4.1 ROLE OF MEDICINAL PLANTS IN THE TREATMENT OF INFLAMMATION

The immediate response of the body against injury or pathogens is inflammation. It is primarily a protective response, which involves healing and restoration of the normal function to the tissue in the body. The most immediate response in an inflammatory trigger is the changes in the vasculature near the site of trigger, enabling the extravasation of plasma proteins and leukocytes, migration of leukocytes, and their accumulation in the site of inflammatory trigger, followed by phagocytosis by competent cells (Garcia-Leme et al 1973, Ryan and Majno 1977, Granger and Kubes 1994, Springer 1994). Migration of leukocytes in tissues toward the site of injury is achieved by locomotion oriented along a chemical gradient, a process called chemotaxis. Thereafter, phagocytosis of offending agents occurs, followed by the release of microbicidal products into the phagolysosome, and the killing or degradation of ingested material (Rabinovitch and De Stefano 1973). After phagocytosis, neutrophils rapidly undergo apoptotic cell death and are either ingested by macrophages or are cleared by the lymphatics (Greenberg 1999, Aderem and Underhill 1999). However, if due to a persistent inflammatory trigger, leukocyte infiltrate itself becomes the offender, since leukocyte-dependent tissue injury underlies many chronic inflammatory diseases.
Thus, all inflammatory and autoimmune diseases, such as Rheumatoid arthritis, are consequences of abnormal functioning of the body’s protective immune system, which is activated and hyper-regulated by unknown agents to attack and destroy the host tissues. The best known approach to treat such patients, by medical practitioners has been to suppress the immune response with immunosuppressive drugs. (Datta et al 2006).

Such immunosuppressive drugs function largely based on interrupting the synthesis or action of mediators that drives and aggravates the host’s response to injury. The role of cytokines and their increased understanding in the inflammatory response has led to the development of treatment strategies based on their selective inhibition (Dinarello 2002, Dinarello 2000, Feige 2000, Lavagno et al 2004, Payvandi et al 2004). Although cytokine functions are complex, cytokine profiles are highly relevant parameters of an immune response. Different cytokines possess biological overlapping functions, and they have the ability to regulate production of other cytokines. For example, many immunomodulators currently under development are screened for their effective inhibition of TNFα overproduction to treat inflammatory disorders (Lipsky et al 2000, Stack et al 1998, Newton and Decicco 1999, Lorenz 2000, Hamilton and Clair 2000). The unfolding of an appropriate inflammatory response is mediated and carefully controlled by different families of cytokines that have either pro-inflammatory (such as TNF-α, IL-1β, IL-8 and IL-12), or anti-inflammatory (such as IL-10 and IL-4) effects (Dinarello 2002, Bai et al 1997, Ogata and Hibi 2003) effects.

The focus of the present study is the identification and development of novel anti-inflammatory agents from the privileged structures available in natural products, which could lead to the identification of new molecules to treat immune related disorders. The basis of this search is the reliance of
modern age drug developers on the enormous knowledge database of traditional healing methods advocating the use of herbs and other plants. There are many reports that traditional medicinal herbs enhance and modulate the immune response. Most secondary metabolites are amongst the phytochemicals which have re-kindled research into the healing and protective efficacy of plants.

The goal of therapeutic strategies for inflammatory disease is development or screening of anti-inflammatory drugs without the reaction on the normal metabolism. Thus, many studies on pharmacological effects and benefits of natural herbs have been performed. The synergistic components found in botanical mixtures represent a largely untapped source of new pharmaceutical products with novel and multiple mechanisms of action.

By establishing an interaction between traditional medicine and modern biotechnological tools, a vastly untapped resource of phytochemicals, can be exploited towards new drug development. Many of the present medicines are derived directly or indirectly from higher plants (eg: Vincristine, Navelbine, Etoposide, Teniposide, Taxol, Taxotere and Topotecan). Thus natural products research continues to provide tremendous variety of lead structures, which are used as templates for the development of new drugs by the pharmaceutical industry (Pezzuto 1997, Grabley and Thiericke 1999, Rates 2001, Simmonds 2003).

There are many approaches to the search for new biologically active principles in higher plants. This random collection, broad screening method is a reasonable approach that eventually should produce useful drugs. The study of the mechanism of action of some of these drugs may provide clues for the production of new therapeutic agents for treatment of diseases such as arthritis and cancer.
The starting point for this research was the knowledge of the traditional use, which assisted in the selection of medicinal plants. Crude extracts of several plants have been used in traditional medicine, as a remedy for the treatment of rheumatoid arthritis, rheumatism, inflammation etc. Based on their activity during initial screening, *Calotropis procera* was selected for the study to isolate lead molecule. The crude extracts of *Calotropis procera* been implicated in various immunomodulatory effects (Figure 1.6) as total crude extracts and the components present in them are poorly understood.

In order to investigate the anti-inflammatory potential of *Calotropis procera* and to elucidate its possible mechanism(s) of action, the plant was evaluated on its ability to regulate cytokines, inflammatory mediators such as NO, cyclooxygenase, chemokines etc., that are critical mediators of inflammation and infection.

### 4.2 DETERMINATION OF THE LEAD STRUCTURE ELICITING THE SPECIFIC BIOACTIVITY

The prospects of finding new drug entities from traditional medicine system, has been enhanced due to the improvement in chemical and biological analytical techniques. Recent advances in molecular and cellular biology have enabled our understanding on mechanism of cell to cell communication, gene expression and cell signalling. This has resulted in creation of suitable experimental designs, to mimic *in vivo* microenvironment *in vitro* perform bio-assay guided fractionation of plant extracts, to identify active components which interact or modulate the bio-target under investigation. The total crude extract of the plant that elicits a positive response in particular assays or on targets, which are presumed to be involved in immune disorders, would then be subjected to bioassay guided fractionation. This enables the direct prioritisation of the extracts for fractionation on the basis of a probability to obtain a known or new biologically active molecule. Bioactivity based *in vitro*
screens is simple and rapid, designed to target specific molecules more rapidly than in conventional animal model based studies. Compounds that elicit a positive response in a particular assay would then be subjected to more in depth analysis of activity-based experiments to confirm the positive effect and this would identify their mechanism of action. Based on this concept ethyl acetate extract of *Calotropis procera* was taken for column purification (Figure 3.3).

