethasone (1, 2 and 3 mg/kg). After 30 min carrageenan (1%, 0.1 mL) was i.p injected and after 4 h of carrageenan-induced peritonitis, mice were sacrificed by cervical dislocation. The animals were dissected and cold saline (1 mL) was introduced in the peritoneal cavity, collected and centrifuged (1000 rpm, 10 min). The supernatant was used to measure the levels of cytokine IL-10 as described earlier (Mizgerd et al., 2001) using commercially available ELISA kit according to the manufacturer’s instructions (Millipore USA). To 50 µL of assay buffer A, 50 µL of samples were added. Cover the plate and incubate at room temperature (25 ºC) with constant shaking (micro plate shaker Grant-bio instrument, England) at 200 rpm. After 2 h wells were washed 4 times with wash buffer (200 µL). Add mouse IL-10 detection antibody (100 µL) to each well and again incubate for 1 h with constant stirring. Wash the wells followed by addition of Avidin-HRP A solution (Avidin is coupled to horseradish peroxidase, 100 µL) to each well and incubate at room temperature for 30 min while shaking. Again wash the wells followed by addition of substrate solution (100 µL), the color begins to turn blue, incubate in dark for 15 min. Finally the reaction was stopped by adding stop solution (sulphuric acid 2N, 100 µL) in each well. The absorbance of yellow color developed was measured at 450 and 570 nm using spectrophotometer and subtracted the absorbance at 570 nm from the absorbance at 450 nm.

To 50 µL of assay buffer add different concentrations of IL-10 (100 µL) (62.5, 125, 250, 500, 1000, 2000 and 4000 pg/mL) was added in wells of 96 well plate and the same procedure as described above was performed. The graph between IL-10 concentrations (X-axis) vs absorbance (Y-axis) was plotted and best fit line generated was used to obtain the concentration of IL-10 in the presence or absence of test agents. The
percent reduction in the levels of PGE$_2$, LTB$_4$, ROS, TNF-$\alpha$, and IL-10 was calculated using following formula:

$$\text{Inflammatory / anti-inflammatory mediators reduction (\%)} = \frac{\text{Control} - \text{treated}}{\text{Control}} \times 100$$

### 2.9 Analgesic assays in mice

Methanol extract, fraction-1 and -2 and opuntiol and opuntioside from cladode of *O. dillenii* were evaluated for peripheral and central analgesic effect in mice by following methods:

#### 2.9.1 Acetic acid-induced writhes

Mice (22-30 g) of either sex (n=3/dose) received either saline (0.9%) or Na$_2$CO$_3$ (1%) or DMSO (10%) serving as vehicle control, or treated with methanol extract (10, 100, 200, 250, 300 and 350 mg/kg), fraction-1 (50, 100, 150, 200 and 250 mg/kg), fraction-2s (10, 50, 100, 150 and 200 mg/kg), opuntiol (10, 25, 50 and 100 mg/kg) and opuntioside (1, 10, 20 and 30 mg/kg) or reference drugs morphine (1, 5 and 10 mg/kg), acetylsalicylic acid (50, 100 and 150 mg/kg), diclofenac sodium (5, 10, 15 and 20 mg/kg), indomethacin (1, 5, 10 and 15 mg/kg) and commercially available $\beta$-sitosterol (10, 20 and 30 mg/kg) administered orally (10 mL/kg). After 30 min acetic acid (0.8%, 10 mL/kg, *i.p*) was administered and mice were transferred immediately into transparent plastic observation box (23x12x13 cm). The writhes were characterized by contraction of abdominal muscles, turning of trunk and stretching of hind limbs. The number of writhes were counted for 30 min using hand counter in treated animals and compared with vehicle control animals. To ascertain the involvement of opioid system in antinociceptive effect, mice received non-selective antagonist naloxone (1 mg/kg, *i.p*) 10 min before administration of minimum and maximum doses of either methanol extract (10 and 350 mg/kg), fraction-1 fraction (50 and 250 mg/kg), fraction-2 (10 and 200 mg/kg), opuntiol (10 and 100 mg/kg) and opuntioside (1 and 30 mg/kg) or morphine (1, 5 and 10 mg/kg), $\beta$-sitosterol (10 and 30 mg/kg) orally followed by the administration of acetic acid (0.8%, *i.p*). The percent inhibition of writhes was calculated as follows (Koster., et al 1959; Rejon-Orantes et al., 2013).

$$\text{Writhes inhibition (\%)} = \frac{\text{Number of writhes in control} - \text{writhes in treated}}{\text{Number of writhes in control}} \times 100$$
2.9.2 Formalin-induced paw licking response

Mice (22-28 g) of either sex (n=3/dose) were pretreated with saline (0.9 %, vehicle control) or DMSO (10%) or treated with methanol extract (100, 200, 300 and 400 mg/kg), fraction-1 (100, 150, 200, 250 and 300 mg/kg), fraction-2 (100, 150, 200 and 250 mg/kg), opuntiol (10, 25, 50 and 75 mg/kg) and opuntioside (1, 10, 20 and 30 mg/kg) or reference drug morphine (1, 5 and 10 mg/kg), acetylsalicylic acid (100, 200 and 300 mg/kg), diclofenac sodium (1, 5, 10 and 15 mg/kg), or β-sitosterol (10, 20 and 30 mg/kg) orally. After 30 minutes formalin (20 µL of 1% solution) was injected subcutaneously (s.c) with syringe (1 mL, 27-guage) into dorsal surface of right hind paw of each mouse and were immediately placed in a plastic observation box (23x12x13 cm) and observed for pain response (licking or biting of the treated paw) for 30 min using timer. To ascertain the possible involvement of opioid receptors mice received naloxone (2 mg/kg, i.p) after 10 min of oral administration of minimum and maximum doses of methanol extract (10 and 400 mg/kg), fraction-1 fraction (100 and 300 mg/kg), fraction-2 (100 and 250 mg/kg), opuntiol (10 and 75 mg/kg) and opuntioside (1 and 30 mg/kg) or morphine (1, 5 and 10 mg/kg), β-sitosterol (10 and 30 mg/kg) followed by injection of formalin (20 µL of 1% solution). The percent inhibition of licking time was calculated as follows (Dubuisson and Dennis 1977; Roome et al., 2011).

\[
\text{Licking time inhibition (\%)} = \frac{\text{Licking time (control)} - \text{licking time (treated)}}{\text{Licking time (control)}} \times 100
\]

2.9.3 Hot plate-induced jumping response

Mice (22-28 g) of either sex (n=3/dose) were pretreated with vehicle control saline (0.9%) or DMSO (10%) or methanol extract (10, 100, 200, 250 and 300 mg/kg), fraction-1 (10, 50, 100 and 150 mg/kg), fraction-2 (10, 50, 100, 150 and 200 mg/kg), opuntiol (10, 25, 50 and 75 mg/kg) and opuntioside (10, 20 and 30 mg/kg) or reference drugs such as morphine (1, 5 and 10 mg/kg), paracetamol (300, 400 and 500 mg/kg) and β-sitosterol (10, 20 and 30 mg/kg). The temperature of the hot plate (Ugo Basile, model-DS 37, 25x25 cm, Italy) was maintained at 50 ± 0.05°C. After 30 min of oral administration of aforementioned, the animals were placed on hot plate and the latency time between the placement and response as licking of the paws or flicking of hind limbs or jumping was recorded at 0, 30, 60, 90 and 120 min with a cut off period of 30 sec to avoid damage of paw. In order to investigate the involvement of opioid receptors mice received naloxone (2 mg/kg, i.p) 10 min prior to administration of minimum and maximum doses of...
methanol extract (10 and 300 mg/kg), fraction-1 (10 and 150 mg/kg), fraction-2 (10 and 200 mg/kg), opuntiol (10 and 75 mg/kg) and opuntioside (10 and 30 mg/kg) or morphine (1, 5 and 10 mg/kg) and β-sitosterol (10 and 30 mg/kg) the latency time was noted as described above. Pain protection was expressed as increase in latency time to thermal stimulus with respect to vehicle control (Eddy and Leimback 1953; Roome et al., 2011).

\[
\text{Pain protection (\%)} = \frac{\text{Treatment latency} - \text{base line latency}}{\text{Base line latency}} \times 100
\]

2.10 Statistical analysis

The statistical comparisons between treated groups and control and between the various treatments were performed by statistical software SPSS using ANOVA (Analysis of Variance). The comparisons of data were conducted using least significant difference (LSD) and Duncan’s multiple range tests. The \( p \) value of < 0.05 was considered statistically significant (\(*p < 0.05\), \(**p < 0.01\) and \(***p < 0.005\)).
3. RESULTS

3.1 Toxicity studies

3.1.1 Acute toxicity in mice

The oral administration of *O. dillenii* cladode methanol extract (1 and 5 g/kg) in mice (n=10 per dose) was well tolerated and had no effect on food consumption (30 ± 0.9 g) as compared to control animals (29 ± 0.8 g). It did not induce any visible behavioral changes (agitation, restlessness, tremors, convulsions, dullness and piloerection) and no mortality occurred during 7 days of observation similar to control. Biochemical Parameters (liver and kidney function) of control mice were total bilirubin (0.48 ± 0.05 mg/dL), direct bilirubin (0.03 ± 0.01 mg/dL), alanine aminotransferase (ALT) (54 ± 6.6 U/L), alkaline phosphatase (ALP) (53 ± 6.4 U/L), γGT (8.0 ± 1.1 U/L) and creatinine (0.15 ± 0.2 mg/dL) were ineffective in the presence of methanol extract (1 and 5 g/kg) and presented similar levels as control (~0.44 mg/dL), (~0.03 mg/dL), (~54 U/L), (~7.9 U/L) and (~0.13 ± 0.3 mg/dL), respectively (Table-4).

The liver and kidney from these animals did not show any macroscopic changes. In hematoxylin and eosin (H&E) stained liver and kidney preparations of control the architecture of liver and morphology of hepatocytes with prominent cytoplasm and nucleus, sinusoids, central vein and portal canal and structure of kidney with cortex, podocyte, capsular space, glomerulus, squamous cells, renal corpulses and distal and convultated tubule were clearly observed at 20x. In methanol extract (1 and 5 g/kg) treated mice structure and morphology of liver and kidney were remain intact and no damage was observed (Figure-3 and 4).

3.1.2 Cellular toxicity on rat neutrophils

The viability of rat neutrophils (5 x 10^6 cells/mL) in untreated animals (control) was 96% from 0-4 h (Table-5). In the presence of *O. dillenii* methanol extract (10-200 µg/mL), fraction-1 (10 µg/mL), fraction-2 (10-200 µg/mL), opuntiol and opuntioside (1-10 µg/mL) and reference drugs NDGA and quercetin (0.1-1.0 µM) the cell viability was not affected from 0-4 h and was similar to control. While methanol extract at (500-1000 µg/mL), fraction-1 (100-200 µg/mL), fraction-2 (200 µg/mL), opuntiol (100 µg/mL) and opuntioside (50-100 µg/mL), NDGA and quercetin elicited dose dependent significant reduction (~81%) in cell viability up to 3 h.
3.2 Inflammatory animal models

3.2.1 Concentration and time dependent effect of AA and TPA on mice ear edema

The topical application of arachidonic acid (AA) or 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced ear edema was reflected as increase in weight of right ear punch as compared to ethanol treated left ear punch representing control. The time and concentration dependent effect of AA and TPA are presented in Figure-5.

