2. Literature Review

2.1. Inflammation

2.1.1. Introduction to Inflammation

It is a dynamic, beneficial and defensive tissue response to protect the host body against invasion by pathogen that eliminates the spread of pathogenic agents and to remove necrosed cells and tissues (Withers, 1992). The various causative factors responsible for inflammations are;

- Physical agents: Heat, Cold, Trauma, Radiation
- Chemical agents: Organic and Inorganic poisons
- Infective agents: Bacteria, Viruses, Toxins
- Immunological agents: Cell mediated reactions and Antigen antibody reactions (Kumar et al, 2003).

The inflammatory response is a temporally and spatially orchestrated event in that inflammatory cells and mediators work together to deactivate and abolish the damaging stimuli to maintain the homeostasis (Medzhitov, 2010; Cotran et al., 1999). Inflammation is primarily a physiological, beneficial process, non-resolving processes that involved in the pathogenesis and progression of many inflammatory diseases such as atherosclerosis, asthma, rhinitis, rheumatoid arthritis, multiple sclerosis and ischemia–reperfusion injury (Waldburger & Firestein, 2009; McFarland & Martin, 2007; Eltzschig & Eckle, 2011; Nathan & Ding, 2010; Chung, 2012; Mandhane et al., 2011; Van-Assche et al., 2011). The inflammatory reaction are characterised by five cardinal signs such as calor (heat), rubor (redness), tumor (swelling), dolor (pain) and function laesa (loss of function), described by Cornelius Celsus in the first century.

2.1.2. Types of Inflammation

Inflammation is divided into two types such as, Acute Inflammation and Chronic Inflammation. Acute inflammation is a physiological innate host response to injury aimed at removing pathological noxae and restoring homeostasis (Majno and Joris, 2004).
enrollment of leukocyte subtypes such as polymorphonuclear neutrophils (PMN) neutrophils is the important feature of acute inflammation, that accumulates in inflamed tissues to attack the pathogenic agents (thermal, microbial, mechanical, etc.) followed by accumulation of monocytes, that distinguish into macrophages within the tissue (Lucas et al, 2010). The acute inflammatory response is initiated by tissue damage or invasion by pathogens. It is protective mechanism and vital to health, but when acute inflammation is unrestrained in amplitude or duration, it can lead to disease. The acute inflammation is mostly self-limited. The alternate non-resolving paths for acute inflammation include abscess formation, fibrosis, or conversion to chronic inflammation (Figure 2.1). Chronic inflammation is of longer duration and occurs either after the causative agents of acute inflammation persists for long time or the stimulus is such that it induces chronic inflammation from the beginning. It is associated with several common diseases, including diabetes, asthma, inflammatory bowel disease, cardiovascular diseases, periodontal disease, psoriasis and rheumatoid arthritis (Kumar et al, 2003).

Figure 2.1 Mechanism and cardinal signs of Inflammation

2.1.3. Pathogenesis of Inflammation

The acute inflammatory response is triggered by infection or tissue injury involves the synchronized delivery of blood components (plasma and leukocytes) to the site of injury (Majno and Joris, 2004; Kumar et al, 2003). This response has been characterized best for
microbial infections (particularly bacterial infections), in which it is triggered by receptors of the innate immune system, such as Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs) (Barton, 2008). This initial recognition of infection is mediated by tissue resident macrophages and mast cells, leading to the production of a variety of inflammatory mediators, including chemokines, cytokines, vasoactive amines, eicosanoids and products of proteolytic cascades. The main and most immediate effect of these mediators is to elicit an inflammatory exudate locally: plasma proteins and leukocytes (mainly neutrophils) that are normally restricted to the blood vessels now gain access, through the postcapillary venules, to the extravascular tissues at the site of infection (or injury). The activated endothelium of the blood vessels allows selective extravasation of neutrophils while preventing the exit of erythrocytes. This selectivity is afforded by the inducible ligation of endothelial-cell selectins with integrins and chemokine receptors on leukocytes, which occurs at the endothelial surface, as well as in the extravascular spaces (where newly deposited plasma proteins form a provisional matrix for the binding of leukocyte integrins) (Pober and Sessa, 2007). When they reach the afflicted tissue site, neutrophils become activated, either by direct contact with pathogens or through the actions of cytokines secreted by tissue-resident cells. The neutrophils attempt to kill the invading agents by releasing the toxic contents of their granules, which include reactive oxygen species (ROS) and reactive nitrogen species, proteinase (Barton, 2008), cathepsin G and elastase (Nathan, 2006). These highly potent effectors do not discriminate between microbial and host targets, so collateral damage to host tissues is unavoidable (Nathan, 2002). A successful acute inflammatory response results in the elimination of the infectious agents followed by a resolution and repair phase, which is mediated mainly by tissue-resident and recruited macrophages (Serhan and Savill, 2005). The switch in lipid mediators from pro-inflammatory prostaglandins to lipoxins, which are anti-inflammatory, is crucial for the transition from inflammation to resolution. Lipoxins inhibit the recruitment of neutrophils and, instead, promote the recruitment of monocytes, which remove dead cells and initiate tissue remodelling. Resolvins and protectins, which constitute another class of lipid mediator, as well as transforming growth factor-β and growth factors produced by macrophages, also have a crucial role in the resolution of inflammation, including the initiation of tissue repair (Serhan and Savill, 2005; Serhan, 2007).
2.1.4. Inducers of inflammation

The Inducers of inflammation can be classified into exogenous or endogenous.

2.1.4.1. Exogenous inducers of inflammation

Exogenous inducers are classified into two groups: microbial and non-microbial. There are two classes of microbial inducer: pathogen-associated molecular patterns (PAMPs) and virulence factors. PAMPs are defined in the sense that the host has evolved a corresponding set of receptors (known as pattern-recognition receptors) that detect their presence. The second class of microbial inducer comprises a variety of virulence factors and is therefore restricted to pathogens. In contrast to PAMPs, they are not sensed directly by dedicated receptors. Instead, the effects of their activity, particularly their adverse effects on host tissues, are responsible for triggering the inflammatory response (Medzhitov and Janeway, 1997).

2.1.4.2. Endogenous inducers of inflammation

Endogenous inducers of inflammation are signals produced by stressed, damaged or otherwise malfunctioning tissues. During necrotic cell death, the integrity of the plasma membrane is disrupted, resulting in the release of certain cellular constituents, including ATP, K^+ ions, uric acid, HMGB1 (high-mobility group box 1 protein) and several members of the S100 calcium-binding protein family (Bianchi, 2007). ATP binds to purinoceptors at the surface of
macrophages, resulting in $K^+$ ion efflux, and can cooperate with other signals to activate the NALP3 inflammasome (Mariathasan, 2006). ATP also activates nociceptors that report tissue injury to the nervous system (Julius and Basbaum, 2001). Platelets are also activated by contact with collagen and produce various inflammatory mediators, including thromboxanes and serotonin1. Another class of endogenous inducer is more relevant to chronic inflammatory conditions that include crystals of monosodium urate and calcium pyrophosphate dihydrate, advanced glycation end products (AGEs) and oxidized lipoproteins. The formation of such crystals is facilitated in certain connective tissues, which provide an appropriate surface for crystal nucleation. The formation of monosodium urate and calcium pyrophosphate dihydrate crystals in the joints and periarticular tissues is responsible for the inflammatory conditions known as gout and pseudogout (Rock and Kono, 2008).

![Inducers of inflammation](image)

**Figure 2.3 Inducers of inflammation**

### 2.1.5. Current drug treatment of inflammation

**Ulcerative Colitis**

Anti-inflammatory: Mesalazine, Prednisolone, Methylprednisolone, Budesonide;

Immunosuppressive: Azathioprine, 6-mercaptopurine, Methotrexate, Cyclosporin, Tacrolimus; Biologics: Infliximab, Adalimumab, Certolizumab Pegol.
Atopic Dermatitis
The biologic agents used for the treatment of Atopic Dermatitis are Rituximab, Omalizumab, Ligelizumab, Dupilumab, Dupilumab, Pitrakinra, Mepolizumab

Bronchial asthma
At present Glucocorticoids and B₂ Adrenoceptor agonists are the most effective drugs for the treatment of airway inflammation and obstruction with Theophylline, Leukotriene receptor antagonists and anticholinergics as second- or third-line therapy.

Rheumatoid arthritis
Non-steroidal anti-inflammatory drugs (NSAIDs) are the first line treatment and disease-modifying anti-rheumatic drugs (DMARDs) are the second line treatment for arthritis. Conventional DMARDs: Methotrexate, Leflunomide, Hydroxychloroquine, Sulfasalazine; Biologic DMARDs: Anti-TNF drugs, Rituximab, Abatacept, Anakinra, Tocilizumab

2.2. Glucocorticoids (GCs)

The powerful anti-inflammatory actions of glucocorticoid hormones (GCs) were discovered in the late 1940s by Philip Hench and his collaborators, who were attempting to treat the chronic and debilitating inflammatory disease, rheumatoid arthritis (RA). Since that time synthetic GCs have become a mainstay in the treatment of diseases such as rheumatoid arthritis, inflammatory bowel disease, asthma, multiple sclerosis and many others. However, severe side effects of GCs have been recognized from the very beginning, and were even detailed in Philip Hench’s Nobel prize lecture (Hench, 1950).