Activity-guided purification with *in vitro* based cellular assays was employed for the isolation and optimisation of the lead molecules. Thus fraction-5 from *Calotropis procera* (Figure 3.4B) was found to be efficient in the preliminary bioassay and thus was further considered for activation-guided purification of its constituents and eventually resulting in optimisation of the lead. Proton NMR and Carbon NMR confirmed the elemental position of the bio-active lead structure from *Calotropis procera* (Figure 3.5). Using Mass Spectroscopy elemental formula of the active compound from *Calotropis procera* was identified as C_{29} H_{48} O_{2} and the molecular weight was found to be 428 and the name of the compound was 4,4,6a,6b,11,11,14b-heptamethyl-1,2,3,4a,5,6,7,8,9,10,12,12a,14,14a-tetradecahydropicene-3,8a-diol or 28-Norolean-12-ene-3,17-diol as shown in Figure 3.6. There were little or no in *vitro* studies that had been done so far to understand the mechanism of action pertaining to the immunomodulatory and anti-inflammatory activity of the pure compound isolated on immune cells. Hence it was necessary to elucidate the potential of crude extracts and pure compounds behind their immunomodulatory and anti-inflammatory activities by *in vitro* based bioassays.
4.3 *IN VITRO* APPROACH TO STUDY THE ANTI-INFLAMMATORY PROPERTY OF MEDICINAL PLANTS

The design of a therapeutic drug is based on the detailed understanding of the basic nature of inter and intra-cellular interactions, in healthy and in diseased conditions. This primarily would enable the new therapeutic drug to reverse the pathological conditions. *In vitro* studies with intact cells may considerably advance our knowledge about the beneficial effects of phytochemical based pharmaceuticals and in some cases may even provide the only source by which such detailed information can be obtained. Target based assays are being considered as the frontline primary screens to assist in the prioritisation of active extracts (Baker et al 1995). Bioactivity-based *in vitro* assays are simple and rapid, enable to identify specific target molecules more rapidly than conventional animal model based studies.

Phytochemicals that have drug like activity could serve as a desirable template for the development of better analytical assays and also allows for a detailed introspection into structure based activity enabling, the enhancement of the activity, by synthetically modifying phytochemicals to obtain better desirable bio-activity. The bioactivity-based screens used in the present study involves measuring the suppressive effect on the proliferative response of lymphocytes using \[^{3}\text{H}]\) Thymidine (Krishnamoorthy et al 2000) and monitoring factors that play an important role in regulating inflammatory mechanisms such as pro-inflammatory and anti-inflammatory cytokines, inflammatory mediators, chemotaxis of immune cells, etc with a view to postulate the possible mechanism by which these extracts could reduce inflammatory and immunological disorders. Signal transduction therapeutics is a new dominant theme of drug discovery and has a major impact in regulating immune disorders (Dong et al 2002, Han et al 2001, Hommes et al 2002). It is also well known that for the production of inflammatory
mediators, multiple signalling pathways are involved and hence it is essential to understand the possible cascades that are either activated or inhibited by a drug, ultimately leading to the anti-inflammatory effect.

4.4 *Calotropis procera* ON REGULATION OF PROLIFERATIVE RESPONSE OF PBMC

4.4.1 Regulation of Lymphocyte Proliferation

Rheumatoid arthritis, inflammatory bowel disease, allergy, etc. are all inflammatory diseases which are characterised by uncontrolled activation and hyper function of the immune responsive cells, particularly lymphocytes. Regulation of lymphocyte proliferation thus is one of the key aspects of immune response and maintenance of homeostasis in the body. (Moss et al 2004, Romagnani et al 2000, Murphy and Reiner 2002)

To adjudge the ability of *Calotropis procera* in modulating the immune response, the effect of the plant on human PBMC of healthy individuals was studied. PBMC resembling primary culture would be considered as the best primary *in vitro* model for studying the immunomodulatory properties of plant drugs. Hence using thymidine incorporation assay the ability of crude extracts of *Calotropis procera* on proliferation of PBMC was analysed and this revealed that the inhibitory effect is more significant in ethyl acetate crude extract when compared to other extracts as shown in Figure 3.2 at 72 hours after treatment.

The central event in generation of immune responses is the activation and clonal expansion of T cells. Interaction of T cells with antigens or phytohemagglutinin (PHA) initiates a cascade of biochemical events and gene expression, which induces the resting T cells, to enter the cell cycle leading to proliferation and then differentiation (Kuby 1997). Thus, growth
modulators or other external events are likely to act by controlling the expression or function of the products of these genes.

The ability of *Calotropis procera* crude extract and bio-active pure compound to inhibit the activated lymphocytes was examined by stimulation with PHA. PHA is a mitogen for T lymphocytes and stimulates T cells to proliferate through interaction with N-acetyl galactosamine glycoproteins expressed on the cells (Kuby 1997). In the present study, T cells were the major proliferating cells in PBMC cultures activated with PHA. Thus, inhibitory effect of *Calotropis procera* ethyl acetate crude and bio-active pure on PHA activated PBMC proliferation could be suggestive of suppression on T cell proliferation (Figure 3.7A).

The inhibitory effect of *Calotropis procera* ethyl acetate crude extract and the bio-active pure on JURKAT T-cells which are cells of lymphoblastic leukaemia, was also assessed. JURKAT T-cells are known to produce copious amounts of IL-2 cytokine which functions in an autocrine fashion to clonally proliferate JURKAT cells. The assessment of *Calotropis procera* ethyl acetate crude and bio-active pure on JURKAT cells would be more accurate as it would mimic the inflammatory condition better (Figure 3.7B).