In control the ear weight 7.8 ± 0.1 mg was increased significantly in the presence of AA (1, 2 and 3 mg/20 µL/ear, n = 10) to 31%, 153% and 190%, respectively. The AA (2 mg/20 µL/ear) causing more than 100% of edema was selected after 30, 60 and 90 min of its application it demonstrated 112% and 153% edema reaching a plateau at 90 min. Thus the concentration of AA (2 mg/20 µL/ear) and time duration (1 h) were selected for AA-induced ear edema in mice in the subsequent experiments.

For TPA, the left ear punch weight (7.8 ± 0.1 mg) of control animals were increased significantly in the presence of TPA (2.5 and 4 µg/20 µL/ear, n = 10) to 165% and 180%, respectively. The TPA (2.5 µg/20 µL/ear) causing more than 100% of edema was selected and edema was noted for different time intervals (2, 4, 6, 8 and 24 h). It begins to appear within 2 h and significantly ear weight was increased by (42%, 101%, and 159%). Between 7-8 h the edema started to decline reaching to 143% and further reduced to only 69% at 24 h. Thus the concentration of TPA (2.5 µg/20 µL/ear) and time duration, 6 h were selected for TPA-induced ear edema in mice in the subsequent experiments.

3.3 Anti-inflammatory assays

Anti-inflammatory activities of test agents were evaluated using ear and paw edema and peritonitis in mice.

3.3.1 Effect of O. dillenii methanol extract, fractions, opuntiol, opuntioside and anti-inflammatory drugs on AA and TPA-induced ear edema in mice using different mode of administration

The AA (2 mg/20 µL/ear) and TPA (2.5 µg/20 µL/ear) concentrations that induced ear edema after topical application in mice ear punches 11.6 ± 0.1 mg (n=50) as described above were used to evaluate anti-inflammatory activity of methanol extract, fraction-1 and -2, opuntiol and opuntioside derived from cladode of O. dillenii. All the test agents were administered using three different routes viz i) topical, ii) intraperitoneal and iii) oral.
3.3.1.1 Topical application

The methanol extract, fraction-1 and -2 (1-20 mg/ear), opuntiol and opuntioside (0.1-10 mg/ear) and control produced similar magnitude of edema in both AA and TPA-induced ear edema. However, the reference drugs, diclofenac sodium (0.5-1.5 mg/ear), indomethacin and NDGA (1-3 mg/ear) and dexamethasone (0.05-0.08 mg/ear) produced significant dose dependent reduction in ear edema reaching ~ 60% with IC50 values of 1.2 mg/ear, 2.5 mg/ear, 3 mg/ear and 0.06 mg/ear respectively.

3.3.1.2 Oral administration

The methanol extract administered orally at low doses (1-25 mg/kg) failed to reduce AA (2 mg/ 20 µL/ear) induced edema and was similar to control (11.6 ± 0.1 mg). However, at higher doses of methanol extract (50-200 mg/kg), fraction-1 (10-200 mg/kg), fraction-2 (1-150 mg/kg), opuntiol (1-100 mg/kg) and opuntioside (0.5-15 mg/kg) demonstrated dose dependent significant attenuation in ear edema with maximum reduction of (~60%) with an IC50 values of 158 ± 5.2 mg/kg, 150 ± 11.5 mg/kg, 112 ± 8.2 mg/kg, 77 ± 4.0 mg/kg and 15 ± 1.4 mg/kg, respectively (Table-6a).

Anti-inflammatory drugs acetylsalicylic acid (10-200 mg/kg), celecoxib (10-100 mg/kg), diclofenac sodium (0.5-25 mg/kg), ibuprofen (1-150 mg/kg) and indomethacin (1-25 mg/kg) also demonstrated dose dependent significant reduction in edema with maximum response reaching ~72% with IC50 values of 168 ± 1.4 mg/kg, 95 ± 5.1 mg/kg, 16 ± 0.9 mg/kg, 146 ± 8.7 and 12 ± 1.0 mg/kg, respectively. The NDGA and dexamethasone (1-50 mg/kg) were ineffective causing only ~ 6% reduction (Table-6b).

The methanol extract at doses of 10-25 mg/kg, fraction-1 (1-25 mg/kg), fraction-2 (1-25 mg/kg), opuntiol (10 mg/kg) and opuntioside (0.5-30 mg/kg) failed to reduce TPA (2.5 µg/ 20 µL/ear) induced ear edema and was similar to control. However, at higher doses methanol extract (50-300 mg/kg), fraction-1 (50-100 mg/kg), fraction-2 (50-400 mg/kg), opuntiol (25-100 mg/kg) and opuntioside (0.5-30 mg/kg) demonstrated significant reduction in a dose dependent manner by 16-55%, 27-50%, 9-54%, 20-59% and 6-52% with an IC50 values of 269 ± 6.6 mg/kg, 203 ± 6.7 mg/kg, 342 ± 12 mg/kg, 85 ± 5.3 mg/kg and 99 ± 2.4 mg/kg, respectively (Table-7a).

Anti-inflammatory drugs indomethacin (1-15 mg/kg), celecoxib (10-50 mg/kg), NDGA (1-75 mg/kg) and dexamethasone (1-20 mg/kg) diminished ear edema significantly in a dose manner by 8-59%, 8-58%, 6-58% and 23-65% with an IC50 values of 12 ± 1.0, 42 ± 3.0 mg/kg, 55 ± 2.9 and 13 ± 0.9 mg/kg, respectively (Table-7b).
The overall potency order obtained against AA-induced ear edema on the basis of IC\textsubscript{50} values was diclofenac sodium = opuntioside = indomethacin > opuntiol > celecoxib > fraction-2 > ibuprofen = methanol extract = acetylsalicylic acid.

In case of TPA-induced ear edema the potency order on the basis of IC\textsubscript{50} values appeared to be indomethacin = dexamethasone > celecoxib = NDGA > opuntiol = opuntioside > fraction-1 > methanol extract > fraction-2 (Table-8).

### 3.3.1.3 Intraperitoneal administration

The highest dose of test agents in oral route of administration after AA or TPA-induced ear edema was used for their anti-inflammatory effect using \textit{i.p} route. Methanol extract (200 and 300 mg/kg), fraction-1 (200 and 250 mg/kg) and fraction-2 (150 and 400 mg/kg), opuntiol (100 mg/kg) and opuntioside (20 and 100 mg/kg) produced significant reduction ~60% in ear edema. The reference drugs, indomethacin (15 and 25 mg/kg) and dexamethasone (15 mg/kg) also produced significant dose dependent reduction (~65%) in ear edema. All test agents produced similar anti-inflammatory effect as produced by oral administration.

### 3.3.2 Effect of \textit{O. dillenii} methanol extract, fractions, opuntiol, opuntioside and anti-inflammatory drugs on histology of AA and TPA sensitized ear punches

\textbf{Figure-6} depicted histological sections of untreated mice ears (control) the keratin, epidermis, epithelial cells, basement membrane, dermis, blood vessels, organized connective tissues and cartilage, sebaceous and sweat glands, fat cells, hair follicles were clearly observable at 20x magnification under the light microscope. After topical application of AA (2 mg/20 µL/ear) keratin, epidermis, basement membrane, sebaceous gland and cartilage were intact, however, thickness of dermis was increased 3x as compared to control which is an indicative of edema formation. The loosening of connective tissues, vasodilatation and recruitment of few neutrophils in the dermis were also evident. In the presence of IC\textsubscript{50} values of test agents methanol extract (158 mg/kg) the thickness of dermis was reduced (3x) and mild vasodilatation, edema and recruitment of inflammatory cells were observed as compared to AA-induced ear edema, fraction-1 (200 mg/kg) mild edema with no vasodilatation and normal thickness of dermis were noted, fraction-2 (112 mg/kg) mild vasodilatation and moderate edema was noted, opuntiol (77 mg/kg) mild edema, opuntioside (15 mg/kg) mild vasodilatation were observed while in the presence of indomethacin (18 mg/kg) the thickness of dermis, vasodilatation, intensity of edema, inflammation and recruitment of neutrophils was
reduced with more pronounced effect as compared to plant derived test agents.

Upon topical application of TPA (2.5 µg/20 µL/ear) the histological analysis indicates that likewise AA the keratin, epidermis, basement membrane, sebaceous gland and cartilage were not affected however, thickness of dermis was increased, vasodilatation, increased intensity of edema, loosening of connective tissues, disorganization of fibers in dermis and recruitment of neutrophils was noted as compared to untreated ear punches. In the presence of IC$_{50}$ values derived from TPA-induced ear edema the methanol extract (269 mg/kg) mild edema and inflammation, fraction-1 (203 mg/kg) no vasodilatation and mild edema with normal thickness of dermis were noted. In the presence of fraction-2 (342 mg/kg), mild vasodilatation, inflammation and moderate edema, opuntiol (85 mg/kg) mild edema, opuntioside (99 mg/kg) mild vasodilatation were observed and dexamethasone reduced the thickness of dermis, vasodilatation, intensity of edema, inflammation and recruitment of neutrophils with more pronounced effect as compared to other test agents (Figure-7).

### 3.3.3 Effect of *O. dillenii* methanol extract, fractions, opuntiol and opuntioside on PLA$_2$-induced paw edema in mice

The initial volume of right hind paw of mice at time 0-min was 30.7 ± 1.7 µL as measured by plethysmometer. After the sub-plantar injection of phospholipase A$_2$ (PLA$_2$) (2 unit/25 µL in saline) the volume increased by 1.6x and 2.2x at 30 and 60 min., respectively which is an indicative of edema formation. However, at 90 min. reversal of edema was noted which was only 1.4x. Similar pattern was obtained after oral administration of saline (0.9%) or DMSO (10%) or Na$_2$CO$_3$ (1%) in which test agents were prepared and hence represent vehicle control groups.

The IC$_{50}$ values of various test agents obtained in either AA or TPA-induced ear edema (Table-8) were used to evaluate their effects in the PLA$_2$-induced hind paw edema for 30, 60 and 90 min. which were orally administered 30 min. prior to PLA$_2$ treatment. Since, maximum PLA$_2$ induced edema was obtained at 60 min therefore it considered to be most reliable.

The IC$_{50}$ values for AA-induced ear edema for methanol extract (158 mg/kg), fraction-1 (203 mg/kg), fraction-2 (112 mg/kg), opuntiol (85 mg/kg) and opuntioside (15 mg/kg) produced a maximum and significant reduction in paw edema respectively, by 31%, 40%, 54%, 45% and 66 % at 60 min as compared to control (Table-9a). The order of potency appeared to be at 30 min opuntioside = fraction-2 = opuntiol = fraction-1 >
methanol extract at 60 min opuntioside = fraction-2 = opuntiol = fraction-1 > methanol extract. At 90 min opuntioside = fraction-2 = fraction-1 = opuntiol > methanol extract.