The word glucocorticoid (GC) is the combination of two words glucose and cortex derived from the outer part (cortex) of adrenal gland. GC has main role in the performance of a variety of fundamental processes such as cell proliferation, inflammation, immune responses, metabolic homeostasis, development and reproduction (Beato and Klug, 2006). The drug hydrocortisone is the most often used human GC in clinical practice. GC represents the most valuable anti-inflammatory drug for the treatment of chronic inflammatory diseases (Barnes, 2006). Topical GCs are used for the treatment of inflammatory diseases whereas inhaled GCs are used for the treatment of asthma and chronic obstructive pulmonary disease (COPD) (Newton et al, 2010; Barnes, 2013) but long-term application of GCs shows severe and
permanent side effects such as osteoporosis, tissue wasting, cataracts, peptic ulcer, diabetes mellitus, Cushing’s syndrome, hypertension, skin atrophy, osteoporosis, psychosis and hypothalamic–pituitary–adrenal (HPA) axis suppression (McDonough et al, 2008). Since the above mentioned severe and permanent side effects reflect functioning of GC receptor (GR), identification of anti-inflammatory GR ligands, which circumvent GC resistance, and illustrate the reduction of GC induced side effect, are the goals of national and multinational pharmaceutical companies. GCs exert their anti-inflammatory and immunosuppressive effects on immune cells, tissues and organs via different genomic and non-genomic mechanisms (Stahn and Buttgereit, 2008).

![Figure 2.4 Structure of Cortisol](image)

**2.2.1. Causative factors of GC resistance**

GC resistance is a most important hurdle in the treatment of numerous inflammatory diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease and COPD (Barnes and Adcock, 2005). The word GC resistance is used to illustrate a decrease in the maximum response of GCs (Hew and Chung, 2010).

**Cigarette smoke**

The cigarette smoking in asthmatic patient shows resistance to the anti-inflammatory actions of GCs (Chaudhuri et al, 2003). Cigarette smoking induces an oxidative stress and can affect several steroid functions such as GR nuclear translocation and effects on nuclear cofactors. The antioxidant therapy may provide useful effects in reversing the oxidative stress caused by cigarette smoke (Okamoto et al, 1999; Ito et al, 2004).
**Immunomodulation**

Th2 cytokines play an important role in corticosteroid resistance (CR) asthma. A recent research study has shown that CD41 T cells from CR asthmatic patients produce lesser amount of IL-10 (an anti-inflammatory cytokine) in response to dexamethasone than cells from corticosteroid-sensitive (CS) asthmatic patients (Hawrylowicz et al, 2002). Hence, therapeutic administration of IL-10 cytokine or IL-10-producing T regulatory cells might be effective in CR asthmatic patients. Furthermore, the addition of vitamin D3 in combination with dexamethasone in the same study can restore the ability of CD41 T cells from CR asthmatic patients to release IL-10 at the same level as those seen in cells from CS asthmatic patients (Xystrakis et al, 2006).

**Viral infection**

The major cause of morbidity and medical expenditure is recurrent exacerbations in asthmatic patients. The respiratory viruses are an important cause of exacerbation triggers (Johnston, 2005). Recent research studies have suggested that rhinovirus infection can reduce GR nuclear translocation and GC function (Bellattato et al, 2003).

**Neutrophilia**

Comparison of Bronchoalveolar lavage fluid (BALF) cell profile has been done between patients of severe CR asthma taking high dose of GCs to CS asthma patients (Wenzel et al, 1997). The eosinophil counts were found to be lowest in the patients with severe CR asthma receiving high dose of GC treatment. In contrast, eosinophil levels were found to be highest in moderate asthma patients not receiving GC therapy. In comparison, the neutrophil levels were found to be significantly higher in the severe CR asthma group as compared to moderate asthma patients, indicating a distinctive form of inflammation in patients with severe CR asthma inspite of receiving treatment with high-dose oral corticosteroids (Camps et al, 2005; Zhang et al, 2002).

**Allergen exposure**

The patients of severe allergic asthma often get an inferior quality during the pollen season and require increased amounts of GC therapy to control the asthma (Leung and Bloom, 2003). The effect of allergen exposure on GR function and GR-binding affinity in peripheral blood mono-nucleated cells (PBMCs) from atopic asthmatic patients has been examined by Denver group. These effects of allergen exposure on GR ligand binding are interrelated with
reduction in T-cell proliferation and could be inverted by antibodies to IL-2 and IL-4 (Nimmagadda et al, 1997).

**Changes in cellular environment**

The mechanism of GC resistance is thought to be the result of significant changes in the cellular microenvironment, which occur as the disease undergoes progression. These cellular changes include alterations in GR translocation, and P-glycoprotein regulation of cellular ligand accumulation (De Iudicibus et al, 2011).

**Oxidative stress**

The oxidative stress is responsible for reducing the GC effect by modifying the activity of histone deacetylase-2 (Barnes et al, 2004). Oxidative stress significantly attenuates HDAC2 activity and expression, thereby limiting recruitment of GC to sites of action in the genome by GR. In case of GC-resistant asthma, there is a marked reduction of HDAC2 expression in PBMCs and alveolar macrophages (Hew et al, 2006). Recent research studies suggested the HDAC2 reduction in COPD patients is due to oxidative stress activation of peroxynitrite and phosphoinositide-3-kinase (PI3K). Both of the above factors are essential for inactivation and degradation of HDAC2 activity by oxidative stress (To et al, 2010).

**Hypoxia**

Hypoxia is observed at many sites of inflammation in inflammatory condition, due to increasing numbers of activated inflammatory cells and subsequently increased oxygen demand (Karhausen et al, 2005). In endothelial cells, chronic hypoxia-induced cellular dysfunction is observed after dexamethasone treatment, and impaired GR transactivation was found under low oxygen tension (Murata et al, 2004; Kodama et al, 2003). On the other hand, similar impairment of GR transactivation was observed in hepatic cells, cultured in a hypoxic environment of 3% O₂ (Wagner et al, 2008).

**Cytokines**

The GC-resistant asthma condition showed improved production of IL-2 and IL-4 cytokines in the airways (Leung et al, 1995). The combined activity of IL-2 and IL-4 diminishes GR translocation and binding affinity in target cells. In monocytes, IL-13 alone was also found to reduce GR activity (Matthews et al, 2004). The impaired GR activity was rescued by inhibiting p38 MAPK activity. The mechanism of cytokine-induced resistance is p38 MAPK-mediated phosphorylation of GR (Irusen et al, 2002). In GC-resistant ulcerative colitis
condition, there are marked increased of TNF-α, IL-6 and IL-8, in mucosal cells which down-regulates the expression of GR (Ishiguro, 1999). Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine, causes GC resistance by blocking GC induction of the dual specificity phosphatase 1 (Roger et al, 2005).

2.2.2. Molecular mechanisms of GC resistance

Several molecular mechanisms of GC resistance have now been identified, including genetic susceptibility, decreased expression of GR, elevated expression of GR-β, defective GR ligand binding and nuclear translocation, reduced HDAC2 expression, collagen signaling, transcription factor activation, raised MIF, increased P-glycoprotein-mediated drug efflux and reduction in number of regulatory T cells.

Genetic susceptibility
GC resistance in asthmatic patients is more common within families, suggesting that genetic factors are responsible for determination of GC sensitivity (Carmichael et al, 1981). The studies of peripheral blood mononuclear cell (PBMCs) of GC-sensitive and GC-resistant asthmatic patients have identified 11 genes that might be possible to develop a genomic test for GC resistance (Hakonarson et al, 2005).

Decreased expression of GR
The increased level of transforming growth factor beta-1 [TGF-β1 (a cytokine released by airway epithelial cells)] has been found in the airway fluid of allergen-challenged asthmatic patients. The effect of TGF-β1 on airway epithelial cells is impairment of GC responses in cells, due to decreased GR expression (Salem et al, 2012). TNF-α is a cytokine found in inflammatory cells of asthmatic patients, shown to decrease GR levels in human monocytes without affecting binding affinity of GR to GCs (Franchimont et al, 1999).

Elevated expression of GR-β
Numerous research reports have shown increased expression of GR-β in GC-resistant patients of several diseases such as asthma, rheumatoid arthritis and inflammatory bowel disease (Sousa et al, 2008; Kozaci et al, 2007; Orii et al, 2002). The expressions of GR-β induced by pro-inflammatory cytokines and improved by microbial super-antigens (staphylococcal enterotoxins) might be responsible for GC resistance in atopic dermatitis patients. Defective GR ligand binding and nuclear translocation IL-2 and IL-4 cytokine expressions are over
expressed in the airways of GC-resistant asthma patients. In vitro, combination of IL-2 and IL-4 cytokines reduces nuclear translocation of GR and binding affinity within the nucleus of T cells. The mechanism of reduction of GR function by IL-2 and IL-4 cytokines might be phosphorylation of the receptor by p38 MAP kinase (Fakhri et al, 2004).