Because of its pivotal role in immune regulation as described above, T cell activation provides a target for pharmacological modulation aimed at achieving clinically useful immunosuppression (Van Den Brande et al 2002). This is well demonstrated by *Calotropis procera* crude and pure by their growth inhibiting activity on PHA stimulated PBMCs and unstimulated JURKAT T-cells.

By thymidine uptake studies, 20 μg/mL of pure showed similar effect to that of 30 μg/mL of crude in case of *Calotropis procera*
(Figure 3.7A and 3.7B). Hence to evaluate the effect on other biological activities, 30 μg/mL of crude and 20 μg/mL of pure was used for Calotropis procera. This inhibition of lymphocyte proliferation has been shown to be through their modulation of the cytokine driven network and not through toxicity on cells, this was confirmed through MTT assay performed at 72 h (Figure 3.8 A and 3.8B).

4.4.2 Reduced IL-2 mRNA expression – A parameter in regulating lymphocyte proliferation

Regulation of T lymphocyte activation, proliferation and cytokine production is one of the mechanisms to reduce hyperactive immune responses. Yet, the immune responses, if abnormally intense or inappropriately prolonged, could aggravate the injury or may even cause death. For instance, exaggerated Th1 response and increased production of IL-2 characterise Crohn’s disease (Fiocchi, 1998; Kugathasan et al., 1998) and increased response to allergens is seen in respiratory disorders like asthma (Renzi et al., 1999).

In this study, RT-PCR analysis revealed the effect of Calotropis procera on IL-2 mRNA expression in JURKAT T-cells. Observations showed that Calotropis procera crude and purified compounds inhibited IL-2 mRNA expression in JURKAT T-cells stimulated with LPS (Figure 3.9) at 12 hours and 24 hours probably suggestive of their tendency to down regulate T cell mediated response. IL-2 is the major T cell growth factor that is required for driving T cells into the proliferation cycle (Smith, 1988). The inhibition of IL-2 would have positively culminated through the inhibition of T cell proliferation. This was also justified by the preliminary results on inhibition of lymphocyte proliferation proliferation at 72 hours (Figure 3.7A).
4.5 TARGETING INFLAMMATORY CYTOKINES FOR POTENTIAL ANTI-INFLAMMATORY DRUG DEVELOPMENT

Inflammation is an aberrant over-response of the innate immune system. An elaborate interaction between the innate and adaptive immune responses is required to sustain an inflammatory response. When actively inflamed, activation of T cells, macrophages, fibroblasts, endothelial cells and plasma cells can be observed. New biological therapies aimed at neutralising cytokines have been very successful and have initiated a new era of rational therapy of inflammatory and autoimmune diseases. In an inflammatory state, copious amounts of cytokines and chemokines have been identified which aid in the tissue damage process. Of these, TNFα, IL-1β, IFNγ, IL-12, IL-6, IL-8 are the major mediators of both primary and secondary cytokine effects and chemokine effects.

4.5.1 Regulation of major Pro-Inflammatory Cytokines TNFα, IL-1β and IL-6 in Inflammation by Calotropis procera

Inflammatory cytokines play an important role in the modulation of acute and chronic inflammation. Over expression of the pro-inflammatory cytokines TNFα and IL-1β has been documented in a number of inflammatory processes, which led to the first successful attempts to block a cytokine therapeutically. TNFα is a versatile, multipotent cytokine which induces production of other inflammatory cytokines such as IL-1, IL-6 and granulocyte macrophage colony-stimulating factor, and chemokines such as IL-8. Interleukin 1β (IL-1β) is a pleiotropic pro-inflammatory cytokine that plays a key role in mediating cartilage degradation in osteoarticular disorders such as osteoarthritis (OA) and rheumatoid arthritis (RA) (Leo et al 1999). A monoclonal antibody to TNFα or infliximab was originally piloted in
rheumatoid arthritis and Crohn’s disease (Lipsky et al., 2000, Rutgeerts 1999). In both cases the therapeutic effect was dramatic with dose-dependent clinical and laboratory responses. While Crohn’s disease and rheumatoid arthritis were the first two disorders in which TNFα and IL-1β inhibition were tested therapeutically, there are now many other disorders in which TNFα and IL-1β inhibition has been tried and found effective (Lamprecht et al 2002, Aeberli et al 2002, Sfikakis 2002, Dinarello 2000, Dinarello 2002). Hence inhibition of proinflammatory cytokines is one of the mechanism by which some of the inflammatory and autoimmune disorders can be regulated.

TNFα is mainly released in monocytic cell lineage and T lymphocytes (Newton and Decicco 1999). For example in Crohn’s disease, T cells of lamina propria cells appear to be largely responsible for elevated production of TNFα (Kontoyiannis et al 1999, Noguchi et al 1998). The monocytic cell lineage and T lymphocytes were mainly mixed in PBMCs, therefore TNFα can be observed in human peripheral blood mononuclear cells (PBMC) after stimulation.

TNFα is released early in copious amounts in response to a wide variety of invasive stimuli, and it’s over production also has been shown to induce the production of various inflammatory mediators such as cyclooxygenase type 2 (COX-2), PGE₂, ROS, iNOS, IL-6, and nitric oxide (Perkins and Kniss 1997, Perkins et al 1998, Wong et al 1996, Dinarello 2002). Thus TNFα and IL-1β activate many inflammatory cells and induce the production of other inflammatory mediators that can modulate important cellular events including gene expression, DNA damage and cellular proliferation and contribute to various inflammatory disorders. Therefore, cellular manipulation of the production of TNFα and IL-1β is of importance in regulating the inflammatory response (Dinarello 2002, Dinarello 1997).
In the present study JURKAT T-cells were activated with LPS to induce proinflammatory cytokines such as TNFα, IL-1β and IL-6 (Figure 3.10). Lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall, is one of the major causative agents of gram-negative sepsis (Meng and Lowell 1997). Stimulation LPS leads to a cascade of intracellular signalling events that ultimately result in production and secretion of cytokines and other inflammatory mediators that constitute the pro-inflammatory response.