The IC50 values for TPA-induced ear edema for methanol extract (269 mg/kg), fraction-1 (203 mg/kg), fraction-2 (342 mg/kg), opuntiol (85 mg/kg) and opuntioside (99 mg/kg) also induced a maximum and significant reduction in paw edema respectively, by 52%, 51%, 81%, 50% and 83% at 60 min as compared to control (Table-9a). The order of potency appeared to be at 30 min and 60 min opuntioside = fraction-2 > methanol extract = opuntiol = fraction-1. At 90 min opuntioside = fraction-2 = fraction-1 = opuntiol = methanol extract.

3.3.4 Effect of anti-inflammatory drugs on PLA2-induced paw edema in mice

The initial volume of right hind paw of mice at time 0-min was 30.7 ± 1.7 µL as mentioned above remained unchanged at 1 mg/kg of cyproheptadine, indomethacin and dexamethasone however, at (5-10 mg/kg) significant dose dependent reduction with maximum reduction of 74%, 79% and 74% at 10 mg/kg was noted during 60 min. with an IC50 values at 30 min 8.5 ± 0.2 mg/kg, 4.0 ± 0.3 mg/kg and 7 ± 0.5 mg/kg. At 60 min 7.6 ± 0.2 mg/kg, 4.5 ± 0.2 mg/kg and 6 ± 0.4 mg/kg. At 90 min 4.3 ± 0.3 mg/kg and 4 ± 0.3 mg/kg, respectively (Table-9b).

3.3.5 Effect of O. dillenii methanol extract, fractions, opuntiol, opuntioside and anti-inflammatory drugs on carrageenan-induced peritonitis in mice

The same IC50 values as mentioned above derived from AA and TPA-induced ear edema were used to observe their effects on vascular permeability by quantifying the Evans blue accumulation in the peritoneal cavity using carrageenan-induced peritonitis. Prior to experiments, a standard curve of Evan blue (2-30 µg/mL) was prepared by measuring its absorbance at 620 nm, a linear graph between concentration vs absorbance is presented in Figure-8.

Upon oral administration of saline or DMSO 10% or Na2CO3 (vehicle control) in mice the concentration of Evans blue accumulated in the peritoneal cavity was 10 ± 0.4 µg/mL. In the presence of the IC50 values derived from AA-induced ear edema the methanol extract (158 mg/kg), fraction-1 (203 mg/kg), fraction-2 (112 mg/kg), opuntiol (85 mg/kg) and opuntioside (15 mg/kg) significantly reduced the Evans blue leakage by 27%, 34% 20%, 48% and 53%, respectively (Table-10). The potency order appeared to be opuntioside = opuntiol > fraction-1 = methanol extract = fraction-2.
Likewise, in the presence of IC$_{50}$ values from TPA-induced ear edema the methanol extract (269 mg/kg), fraction-1 (203 mg/kg), fraction-2 (342 mg/kg), opuntiol (85 mg/kg) and opuntioside (99 mg/kg) also significantly reduced the Evans blue leakage by 47%, 34%, 65%, 48% and 69%, respectively as compared to control. The potency order appeared to be opuntioside = fraction-2 > opuntiol = methanol extract = fraction-1.

The reference drugs, indomethacin (1, 5 and 10 mg/kg) and dexamethasone (1, 3, and 5 mg/kg) also significantly reduced the Evans blue dye leakage into the peritoneal cavity in a dose dependent manner with an IC$_{50}$ value of 10 ± 0.2 mg/kg) and 5 ± 0.1 mg/kg, respectively. Thus the order of potency order for vascular permeability reduction in mice appears to be dexamethasone = indomethacin = opuntioside > opuntiol > fraction-2 = fraction-1 > methanol extract (Table-10).

3.3.6 Effect of O. dillenii methanol extract, fractions, opuntiol, opuntioside and anti-inflammatory drugs on PGE$_2$ levels using AA and TPA-induced ear edema in mice

The same IC$_{50}$ values as mentioned above derived from AA and TPA-induced ear edema were used to observe their effects on PGE$_2$ levels using PGE$_2$ monoclonal ELISA kit. Prior to experiments, a standard curve of PGE$_2$ (7.8-1000 Pg/mL) was prepared by measuring its absorbance at 412 nm, a linear graph between concentration vs logit (B/B$_0$) to obtained lineralized data is presented in Figure-9. The intensity of yellow color is inversely proportion to the amount of free PGE$_2$ present in wells. The basal levels of PGE$_2$ were 42 ± 1.0 pg/mL. After topical application of AA or TPA levels of PGE$_2$ were significantly increased (164 ± 7.0 pg/mL). Treating the mice with oral administration of IC$_{50}$ values derived from AA-induced ear the methanol extract (158 mg/kg), fraction-1 (203 mg/kg), fraction-2 (112 mg/kg), opuntiol (85 mg/kg) and opuntioside (15 mg/kg) reduce the levels by 41%, 35%, 53%, 54% and 63%. Celecoxib (42 mg/kg) and indomethacin (18 mg/kg) also significantly reduce the production by ~65%.

Likewise, in the presence IC$_{50}$ values for TPA-induced ear edema the methanol extract (269 mg/kg), fraction-1 (203 mg/kg), fraction-2 (342 mg/kg), opuntiol (85 mg/kg) and opuntioside (99 mg/kg) also reduce the levels by 41%, 35% 53%, 54% and 63%. Celecoxib (95 mg/kg) and dexamethasone (13 mg/kg) also significantly reduced the PGE$_2$ levels by 72% (Figure-10). The order of potency appeared to be dexamethasone > indomethacin = celecoxib = opuntioside > fraction-2 = opuntiol > methanol extract and fraction-1.
3.3.7 Effect of *O. dillenii* methanol extract, fractions, opuntiol, opuntioside and reference drugs on LTB₄ levels in rat neutrophils

LTB₄ stimulated by calcium ionophore A23187 generated from rat neutrophils and measured by HPLC. Prior to experiments, a standard curve of LTB₄ (0.05, 0.1, 0.3, 0.5, 0.7 and 1.0 ng/mL) was prepared by measuring its absorbance at 280 nm, a linear graph between concentration vs absorbance and retention time at 7.8 min is presented in Figure-11. Control animals represented levels of LTB₄ = 8.06 ± 0.5 ng/mL which was significantly reduced in the presence of test agents. Methanol extract, fraction-1 (10 µg/mL), opuntiol (1.0 µg/mL) were ineffective in suppressing the LTB₄ levels while at their high doses methanol extract, fraction-1 (20-50 µg/mL), fraction-2 (20-100 µg/mL), and opuntiol (20-50 µg/mL) significantly reduce the LTB₄ levels in a dose dependent manner with an IC₅₀ values of 49 ± 6.0, 25 ± 2.7, 69 ± 2.2 and 19 ± 3.3 µg/mL, respectively while opuntioside (1-30 µg/mL) failed to suppress its levels. Reference drugs NDGA (0.1-2 µM) and quercetin (1.0-10 µM) also presented dose dependent significant reduction with an IC₅₀ values of 1.6 ± 0.3 and 5.5 ± 0.3 µM, respectively (Table-11). The representative chromatograms of each treatment have been shown in (Figure-12).

3.3.8 Effect of *O. dillenii* methanol extract, fractions, opuntiol, opuntioside and NDGA on ROS levels using TPA-induced ear edema in mice

The same IC₅₀ values as mentioned above derived from TPA-induced ear edema were used to observe their effects on production of ROS from mice ear punches using DCFH-DA dye. The basal levels of ROS in mice ear punches were 4596 ± 198 RFU which were significantly increased 19308.87 ± 419 RFU upon topical application of TPA. The IC₅₀ values obtained from TPA-induced ear edema in the presence of methanol extract (269 mg/kg), fraction-1 (203 mg/kg), fraction-2 (342 mg/kg), opuntiol (85 mg/kg) and opuntioside (99 mg/kg) the levels were significantly decrease by 9%, 25%, 19%, 37% and 30%, respectively as compared to control. In addition NDGA (55 mg/kg) also inhibited the production by 40% (Figure-13). The potency order appeared to be NDGA = opuntiol > opuntioside = fraction-1 = fraction-2 > methanol extract.

3.3.9 Effect of *O. dillenii* methanol extract, fractions, opuntiol, opuntioside and dexamethasone on TNF-α levels using TPA-induced ear edema in mice

The same IC₅₀ values as mentioned above derived from TPA-induced ear edema were used to observe their effects on production of TNF-α from TPA-sensitized ear punches using TNF-α ELISA kit. Prior to experiments, a standard curve of TNF-α (0-1000 Pg/mL)
was prepared by measuring its absorbance at 450 nm, a linear graph between concentration vs absorbance is presented in Figure-14. The basal levels of TNF-α in mice ear punch were 227 ± 4.3 pg/mL which were significantly increase after the topical application of 512 ± 15.7 pg/mL. Treating the mice with oral administration of IC₅₀ values derived from TPA-induced ear edema with methanol extract (269 mg/kg), fraction-1 (203 mg/kg), fraction-2 (342 mg/kg), opuntiol (85 mg/kg) and opuntioside (99 mg/kg) significantly reduced the concentration by 30%, 43%, 29%, 49% and 21%, respectively. Dexamethasone (13 mg/kg) also suppresses its levels by 55% (Figure-15). The potency order appeared to be dexamethasone = opuntiol = fraction-1 > methanol extract = fraction-2 = opuntioside.

3.3.10 Effect of O. dilleniï methanol extract, fractions, opuntiol, opuntioside and dexamethasone on IL-10 levels using carrageenan-induced peritonitis in mice

The same IC₅₀ values as mentioned above derived from AA and TPA-induced ear edema were used to observe their effects on levels of IL-10 levels using carrageenan-induced peritonitis in mice. Prior to experiments, a standard curve of IL-10 (62.5-4000 Pg/mL) was prepared by measuring its absorbance at 450 nm, a linear graph between concentration vs absorbance is presented in Figure-16. The basal levels of IL-10 were 272 ± 7.1 pg/mL which were significantly increased by intraperitoneal administration of carrageenan 1171 ± 8.7 pg/mL.

The IC₅₀ values of test agents obtained from AA-induced ear edema were used to determine their effect on IL-10. In the presence of methanol extract (158 mg/kg) the levels of IL-10 were significantly reduced by 26% while fraction-1 (203 mg/kg), fraction-2 (112 mg/kg), opuntiol (85 mg/kg) and opuntioside (15 mg/kg) were failed to change its levels.