**Defective histone acetylation**
Histone acetylation plays a significant role in the regulation of inflammatory genes and the mechanism of action of GCs. From various research findings, it has been proved that GCs switch on GC-responsive genes (MKP1), via acetylation of specific lysine residues (Lys5 and Lys16) on histone 4 (Ebina et al, 1993). The major mechanism of gene repression by GCs is employment of HDAC-2 gene to activated inflammatory genes. HDAC2 expression is reduced in GC-resistant patients with inflammatory diseases (rheumatoid arthritis and inflammatory bowel disease) (Bonacci et al, 2006).

**Collagen signaling**
The key features of airway remodeling in asthma patients are hyperplasia and hypertrophy of airway smooth muscle cells and increased collagen deposition (Li et al, 2006; Loke et al, 2006). The mitogenesis of airway smooth muscle cells can be inhibited by GCs. Culturing of airway smooth muscle cells on denatured type I collagen develops resistance to the antimitogenic and antimigratory property of GCs. A collagen-induced GC resistance has been recognized to integrin-extracellular matrix activation signaling pathway which impairs the GC ability of reducing Cyclin D1 levels, a cell cycle and migration regulatory protein (Lane et al, 1998; Flaster et al, 2007).

**Transcription factor activation**
Extreme activation of a transcription factor has been found as a mechanism of GC resistance in asthmatic patients (Ishiguro et al, 2006). It is a heterodimer of Fos and Jun proteins and can be activated by pro-inflammatory cytokines such as TNF-α through the JNK pathway. In PBMCs and bronchial biopsy samples of GC-resistant asthma patients, JNK is activated and higher expression of c-Fos is found than in PBMCs and bronchial biopsy samples of GC-sensitive asthma patients (Farrell et al, 2000).

**Macrophage migration inhibitory factor**
It is a pro-inflammatory cytokine that has potent anti-GC effects and associated with a variety of inflammatory diseases (Farrell and Kelleher, 2003). It is induced by GC and inhibits their...
anti-inflammatory effects through inhibition of MKP1 induction (Tsujimura et al, 2008). Elevated expression of MIF has been found in colonic mononuclear cells in GC-resistant ulcerative colitis patients. MIF antibody restores the anti-inflammatory response of GC in colonic mononuclear cells (Hawrylowicz, 2005).

**Increased P-glycoprotein**

It is known as permeability glycoprotein or multidrug resistance protein 1 (MDR1). It is an important protein of the cell membrane that pumps many foreign substances including GC out of cells. Pumping of foreign substances out of the cell might be an important mechanism for GC resistance in inflammatory diseases (Stanczyk et al, 2008). Elevated levels of MDR1 expression have been found in circulating lymphocytes in GC-resistant inflammatory bowel disease and rheumatoid arthritis patients (Cosio et al, 2004; Fox et al, 2007).

**Reduction in regulatory T cells**

IL-10 is an important anti-inflammatory and immunoregulatory cytokine secreted by regulatory T cells in response to GC (Failla et al, 2007). An in vitro study showed that T-helper cells from patients of GC-resistant asthma did not secrete IL-10 after culture with GC; however, after addition of vitamin D$_3$ (calcitriol), secretion of IL-10 rises to levels seen in cells from GC-sensitive patients cultured with GC alone. Vitamin D is an important immune system regulator, particularly in the control of regulatory T cells. Therefore, low dietary vitamin D intake or lack of sunlight might be causative factors to reduced GC responses in inflammatory diseases (Rahman and Adcock, 2006).

**2.2.3. Newer strategies for reversal of GC resistance**

A number of alternative anti-inflammatory drugs are available for the treatment of GC-resistant diseases. Resistance to the anti-inflammatory action of GC is a major obstacle for the effectual treatment of asthma and chronic obstructive pulmonary disease (COPD). The superior understanding of molecular mechanisms of GC resistance has identified a variety of therapeutic strategies for better treatment of asthma and COPD. A newer strategy for the treatment of GC-resistant asthma and COPD is the reversal of GC resistance by the development of a GC-sensitizing or GC-sparing agent. Another treatment strategy for reversal of GC resistance is blockade of following mechanisms. Some of the examples of these mechanisms are use of vitamin D to restore IL-10 response, inhibition of p38 MAP kinase,
activation of HDAC2 expression by use of theophylline, antioxidants, or PI3K-δ inhibitors and inhibition of P-glycoprotein and MIF.

**p38 MAP kinase inhibitors**
Several p38 MAP kinase inhibitors are used in the treatment of GC-insensitive inflammatory diseases such as inflammatory bowel disease, rheumatoid arthritis and COPD in which p38 MAP kinase undergoes activation. In future, reversal of GC resistance might be possible in GC-resistant asthma patients by p38 MAP kinase inhibitors, JNK inhibitors and vitamin D₃ treatment (Nobili et al, 2006).

**Histone deacetylase 2**
Activation of HDAC2 can be achieved with theophylline, which restores HDAC2 activity in macrophages of COPD to normal levels and reverses GC resistance (Huschtscha et al, 2009). Oral administration of theophylline reversed the GC resistance in mice exposed to cigarette smoke that developed GC-resistant inflammation (Feagan et al, 1995). Current research studies suggested that the mechanism of theophylline in restoring HDAC2 is by selective inhibition of PI3Kδ, which is activated by oxidative stress in COPD patients (Stack et al, 1999).

**Oxidative stress (Vitamin C and E)**
Oxidative stress is an important mechanism in the reduction of HDAC2 and leads to GC resistance. Hence, antioxidants such as vitamin C and E should be effectual in the reversal of GC resistance (Jewell and Truelove, 1970).

**P-glycoprotein**
There are several therapeutic strategies for inhibiting P-glycoprotein to prevent the efflux of GC outside the cell, some of which are based on the finding that verapamil and quinidine act as efflux blockers (Gordon et al, 2001).

**2.2.4. Treatments for reversal of GC resistance**
The GC resistance can be treated with alternative anti-inflammatory treatments, such as immunomodulators (methotrexate, azathioprine (AZP) and mercaptopurine (MP), calcineurin inhibitors or novel anti-inflammatory treatments, such as phosphodiesterase 4 (PDE4)
inhibitors, cytokine inhibitors, chemokine antagonists, p38 MAPK inhibitors, PI3K inhibitors, Toll-like receptor (TLR) inhibitors or NF-κB inhibitors.

**Immunomodulators**

Methotrexate is a structural analog of folic acid. It competitively inhibits the binding of dihydrofolate to the enzyme dihydrofolate reductase, thereby interfering with purine and pyrimidine synthesis required for the cell proliferation (Van Assche et al, 2004). The effects of methotrexate on cytokine production interrupt the cell cycling process and leads to apoptosis (Kitahara and Kawai, 2005). Immunomodulator agent such as methotrexate might be ineffective in GC-resistant inflammatory bowel disease and rheumatoid arthritis caused by increased expression of P-glycoprotein. This finding has led to a search for novel anti-inflammatory agents, particularly for GC-resistant diseases such as COPD and acute respiratory distress syndrome. AZP and 6-MP drugs are purine analogs that exert their immunomodulatory effect by competitively inhibiting the biosynthesis of purine nucleotides. In cultures of intestinal cell line, these drugs cause a G2 phase cell cycle arrest, apoptosis of intestinal cells and inhibit intestinal epithelial cell proliferation (Dastidar et al, 2007). AZP and 6-MP drugs are widely used for the treatment of inflammatory bowel disease (Stadtmann and Zarbock, 2012). The biological products and immunosuppressant agents are used in steroid resistance as an alternative to steroids including tissue isolates, blood isolates, recombinant peptides, cell and gene therapies and antibody-based therapies such as anti-TNF-α (Chung, 2011) and anti-adhesion molecules, such as natalizumab (anti-α4 integrin) in Inflammatory Bowel Diseases (IBD) (Fung-Leung, 2011).

**Calcineurin inhibitors**

Cyclosporin A and tacrolimus are calcineurin inhibitors and are effective in GC-resistant inflammatory bowel disease and rheumatoid arthritis patients, but they have not shown their profound effect in GC-resistant asthma (Marwick et al, 2010).