Analysis of ethyl acetate crude extract and bio-active pure compound of *Calotropis procera* on their capacity to down regulate the LPS stimulated production of TNFα showed that the crude extract and its bio-active pure compounds inhibited the production of TNFα right from 6 hours up to 24 hours (Figure 3.10A). Though there were not much efficient modulations in the expression at varied duration of incubation, the study proved their capacity to markedly reduce TNFα at an early time point (as indicated by Lanes 6 and 9). There is now abundant evidence that TNFα inhibition dramatically improves patient outcomes in RA, as well as in other autoimmune systemic inflammatory conditions (Kavanaugh 2006, Kavanaugh et al 2006). Hence the down regulation of TNFα by both the pure compounds suggests their possible role in regulating inflammation.

Similarly to interpret the inhibitory effects on potential pro-inflammatory cytokine IL-1β *in vitro*, the responsiveness of these plants on its expression was also studied. In parallel with previous studies on TNFα, the effect of *Calotropis procera* crude extract and bio-active pure on IL-1β expression was also promising. From Figure 3.10B the efficacy of the plant extracts to reduce IL-1β expression revealed their possible role in inhibiting down stream mediators of inflammation. IL-1β and TNFα play an important role in amplification loop of the inflammatory response as they can stimulate
their own and each others production. Hence the early down regulation of TNFα, which occurs at 6 hours on treatment with pure compound of *Calotropis procera*, might partly contribute to the reduction of IL-1β at later stages (predominantly reduced at 12 h and 24 h time point than 6 h).

Interleukin-6 is a pro-inflammatory cytokine produced by multiple cell types in response to diverse stimuli (Heinrich et al 1990). Recent studies demonstrated that inhibition of IL-6 is an effective treatment for various inflammatory diseases, including rheumatoid arthritis (Goldblatt and Isenberg 2005). When assessed for it’s potential inhibitory role in the expression of IL-6 it was observed that only the crude extract of *Calotropis procera* showed moderate inhibition, while the bio-active pure does not have any significant inhibition. (Figure 3.10C).

4.6 REGULATORY EFFECT OF *Calotropis procera* ON CHEMOKINE - IL-8

Chemokines are essential in recruitment of leukocytes for the clearance of infectious agents and promoting of wound healing, and they also contribute to many pathological inflammatory reactions and autoimmune reactions (Wang et al 1997). Chemokines and chemokine receptors play essential roles in leukocyte trafficking under inflammatory condition as well as many other immune responses including cell growth and differentiation (Loetscher et al 1996; 2000). Therefore, regulation of the expression levels of chemokine receptors probably results in alteration of immune responses against infection, inflammation, and immune diseases.

Among the chemokines, interleukin-8 (IL-8) is a known putative marker for several inflammatory diseases (An et al 2004, Babu et al 2004,
Goldenberg-Cohen et al 2004, Sukedai et al 2004, Baggiolini et al 1997), and has an important role in mediating the inflammatory processes by activating neutrophils and eosinophils (Teran et al 1996).

From the study on IL-8, it was clear that *Calotropis procera* crude and pure do down regulate the expression of IL-8 mRNA effectively (Figure 3.21A). The up regulation of IL-8 by pro-inflammatory cytokines like IL-1β and TNFα was demonstrated in various studies. In the present context, the reduction of IL-8 by *Calotropis procera* crude and pure extracts can be attributed to the early attenuation of pro-inflammatory cytokine production at 6 h (Figures 3.10).

### 4.7 REGULATORY EFFECT OF *Calotropis procera* ON Th1 AND Th2 CYTOKINES

One theory of immune regulation involves homeostasis between T-helper 1 (Th1) and T-helper 2 (Th2) activity. Differentiation of naive, uncommitted T cell precursor cells into Th1 or Th2 cells is a complex developmental process, and molecular mechanisms underlying these processes may provide a conceptual framework in developing immune modulation therapies against allograft rejection, autoimmune diseases, and allergic diseases.

The dominant factors that control the differentiation program are now recognised to be cytokines. T cells activated in the presence of IL-12 differentiate into Th1 cells, predominantly secrete IFNγ and TNFα and promote delayed-type hypersensitivity responses (Trinchieri et al 1995). In contrast, T cells that are activated in the presence of IL-4 differentiate into Th2 cells, produce mainly IL-4 and IL-5, and promote humoral and allergic responses (Lederer et al 1996, Nelms et al 1999, Romagnani et al 2000). These two systems are interrelated, and an increase in IL-12, will inhibit IL-4
production, shifting the immune response to a mainly Th1 type, and thus a cellular immune response. Over activation of either pattern can cause disease, and either pathway can down-regulate the other (Mueller et al 1996).

Th1 or Th2 types are associated with varied autoimmune and inflammatory disorders. For instance, on the negative side, the Th1 pathway is often portrayed as being the more aggressive of the two, and apparently, when it is over active, can generate inflammatory and organ-specific autoimmune diseases (e.g., arthritis, Crohn's disease, multiple sclerosis, type 1 diabetes) (Nicoletti et al 1996, Karlsson et al 2000, Tsiavou et al 2004). Similarly exaggerated Th2 responses characterise allergic and respiratory disorders such as asthma (Romagnani et al 2000).

Maintaining the delicate balance between Th1 and Th2 cells is vital. Many researchers are currently attempting to enhance the activity of Th1 helper cells in case of allergic disorders and Th2 in case of inflammatory disorders. Hence the ability of *Calotropis procera* to modulate Th1 and Th2 was evaluated.