Likewise, IC₅₀ values from TPA-induced ear edema obtained were used to measure their effect on IL-10. In the presence of methanol extract (269 mg/kg) IL-10 levels were significantly reduced by 36% while fraction-1 (203 mg/kg), fraction-2 (342 mg/kg), opuntiol (85 mg/kg) and opuntioside (99 mg/kg) were remained unchanged (Figure-17). The reference drug dexamethasone (1-3 mg/kg) significantly reduced its levels in a dose dependent manner with an IC₅₀ value of 1.9 ± 0.03 pg/mL.
3.4 Analgesic assays

3.4.1 Effect of *O. dillenii* methanol extract, fractions, opuntiol and opuntioside on acetic acid-induced writhes in mice

The number of abdominal writhes induced by acetic acid (0.8%, n= 20) is presented as mean ± SEM values and percent inhibition of writhes in the presence of test agents and reference drugs were calculated as compared to control. Control animals exhibited 50 ± 0.8 numbers of writhes. In the presence of methanol extract (10-350 mg/kg) and fraction-1 (50-250 mg/kg) demonstrated significant (*p* < 0.05) dose dependent reduction in number of writhes reaching to a maximum of ~58% with an IC$_{50}$ of 310 ± 10 mg/kg and 248 ± 6.0 mg/kg, respectively. The fraction-2 at 10 mg/kg produced similar number of writes as control while at 50-200 mg/kg a significant dose dependent attenuation (18-51%) with an IC$_{50}$ of 150 ± 5.8 mg/kg were noted. Opuntiol (10-100 mg/kg) and opuntioside (1-30 mg/kg) also significantly diminished the number of writhes (10-52%) and (16-58%) with an IC$_{50}$ of 100 ± 5.8 and 26 ± 0.9 mg/kg, respectively (Figure-18).

3.4.2 Effect of reference analgesic drugs on acetic acid-induced writhes in mice

Among the reference drugs morphine (1-10 mg/kg), acetylsalicylic acid (50-150 mg/kg), diclofenac sodium (5-20 mg/kg) and indomethacin (1-15 mg/kg) demonstrated significant reduction 22-72%, 30-64%, 10-60% and 12-56% in the number of writhes with an IC$_{50}$ values of 4.0 ± 0.4 mg/kg, 108 ± 4.4 mg/kg, 16 ± 0.9 mg/kg and 11.0 ± 1.0 mg/kg, respectively. Phytosterol β-sitosterol (10-30 mg/kg) also showed significant reduction in abdominal writhes (26-54%) with an IC$_{50}$ value of 27 ± 1.5 mg/kg (Figure-18).

On the basis of IC$_{50}$ values potency order appeared to be morphine = indomethacin = diclofenac sodium = opuntioside = β-sitosterol > opuntiol = acetylsalicylic acid > fraction-2 > fraction-1 > methanol extract (Table-12).

3.4.3 Effect of *O. dillenii* methanol extract, fractions, opuntiol, opuntioside, analgesic drugs and β-sitosterol in the presence of naloxone on acetic acid-induced writhes in mice

Abdominal writhes of mice in the presence of non-selective opioid receptor antagonist naloxone were (48 ± 0.6) which were similar to control (Figure-19) and also remained unchanged in the presence of minimum and maximum doses of methanol extract (10 and 350 mg/kg), fraction-1 (50 and 250 mg/kg) and fraction-2 (10 and 200 mg/kg), morphine (1, 5 and 10 mg/kg) and β-sitosterol (10 and 30 mg/kg). However, indomethacin (15
(15 mg/kg), opuntiol (100 mg/kg) and opuntioside (30 mg/kg) reduced the abdominal writhes by ~50%.

3.4.4 Effect of *O. dillenii* methanol extract, fractions, opuntiol and opuntioside on formalin-induced paw licking in mice

In control animals formalin-induced paw licking time during early phase (1-5 min.) was 56 ± 1.9 sec and in late phase (15-30 min.) it was 106 ± 2.5 sec. During early phase the methanol extract, fraction-1, fraction-2 (100 mg/kg) did not affect the paw licking while methanol extract (200-400 mg/kg), fraction-1 (150-300 mg/kg), fraction-2 (150-200 mg/kg), opuntiol (10-75 mg/kg) and opuntioside (1-30 mg/kg) caused dose-dependent reduction in paw licking with an IC$_{50}$ values (mg/kg) of 393 ± 6.7, 268 ± 9.2 and 245 ± 8.7 71 ± 2.2 and 28 ± 1.1, respectively. During late phase the methanol extract, fraction-1 and fraction-2, opuntiol and opuntioside also reduced paw licking in a dose dependent manner with IC$_{50}$ values (mg/kg) of 233 ± 12, 145 ± 7.6, 190 ± 10, 53 ± 2.9 and 24 ± 1.2 mg/kg, respectively (Table-13a).

3.4.5 Effect of reference analgesic drugs and β-sitosterol on formalin-induced paw licking in mice

The reference drug morphine (1-10 mg/kg) presented 23-76% and 25-81% attenuation in paw licking in early and late phase with IC$_{50}$ values of 4 ± 0.2 and 5 ± 0.2 mg/kg respectively. Pretreatment with anti-inflammatory drugs acetylsalicylic acid (100-300 mg/kg), diclofenac sodium (1-15 mg/kg) and indomethacin (1-20 mg/kg) produced same effects as produced by control in early phase while in second phase reduction in paw licking by 29-60%, 15-50% and 11-53% with IC$_{50}$ values of 205 ± 2.9, 15.1 ± 1.2 and 20 ± 2.4 mg/kg, respectively (Table-13b).

Thus potency order of test agents in early phase was morphine > β-sitosterol = opuntioside > opuntiol > fraction-2 > fraction-1 > methanol extract. And in late phase morphine > diclofenac sodium = indomethacin = β-sitosterol = opuntioside > opuntiol > fraction-1 > fraction-2 = acetyl salicylic acid > methanol extract (Table-13c).

The naloxone (2 mg /kg) displayed early phase (56 ± 1.9 sec) and late phase (106 ± 2.5 sec) paw licking response of mice that remained unchanged in the presence of minimum and maximum doses of methanol extract (100 and 400 mg/kg), fraction-1 (100 and 300 mg/kg), fraction-2 (100 and 250 mg/kg), morphine (1, 5 and 10 mg/kg) and β-sitosterol (10 and 30 mg/kg). However, opuntiol and opuntioside at their higher doses of 75 and 30 mg/kg in both phases reduced the paw licking by ~43% (Table-13d).
3.4.6 Effect of *O. dillenii* methanol extract, fractions, opuntiol and opuntioside on hot plate-induced jumping response in mice

As presented in Table-14a the response of control animals (n=22) to thermal stimulus exhibited base line latency time of about 12-16 sec was measured from 0-120 min. In the presence of test agents (n=9) increased in latency time (sec) from 30-60 min and this response began to slowly decline at 90 min and further decrease at 120 min. Methanol extract at 10 and 100 mg/kg failed to change the latency time while 200-300 mg/kg elicited significant analgesic activity with maximum increase of latency time 62% at 60th min of 300 mg/kg with IC50 values 289 ± 8.6, 243 ± 8.8, 297 ± 7.6 and 408 ± 4.4 mg/kg from 30-120th min. The fraction-1 had no analgesic effect at 10 mg/kg while 50-150 mg/kg demonstrated increase in latency time in dose-dependent manner with maximum pain protection 61% at 60th min of 300 mg/kg with IC50 values of 133 ± 8.8, 100 ± 5.8, 155 ± 8.6 and 213 ± 8.8 mg/kg at 30, 60, 90 and 120 min respectively. Likewise, fraction-2 non-analgesic response at 10 mg/kg and at 100-200 mg/kg significant dose dependent response with peak protection (70%) against thermal pain was achieved at 200 mg/kg at 60th min with an IC50 values of 183 ± 6.7, 133 ± 8.8, 207 ± 6.6 mg/kg and 293 ± 11.6 at 30, 60 and 90th min. The opuntiol (10-75 mg/kg) also significantly increased the pain protection with maximum analgesic effect of 63%. IC50 values were 71 ± 4.7, 58 ± 2.7, 86 ± 6.8 and 105 ± 2.9 mg/kg from 30-120th min. The opuntioside (10-30 mg/kg) also increased the hot plate reaction time in dose dependent significant manner with maximum pain protection of 68% and estimated IC50 values were 24 ± 3.2, 19 ± 1.2, 33 ± 1.4 and 35 ± 2.6 mg/kg at 30, 60, 90 and 120 min (Table-14a).

3.4.7 Effect of reference drugs and β-sitosterol on hot plate-induced jumping response in mice

Morphine at 1 mg/kg was similar to control while 5-10 mg/kg significant dose dependent analgesic effect with maximum increase in latency time pain of about 81% at 120th min of 10 mg/kg with an IC50 values of 7 ± 0.3, 5 ± 0.5, 5 ± 0.6 and 6 ± 0.1 mg/kg, at 30, 60, 90 and 120th min. Paracetamol (300-500 mg/kg) also presented significant dose dependent increase latency time and peak pain protection was 62% at 30th min of 500 mg/kg. The IC50 values were 417 ± 8.8 and 470 ± 5.8 mg/kg at 30 and 60th min and its effect declined from 90-120 min. β-sitosterol (10-30 mg/kg) also presented significant dose dependent reduction with maximum analgesic effect 76% at 120th min with an IC50 values of 27 ± 1.7, 8 ± 0.3 and 7 ± 1.4 mg/kg at 60, 90 and 120 min (Table-14b).
The order of potency at 30th min appeared to be morphine = opuntioside > opuntiol > fraction-1 > fraction-2 > methanol extract > paracetamol. At 60th min the morphine = opuntioside = β-sitosterol > opuntiol > fraction-1 > fraction-2 > methanol extract > paracetamol. At 90th min morphine = β-sitosterol = opuntioside > opuntiol > fraction-1 > fraction-2 > methanol extract. At 120th min morphine = β-sitosterol > opuntioside > opuntiol > fraction-1 > fraction-2 > methanol extract. (Table-14c).

3.4.8 Effect of *O. dillenii* methanol extract, fractions, opuntiol and opuntioside in the presence of naloxone on hot plate-induced jumping response in mice

The latency time in the presence of naloxone (2 mg /kg) was 12-16 sec from 30-120 min and similar to control which was also remained unchanged in the presence of minimum and maximum doses of extract (10 and 300 mg/kg), fraction-1 (10 and 150 mg/kg and fraction-2 (10 and 200 mg/kg), opuntiol, opuntioside (10 mg/kg), morphine (1-10 mg/kg) and β-sitosterol (1 and 30 mg/kg). While maximum doses of opuntiol (75 mg/kg) and opuntioside (30 mg/kg) presented significant increase in latency time (~51%) in the presence of naloxone (Table-14d).
4. DISCUSSION

*O. dillenii* is being used in traditional medicine against inflammation in many countries around the world including Pakistan but its mechanism of action has not yet been clearly understood. Therefore, in the present investigation its methanol extract, fractions-1 and -2 as well as α-pyrones, opuntiol and opuntioside were evaluated for their anti-inflammatory properties using a variety of animal models such as AA and TPA-induced ear edema, PLA2-induced paw edema and carrageenan-induced vascular permeability in mice. Thus allowing the assessment of panel of inflammatory mediators such as PGE2, TNF-α and ROS from ear edema, levels of LTB4 as well as anti-inflammatory mediator IL-10 to elucidate its mechanism of action. These studies were accompanied by histological changes in mice ear sections after edema induction in the presence and absence of test agents.