**PDE4 inhibitors**

PDE4 inhibitors such as Theophylline, Rolipram, Cilomilast and Roflumilast are broad-spectrum anti-inflammatory agents used clinically for the treatment of COPD and inflammatory bowel disease. But, these agents have proved disappointing because of several side effects such as nausea, diarrhea and headaches (Brusselle et al, 2011).
Cytokine inhibitors

TNF-α is an important mediator of neutrophilic inflammation in COPD patients. Hence, TNF-α inhibition has gained much interest as an anti-inflammatory agent. However, the TNF-α inhibitor infliximab showed little effect on bronchoalveolar lavage and serum inflammatory mediator levels in severe COPD patients (Barnes, 2013)

Chemokine antagonists

Small molecule chemokine receptor antagonists have been proposed as drug targets in COPD patients. The levels of CXCL8, CXCL1 and CXCL5 are increased in COPD leading to up-regulation of neutrophil and monocyte activation by binding to their common CXCR2 receptor (Pace, 2008).

p38 MAPK inhibitors

The p38 MAPK signaling pathway is activated in response to several inflammatory responses such as cytokines, growth factors and oxidative stress and plays a central role in regulation of inflammation. Activation of p38 MAPK in COPD patients correlates with the degree of lung function impairment and alveolar wall inflammation (Basith et al, 2011).

PI3K inhibitors

The PI3K signaling pathway regulates both innate and adaptive immune responses. The PI3Kg isoform is involved in modulation of neutrophil recruitment having documented reduction of neutrophil migration, macrophage function, T-cell activation and protection in models of airway inflammation (Kwak et al, 2011). As a result, PI3Kg inhibitors may act as a potential anti-inflammatory therapy for COPD (Bakke et al, 2011).

TLR inhibitors

The innate immune system, particularly TLRs, has important role in the pathogenesis of COPD. Cigarette smoke induces the expression of TLRs in bronchial epithelial cells (Barnes, 2005). As a result, TLR inhibitors such as TLR2, TLR4 and TLR9 are attractive target not only for anti-viral/bacterial approaches reducing exacerbations but also for inhibition of cigarette smoke-induced inflammatory responses (Usmani et al, 2005).

NF-κB inhibitors

The NF-κB family of transcription factors regulates the expression of multiple pro-inflammatory mediators [cytokines, chemokines, adhesion molecules, matrix inhibitory proteins, etc.], playing a crucial role in the chronic inflammatory state of COPD. NF-κB inhibition could potentially provide a novel strategy for the treatment of COPD by reducing chronic inflammation.

Pharmacological evaluation of combination of plant steroids and low dose Glucocorticoid for treatment of inflammatory disorders in experimental animals
metalloproteinase (MMPs) and cyclooxygenases] in response to both cigarette smoke and inflammation (Giembycz et al, 2008). The main concern with long-term inhibition of NF-κB is the immune system suppression and impairment of the host defense mechanisms (Kobayashi et al, 2012).

**Janus Kinase/Signal Transducers and Activators of Transcription inhibitors (JAK/STAT inhibitors)**

Several cytokine and inflammatory mediators in COPD signal via the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway and members of the JAK/STAT pathway are elevated in COPD patients. JAK/STAT inhibitions may represent a potential therapeutic mechanism for inhibition of pulmonary inflammation (Ito et al, 2000).

**Inhaled long-acting β₂-agonists (LABAs)**

The function of GCs might be enhanced by LABAs by increasing nuclear translocation of GR, which increases the anti-inflammatory effects of GCs (Redington et al, 2000; Bonacci et al, 2006; Tsujimura et al, 2008). The drug formoterol produces its action by activation of phosphatase protein phosphatase A2, which reverses the phosphorylation of GR and JNK1. They also reverse the steroid resistance induced by oxidative stress through a mechanism that involves inhibition of PI3K (Rahman and Adcock, 2006).

**2.2.5. Side effects of GC**

When GCs are prescribed at high doses and/or for prolonged periods of time, they can induce abundant adverse reactions. Clinically unwanted endocrine and metabolic effects includes diabetes mellitus, redistribution of body fat and increased body weight. It also has effects on bone (e.g. osteoporosis), muscle (e.g. myopathy) and the cardiovascular system (e.g. atherosclerosis and hypertension) as shown in Figure 2.5 (Solito et al, 1998). The adverse effects on dermis include atrophy of the epidermis and dermis, leads to improved skin permeability, trans-epidermal water loss and impaired wound healing. Other remarkable adverse effects include unwanted effects on the central nervous system, such as mood swings, euphoria and depression, increased risk of bacterial, viral parasite and fungal infection, and on eyes (e.g. glaucoma and cataract) (Croxtall et al, 1995; Grutz, 2005). Both the desirable and undesirable GC effects are mediated by genomic and non-genomic mechanisms of action (Roffe et al, 2012; Auphan et al, 1995; Adcock 2000).
Pharmacological evaluation of combination of plant steroids and low dose Glucocorticoid for treatment of inflammatory disorders in experimental animals

2.2.6. Role of HPA axis in inflammation

GCs are the hormones synthesized from the adrenal cortex of the adrenal gland and carried into the blood circulation. Hydrocortisone (cortisol) is the naturally occurring GC synthesized from its precursor cortisone. The production of GC occurs diurnally in a pulsatile manner. The release of GCs is regulated by a hierarchy of endocrine organs. The hypothalamus secretes the corticotropin-releasing hormone (CRH) controlling the function of pituitary gland, which in turn triggers the release of adrenocorticotropic hormone (ACTH). The ACTH thus stimulates the adrenal cortex of adrenal gland and leads to synthesis of GC. The hormonal link between these endocrine tissues is considered as the HPA axis. The input to the hypothalamus is given by the central circadian clock, psychological stress, physiological stress or diverse systemic inflammatory reactions (Kleiman and Tuckermann, 2007) (Figure 2.6).
2.2.7. GCs and inflammation

Inflammation is the local reaction of living tissue to injury. Inflammation is of two types: acute and chronic. Acute inflammation is characterized by increased blood flow and vascular permeability with accumulation of fluid, leukocytes infiltration and release of inflammatory mediators such as cytokines at the site of inflammation. Chronic inflammation is characterized by the development of cellular and humoral immune responses to the pathogenic microorganisms at the site of injury. GCs impede many of the primary events of the inflammatory process. They also endorse the resolution of inflammation process by producing vasoconstriction of blood vessels and improved vascular permeability that results from an inflammatory insult. GC also decreases migration of leukocytes at the inflammation sites and leads to synthesis of new protein moiety (Perretti and Ahluwalia, 2000). They also alter distribution/trafficking of leukocyte (McEwen et al, 1997), death/survival of leukocytes (Herold et al, 2006; McColl et al, 2007) and cellular differentiation programs, thus framing the successive inflammatory response. By transrepression mechanism, GC causes release of various pro-inflammatory mediators at the site of inflammation such as cytokines,
chemokines, cell adhesion molecules and inflammatory enzymes (Perretti and Ahluwalia, 2000; Smoak and Cidlowski, 2004).

### 2.2.8. Mechanism of action of GCs

Glucocorticoids are members of a class of molecules known as steroids, which also includes mineralocorticoids and sex steroids that are derived from cholesterol. In the cytoplasm, steroids bind to ligand-specific receptors, and the receptor-bound steroid molecule translocates into the nucleus, as shown in Figure 2.7. The effects of the steroids and their receptor complexes can be classified into genomic and non-genomic effects (Dietrich et al., 2011). Genomic effects include transactivation, transrepression, and post-transcriptional modification, whereas non-genomic effects include interference with the activation of signaling cascades via protein-protein interactions.

Most cellular responses to glucocorticoids are attributed to their binding to the intracellular glucocorticoid receptor (GR). In the cytoplasm, glucocorticoids bind to GR, found in its inactive form in a complex with molecular chaperones including the heat shock protein 90 (Hsp90), Hsp70, the p59 immunophilin molecule, and the small p23 phosphoprotein (De Bosscher et al., 2003; Lovgren et al., 2007). After binding to a glucocorticoid molecule, the GR detaches from this multi-protein complex and translocates into the nucleus, where the majority of glucocorticoid actions to modulate target gene expression are exerted. The following sections outline the multiple mechanisms of glucocorticoids on inflammatory and immune cell functions.
Pharmacological evaluation of combination of plant steroids and low dose Glucocorticoid for treatment of inflammatory disorders in experimental animals

**Figure 2.7** Mechanism of Action of Glucocorticoids

**Transcriptional effects of glucocorticoids**

The steroid-activated receptor is able to bind to specific steroid response elements in DNA chromatin, such as glucocorticoid response elements (GRE). The binding of glucocorticoid-glucocorticoid receptor (GC/GR) complex to GRE results in gene transcription, the production of messenger RNA (mRNA) molecules and the synthesis of specific proteins. As recently reviewed the GC/GR complex modulates gene transcription by three different molecular mechanisms involving binding to three specific types of GRE (Chinenov and Rogatsky, 2007). One mechanism involves the activation of gene transcription by direct binding of simple GREs in the promoter regions of target genes that typically bind homodimeric GR. Composite GREs in gene promoters also bind the GC/GR complex, but do so together with other transcription factors. In contrast, tethering GREs are in fact sites on DNA for other transcription factors (such as NF-κB, AP-1, and Stat3) which in turn bind the GC/GR complex via protein-protein interactions (Chinenov and Rogatsky, 2007).