Initial observations using JURKAT T-cells showed *Calotropis procera* crude and pure could influence the cellular proliferation of T lymphocytes *in vitro* (Figure 3.7B). When the profile of cytokine secretion by activated T cells was analysed by RT-PCR, it revealed that the pure compound of *Calotropis procera* was selective in the above-mentioned activities. Cytokines belonging to Th1-type helper cells were inhibited, whereas those associated with Th2-type helper cells were increased (Figure 3.11). On the other hand, crude suppressed both Th1 and Th2 cytokines. The inhibitory effect on IL-12 and IFNγ (Figure 3.11A and 3.11B) and specificity toward certain T-helper cells indicates *Calotropis procera*
pure could have important regulatory and modulatory activities in diseases where there is enhancement of the Th1-type responses.

In addition, *Calotropis procera* pure compound enhanced Th2 cytokines IL-10 and IL-4 (Figures 3.11C and 3.11D). Different disease manifestations are associated with prominence of one or the other of the Th1 and Th2 phenotypes. Th2 cytokines are known to down-regulate IL-1β and TNFα in a variety of experimental conditions (Miossec 2004, Briolay et al 1992). Several studies in animal models have suggested that Th2 cytokines attenuate Th1-dependent autoimmune disorders (Mueller et al 1996, Liblau et al 1995, Bessis et al 1995). Therefore the increased Th2 cytokines by *Calotropis procera* pure can further aid in its anti-inflammatory action by negatively regulating Th1 cytokines (Figure 3.11), macrophages and inflammatory cytokines such as TNFα, IL-1-β and Nitric oxide (Figure 3.12). Thus *Calotropis procera* pure may be a useful tool in the treatment of Th1-mediated diseases.

4.8 DIFFERENTIAL EFFECTS OF *Calotropis procera* ON ARACHIDONIC ACID PATHWAY ENZYMES

Enzymes involved in the arachidonic acid pathway play an important role in the pathophysiology of a variety of inflammatory disorders. Synthesis of prostaglandins (PGs) is dependent on two key enzymes, phospholipases A₂ that generate arachidonic acid from membrane phospholipid, and cyclooxygenases (COX) that convert AA to a variety of PG in a cell specific manner (Cummings et al 2000). The PLA₂ reaction is the primary pathway through which arachidonic acid (AA) is liberated from phospholipids. Free AA is the precursor of the eicosanoids, which include the prostaglandins, generated through the cyclooxygenase reaction, and the
leukotrienes, generated through the lipoxygenase reaction (Smith et al. 2000). Thus, PLA₂ is an important signalling enzyme, through which multiple downstream effectors are generated.

4.8.1 *Calotropis procera* on Regulation of PLA₂ and COX-2

The inhibitory effect of *Calotropis procera* on the cytosolic PLA₂ enzyme was studied using RBL-2H3 mast cell line. Activation of mast cells induces the synthesis and release of leukotrienes and of prostaglandin D₂ (Kawata et al. 1995). Of the various classes of PLA₂, the cPLA₂ is the major class of enzyme, most notable for eicosanoid generation. It was noted to preferentially cleave AA from phospholipids (Clark et al. 1991, Sharp et al. 1991). The basal level expression of this enzyme is low but becomes highly expressed during inflammation and sepsis as a result of LPS, cytokine and NF-kappa B induction. This enzyme has become associated with allergic rhinitis, rheumatoid arthritis and septic shock.

The anti-inflammatory activity of glucocorticoid appears to be due in part to the down-regulation of PLA₂-IIA expression, and together this body of evidence suggests that inhibition of PLA₂-IIA represents a target for the treatment of inflammatory disease. It was observed that the ethyl acetate crude extract of *Calotropis procera* showed a moderate down regulation in the PLA₂ activity while the pure did not show significant inhibition as studied by [¹⁴C] Arachidonic acid incorporation (Figure 3.13A). The crude extract and pure compound showed a significant down regulation of the enzyme COX-2 in RAW 264.7 macrophages (Figure 3.13B). The ability of *Calotropis procera* crude extract and pure compound to inhibit these potent inflammatory enzymes reveals a possible mechanism of action in controlling inflammation. Accumulating evidences in the literature suggest that cytosolic PLA₂ together with two sPLA₂ isozymes (sPLA₂-IIA and sPLA₂-V) are functionally coupled with cyclooxygenase-1 and 2 pathways,
respectively, for immediate and delayed PG biosynthesis. This spatio-temporal coupling of cyclooxygenase enzymes with PLA2s may represent a key mechanism in the propagation of inflammatory reaction. The proinflammatory cytokines TNFα, IL-1β and IL-6 have been shown to induce sPLA2 in a ubiquitous manner (Lhousseine and Mounia 2001). Thus the pure compound by its regulatory effect on the two major enzymes of the pathway helps in the inhibition of prostaglandin biosynthesis thereby diminishing inflammation at sites of injury.

4.8.2 Analysis Molecular Modelling Studies of 28-NOROLEAN-12-ENE-3,17-DIOL with Cyclooxygenase -2

There are two isoforms of the enzyme cyclooxygenase, COX-1 and COX-2. COX-1 is constitutive, while COX-2 is inducible. The structure of COX-1 was elucidated by Picot et al (1994). Many inhibitors popularly known as non-steroidal anti-inflammatory drugs (NSAID) inhibit cyclooxygenase enzyme. Some of these drugs inhibit both COX-1 and COX-2 (aspirin). Other drugs (like SC-558, rofecoxib) selectively inhibit COX-2 over COX-1. There are four broad classes of NSAIDs. The first class irreversibly inactivates the enzyme by covalent modification. Acetylsalicylate (aspirin) is an example of this class. The second class of NSAIDs work by reversible competitive inhibition with the substrate. An example of this second class is ibuprofen, diclofenac. The third class of NSAIDs work by forming salt bridges with the enzyme that results in a slow, time-dependent inhibition. Examples include flurbiprofen and indomethacin. The fourth class of NSAIDS are selective for COX-2 and inhibit in a slow, time-dependent process.