Herbal preparations are used globally by ~ 60% (Mondal et al, 2012) of population for the treatment of various diseases and in Pakistan ~ 75% (Qureshi et al., 2007) people rely on it as a main health care system. Despite of its popularity numerous cases of herbal toxicity have been reported (Kunle et al, 2012) therefore to minimize such incidents and ensuring their safety Organization of Economic Cooperation Development has set guidelines for the herbal products before they are marketed and reach to the people (OECD, 2001). *O. dillenii* cladodes methanol extract (1 g/kg) orally administered to mice was reported earlier to be non-toxic in mice (Saleem et al., 2005). In the present study, its higher dose (5 g/kg) did not elicit a noticeable significant change in the mice body weight (30 ± 0.9 g) up till 7 days of observation as compared to control suggesting that it has no adverse effect on the metabolic processes of the mice involved in the physiology of body weight (Cajudy and Possidio 2010). Furthermore, no abnormal behavioral or mortality occurred at this dose and hence is non-toxic.

The biochemical parameters affiliated with liver (total and direct bilirubin, alanine aminotransferase, alkaline phosphatase and gamma glutamyl transferase) and kidney (creatinine) function after treatment with *O. dillenii* did not differ significantly from control values. Furthermore, the histological studies of liver from mice at 1 g/kg *O. dillenii* methanol extract were ineffective and free of any adverse effects (Saleem et al. 2005). In our studies, mice receiving 5 g/kg *O. dillenii* methanol extract consistently for 7 days did not elicit any detectable visual changes upon autopsy to the vital organs (lungs, heart, liver and kidneys). Additionally, the architecture or morphological features of hepatic and renal cells using hematoxylin and eosin stain were intact and similar to tissues
from vehicle control animals. Thus *O. dillenii* methanol extract at 5 g/kg in mice which is equivalent to 24 g/60 kg/day in humans (Reagon-Shaw et al., 2007) is an indicative that it is neither hepato- nor nephrotoxic also donot induces behavioral changes favoring that it is non-toxic. The protective role of *O. dillenii* polysaccharides was also evident against carbon tetrachloride-induced injury of liver in mice as reflected by 70% reduction in the raised enzymatic levels (Yang et al., 2009). Consistently, another member of *Opuntia* species, *O. ficus* cladode extract (1.5 g/kg) had no adverse effects on levels of liver enzymes and also provided protection by 34% against chlorpyrifos-induced hepatotoxicity (Ncibi et al., 2008). Likewise, *O. ficus* fruit juice (3 mL/rat, p.o) was reported to reduce CCl₄-induced hepatotoxic damage in rats (Galati et al., 2005) supporting hepatoprotection but mechanism of action is not clear. One of the most popular anti-inflammatory and antioxidant spice, turmeric (*Curcumin longa*) reduced CCl₄-induced elevated levels of ALT and AST in rats (Park et al., 2010) that were associated with its active constituent curcumin. In rats *H. perforatum* (St. John’s worth, an anti-depressant) and *Camelia sinensis* extracts (green tea) reduced the raised levels of liver enzymes by ~50% and attenuated liver necrosis (Bitiren et al., 2010). It is of great concern that 34 hepatitis cases had been affiliated by frequent consumption of commercially available preparations of green tea that elevate the levels of ALP, γGT and bilirubin levels by 8-25 folds as compared to normal levels and causes liver inflammation and necrosis in humans (Mazzanti et al., 2009) implying that natural products though safe to use but at higher dosages or consumptions could manifest serious side effects emphasis that maximum tolerated dose should be taken into account. Likewise, drug such as paracetamol a popular analgesic agent (Graham and Scott 2005) at high doses also induces hepatotoxicity by raising the enzymes levels which were suppressed by established anti-inflammatory extract of *B. serrata* (Ibrahim et al., 2011).

Thus to avoid such incidences of adverse effect by *O. dillenii* consumption a detailed pre-clinical trials addressing toxicological studies including chronic toxicity, genotoxicity and teratogenicity along with its effect on reproductive, nervous and cardiovascular systems are required before it is registered as a herbal drug or else promoted as edible plant.

Considering the small amounts of fractions and pure compounds instead of animal toxicity the cellular toxicity were subjected in which methanol extract (10-1000 µg/mL), fraction-1 and -2 (10-200 µg/mL), opuntiol and opuntioside (1-100 µg/mL) were non-cytotoxic up to 1 h of incubation against rat neutrophils using tryphan blue exclusion assay and these non-cytotoxic concentrations were further used in anti-inflammatory
assays. The non-cytotoxic effect of *O. dillenii* may be due to presence of non-toxic phenolics and flavonoids such as quercetin, isorhamnetin, kaempferol and kaempferide (Yingkun et al., 2000; Ahmed et al., 2005) which also resides in extracts from other plants such as *Helichrysum italicum* (Sala et al., 2003), *Macrosiphonia longiflora* (Silva et al., 2014) and edible *Artemisia asiatica* (Jeong et al., 2014) which were also non-cytotoxic. Some constituents such as pyrrolizidine alkaloids from *Borago officinalis* *Heliotropium*, atractylosides, gummiferin from *Atractylis gummifera*, neo-clerodane diterpenoids from *Teucrium chamaedrys*, epigallocatechin gallate from *C. sinensis* (Licata et al., 2013) and aristocholochic acid from *A. fangchi* (Nortier et al., 2000) are associated with various types of toxicity while *O. dillenii* is free of such constituents and therefore also supports its non-toxic phytoconstituents nature.

Usually the acute inflammation in animals is induced by a variety of irritants applied topically such as arachidonic acid (AA) (Young et al., 1984), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), xylene (Saraiva et al., 2011), croton oil (Tonelli et al., 1965), phorbol myristate acetate (Kuehl et al., 1977), capsaicin (Gabor and Razga 1992), mustard oil (Inoue et al., 1997), ethyl phenylpropiolate (Brattsand et al., 1982) or histamine (Gabor, 2000). However, AA and TPA are most popularly employed to assess the anti-inflammatory properties of the test agents and hence were also used in the present study.

The local application of AA or TPA (phorbol ester) to mice ear caused edema and an increase in its weight (11.6 ± 0.2 mg) that was reflected by 1.5 x increase as compared to control which was within (8.5-17.8 mg) the reported limits (Bralley et al., 2008; Palacios-Espinosa et al., 2014; Garrido et al., 2006). This swelling /edema was associated with visible cardinal signs of inflammation such as redness (erythema) with marked vasodilatation visually observable as prominent blood vessels in the confined area mainly due to release of PGE\(_2\) that acts at the site of inflammation (Opas et al., 1985). It is followed by plasma extravasation (Young et al., 1984) which was noted by release of fluid after pricking the edematous tissue and was used for quantifying the inflammatory mediators as described later.

The major differences between these two edemogens are in their onset of inflammatory process and its total duration. The AA induced rapid onset of redness (erythema) occurring within 5 minutes followed by edema appearing within 15 min that persisted for shorter duration i.e. 60-90 min in agreement to previous findings (Opas et al., 1985; Rao et al., 1993; Leite et al., 2011). This rapid action is more likely due to biosynthesis of PGE\(_2\) in substantial amounts within short duration which is sufficient to
induce edema (Wu, 1998). On the other hand, TPA produced redness after 1 h of its application followed by slow and long lasting edema beginning after 1.5 h that lasted for 6-8 h and is also in agreement with the pattern reported by other investigators (Clark et al., 1985; Rao et al., 1993). This delayed action might be due to its complexity in edema induction as multiple pathways are involved including PLA₂ induction and free radical generation (Finen et al., 1984).

This edema and damages were confirmed by histological profiles of mice ear after treatment with AA and TPA which did not induce any damage to epidermis. However, both caused prominent changes including thickness of dermis, vasodilatation, neutrophils infiltration, loosening of connective tissues and disorganization of extracellular matrix fibers which was evident in the edematous tissue. Similar features have also been reported in mice ear by various investigators (Boller et al., 2010; Saraiva et al., 2011; Chibli et al., 2014). These features have also been displayed by other irritants such as mustard oil (Inoue et al., 1997), xylene (Kim et al., 2007) and after repeated exposures to UV radiations (Lavker et al., 1995). Thus both AA and TPA increased ear edema weight and also damaged histological evidences confirmed inflammation of mice ear.

These inflammatory phenotypes are highly orchestrated events that are manifested due to release of mediators like histamine, prostaglandins and leukotrienes that has been linked with AA (Opas et al., 1985; Leite et al., 2011) which enhances vasodilatation accompanied by vascular changes leading to ear edema (Murphy and Ward 2005). Similar phenomena were also demonstrated by TPA but the magnitude of damage was more pronounced similar to previous studies (Saraiva et al., 2011). This effect is more likely due to the involvement of multiple biochemical pathways as TPA stimulates protein kinase C initiating cascade of events including activation of phospholipase A₂ releasing free AA that triggers PGE₂ and LTB₄ synthesis (Clark et al., 1985; Otuki et al., 2011). Additionally, TPA also increases the production of NF-κΒ which regulates the production of other pro-inflammatory cytokines such as TNF-α, IL-1β and 6 as well as iNOS and COX-2 enzyme (Ali and Mann 2004). The histamine release (Leite et al., 2011), generation of ROS, activation and proliferation of oncogenes are also associated with TPA action (Garg et al., 2008). On the contrary, topical application of O. dillenii cladode derived methanol extract, fractions, opuntiol and opuntioside to mice ear did not elicit either irritation or redness or edema and therefore is not an irritant or causes allergic reactions. The mice ear punch weight (7.9 ± 0.3 mg) also remained unaffected implying that it is devoid of edematous properties further supporting its non-toxic nature.
The topical application of both non-steroidal (indomethacin) and steroidal (dexamethasone) anti-inflammatory drugs elicited a marked reduction (60%, at 0.08 and 4 mg/ear, respectively) in edema suggesting that both are easily absorbed and able to penetrate in the skin but former being 46x more effective. Using oral route of administration both non-selective and selective COX inhibitors also reduced AA-induced ear edema with diclofenac sodium (16 ± 0.9 mg/kg) and indomethacin being most active similar to earlier studies (Siqueira-Junior et al., 2003, Saraiva et al., 2011) following their interaction with COX pathway. Likewise, indomethacin, NDGA (LOX inhibitor) and dexamethasone (PLA2 inhibitor) also reduced TPA-induced ear edema in mice with indomethacin (12 ± 1.0 mg/kg) and dexamethasone were most potent and in agreement with the results were reported by other investigators (Palacios-Espinosa et al., 2014), Huang et al., 1991 and Saraiva et al., 2011).