This ability of the GC/GR complex to interact with DNA as a transcription factor, and with proteins to affect the function of other transcription factors, provides a great diversity of possible cellular responses to glucocorticoids. In addition, transcriptional coregulators, such as Src homologous members (Src-1, Src-2, and Src-3), methyl transferases, and histone acetyl transferases, are also reported to play a crucial role in chromatin remodeling, assembly of...
transcriptional factors and target gene transcription in response to glucocorticoids (Xu and Li, 2003). A number of genes whose products function as pivotal anti-inflammatory factors, such as annexin-1, MAPK phosphatase 1 (MKP-1), glucocorticoid-induced leucine zipper (GILZ) and secretory leukocyte peptidase inhibitor (SLPI) are activated via GC/GR interaction with GRE (Barnes, 2006b).

The GC/GR complex physically interacts with the transcription factor NF-κB in the cytoplasm to block its nuclear translocation (Widen et al., 2003) or in the transcription complex to prevent gene transcription activated by NF-κB (McKay and Cidlowski, 1998; Ray and Prefontaine, 1994). The GC/GR complex also represses transcriptional activation mediated by AP-1, through a direct interaction between GR and c-Jun/c-Fos, the two subunits which comprise AP-1 (Schule et al., 1990; Touray et al., 1991). Protein-protein interactions between the GC/GR complex and NF-κB and Activator protein 1 (AP-1) result in repression of the production of cytokines relevant to autoimmune disease, including IL-1β, IL-2, IL-3, IL-6, IL-8, TNF, and granulocyte macrophage colony stimulating factor (GM-CSF) (Almawi and Meledjian, 2002) as well as other mediators of inflammation such as cyclooxygenase-2. In short, negative transcriptional regulation by GR, either through nGRE binding or transcriptional interference, is a significant alternative means through which the anti-inflammatory and immune suppressive actions of glucocorticoids are mediated.

**Post-transcriptional effects of glucocorticoids**

After transcription, mRNAs encoding many pro-inflammatory genes, such as IL-1β, IL-6, IL-8, TNF, and GM-CSF are unstable and are rapidly degraded by RNases (Anderson et al., 2004; Smoak and Cidlowski, 2006). All these mRNAs contain AU-Rich elements (ARE) in 3′ un-translated gene regions (Barnes, 2010). In active inflammation, inflammatory factors such as IL-1β and TNF activate pathways such as p38 MAP kinase, which results in the stabilization of mRNA via effects on ARE and hence enhance translation of pro-inflammatory cytokines (Chikanza and Kozaci, 2004). Glucocorticoid induction of MKP-1 is associated with reduced p38 MAP kinase activity (Toh et al., 2004) and this results in impaired pro-inflammatory mRNA stability and reduced cytokine expression (Yang et al., 2006). Glucocorticoids can also reduce mRNA stability through increasing the expression of proteins, such as tristetraproline (TTP), HuR, and T cell intracellular antigen-1 (TIA-1), that destabilize mRNAs encoding inflammatory proteins (Anderson et al., 2004; Barnes, 2006a).
Non-genomic effects of GCs

Apart from classic genomic effects, GCs are also reported to have rapid non-genomically mediated effects through alternative pathways. So far, four alternative mechanisms have been proposed: (i) a signaling pathway through a membrane-bound GR (mGR), (ii) direct membrane effects, (iii) interaction of the GR with other proteins in the cytoplasm, and (iv) GR translocation on the mitochondria. With regard to the first mechanism, cell membrane glucocorticoid binding was first reported in mouse and human lymphoid cell lines (Gametchu et al., 1999) and termed mGR. Synthetic glucocorticoid analogues were reported to increase membrane lipid mobility in lymphocytes and some cancer cell lines suggesting that glucocorticoids can directly modulate plasma membrane physicochemical properties to regulate cell functions such as membrane Na⁺ and Ca²⁺ ion channels, cell fluid shear response, and cell tight junction formation (Keating et al., 1985). The binding of glucocorticoids to the cytosolic GR has been reported to lead to rapid intracellular signaling events that are independent of interaction with DNA-bound transcription factors (Croxtall et al., 2000). Finally, the GC/GR complex can directly translocate into the mitochondria, impacting on sensitivity to glucocorticoid-induced apoptosis (Talaber et al., 2009).

2.2.9. Novel anti-inflammatory targets

2.2.9.1. Annexin-1 (Anx-1)

It is a calcium and phospholipid binding protein (37 kDa made up from 346 amino acids) have diverse physiological roles such as, anti-inflammatory responses, interaction with cytoskeleton proteins, exocytosis, cell proliferation and cell differentiation (Gerke and Moss, 2002; Raynal and Pollard, 2004). It was originally found as a glucocorticoid-inducible protein in leukocytes and known as lipocortin (Blackwell et al, 1984). It is a member of annexin superfamily, which plays the role of anti-inflammatory mediator (Yang and Morand, 2006). It is mostly found in white blood cells, stromal cells, and biological fluids expressed in variety of cells including white blood cells, endothelial cells, fibroblasts and gut epithelial cells (Perretti & D’Acquisto 2009). It inhibits the formation of lipid mediator by blockade of enzyme phospholipase A₂, thereby regulating cellular growth and differentiation, neuroendocrine secretion, central nervous system response to cytokines, and accumulation of neutrophil to different tissues (Kamal et al, 2005; Parente and Solito, 2004). It blocks the release of inflammatory mediators by inhibition of cytosolic phospholipase A₂ enzyme, thus prevents the formation of pro-inflammatory eicosanoid, inducible cyclooxygenase and nitric oxide.
oxide synthase enzymes (Minghetti et al, 1999). It may also affects on inducible nitric oxide synthase (iNOS) expression in rats with septic shock.

Dexamethasone and N-terminal fragment of Anx-1 inhibits the induction of iNOS in a lipopolysaccharide (LPS) activated macrophage cell line (Wu et al, 1995). Various research reports have shown that dexamethasone and Anx-1 can inhibit the expression of COX-2 and iNOS along with the release of PGE₂ (Minghetti et al, 1999). Similar to GCs, Anx-1 inhibits eicosanoid synthesis, obstructs leukocyte migration (Perretti et al, 1996), and induces inflammatory cells death (Solito et al, 2001). It has been proposed that the above described effects of Anx-1 may be attributed to release IL-10 and a concurrent reduction of IL-12 (Ferlazzo et al, 2003). It may also inhibit the migration of leukocyte to vascular endothelium by impairing with adherence of neutrophil and monocyte to endothelium (Solito et al, 2000). It also promotes the apoptosis of inflammatory cells which may contribute to anti-inflammatory properties of Anx-1 (Solito et al, 2001). In rheumatoid arthritis, Anx-1 acts through inhibition of mitogen activated protein (MAP) kinases, thereby inhibiting pro-inflammatory pathways in RA (Patel et al, 2012). In patients suffering with RA, discrete Anx-1 expression has been observed in monocytes of the synovial membrane. In adjuvant-induced arthritis rat model, the endogenous Anx-1 mediates the anti-inflammatory effect of dexamethasone through the modulation of synovial TNF-α release and neutrophil recruitment (Yang et al, 1999).

2.2.9.2. Glucocorticoid induced leucine zipper (GLIZ)

It is a chain of 137 amino acid protein with a central leucine zipper dimerization domain. It is dispersed in spleen, thymus, bone marrow and lymph nodes (Cannarile et al, 2001). GCs upregulate the expression of GILZ in mast cells (Godot et al, 2006), T cells (Asselin-Labat et al, 2004), eosinophils, epithelial (Eddleston et al, 2007) and myeloid cells (Ehrchen et al, 2007). It is a newer protein that mediates anti-inflammatory effects of GCs in leukocytes (Beaulieu and Morand, 2011). GCs induced upregulation of GILZ inhibits the activation of macrophages by lipopolysaccharides leads to production of inflammatory mediators and pro-inflammatory cytokines (Hamdi et al, 2007). The current research studies have confirmed the anti-inflammatory and immunosuppressive roles of GILZ by inhibition of NF-κB gene transcription in macrophages and T cells (Ayroldi et al, 2001; Berrebi et al, 2003). GILZ inhibits numerous inflammatory pathways such as transcription factors NF-κB, AP-1 and mitogen-activated protein kinase (MAPK) which results into inhibition of inflammation (Ayroldi and Riccardi, 2009).
The various research studies have reported to mediate the dexamethasone induced anti-inflammatory effect in human airway epithelial cells (Eddleston et al, 2007). In lipopolysaccharide induced pleuritis mice model, the expressions of GILZ was elevated in inflammatory exudates after anti-inflammatory drugs treatment and was associated with neutrophil apoptosis (Yang et al., 2009). Hence, GILZ is a key molecule in GC biology, which represents a candidate mediator of GC regulation of immune and inflammatory responses. From the various research studies it has been concluded that GILZ could be an ideal drug candidate for the development of new anti-inflammatory therapy.