To validate the docking method used in this study, modelling of cyclooxygenase-2 with standard inhibitors and substrate was evaluated.
Keifer et al (2003), have evaluated the binding efficacy of diclofenac with Cyclooxygenase-1. It is found that diclofenac binds to the active site residue SER 530 and is in close proximity to TYR385. SER530, is the acetylation site of aspirin and thus inhibits the activity of COX-1 and COX-2 non-selectively (Figures 3.15 and 3.17 A, B). Kurumbail et al (1996), have evaluated the binding efficacy of SC-558. The selectivity shown by SC-558 seems to result from the phenylsulphonamide moiety, which binds in a pocket that is more restricted in COX-1 and is unoccupied in the complexes of COX-2 with non-selective inhibitors. The pocket usually referred to as the “side pocket” branches of from the main hydrophobic channel that leads to the cyclooxygenase active site and is more accessible in COX-2 primarily because of a substitution of isoleucine to valine at position 523. The valine side chain being smaller than that of isoleucine opens up the pocket that comprises Leu 352, Ser 353, Tyr 355, Phe 518 and Val 523. (FIGURE 3.18)

On the other hand, the modelling studies with arachidonic acid (Figures 3.14 and 3.16) which is a precursor for the formation of prostaglandins reveals that the arachidonic acid is held with its carboxylic moiety by ARG120, while the non-polar end extends into the hydrophobic channel. It places its 13th carbon in close proximity to TYR385 for abstraction of the corresponding hydrogen and oxygenation takes place at this position. In all our docking studies, the two inhibitors, non-selective diclofenac, selective SC-558, and substrate arachidonic acid, were modelled and were much similar to the reported PDB id, 6COX, 1PXX, 1CVU respectively.

Molecular Modelling studies of 28-norolean-12-ene-3,17-diol reveals that it does fits snuggily in the hydrophobic channel leading to the active site (Figures 3.19 and 3.20). Further analysis reveals that it’s polar OH at positions, 3 and 17 interact with key residues, TYR355, which guards the entrance to the channel, and TYR385 which is the active site residue. This is
indicative of the fact that it fits in the same interacting zone displayed by the substrate arachidonic acid, possibly this is indicative of competitive binding behaviour of Norolean.

4.9 ANALYSIS OF *Calotropis procera* ON NITRIC OXIDE PRODUCTION

The intercellular messenger nitric oxide (NO) is a short lived free radical that participates in the physiology and pathophysiology of many systems (Moncada et al 1991). Macrophages are one of the major immune cells that produce nitric oxide on induction of iNOS gene and are essential for the control of a variety of microbial infections (Bogdan et al 2000). However, uncontrolled NO production by macrophages during inflammatory disorders is detrimental, resulting in tissue damage and cell dysfunction or death (Murphy 1999).

A variety of exogenous stimuli, including LPS, PMA, IFNγ and TNFα stimulate the expression of iNOS. Hence in this study macrophages were induced with LPS that resulted in the production of high levels of nitric oxide and iNOS (Figures 3.12A and 3.12B) and the effect of *Calotropis procera* crude and pure on nitric oxide production was then evaluated under these induced conditions.

Three isoforms of NOS have been identified and are classified into two major categories: constitutive and inducible NOS. Inducible nitric oxide synthase (iNOS), which is normally not present in resting cells, is expressed in several pathophysiological conditions, and it produces large amounts of NO in response to inflammatory signals, such as cytokines and lipopolysaccharide (LPS) (Alderton et al 2001, Liu and Hotchkiss 1995, MacMicking et al 1997, Moncada et al 1991). Thus, NO over production by iNOS can be considered as pathological, depending on the ability of cells or
tissues to control both the expression of iNOS activity and the nonspecific effects of NO. In animal models of inflammatory diseases inhibition of iNOS activity often reduces severity of the disease (Clancy et al 1998).

In the current study, LPS induced macrophages (RAW 264.7) were found to produce high level of NO. The effect of the crude extracts and pure compounds of Calotropis procera on NO production was evaluated under the above-mentioned induced conditions. A significant decrease in the NO production was observed on treatment with the plant extract at 24 hours and it was presumed to result from the suppression of iNOS gene induction, which was studied by RT-PCR (Figures 3.12B). The expression levels of iNOS were down regulated right from 6 hours (Lanes 6) when compared to LPS treated control lanes suggesting that the reduced iNOS would have resulted in the inhibition of NO in LPS induced macrophages at 24 hours.

Nitric oxide also appears to mediate or augment the synthesis of TNFα, IL-1β and chemokines. Studies also show that, reduced levels of IL-1β have been observed after inhibition of iNOS activation (Southey et al 1997). TNFα and IL-1β also have been shown to have direct regulation on NO release through iNOS gene expression (Perkins et al 1998). Therefore the inhibition of production of these proinflammatory mediators has proven to have tremendous therapeutic value. Keeping with the above line of studies, the present study on Calotropis procera crude extract and bio-active pure compound showed reduced NO production (Figures 3.12) which substantiated the fact that it might in turn have an inhibitory role on induced proinflammatory cytokines TNFα and IL-1β and vice versa (Figures 3.10).
4.10 MITOGEN ACTIVATED PROTEIN KINASES (ERK1/2, P38 AND JNK)-ROLE IN ANTI-INFLAMMATORY EFFECT OF *Calotropis procera*

Mitogen activated protein kinases (MAPK) are central to many immune responses, including the regulation of cytokine responses, chemokine responses and cell proliferation. MAP kinases, signal transduction pathways in mammalian cells include the extracellular signal related kinase (ERK), c-Jun N-terminal kinase (JNK/SAPK) and p38 MAP. All the three kinases can regulate the initiation and progression of inflammation and immune responses (Kyriakis and Avruch 2001, Rao 2001). All MAP kinases cascades cooperate in the orchestration of inflammatory responses, and their extensive cross talk with other inflammatory pathways, such as NF-κB and Janus kinase/STAT signaling has been described (Rao 2001, Tibbles and Woodgett 1999, Kyriakis and Avruch 2001). As a result, MAP kinases provide potential targets for therapeutic immunomodulation. Since *Calotropis procera* was effective in regulating various mediators of the immune response their effect on MAPK was studied.