On the contrary to all the reference drugs topical application of methanol extract, fraction-1 (opuntiol-rich) and -2 (opuntioside-rich) (1-20 mg/ear), opuntiol or opuntioside (0.1-10 mg/ear) failed to reduce ear edema (11.8 ± 0.2 mg). It is either due to low penetration or weak percutaneous absorption by stratum corneum, the first layer of skin, thereby restricting with its entry into the systemic circulation (Trotta et al., 2002). Likewise, extracts of Mangifera indica L. (Garrido et al., 2006) and Flos populi (Xu et al., 2014) and protein phycocyanin from microalgae (Romay et al., 1998) and luteolin (flavonoid) derived from Perilla frutescens (Ueda et al., 2002) also failed to reduce ear edema in mice when applied topically, however, produced their anti-inflammatory effects when administered orally. It is well established that orally administered drugs are absorbed by gastrointestinal tract and may require metabolic activation in the liver producing pharmacological effects (Russo et al., 2013). The orally administered methanol extract, fraction-1 and -2, opuntiol and opuntioside reduced the erythema and significantly attenuated AA-induced ear edema in a dose dependent fashion. The AA-induced ear edema is associated with activation of COX pathway (Arner et al., 1985; Opas 1985; Carlson et al., 1985; Pignat et al., 1986; Gabor, 2000) without being involvement of LOX (NDGA) and PLA2 (dexamethasone) inhibitor. Therefore; it is suitable model for COX pathway. On the basis of IC50 values opuntioside (15 ± 1.7 mg/kg) was most potent among plant derived test agents and equipotent to non-selective COX inhibitor indomethacin (18 ± 0.9 mg/kg) and to commonly prescribed anti-inflammatory diclofenac sodium (NSAIDs) but it was 6 folds more potent than the selective COX-inhibitor celecoxib (95 ± 5.1 mg/kg) implying that anti-inflammatory action of O. dillenii derived test agents predominantly opuntioside is also directed
towards COX pathway. Previously reported *O. dillenii* aqueous ethanol extract of cladode produced anti-edematous response against xylene-induced ear edema in mice (Qiu et al., 2000) and its fruit extract inhibited the edema by 53% (Loro et al., 1999) however, its isolated compounds were not identified yet for their anti-inflammatory activity. Other plant extracts from *M. indica* L (Garrido et al., 2006), *Forsythiae fructus* (Kang et al., 2008) and patuletin and patulitrin (flavonoids) from French marigold (Yasukawa and Kasahara 2013) and curcumin (diarylheptanoid) from turmeric (Huang et al., 1991) also reduced AA-induced ear edema in mice favoring that they are interfering with the COX pathway.

In case of TPA all plant derived test agents administered orally also significantly reduced the ear edema supporting their anti-inflammatory properties with opuntiol and opuntioside being most potent in augmenting the edema reduction with similar magnitude (IC$_{50}$ = 85 ± 5.3 mg/kg). All the reference drugs used i.e. indomethacin, dexamethasone, celecoxib and NDGA were 1-7x more effective in antagonizing the TPA induced edema as compared to *O. dillenii* derived test agents. Thus the reduction in TPA-induced edema by diverse group of reference drugs used clearly suggest that multiple pathways such as PLA$_2$, COX and LOX are involved in their anti-inflammatory action and these results are in agreement with those reported previously (Murakawa et al., 2006) and also suggestive that *O. dillenii* derived test agents may also act via these mechanisms. TPA-induced ear edema in mice via aforementioned pathways has also been demonstrated by other plants extracts including *Cortex phellodendri Chinensis, C. phellodendri Amurensis* (Xian et al., 2011), *Polygonoum cuspidatum* (Bralley et al., 2008) and flavonoids, patuletin and patulitrin from French marigold (Yasukawa and Kasahara 2013), curcumin from turmeric (Huang et al., 1991), boswellic acid from *B. carteri* (Banno et al., 2006) and β-sitosterol (sterol) from *Buddleja globosa* (Backhouse et al., 2008) supporting their anti-inflammatory properties.

The IC$_{50}$ values of *O. dillenii* methanol extract, fraction-1 and -2, opuntiol, opuntioside and reference drugs derived from AA- and TPA-induced ear edema were used in further experiments as described below:

**a)** The histological profiles of AA and TPA sensitized ear punches revealed that all test agents at their corresponding IC$_{50}$ values reduced the thickness of dermis, vasodilatation, intensity of edema, loosening of connective tissues, disorganization of fibers and recruitment of neutrophils which are in parallel with reduction in ear weight described above thereby confirming their anti-inflammatory action histologically. However, these effects were more pronounced with indomethacin and dexamethasone as
compared to plant derived agents. Some of these pathological changes were also suppressed by other plant extracts originated from extracts of Baccharis illinita (Boller et al., 2010), Protium kleinii (Otuki et al., 2005), Bryphyllum pinnatum (Chibli et al., 2014) and also fixed oil extracted from Caryocar coriaceum (Saraiva et al., 2011).

b) Since, TPA activates phospholipase A2 (PLA2) therefore PLA2-induced paw edema in mice in the presence of O. dillenii derived test agents was also investigated. It is well known that PLA2 is precursor of biosynthesis of various inflammatory mediators such as eicosanoids (Dennis et al., 2011) and also trigger the release of histamine, serotonin from mast cells (Cirino et al., 1989). The sub-plantar injection of PLA2 (2 units/25 µL) produced redness and swelling as reflected by increase in paw volume in a time dependent manner reaching its maximum after 60 min followed by a 37% decline at 90th min. Similar pattern of paw edema has been noted earlier (Sala et al., 2003; Giner-Larza et al., 2001). All the reference drugs including dexamethasone, indomethacin and cyproheptadine (combined antagonist of histamine and serotonin) reduced the paw edema as expected by blocking PLA2 action which mediated AA pathway and suppressed the release of histamine and serotonin (Giner-Larza et al., 2001), respectively. The O. dillenii derived test agents also significantly reduced paw edema with opuntioside being most potent either by inhibiting either PLA2 enzyme directly consequently blocking AA pathway or indirectly by interfering mast cell degranulation. Flavonoid tiliroside from Helichrysum italicum (Sala et al., 2003) also reduced paw edema by same mechanism. To confirm similar findings in O. dillenii further experiments on mast cell models, quantification of PLA2 levels (Dorsam et al., 1995) and histamine-induced paw edema (Billci et al., 2001) are required. Although, O. dillenii is effective against acute inflammatory animal models but its effect on chronic inflammatory animal models such as cotton-pellet-induced granuloma (Swingle and Shideman 1972) and arthritis-induced by Freund’s adjuvant (Latha et al., 1998) needs to be evaluated.

c) It is well established that PGE2 stimulates erythema, increase blood flow, promotes edematous response (Williams and Peck, 1977) and involved in several inflammatory diseases such as rheumatoid arthritis and atopic dermatitis (Sheng et al., 1998). Its basal levels after AA or TPA application on mice ear were increased by 4 folds (164 ± 7.0 pg/punch) justifying the cardinal signs of inflammation i.e. redness, vasodilatation and edema. However, these levels were ~55x lower than the reported (~9000 pg/punch) values range (Sanchez and Moreno 1999, Lloret and Moreno 1995). This tremendous variation in the levels of PGE2 is more likely due to 4x higher concentration of TPA (10 µg/ear) used but the differences in the strains of mice such as
Swiss Webster (Lloret and Moreno 1995) used by them cannot be ignored which may also affect their response to the inflammatory agents. The release of PGE2 at inflammatory site is an indicator of involvement of COX activity (Carlson et al., 1985; Wu, 1998; Gabor 2000) and is a most reliable molecule used for screening of anti-inflammatory agents. Nevertheless, the increased levels of PGE2 were significantly reduced by dexamethasone, celecoxib and indomethacin favoring the earlier reports (Siqueira-Junior et al., 2003; Saraiva et al., 2011). Similar pattern was also demonstrated by O. dillenii derived test agents among which opuntioside was most potent (63%) and 1.3x better than opuntiol. Consistently other plant extracts from C. phelloendri and C. phelloendri Amurensis (Xian et al., 2011), curcumin (Huang et al., 1991) and resveratrol from red grapes (Richard et al., 2005) also reduced the PGE2 levels and hence are reputable COX pathway inhibitors. It is noteworthy, that opuntioside (a glucoside) was 1.3x better in its PGE2 inhibitory action as compared to its aglycone partner, opuntiol possibly due to presence of sugar moiety which possibly plays an important role in its faster absorption in the gastrointestinal tract (Hempel et al., 1997; Guardia et al., 2001) and hence more effective at low doses. However slower absorption of opuntiol may due to presence of one –OH group as previously described in aglycone moiety the position and number of –OH group plays important role in absorption (Tian et al., 2009). Similar phenomenon has been observed with aglycone quercetin which was also absorbed slowly compared to its glucoside moiety (Erlund et al., 2000). The preference of glucoside moiety towards COX pathway particularly for PGE2 inhibition was also reported for compounds belonging to different chemical classes as phenylpropanoid glycosides (angoroside A, -C, -D and isoacetoside) derived from Scrophularia scorodonia L that selectively inhibited COX pathway without affecting the LOX pathway (Diaz et al., 2004) further emphasizing the importance of glucose moiety in the former case. Since, opuntioside was comparable to indomethacin and celecoxib in suppressing PGE2 levels via their interaction with COX pathway thereby alleviating inflammation emphasizes that its effect on COX-2 gene expression should be investigated using real time-polymerase chain reaction method (Xian et al., 2011) which will shed light on its anti-inflammatory action at the molecular level.

d) During the process of inflammation LTB4 has a major contribution as a chemotactic agent for neutrophils, promoting its movement (Casale et al., 1992) and also increasing the vascular permeability (Feinmark et al., 1981). Its elevated levels are linked with several inflammatory diseases such as asthma and chronic obstructive pulmonary diseases (COPD) (Wilborn et al., 1996). The glycogen-induced peritonitis in rats has been
shown to increase concentration of neutrophils in the peritoneal cavity by 4 fold reaching to ~14-23 million (Yamashita et al., 1982) providing its sufficient numbers and hence high levels of LTB₄ is made available as compared to PGs levels under same conditions (Lewis and Austen 1988) thereby it is a most suitable model for LTB₄ study. The raised levels (8.06 ± 0.5 ng/mL) of LTB₄ after glycogen administration were significantly reduced in the presence of NDGA (LOX inhibitor) and quercetin (an anti-oxidant) with IC₅₀ values of 0.5 µg/mL and 1.7 µg/mL respectively, due to suppression of the LOX activity as described earlier (Ammon et al., 1991). Likewise, the methanol extract, fraction-1 and -2 and opuntiol also suppressed the LTB₄ levels with opuntiol (IC₅₀ = 19 ± 3.3 µg/mL) being most potent while opuntioside was ineffective. Other plant extracts with reputed anti-inflammatory activities e.g. gum resins of B. serrata (Ammon et al., 1991), A. corniculatum (Roome et al., 2008b) and oleanonic acid from Pistacia (Giner-Larza et al., 2001) also suppressed LTB₄ levels. Thus confirming that reduction in their levels also contributes to their anti-inflammatory action.