2.2.9.3. Mitogen activated protein kinase phosphatase-1 (MKP-1)

It is also called as Dual specificity phosphatase 1 (DUSP1). It is member of phosphatases family that catalyzes the removal of phosphate group from threonine, serine, or tyrosine amino acid residues (Dickinson and Keyse, 2006). It is an inducible nuclear phosphatase that dephosphorylates Mitogen-activated protein kinases (MAPKs) (Liu et al., 2007). MKP-1 proteins are important regulators of immune responses and inflammatory process and were found to regulate p38 MAPK and JNK signaling (Korhonen and Moilanen, 2014). MKP-1 proteins are activated by phosphorylation process and linked to many physiological and pathophysiological processes, such as CNS disorders, skeletal muscle metabolism, malignancy, atherosclerosis, asthma, psoriasis and rheumatoid arthritis (Vicent et al, 2004; Boutros et al, 2008; Wu et al, 2006; Duric et al, 2010; Bhavsar et al, 2008; Rauhala et al, 2005; Kjellerup et al, 2012). The GC-induced expression of MKP-1 has also been associated with inhibition of extracellular signal-regulated kinase (ERK) pathway in certain cells (Wu et al., 2006).

The phosphorylation of transcription factors and RNA binding proteins, regulates the expression of inflammatory gene at both transcriptional and post-transcriptional levels (Clark et al., 2003; Dean et al., 2004). MKP-1 is induced by pro-inflammatory stimuli and forms a negative feedback loop to limit MAPK signaling and the expression of inflammatory mediators. Hence, over expression of MKP-1 protein attenuates JNK and p38 MAPK pathways signaling and inhibits the expression of several inflammatory genes (Zhao et al., 2005; Nimah et al., 2005). Hence, MKP-1 would be a valuable healing target for the development of pro-resolving anti-inflammatory therapies that contributes to the resolution of inflammation in vivo.
**2.2.9.4. Interleukin 10 (IL-10)**

It is an anti-inflammatory cytokine, expressed by variety of cells such as lymphocytes and monocytes (Grutz, 2005). It has been linked to family of α-helical cytokines responsible for regulation of innate and adaptive immunity that shows pleiotropic effects in the immunoregulation and inflammation process (Franchimont, 2004). It acts as a natural immunosuppressant of tumor necrosis factor (TNF) that inhibits the production of inflammatory mediators.

IL-10 expressions can be upregulated by GCs and on the contrary side, it acts synergistically with GCs, with improved ability of dexamethasone to reduce IL-6 secretion in whole-blood cell cultures (Middleton et al, 1998; Galon et al, 2002). The attachment of IL-10 to its receptor, leads to stimulation signal transducer and activator of transcription 3 (STAT3) (Glocker et al., 2011). The mechanisms of action of IL-10 are inhibition of NFκB, activation of PI3K, down-regulation of TLR4 and up-regulated expression of suppressor of cytokine signaling 3 (SOCS3) proteins (Saraiva & O'Garra 2010; Muzio et al., 2000).

GCs exhibits anti-inflammatory activity in various diseases conditions such as rheumatoid arthritis (Verhoef et al., 1999), cardiac bypass surgery (Tabardel et al., 1996), and asthma (Karagiannidis et al., 2004) has been related with induction of IL-10 expression which dependent on p38 MAPK (Ma et al., 2001). The synthesis of pro-inflammatory cytokines (IL-1, IL-6, IL-8, IL-12 and TNF-α) has been inhibited by GCs and also it increases the production of inflammatory mediator. It also decreases the expression of co-stimulatory and major histocompatibility class (MHC) II molecule (Zhang et al., 2010).

**2.2.9.5. Interleukin-12 (IL-12)**

IL-12 was previously known as natural killer cell stimulatory factor (NKSF) and cytotoxic lymphocyte maturation factor (CLMF). It is a heterodimer made up of two subunits each of 35 and 40 kDa (Gately et al, 1992). IL-12 is secreted by macrophages, activated B cells, and antigen-presenting cells (Kiniwa et al, 1992). The biological activities of IL-12 include enhancement of lymphokine activated killer (LAK) cell, induction of activated T cell, cytotoxic T cells activation, increased natural killer (NK) cell cytotoxicity, NK cell proliferation, induction of IFN-γ production by NK cells and T cells, and inhibition of Immunoglobulin (IgE) synthesis by lymphocytes via IFN- γ dependent and independent mechanisms. Hence, IL-12 plays an important role in cell-mediated inflammation and regulation of immunoglobulin production (Scott, 1993).
2.2.9.6. IL-1 receptor antagonist (IL-1Ra)

The interleukin-1 receptor antagonist is a member of the IL-1 family that binds to IL-1 receptors. There are two structural variants of IL-1Ra: 17-kDa form secreted from neutrophils, macrophages and monocytes (sIL-1Ra) and 18-kDa form present in the cytoplasm of monocytes, keratinocytes, fibroblasts and epithelial cells (icIL-1Ra) (Arend et al, 1998). IL-1Ra is used for the treatment of inflammatory diseases as a non-glycosylated, recombinant and N-terminal methionine protein. IL-1Ra appears as of considerable benefit in the management of Juvenile arthritis (Still's disease) and in the treatment of auto-inflammatory illnesses (Dinarello, 1998).

2.2.9.7. Secretory leukocyte inhibitory protein (SLPI)

It is 11.7 kDa nonglycosylated, cysteine-rich protein associated with innate immunity (Thompson and Ohlsson, 1988). It is expressed in mucosal tissues and immune cells such as neutrophils, monocytes and macrophages (Odaka et al, 2003). Bronchial secretions, saliva, seminal fluid, and breast milk also contains SLPI (Fakioglu et al, 2008). It exhibits anti-inflammatory, antimicrobial (Sagel et al, 1999), and antifungal functions (Tomme et al, 1997). It also improves cutaneous wound healing (Thuraisingam et al, 2006), activity of matrix metalloproteinases (Ramadas et al, 2009), and HIV-1 infection prevention (Py et al, 2009). It plays the role of anti-inflammatory mediator in chronic obstructive pulmonary disease (Wang et al, 2000) and cystic fibrosis (Weldon et al, 2007).

It also protects the local tissue against the harmful consequences of inflammation. There is release of toxic product such as serine proteinases from stimulated leukocytes and leads to subsequent deprivation of tissues. It protects the tissues by inhibiting the release of proteases, such as elastase, cathepsin G and trypsin from neutrophils; trypsin and chymotrypsin from pancreatic acinar cells; and tryptase and chymase from mast cells (He et al, 2003; Gipson et al, 1999).

Its main function is inhibition of neutrophil elastase based on the enzyme kinetic studies (Vogelmeier et al, 1996; Travis and Fritz, 1991). The hepatic expression of intravenous infusion of SLPI during hepatic ischemia and reperfusion in mice is characterised by reduced lung and liver damage, and diminished neutrophil accumulation to these sites (Lentsch et al, 1999).
2.2.9.8. Tristetraprolin (TTP)

The variety of proinflammatory cytokine mRNAs are destabilizes by zinc finger protein TTP by binding to AU-rich elements (ARE) subjecting them for degradation. It is a protein that destabilizes the mRNA that identifies specific transcripts and produces degradation by exonucleases enzymes (Carrick et al., 2004). A TTP knockout inflammatory arthritic mouse model is characterised by elevated stability of TNF mRNA and augmented TNF protein expression (Carballo et al., 2001). Colony stimulating factor-2 (CSF2), Cyclooxygenase-2 (COX-2) and Interleukin-2 (IL-2) genes are dysregulated in the deficiency of TTP and the expressions of further inflammatory mediators will be controlled by TTP (Ogilvie et al., 2005).

The stabilization of inflammatory mRNAs can be by phosphorylation and inactivation of TTP by MAPK-activated protein kinase 2 (Chrestensen et al., 2004). The dexamethasone action on various cells such as pulmonary epithelial cells (A549 and BEAS-2B), human keratinocytes (Stojadinovic et al., 2007) or HeLa cells elevates the expression of TTP mRNA. Diminution of TTP expression by siRNA decreases the inhibitory effect of dexamethasone on endogenous TNF mRNA expression (Smoak and Cidlowski, 2006). Hence, stimulation of TTP gene may responsible for the post- transcriptional inhibitory effects of GCs that will leads to development of novel anti-inflammatory approaches to battle against inflammatory disease.

2.2.9.9. Inhibitor of NF-κB

It is a nuclear factor that binds to the enhancer element of the immunoglobulin kappa light-chain of activated B cells that blocks the transcription factor NF-κB in various inflammatory conditions (Sen and Baltimore, 1986). In inflammatory and cellular stress processes NFκB plays a significant role by controlling cytokine inducible gene expression and stimulation of lymphocyte by antigens. The different stimuli (inflammatory cytokines, viral infection, and ultraviolet radiation) NF-κB, undergoes phosphorylation by protein kinase complex IKK, and degradation by proteolysis process (Auphan et al, 1995).