4.10.1 Regulation of Mitogen Activated Protein Kinases by *Calotropis procera* crude and pure

It is prominent that many genes that are positively regulated at transcriptional level by MAPKs are negatively regulated by *Calotropis procera*. Prompted by this observation, the effect of *Calotropis procera* upon the MAPK pathway was investigated. Effect of *Calotropis procera* crude and pure show that ERK, JNK and p38 MAP kinases are differentially regulated by crude and pure in LPS stimulated JURKAT T-cells. *Calotropis procera* crude had inhibitory effect on phosphorylation of all the three mitogen activated protein kinases whereas pure had no effect on inhibition of ERK
(Figures 3.2, 3.23 and 3.24). The inhibitory effect on all the three MAPK suggests the regulatory role of crude on multiple targets when compared to pure. *Calotropis procera* pure demonstrated significant inhibition of JNK (Figure 3.23) while it had moderate effect on p38 (Figure 3.24). JNK is one of the major kinases responsible for IL-2 production (Chen et al 1998). Hence the reduced phosphorylation of JNK by *Calotropis procera* pure could influence its effect on lymphocyte proliferation (Figure 3.7). However, it is likely that inhibition of JNK signaling is not the sole mechanism by which *Calotropis procera* pure interferes with lymphocyte proliferation because the inhibitory effect of pure on lymphocyte proliferation was similar to the crude which was effective on all the three MAPKs (Figures 3.2, 3.23 and 3.24).

However, JNK and p38 MAPK positively regulate a variety of genes involved in inflammation, such as TNF-α, IL-1, IL-6, IL-8 and several studies have described the participation of JNK and p38 MAP kinase in inflammation (Swantek et al 1997, Ip and Davis 1998, Badger et al 1996 and 2000, Hallsworth et al 2001, Hommes et al 2002, Hommes et al 2003). It is likely the significance of inhibition of JNK and p38 MAP kinase pathways by *Calotropis procera* pure lies in it being the probable mechanism of inhibition of the expression of pro-inflammatory genes (Figure 3.10).

### 4.11 REGULATION OF C-JUN AND C-FOS BY MAPK

Transcription factor AP-1, a heterodimer composed of *c-jun* and *c-fos* performs various functions in the cells related to proliferation, differentiation, survival, response to stress and regulation of inflammation (Shaulian and Karin 2001, Rincon and Flavell 1994). It is well established that AP-1 activation, resulting from the engagement of several different surface receptors with their extracellular ligands, usually involves MAPK intermediates. Hence the work on down-regulation of inflammatory responses
by *Calotropis procera* was furthered by looking at the transcriptional regulation of *c-jun* and *c-fos*, which are known to be important for activation of immune response. Both the level and the activity of AP-1 are regulated by ERK, JNK and p38 MAP kinases (Cook et al 1999, Karin 1995, Whitmarsh and Davis 1996, Ip and Davis 1998). ERK has been shown to mediate its effects on AP-1 by phosphorylating and increasing the activity of the protein Elk-1, a transcription factor involved in the up-regulation of the *c-fos* gene (Gille et al 1995, Gille et al 1992). JNK has been shown to phosphorylate *c-jun* on two N-terminal serine residues (Ser63 and Ser73) critical for its transactivational activity (Minden et al 1994, Derijard et al 1994). In addition to the ERK and JNK pathways, the p38 pathway is also up regulated during the T cell and macrophage responses.

Activation of p38 causes increased reporter gene expression mediated by the transcription factors ATF-2 and Elk-1 (Tan et al 1996). Since Elk-1 has been implicated in the up-regulation of the *c-fos* gene, which can form heterodimers with *c-jun* subunits, this suggests some involvement of the p38 MAP kinase pathway in AP-1 binding and cytokine synthesis. Therefore the inhibitory effect on MAPK pathway by *Calotropis procera* was assessed further by looking at the expression of *c-jun* and *c-fos*, which are known to be transcriptionally regulated by different MAPKs.

### 4.11.1 Inhibition of JNK and p38 by *Calotropis procera* pure has an Effect on AP-1 Through Down Regulation of *c-jun* and *c-fos* Expression

*Calotropis procera* pure compound showed inhibition of JNK activation and not ERK activation (Figures 3.22 and 3.23), hence it was hypothesised that the anti-inflammatory effect of *Calotropis procera* could indeed take place through reduced levels of *c-jun*. The cytokine transcriptional factor *c-jun*, has been shown to get activated mainly by JNK
Several studies have demonstrated the significant role of JNK in cytokine signalling, in mediating inflammatory responses and mediation of activation of transcription factors such as c-jun (Han et al 2001, Vincenti and Brinckerhoff 2001, Swantek et al 1997). RT-PCR analysis of Calotropis procera pure treated cells revealed down regulation of c-jun mRNA levels from 3h onwards post treatment and persistent reduction could be noticed even at 6 h after treatment (Figure 3.25B). This data along with inhibition of JNK activation (Figure 3.23) when correlated with the earlier observations of sustained down regulation in levels of TNFα and IL-1β (Figure 3.10) proves a possible interrelation between JNK and c-jun in the regulation of inflammatory mediators. On the other hand, Calotropis procera pure had no significant effect on ERK. Observations made on c-fos expression show that treatment of Calotropis procera pure could only moderately down regulate c-fos expression at later time points (Figure 3.25A). In summary, the present study demonstrates that immunomodulatory effect of Calotropis procera pure is mainly mediated through the JNK pathway, which leads to the decrease of c-jun and thus regulates AP-1 expression and subsequently brings about down regulation of inflammatory mediators.