Following earlier contention opuntioside, an esterified product of opuntiol lack of free OH group but containing glucose moiety was completely ineffective against LOX pathway suggesting that its anti-inflammatory action is not via LOX pathway. On the contrary it was most effective in reducing PGE₂ levels as compared to opuntiol emphasizing that it is selective for COX pathway as described above. Thus opuntiol bearing free hydroxyl group at position 7 is more likely to be responsible for LOX inhibition as has also been described by Kim et al. (2004) and Werz and Steinhilber (2006). This trend has been displayed by other molecules as one of the derivative of coumarin, an esculetin (6,7-dihydroxycoumarin) an aglycone was 445x more potent LOX inhibitor in comparison to its esterified product i.e. esculin (Sekiya et al., 1982) further strengthening the contention that the OH group is crucial for LOX activity (Torres et al., 2013) it may due to association between LOX inhibiting and anti-oxidant activity. The hydrogen atoms of hydroxyl groups may react with ROS (Lu et al., 2010). In traditional medicine the LOX inhibitor, NDGA is used in the management of inflammation and pain (Rahman et al., 1992) but various side effects such as nausea, memory disturbance and hepatic dysfunction (Hope et al., 1983) are associated with it due to its inhibitory effect on multiple enzymatic pathways. Other frequently used LOX inhibitors such as montelukast and zafirlukast which are effective against chronic asthma but also induces adverse effects like fever and infection of upper respiratory tract system (Knorr et al., 2001). Thus opuntiol could be an appropriate candidate to be included in the discovery of new LOX inhibitor research parade however, experiments with animal model for asthma
(Wu et al., 2003) are required to confirm its in vivo action. The reason that it is a most promising candidate in the treatment of asthma is that O. dillenii is already being used in traditional medicine against asthma (Nadkarni’s, 1976).

e) The ROS stimulates production of a variety of cell mediators such as LTB₄, TNF-α, IL-1 and -8 responsible for increasing vascular permeability and also damages other macromolecules like lipids and DNA ultimately leading to cellular and tissue impairments and contribute in promoting various inflammatory diseases such as atopic dermatitis (Okayama, 2005), atherosclerosis and neurodegenerative disorders (Manton et al., 2004). The basal levels of ROS were raised by 4 folds (19308.87 ± 419 RFU) after TPA application which was within the reported range (~26000 RFU) (Xian et al., 2011). These high levels were attenuated in the presence of O. dillenii derived test agents with opuntiol being most potent indicating that they all possess anti-oxidant properties. The NDGA as reported earlier (Rameron et al 1998) also caused maximum ~40% reduction in ROS levels due to the presence of -OH group (Rice-Evans et al., 1996; Wang et al., 2006). Since opuntiol and NDGA were equipotent against ROS due to the presence of free -OH group in their skeleton and may be responsible for their anti-oxidant effect. In this regard, opuntiol was 1.1 fold better than opuntioside (an esterified product) and emerged as a potent LTB₄ inhibitor. Earlier α-pyrene containing compounds have been shown to interfere with 5-LOX inhibition due to their anti-oxidant activities by uncoupling redox cycle at the 5-LOX active site containing iron (Napagoda et al., 2014). The 5-LOX inhibiting activity has been reported to be interlinked with anti-oxidant properties (Werz and Steinhilber 2006). The fruit extract of O. dillenii (Chang et al., 2008), O. ficus cladodes extract (Lee et al., 2002) as well as both opuntiol and opuntioside (Qiu et al., 2002) were described as anti-oxidant. However additional experiments in future are needed to identify the nature of free radical species scavenged by O. dillenii such as hydroxyl radical (OH⁻), hydrogen peroxide (H₂O₂), hydroxide anion (OH⁻) and hypochlorous acid. Other plant extracts which reduced the TPA-induced ROS generation are C. phellodendri Chinensis, C. phellodendri Amurensis (Xian et al., 2011) and Garcinia gardneriana (Otuki et al., 2011).

f) Since, histological profiles of AA and TPA sensitized ear punches qualitatively demonstrated vasodilatation and infiltration of neutrophils at the site of inflammation, further prompted us to conduct vascular permeability experiments. The intensity of leakage of Evans blue dye into the peritoneal cavity of mice is generally considered as an index of vessels leakage which is quantifiable. In control mice it was (10 ± 0.4 µg/mL) within the reported limits (1-17.2 µg/mL) (Longhi-Balbinot et al., 2012;
Smiderle et al., 2008; Silva et al., 2003). This dye leakage is accompanied by the release of PGE₂, LTB₄, histamine, serotonin (Anderson et al., 2000) and TNF-α (Kolaczkowska et al., 2002) which were reduced in our study by indomethacin and dexamethasone (IC₅₀: 5 ± 0.1 mg/k) with a similar magnitude and has also been reported earlier (Longhi-Balbinot et al., 2012). The opuntioside and fraction-2 (opuntioside-rich) were twice more potent in reducing vessels leakage than opuntiol. This important observation again indicates that opuntioside which predominantly inhibited PGE₂ is mainly responsible for this activity. Likewise, isolated triterpene from Combretum leprosum and arctigenin from F. fructus reduces vascular permeability (Kang et al., 2008).

g) It is well known that TNF-α stimulates PGE₂, nitric oxide (NO) production and enhances nociceptors sensitization (Cunha et al., 1992) and all of them participate in increasing vascular permeability that is reflected in the form of fluid extravasation and leukocytes infiltration (White, 1999; Aggarwal et al., 2012) at the site of inflammation. High levels of TNF-α have been implicated in several inflammatory diseases such as inflammatory bowel disease (Carswel et al., 1975), cancer (Aggarwal, 2003) and Alzheimer’s disease (Gaur and Aggarwal 2003). It basal levels were raised by two folds in TPA-induced ear edema (512 ± 15.7 pg/punch) which were 25x higher than the reported values (Xian et al., 2011). This variation in the levels of TNF-α is more likely due to different strains of mice (NMRI vs ICR). These raised levels were significantly reduced in the presence of O. dillenii derived test agents with ~ 50% reduction by opuntiol and fraction-1 (opuntiol-rich) which was comparable to dexamethasone. The reduction in TNF-α level by dexamethasone has been linked with the NF-κB attenuation (Borges et al., 2014) via cytokines genes regulation (Barnes, 1999). Other plant extracts of C. phellodendri chinensis and C. phellodendri amurensis (Xian et al., 2011), isolated allantopyrone A (Yokoigawa et al., 2014), turmeric (Lantz et al., 2005) and resveratrol inhibited TNF-α levels (Heynekamp et al., 2006). It is noteworthy that both TNF-α and LTB₄ are predominant inflammatory mediators of chronic obstructive pulmonary disease (COPD) and poorly respond to anti-inflammatory drugs (Pang et al., 2011), thus opuntiol could be an appropriate choice to be included for further experiments of COPD animal models (Demeds et al., 2006).

Thus the raised levels of TNF-α in TPA-induced ear edema is responsible for vessels leakage that could be clearly depicted in histological profiles of mice ear. It also increased vascular permeability in carrageenan-induced peritonitis thereby promoting the inflammation. The α-pyrone, opuntiol predominantly attenuated TNF-α levels supporting its anti-inflammatory role. On the other hand, opuntioside also reduced TNF-α levels by
1.4x less potently than opuntiol but in case of carrageenan-induced vascular permeability the opuntioside was 2x better in reducing vessels leakage than opuntiol thereby, it is speculated that perhaps differences in the processes of vascular inflammatory mechanisms may be responsible for this differentially affected responses i.e. opuntioside suppresses the vascular permeability via COX pathway whereas opuntiol via cytokines attenuation. TNF-α suppression is one of the consequences of down regulation of transcription factor NF-κB which can be performed by western blotting (Choi et al., 2013) in future experiments.

h) The pro-inflammatory response of body against foreign stimuli is essential for defense against the pathogens. Conversely, it is necessary to limit and resolve the inflammatory process to avoid damaging the host itself and hence blocked by various anti-inflammatory cytokines including IL-10 (Asadullah et al., 2003) by controlling the homeostatic mechanisms. Although it is important for the resolution of inflammation but its excessive production may also compromise the host defenses mechanism leading to pathogens persistence (Jusek et al., 2012). After the carrageenan-induced peritonitis the basal levels of IL-10 were increased by 4 folds (1171 ± 8.7 pg/mL) which were within (60-1500 pg/mL) the reported range (Longhi-Balbinot et al., 2012; Silva et al., 2014). These levels were reduced by dexamethasone similar to earlier reports (Silva et al., 2014). *O. dillenii* derived methanol extract significantly reduced its levels by 36% which was equivalent to dexamethasone, however, fraction-1 and -2, opuntiol and opuntioside were unchanged suggesting extract which consist of mixture of phytoconstituents may produced synergestic effect in attenuation of raised levels. Similarly *Macrosiphonia longiflora* extract attenuated its levels (Silva et al., 2014).

Anti-inflammatory and analgesic activities are interrelated (Panday and Rizvi 2009) as they share many damaging molecules such as PGE$_2$, nitric oxides, ROS, leutotrienes, serotonin, histamine and TNF-α involved in events common in eliciting inflammation and pain that sensitize the peripheral nociceptors (Coutaux et al., 2005; Zakaria et al., 2010). It has also been noted that most of anti-inflammatory agents also display analgesic effect (Dewanjee et al., 2009) therefore *O. dillenii* derived test agents were also evaluated for their analgesic potential using chemical (acetic acid-induced writhing and formalin-induced paw licking test) and for thermal nociceptive stimuli (hot plate-induced jumping response) in mice.

Since writhing test alone cannot ascertain the analgesic action due to its low specificity to differentiate between peripheral or central effect (Sianchez-Mateo et al., 2006) by the involvement of sympathetic system *via* release of biogenic amines and AA
metabolites (Andrade et al., 2007) making it difficult to draw definite conclusions therefore, other paradigms such as formalin-induced paw licking and hot plate-induced jumping response were also performed.

It is well established that acetic acid-induced vicosomatic pain in the form of writhes mediated by mast cells of peritoneal cavity (Ribeiro et al., 2000) and late phase of formalin test (paw licking response) promotes tissue injury, inflammation and peripheral pain via stimulation of primary afferent sensory Aδ and C nerve fibers (Shibata et al., 1989). These responses are associated with the release of PGE₂ by sensitizing peripheral pain through activation of their receptors residing on the peripheral terminals of sensory neurons (Lin et al., 2006) and are accompanied with release of other mediators such as histamine, bradykinin, LTs and TNF-α (Ribeiro et al., 2000; Shibata et al., 1989). The acetic acid induced writhes (50 ± 0.8) in mice and formalin induced paw licking response (106 ± 2.5 sec) were within corresponding reported limits (32-80 and 70-200 sec) (Longhi-Balbinot et al., 2012; Silva et al., 2003; Abdollahi et al., 2003; Khan et al., 2010 and Rocha et al., 2005; Santos et al., 2010). Previously, lyophilized fruit extract of O. dillneii demonstrated analgesic actions against acetic acid induced writhes in mice (Loro et al., 1999). Consistently, in the present study both peripherally mediated writhes and paw licking responses were significantly inhibited by the methanol extract of O. dillenii cladodes and its fractions and derived pure compounds. Among which opuntioside emerged as 4 folds better anti-nociceptive than opuntiol possibly due to its more selectivity towards PGE₂ inhibition. It is further supported by 1.3x greater reduction in PGE₂ levels by opuntioside as compared to opuntiol emphasizing and confirming its selectivity towards COX pathway. However, the role of absorption factor cannot be undermined which may also contribute to its profound analgesic effect due to presence of glucose moiety whose importance has been highlighted earlier by GI tract (Guardia et al., 2001). Myricitrin a flavonol glycoside of myricetin (aglycone) significantly reduced the paw withdrawal response in a chronic inflammatory pain than its aglycone partner (Meotti et al., 2006). In addition the reduction in pain by O. dillenii derived test agents may also due to the suppression in the levels of LTB₄ and TNF-α but the effect of opuntioside was more pronounced against PGE₂. In parallel, indomethacin, diclofenac sodium, acetyl salicylic acid and morphine and β-sitosterol also reduced the aforementioned peripheral responses which were also described by (Rao et al., 2007; Roome et al., 2011 and Villasenor et al., 2002) supporting their actions via COX pathway and peripherally mediated opioid receptors (Farshchi et al., 2009; Trongsaskul et al., 2003). Other plant extracts such as A. corniculatum (Roome et al., 2011), A. catechu
(Khan et al., 2011 as well as α and β amyrin from B. globosa (Backhouse et al., 2008) also attenuated writhes in mice probably via PGs inhibition.