The mutilation of NF-κB occurs due to mutations of IκB-α gene leads to decreased production of proinflammatory cytokines and interferons that increases the danger of infection (Bondeson et al, 2007). The degradation of IκB proteins occurs under the influence of pro-inflammatory signaling pathways and leads to release of NFκB that enters the nucleus to activate the target genes. Two groups independently identified the upregulation of IκB as a putative mechanism for the development of novel anti-inflammatory approaches...
Pharmacological evaluation of combination of plant steroids and low dose Glucocorticoid for treatment of inflammatory disorders in experimental animals

Chapter 2, Section 2.3

by which GCs could impair NFκB function and inhibit expression of immune or inflammatory mediators (Scheinman et al., 1995a,b).

The upregulation of IκB in different cells by GC either did not occur or was not adequate to change the IκB degradation, and nuclear translocation of NFκB in response to pro-inflammatory stimuli (Nissen and Yamamoto, 2000; Pruett et al., 2003). Activation of NF-κB by nuclear translocation of cytoplasmic complexes plays a pivotal role in inflammation process by inducing transcription of proinflammatory genes (Baldwin, 1996). The various cytokines (TNF-α, IL-6, IL-8, and IL-1β) are produced by NF-κB by the expression of enzyme cyclooxygenase 2 (Cox-2). NF-κB translocation can improve the transcription of collagenase-3 (MMP13) gene in IL-1 stimulated synoviocytes (Mengshol et al, 2000). The activation of NF-κB elevates the production of pro-inflammatory cytokines in nephritic glomeruli, resulting in glomerulonephritis in rat (Sakurai et al, 1996).

2.2.9.10. Mast cell signaling effectors

The activation of mast cells by Ig receptors initiates a cell signaling cascade that includes stimulation of src-like tyrosine kinases, activation of PI3K-Akt, Raf-MEK-ERK pathways and release of calcium from intracellular stores. Mast cell signaling effectors up regulates pro-inflammatory gene expression as well performs speedy release of inflammatory mediators from storage granules. GCs can inhibit both immediate and delayed responses to antigen exposure under allergic condition of asthma (Kassel and Cato, 2002).

The expression of DUSP1 was concerned with dexamethasone mediated inhibition of mast cell extracellular signal-regulated kinases (ERK) signaling proteins (Kassel et al., 2001). The Downstream of Tyrosine Kinase-1 (Dok-1) is associated with the activation of Ras GTPase activating protein and inhibits the activation of Raf and extracellular signal-regulated kinases (ERK) (Di Cristofano et al., 2001). The increased expression of Dok-1 is partly mimicked the dexamethasone effects on ERK pathway, whereas RNAi mediated reduction of Dok-1 levels improves the mast cells inflammatory response (Hiragun et al., 2005).

2.3. Fluticasone (FC)

Fluticasone propionate (androstane nucleus) (Figure 2.8) is a topically active corticosteroid molecule (Phillipps, 1990). It is greatly lipophilic in nature (3-fold more lipophilic than
Inhibitory effects of Fluticasone:

- More potent in vitro than beclomethasone, dexamethasone and budesonide in inhibiting anti-CD3-induced human T-lymphocyte proliferation, in growing levels of secretory leucocyte protease inhibitor and attenuating tumour necrosis factor-α induced expression of endothelial cell adhesion molecule in bronchial epithelial cells.
- More potent and longer-acting than other corticosteroids in inhibiting interleukin-5 induced blood eosinophilia, oedema formation, and IL-5 stimulated eosinophil lung tissue accumulation.

Therefore, FC has increased intrinsic glucocorticoid potency and elevated topical anti-inflammatory activity (Johnson, 1995). FC has been shown to attenuate pulmonary inflammation in laboratory animals (Lapae Silva et al, 1994) and human beings (Booth et al, 1995), and inhibit chemotaxis of neutrophil (Llewellyn et al, 1994), endothelial cell adhesion molecule expression (Johnson, 1995), and cytokine production (Masuyama et al, 1994).

2.4. Plant steroids

The plant is a biosynthetic laboratory for huge number of compounds like resins, tannins, alkaloids, glycosides, saponins, flavonoids, steroids and sesquiterpene lactones that shows physiological and curative effect on the human body. The compounds present in plant exhibits medicinal property are generally called as secondary metabolites that have distinct chemical structure. Amongst all these available compounds, steroids have four carbon rings as the main skeleton called as steroid nucleus. The addition of different chemical groups at different

Figure 2.8 Structure of Fluticasone
positions on main skeleton leads to creation of many diverse types of steroidal compounds such as; sex hormones testosterone, progesterone, the anti-inflammatory steroidal compounds like corticosteroids, cardiac steroids digoxin and digitoxin, animal steroid like steroidal glycosides, cholesterol.

The plant steroids have many medicinal activities like antibacterial, hepatoprotective, cytotoxic, anti-tumor, immunosuppressive, plant growth hormone regulator, anti-helminthic and cardiotonic activity. Glucocorticoids are the steroidal agents used to treat variety of inflammatory disorders but, on long term usage it shows severe side effects. In order to overcome these undesirable side-effects, the research work is going on to identify novel bioactive phytoconstituents with therapeutic potential with no or minimum side effects. The various plant steroids have structural similarity with glucocorticoids such as Hecogenin, Diosgenin, Solasodine, Glycyrrhizin, Boswellic acid, Guggulsterone, Sarsasapogenin or Withaferin-A.

2.4.1. Hecogenin (HG)

Hecogenin (Figure 2.9) is a steroidal sapogenin present in the leaves of *Agave genus* species such as *Agave sisalana*, *Agave cantala*, *Agave aurea* and many more (Paik et al, 2005). The extracts obtained from above plants have been used for their cardioactive or larvicidal activity (Achenbach et al, 1994). The triterpene saponins exhibited suppressive effect on ethanol induced gastric mucosal lesions in rats and also on ethanol and indomethacin induced gastric damage in rats by inhibiting gastric acid secretion and activation of mucous membrane protective factors (Morikawa et al, 2006; Ramasubramancaraja and Babu, 2011). The anti-cancerous activity of HG was also conducted and found to present an anti-proliferative activity in human osteosarcoma cells (Corbiere et al, 2003). HG was effective against broad spectrum of pharmacological activities such as hypotensive and antifungal activity (Gondim, 2006). A research report (Hashizume et al, 2008) showed that HG is a selective inhibitor of human UDP-glucuronosyl transferases enzymes accountable for the detoxification of several chemical toxins in the body (Basu et al, 2004). It also showed an anti-inflammatory effect on ethanol induced gastric mucosal inflammation in rats (Morikawa et al, 2006). Various other saponins have also shown anti-inflammatory effects on indomethacin and ethanol induced gastric damage. Their protective effects are not due to the inhibition of gastric acid secretion, but possibly to the activation of protective factors present in the mucous membrane (Ramasubramancaraja and Babu, 2011). The documented reports of Cerqueira et al (2012)
shown the effect of HG on oxidative stress, lipid peroxidation and myeloperoxidase, a biomarker of inflammation, as well as its gastric ulcer protective effect was confirmed with histological and COX-2 immunohistochemistry studies (Cerqueira et al, 2012).

![Figure 2.9 Structure of Hecogenin]

### 2.4.2. Diosgenin (DG)

It is a steroidal saponin obtained from the plants *Dioscorea nipponoca, Solanum incanum, Solanum xanthocarpum* and *Trigonella foenum graecum* (Figure 2.10). It is biologically active phytochemicals have been used for the treatment of various types of disorder such as leukemia, inflammation, hypercholesterolemia and cancer. It is also able to prevent bone loss to the same extent as that of oestrogen. It is a typical initial intermediate for synthesis of steroidal compounds, oral contraceptives and sex hormones (Patel et al, 2012). It possesses various pharmacological activities such as anti-inflammatory activity against rat paw inflammation induced by formalin (Ahmadiania et al, 2001) and rheumatoid arthritis (Shishodia and Aggarwal, 2006; Liagre et al, 2004).

![Figure 2.10 Structure of Diosgenin]
2.4.3. Solasodine (SS)

It is a steroidal glycoalkaloids obtained from the plant Solanum xanthocarpum, belonging to family Solanaceae (Figure 2.11). It has been used for the treatment of bronchial asthma (Govindana et al, 2004) and anti-allergic activity (Gupta, 1994), immuno-suppressive, liver diseases and membrane stabilizing activity (Chitrvanshi et al, 1990). It has is used as starting material for the synthesis of oral contraceptive, steroidal hormone (Solouki et al, 2011).

![Figure 2.11 Structure of Solasodine](image)

2.4.4. Glycyrrhetinic acid (GA)

It is a pentacyclic triterpenoid derivative of the beta-amyrin isolated from Glycyrrhiza glabra (Figure 2.12). It is reported to have allergic action by the suppression of PAF production (Ichikawa et al, 1989), anti-inflammatory activities by inhibiting the release of serotonin, histamine and bradykinin (Akamatsu et al, 1991) and lowers vascular permeability (Abe et al, 2003). It also shows inhibition of granuloma weight, exudate and formalin induced paw edema (Nasyrov and Lazareva, 1980).