4.12 ROLE OF TRANSCRIPTION FACTORS IN THE ANTI-INFLAMMATORY POTENTIAL OF Calotropis procera

Inflammation is mediated by a vast array of soluble factors, cell surface molecules and enzymes that recruit cells to the site of inflammation and activate their gene expression machinery to produce more inflammatory mediators. Transcription factors are central to this process. The regulation of gene expression by transcription factors is fundamental to the phenotype of all cells. After a signal has been transduced from the cell surface to the nucleus, the transcription factors respond in combinatorial fashion to bind the promoter regions of genes containing appropriate recognition sequences to induce the
level of mRNA synthesis. Because the genes for many inflammatory mediators contain similar recognition sequences, or response elements, it is likely that inhibition of a limited number of key transcription factors may simultaneously control many mediators of inflammation. Transcription factors that are necessary for the expression of a large number of inflammatory mediators include NF-κB, AP-1 and STATs.

4.12.1 Differential Effects of *Calotropis procera on Nf-κB*

The transcription factor NF-κB stands out as one of the most important inducers of inflammation. Analysis of IκB degradation and NF-κB p65 translocation by western blotting revealed considerable degradation of IκBα in the cytosolic fraction of RAW 264.7 macrophages stimulated with LPS. Co-incubation with *Calotropis procera* crude extract or pure compound showed marked inhibition of IκBα degradation in the cytosolic fraction (Figure 3.26A) and only basal levels of NF-κB p65 was present in the nuclear fraction (Figure 3.26B). NF-κB is involved in the expression of numerous inflammatory mediators, including the cytokines TNFα and IL-1β, IL-6, IL-8, enzymes iNOS, COX-2, cPLA2 and cell adhesion molecules ICAM-1 AND VCAM-1 (Baldwin 2000) and has a central role in a wide variety of inflammatory diseases, including RA, inflammatory bowel diseases and asthma (Miagkov 1998). Hence the present study clearly demonstrated that the earlier inhibition seen in pro-inflammatory cytokines and mediators is due to the inactivation of NF-κB and inhibition of MAP kinase pathway in producing the anti-inflammatory effect.
4.12.2 Observations Made on STAT 3 AND SOCS 3

Present study has shown that there is a marked suppression in Th1 type cytokines and induced expression of Th2 cytokines on *Calotropis procera* pure treatment (Figure 3.11). To understand the mechanism by which this occurs, the effect of *Calotropis procera* on STAT3 and SOCS-3 was studied. Cytokines regulate the growth and differentiation of cells by binding to cell-surface receptors and activating intracellular signal transduction cascades such as the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling pathways (Nakajima et al 1995). The suppressors of cytokine signaling (SOCS) family of proteins negatively regulate cytokine signaling. Transcripts encoding CIS, SOCS1, SOCS2, and SOCS3 are transiently up regulated in response to a wide variety of cytokines, and over expression of these proteins in cell lines results in inhibition of cytokine signaling. SOCS proteins therefore form part of a classical negative feedback circuit (Krebs DL 2001).

Observations on STAT3 and SOCS 3 revealed considerable up regulation of STAT3 and SOCS 3 by both crude extract and pure compound of *Calotropis procera* (Figure 3.28). In the previous experiments on JURKAT *Calotropis procera* showed marked inhibition of the Th1 cytokines IFN\(\gamma\) and IL-12 which would have resulted from the up regulation of the anti-inflammatory cytokine IL-10. IL-10 was reported to have the remarkable property of blocking cytokine, chemokine and nitric oxide production from macrophages activated by bacterial lipopolysaccharide (LPS) (Bogdan et al 1991, de Waal Malefyt et al 1991). IL-10 derived from either Th1- or Th2-polarized subtypes are important in regulating inflammation in Th1- or Th2-driven immune responses. The IL-10R activates Janus kinase (JAK) 1, the only JAK family member required for IL-10 signaling (Riley et al 1999, Rodig et al 1998). Activation of JAK1 initiates phosphorylation of the
IL-10Ra chain, on two tyrosine residues that dock the SH2 domains of STAT3, this recruitment initiates the anti-inflammatory cascade (Weber-Nordt et al 1996). More definitive studies have shown that STAT3 is the only STAT protein required for the anti-inflammatory effects of IL-10 (Lang et al 2002, Takeda et al 1999, Williams et al 2004). Hence the up regulation of STAT3 and SOCS3 by *Calotropis procera* most likely through IL-10 synthesis, is clearly in line with the inhibition seen, of Th1 cytokines.

### 4.13 SUMMARY OF ANTI-INFLAMMATORY POTENTIAL OF *Calotropis procera* PURE COMPOUND

The bio-active lead molecule from *Calotropis procera* exhibits significant anti-inflammatory potency by targeting directly the pro-inflammatory cytokines TNFα, IL-1β, IFNγ and IL-12 and the pro-inflammatory mediator nitric oxide. The pure compound also has a potent regulatory effect on the chemokine system and Th1 / Th2 balance possibly via the STAT system. All these effects can be attributed to the MAP kinase cascade as the pure compound showed very good inhibition of the two major MAP kinases, JNK and p38 (Figure 4.1). The bio-active pure moderately inhibited PLA2 and down regulated the expression of COX-2. Analysis of signaling events suggests that the pure compound also exhibits inactivation of NF-κB. This is indicative of a complete down regulation of the hyper immune response.

Thus, isolation of anti-inflammatory molecules targeting potential targets and assessing their biological activity in various combinations, for different inflammatory disorders, augurs well for the future of anti-inflammatory therapy.
Figure 4.1  Possible mechanism of action of the crude extract and the lead compound from *Calotropis procera*