The neurogenic (spinal/supraspinal) responses representing the early phase of biphasic formalin test promotes the release of variety of mediators such as substance P, bradykinin, histamine and serotonin (Hunskaar and Hole 1987; Shibata et al., 1989). Likewise, hot plate test is an acute, non-inflammatory and supraspinal reflex (Pini et al., 1997) exhibits jumping response. It is to be emphasized that COX-2 is constitutively expressed in the dorsal horn region of spinal cord which is up-regulated by the action of stimuli and therefore facilitates the transmission of nociceptive input (Luo et al., 2005) affecting the levels of various PGs particularly PGE2. Various studies described that prostaglandin administration into the spinal cord enhances nociception (Tegeder et al., 2001). During inflammatory state COX-2 is also expressed in microglial cells thereby providing important source of PGs synthesis (Kang et al., 2004). In the current study both centrally mediated pain-induced responses viz paw licking (106 ± 2.5 sec) and jumping latency time (12-16 sec) were within corresponding (70-200 sec and 8-20 sec) reported limits (Rocha et al., 2005; Khan et al., 2010; Santos et al., 2010 and Alvarenga et al., 2013; Wei et al., 2011). However, in the presence of O. dillenii derived test agents the paw licking responses were reduced while jumping latency time was increased. Among all, the opuntioside were ~3 fold more potent in relieving pain as compared to opuntiol possibly due to its more selectivity towards PGE2 inhibition via reduction in PGE2 levels as described previously by (Smith et al., 1998). The centrally mediated pain induced by hot plate was also reported to be reduced by lypholized fruit extract of O. dillenii (Loro et al., 1999). This led us to suggest that O. dillenii derived test agents particularly opuntioside interrupts centrally mediated pain via its PGE2 inhibitory properties as explained above in mice ear punches. All the reference drugs such as morphine, paracetamol and β-sitosterol demonstrated pain relieving effects in both animal models which are in line with previous studies (Chen et al., 2013, Khan et al., 2010 and Villasenor et al., 2002).

Naloxone, a non-selective opioid receptor viz μ, δ and κ receptors (Almeida et al., 2001) antagonist reversed the centrally mediated analgesic effect induced by morphine in all the aforementioned animal pain models and is in complete agreement with earlier reports (Villasenor et al., 2002; Roome et al., 2011). Likewise, it also completely blocked the analgesic effect elicited by O. dillenii methanol extract, fraction-1 and -2 and also by β-sitosterol, implying that their analgesic actions are also neurogenic in nature via opioid receptors at spinal/supraspinal level. However, specific receptor type(s) involved needs to
be identified. On the contrary, the centrally mediated analgesic effect elicited by opuntioside and opuntiol could not be antagonized by naloxone indicating that their effect is opioid independent but involvement of other centrally mediated control such as analgesic effect via COX-2 pathway cannot be ignored (Smith et al., 1998). Assuming that *O. dillenii* test agents crosses the blood brain barrier it is suggested that they exert dual pain relieving analgesic effect by acting at both peripheral and central level via inhibition of prostaglandin synthesis.

The most prescribed anti-inflammatory and analgesic drugs are NSAIDs acts via COX pathway and also exhibit antipyretic activity (Ganong, 2001). Therefore, the *O. dillenii* derived test agents predominantly opuntioside which also act via COX pathway (inhibition of PGE$_2$) and may be endowed with anti-pyretic properties cannot be ruled out therefore, needs to be investigated. However, there are many undesirable effects associated with NSAIDs such as ulcer, erosion of stomach lining (Ehsanullah et al., 1988) and liver dysfunction (Lin et al., 2006). On the other hand the present study advocates that *O. dillenii* is an anti-inflammatory via inhibition of PGE$_2$, LTB$_4$, TNF-$\alpha$ and ROS and also an analgesic (peripheral and centrally mediated) but free of hepato- or renal toxic effects. Beside these scientific evidences the use of *O. dillenii* traditionally in the management of inflammation, wound healing as well as in gastric ulcer provides support that it may be a better option than NSAIDs and requires in depth pre-clinical studies.

The contention that anti-inflammatory and analgesic activities are interrelated was also maintained in the present study conducted on *O. dillenii* most probably due to common phytoconstituents responsible for these activities. The cladodes of *O. dillenii* contains a variety of compounds including flavonoids: quercetin, 3-O-methyl quercetin, isorhamnetin, kaempferol and kaempferide (Yingkun et al., 2000), isorhamnetin (Ahmed et al., 2005), isorhamnetin-3-O-galactoside and isorhamnetin-3-O-rutinoside (Gupta et al., 2002); phenolics (Zhao et al., 2007); $\alpha$-pyrones opuntiol and opuntioside (Qiu et al., 2000); $\beta$-sitosterol (Stintzing and Carle 2005); betalains (Feugang et al., 2006), vitamin C (Zhao et al., 2007). In the present studies the methanol extract of *O. dillenii* cladodes and its petroleum ether, ethyl acetate and methanol fractions besides being rich in opuntiol and opuntioside also flavonoids and phenolics extracted in these solvents as previously described these solvents prefer recovery of flavonoids and phenolics (Hincapie et al., 2011; Saeed et al., 2010) which appears to be responsible for various pharmacological activities. This was reflected by *O. dillenii* alcohol extracts derived from flowers, fruits and cladodes demonstrating anti-inflammatory and centrally mediated analgesic activity in animal models. Among them flowers bearing high levels of flavonoid glycosides
kaempferol 3-O-α-arabinoside, isorhamnetin-3-O-rutinoside and isorhamnetin-3-O-glucoside) eliciting most pronounced effect (Ahmed et al., 2005). An association between anti-inflammatory (Guevence et al., 2009) and analgesic activity (Rajnarayana et al., 2001) has been proposed to be associated with flavonoids (quercetin, isorhamnetin and kaempferol), polyphenols (Kao et al., 2007) via inhibition of PLA₂ (Nijveldt et al., 2001) and COX and LOX pathways (Rotelli et al., 2003). Thus the anti-inflammatory activities of O. dillenii via PLA₂, COX and LOX pathways, in addition to the aforementioned compounds is also due to the presence of opuntiol and opuntioside reported for the first time here. The pyrone ring is common to opuntiol, opuntioside quercetin and kaempferol (Loggia et al., 1988) and arzanol (Kothavade et al., 2013) and all of them attenuated PGE₂ and LTB₄ production in several ear and paw edema and peritonitis models in rodents including the present study. The pyrone-rich extracts from other plants viz Torresea cearensis (Leal et al., 1997) and Justicia pectoralis Jacq a popular medicinal herb in Brazil used for the treatment of pain (Lino et al., 1997) and isolated pyrone containing compounds such as esculentin (6,7 dihydroxycoumarin), fraxetin and daphnetin (Neichi et al., 1983) also inhibited COX (PGs) and LOX (LTs) metabolites. Interestingly, opuntioside (glucose moiety) displayed potent COX inhibiting activity via inhibition of PGE₂ while opuntiol (aglycone) though emerged as a dual inhibitor of COX/LOX pathway with preference toward LTB₄ a LOX pathway metabolite thereby, contributing in both anti-inflammatory and analgesic effects.

A phytosterol, β-sitosterol along with α-pyrene coumarin and styrylpyrones rich Polygala sabulosa extract showed anti-inflammatory effects (Borges et al., 2014) while β-sitosterol from B. globosa (Backhouse et al., 2008) and from leaves of Nyctanthes arbortristis (Nirmal et al., 2012) also exhibited peripheral and centrally mediated pain relieving properties (Villasenor et al., 2002). Thus O. dillenii extract and fractions containing phytosterol and α-pyrene opuntiol and opuntioside may synergistically be acting as anti-inflammatory and as analgesic.

A pro-inflammatory mediator TNF-α was attenuated by flavonoid, isorhamnetin derived from Oenanthe javanica (Yang et al., 2013). Furthermore, its antioxidant effects along with that of quercetin and rutin were also evident by stimulating immune system against ROS production (Paya et al., 1992; Nakamura et al., 2003). Other pyrone containing compounds, allantopyrone A consistently attenuated TNF-α levels (Yokoigawa et al., 2014) while fraxetin and daphnetin (Fylaktakidou et al., 2004) reduced levels of free radicals supporting the anti-oxidant activity of pyrones. Furthermore, the dietary components like betalains (indole derived pigments) in endothelial cells model
(Gentile et al., 2004) and vitamin C (Zielinska et al., 2001) residing in extract of *O. ficus indica* as well as opuntiol and opuntioside (Qiu et al., 2002) displayed appreciable antioxidant activities. Therefore, in present study reduction in the reduction in LTB₄ and ROS levels by *O. dillenii* may due to its anti-oxidant properties.

Therefore, in the light of literature reports and present investigation it is speculated that flavonoids, phenolics, pyrone ring particularly opuntiol and opuntioside, indole derived pigments and sterols residing in *O. dillenii* are contributing to its anti-inflammatory and analgesic effects. The findings of present study on *O. dillenii* are summarized in Figure-20 and 21 and the proposed mechanism of anti-inflammatory and analgesic action is highlighted in Figure-22.

It is concluded that the methanol extract, fraction-1 and -2, opuntiol and opuntioside derived from cladodes of *O. dillenii* significantly attenuated the levels of inflammatory COX (PGE₂) and LOX (LTB₄) metabolites as well as pro-inflammatory mediators ROS and TNF-α. However, levels of LTB₄ were not reduced by opuntioside possibly due to replacement of -OH group in its structure by glucose at position 7 and its selectivity towards COX inhibitors like celecoxib, diclofenac sodium and indomethacin. On the other hand opuntiol has emerged as a dual COX/LOX inhibitor and also attenuated ROS and TNF-α like NDGA and quercetin supporting its preference towards LOX pathway. The opuntioside effectiveness than opuntiol as an analgesic agent acting at both peripheral and central levels is possibly due to structural differences between them and preference towards COX pathway. Thus present findings have demonstrated that edible *O. dillenii* cladodes exhibited anti-inflammatory and analgesic activities which are mediated via multiple mechanisms of actions and hence provide scientific justification for its traditional use against inflammation and pain and qualify to be included in the natural product drug discovery program.