![Figure 2.12 Structure of Glycyrrhetinic acid](image)
2.4.5. Boswellic acid (BA)

It is pentacyclic triterpene molecule having steroid like structure obtained from the plant *Boswellia serrata* (Figure 2.13). It has been demonstrated to be a potent anti-inflammatory drug in in-vivo animal models as well as in clinical studies (Dahmen et al, 2001; Chrubasik et al, 2007). It decreases infiltration of polymorphonuclear leukocyte and migration, inhibition of primary antibody synthesis and inhibition of complement pathway (Sharma et al, 1989; Shah et al, 2009).

![Figure 2.13 Structure of Boswellic acid](image)

2.4.6. Guggulsterones (GS)

Guggul contains particular group of steroidal compounds called as guggulsterones obtained from the plant *Commiphora mukul* (Figure 2.14). It has been reported to have potent anti-inflammatory and anti-arthritis activity (Gebhard et al, 2009; Duwiejua et al, 1993; Satyavati, 1988). The guggulsterone Z obtained from HPLC analysis of plant extract is responsible for anti-inflammatory activity (Sosa et al, 1993).

![Figure 2.14 Structure of Guggulsterone](image)
2.4.7. Sarsasapogenin (SG)

It is a saponin glycosides obtained from the *Smilax officinalis* (Figure 2.15). It possesses anti-inflammatory, anti-arthritis, antirheumatic and used for the treatment of psoriasis (inflammatory skin disorder) (Peana et al, 1997; Ageel et al, 1989; Shao et al, 2007; Thurman, 1988).

![Figure 2.15 Structure of Sarsapogenin](image)

2.4.8. Withaferin A (WF)

Withaferin A is a steroidal lactone (Figure 2.16) isolated from the herb *Withania somnifera* (Indian Ginseng and Ashwagandha) widely used in traditional Indian medicine as an anti-inflammatory agent (Mishra et al, 2000). It is reported to have antioxidant, anti-stress, hemopoietic, immunomodulatory and rejuvenating properties (Rasool and Varalakshmi, 2006).

![Figure 2.16 Structure of Withaferin-A](image)
2.5. Recent documented reports on various anti-inflammatory models

2.5.1. Croton oil single and multiple applications

The Caryocar coriaceum Wittm. fruit pulp fixed oil (20 µL/ear) exhibits significant topical anti-edematous effect against Croton oil single (inhibition of 32.0 %; p<0.05) and multiple (41.4 % after 9 days, p<0.001) applications. Histological analysis also revealed that fruit pulp oil was able to reduce the edema and the influx of inflammatory cells in mice ears sensitized with Croton oil (Rogerio et al, 2011).

Topical application of ethanol extract of Bryophyllum pinnatum leaves significantly (P<0.001) inhibited the ear edema induced by Croton oil single application (inhibition of 57 %), arachidonic acid (inhibition of 67 %), phenol (inhibition of 80 %), capsaicin (inhibition of 72 %) and Croton oil multiple application (55% after 9 days). Histopathological analyses also confirmed the topical anti-inflammatory effect of extract with reduction of edema, inflammatory cells infiltration, epidermal hyperplasia and vasodilation (Chibli et al, 2014)

2.5.2. Cotton pellet induced granuloma

The methanol extract of Plantago erosa leaves at a dose of 300 to 600 mg/kg, orally inhibited the formation of granulomatous tissue in Cotton pellet induced granuloma in rats (Barua et al, 2011).

The aqueous extract and ethanolic extract of A. lebbeck leaves at a doses of 50, 100, 200 mg/kg p.o. significantly (p<0.05) inhibited the granuloma formation with PI values of 19.07, 27.57, 38.55 and 23.93, 32.23, 42.33, respectively. Hence, the aqueous extract and ethanolic extract A. lebbeck leaves showed significant anti-inflammatory activity (Meshram et al, 2015).

The aqueous extract of Flos populi at dosages of 50, 100 and 200 mg/kg b.w. produced significant dose dependant anti-inflammatory activity against cotton pellets induced granuloma (Xu et al, 2014).
2.5.3. Tri-Nitro-Benzene-Sulphonic acid induced colitis

Camel’s milk (CM) at a dose of 10 ml/kg, b.i.d. by oral gavage effectively suppressed the severity of colon injury as evidenced by amelioration of macroscopic damage, colon weight/length ratio, histopathological alterations, leukocyte influx and myeloperoxidase activity along with suppression of TNF-α and IL-10 cytokines level in colonic mucosa. Thus, it is proved that CM may be an interesting complementary approach for the management of Inflammatory Bowel Diseases (Arab et al, 2014).

The onopordopicrin enriched fraction at a dose of 25 and 50 mg/kg, orally significantly reduced the macroscopic inflammation scores (p<0.05 and p<0.01, respectively) and morphological alterations associated with an increase in the mucus secretion along with significant amelioration of neutrophil infiltration and cytokine levels (de Almeida et al, 2013).

2.5.4. Di-Nitro-Fluro-Benzene induced contact dermatitis

On topical application of DNFB on the ear provoked allergic contact dermatitis with ear swelling and ear erythema in Balb/c mice. Treatments of mice with 1% aloperine suppressed the DNFB induced increase in ear thickness and ear erythema. Also, it significantly decreased the up-regulated mRNA expression and TNF-α, IL-1β and IL-6 induced by DNFB in ear biopsy homogenates (Yuan et al, 2010).

In this model AD-like skin lesion was induced by repetitive application of DNFB on skin of NC/Nga mice and the effects of Resolvin E1 were evaluated on the basis of ear swelling, cytokine production and histopathological findings of mice ear tissue. Intraperitoneal injection of Resolvin E1 for 1 week after DNFB challenge significantly lowered the ear swelling and improved back skin lesions. In addition, it also significantly suppressed the production of interferon-gamma, IL-4 and serum IgE level (Kim et al, 2012).

2.5.5. Ovalbumin induced asthma

The P. scandens ethanolic extract and P. scandens aqueous extract were administered orally prior to challenge with aerosolized 2.5% w/v OVA. The results reveal a significant increase in total and differential leucocyte count, TNF-α, IL-6, and IL-13 in OVA induced AHR. Similar,
observations were obtained for MPO and MDA in lungs. Histopathological analysis of lung tissue revealed the effectiveness of treatment drug (Gupta et al, 2014).

In OVA-sensitized mice, both the oral administration of white ginseng (WG) and red ginseng (RG) reduced airway hyperresponsiveness (AHR) and suppressed immune cell infiltration in BALF. The treatment of mice with WG and RG significantly lowered the OVA-specific IgE levels, inflammatory cytokine production by peribronchial lymphocytes. Histopathological examination of mice lung tissue showed reduced inflammatory cell infiltration and epithelial hyperplasia in WG and RG treated OVA mice compared with OVA control mice (Liu et al, 2015)

2.5.6. Complete Freund’s adjuvant induced arthritis

The results of CFA treated rats shows significant increased (p<0.001) in paw volume and joint diameter. It significantly increased spleen weight, WBCs and ESR along with urinary hydroxyproline, nitric oxide and myeloperoxidase. There was significant decreased (p<0.001) in thymus weight, RBCs and Hb in CFA treated rats along with behavioral, biochemical and histopathological alteration induced by the CFA (Patil et al, 2012).

The treatment of rats with Methanolic Extract of Ficus Bengalensis at 400 mg/kg, oral elicited reduction in paw edema and arthritic score, amelioration of oxidative stress, prevention of elevation of LPO and NO, restoration of antioxidants (in edematous and liver tissues), inhibition of serum lysosomal enzymes, biomarkers of connective tissue, and pro-inflammatory cytokines along with improvement of radiographic features of hind legs (Thite et al, 2014).

The treatment of rats with extract of Vitex negundo seeds (EVNS) with abundant phenylnaphthalene-type lignans, significantly inhibited the paw edema, decreased the arthritis score and spleen index, and reversed the weight loss of CFA-injected rats. Histopathological studies showed a marked decrease of synovial inflammatory infiltration and synovial lining hyperplasia in the joints of EVNS treated animals. The remarkable decrement of serum inflammatory factors (TNF-, IL-1β and IL-6) were observed in EVNS treated rats, whereas, IL-10, an anti-inflammatory cytokine, was found to be significantly increased by EVNS. The expressions of Cyclo-oxygenasase-2 and 5-
Lipo-oxygenase in PBMC were also inhibited by administration of EVNS. Therefore it may be an effective cure for the treatment of human rheumatoid arthritis (Zhenga et al, 2014).

The standardized aqueous extract of *Phyllanthus amarus* extract (PAE) was tested against Freund’s complete adjuvant induced arthritic rats. Arthritis assessment, paw volume, joint diameter, mechanical hyperalgesia and nociceptive threshold were measured. On day 28, the animals were sacrificed, tibiotarsal joint was extracted for histopathology. PAE significantly decreased the arthritis which was evident with arthritis index, paw volume and joint diameter. The histopathology also revealed the control in inflammation with PAE (Mali et al, 2011).