Part III

Effect of Bioactive Fraction of *J. cordifolia* on production of proinflammatory cytokines by LPS stimulated monocyte and monocyte derived dendritic cells
Chapter 1

Introduction
3.1.1 Cell based assays: introduction

Bioactivity testing has used animals in the forefront for routine experimentation including for those that are intended for human consumption. Although it is mandatory to test in animals before drug release (Taylor et al., 2005), the results are questioned for applicability in humans due to several reasons like false positive correlation of tested chemicals (Hartung, 2009), differences in human and other species regarding physiology and drug metabolism (Smith, 1991). Differences of inter related species can mask the effect of drugs in preclinical studies (Astashkina et al., 2012). Animal based-studies are generally costly, have associated ethical issues, unreliable results and thereby suggest that tissue and cell-based studies are useful for bioactivity screening of new compounds. Cell-based assays are a key technology that can be used to predict human physiology and has an increasing role in clinical testing in the past few years. The identity of a cell and its behaviour, or survival in an in vitro system depends on the neighbouring cells and on other biological processes that occur within the organism (Blaauboer, 2008). The complexity of the whole organism as an animal model and the data obtained from such models cannot fully understand human responses (Worth and Balls, 2002). Scientific studies require physiologically relevant human cell-based assays that provide biomedical and biochemical data which can better predict the human state.

3.1.2 Function of cell based assays

1. Efficacy of drugs

The antibody dependent cellular cytotoxicity of an antibody to target tumour cells in the presence of lymphocytes or effector cells can be studied by the use of lymphocyte populations prepared from human blood or mouse
spleen. Another similar study called the complement-mediated cytotoxicity of an antibody uses dilutions of human or rabbit blood to target tumour cells in the presence of complement proteins (Festing and Wilkinson, 2007).

2. Screening of inflammatory response and inflammation

The safety of a drug, the presence of a trace or a microbial contaminant, the ability of the drug to cause inflammation, the production of inflammatory cytokines or inflammatory mediators are the different factors that can be studied in detail. Drug based induction of inflammatory cytokines, such as IL-1, TNF, or interferons can use the blood mononuclear cells or monocytes as an assay system while studies on the enhancement of the immune response to infections require monocytes or neutrophils (Mosmann, 1983). A different approach to study drug-induced inflammatory response involves dosing cells and the quantitative determination of cytokine production (e.g. IL-1).

3. Pharmacodynamic biological markers

Pharmacodynamic studies are important for gaining information about the efficacy, toxicology and mechanism of action of the candidate drugs in a preclinical or clinical study. Several parameters that are studied are the circulating cytokines, antibodies or cell surface proteins. The mechanisms of action of the drug in the host system and immunomodulatory responses of the drug require the use of cell based studies for effectiveness. Recent advances in cell based studies have resulted in the use of cells for gene analysis in toxicity and resultant microscopic changes in the cell cytoskeleton (Hillegass et al., 2010).

Certain drugs that are specifically targeted for human use can induce or elicit an immune response in the host animals and can even produce
neutralizing antibodies. The antibodies produced can affect the total antibody titre or limit toxicity. Anaphylaxis in the test animal can also increase antibody titre. The pharmacokinetics which deals with the fate of the drug inside the host body and toxicokinetics of the drug which deals with its toxicity in the host body are altered with relation to antibodies (Zong and Thompson, 2006).

3.1.3 The Mononuclear Phagocyte System

The mononuclear phagocytic system consists of a family of cells that includes precursors in the bone marrow that are committed to the formation of circulating blood monocytes and tissue macrophages in every organ in the body (Hume, 2006). The monocytes circulate in blood and are recruited into the tissues as a result of a stimulus like infection. These cells are distinctively different from the polymorphonuclear cells (Dale et al., 2008). In the tissues the monocytes differentiate into macrophages which comprise 20% of the mononuclear population. The committed cells in the mononuclear phagocytic system undergo a series of morphological changes where a myeloid progenitor becomes a monocyte through monoblastic, and promonocytic stages (Hume, 2006) requiring a host of growth factors like colony-stimulating factor (CSF-1) and granulocyte macrophage colony-stimulating factor (GM-CSF) (Stanley, 2000).

3.1.4 Monocytes and macrophages

The two major subsets of monocytes that circulate in the blood are the classical CD14++ monocytes, comprising the majority (80-90%) in the circulation and a subset of CD14+CD16+ monocytes, which comprises 13% of CD14+ cells (Passlick et al., 1989). Human CD14++ CD16- monocytes exhibit a higher phagocytotic activity. Stimulation with lipopolysaccharide,
results in the production of interleukin (IL)-10 at higher concentrations with tumor necrosis factor (TNF)-α. In comparison, the human CD16\(^{+}\) monocytes can lead to the production of TNF-α on LPS stimulation (Sanchez-Torres \textit{et al.}, 2001). There have been several reports on the presence of large numbers of CD16\(^{+}\) monocytes in acute inflammation patient blood and also in infectious diseases (Mizuno \textit{et al.}, 2005). These cells are found increased in cases of auto-immune disorders like systemic lupus (Cairns \textit{et al.}, 2002) and rheumatoid arthritis (Rossol \textit{et al.}, 2012).

Monocytes and macrophages function in important immunological aspects like phagocytosis of infection causing pathogens and have other functions like homeostasis and embryonic development. The depletion of macrophages in CSF deficiency has highlighted the importance of these cells in male and female fertility and development of the pancreas and nervous system (Pollard, 2009; Gow, \textit{et al.} 2010). Macrophages that are resident in a tissue adapt to perform the particular function of the particular organ. The entry of the monocyte into tissue by chemotaxis requires the adhesion of the cells to the vessel walls with the resident macrophages playing a protective role. The release of cytokines by these residents helps the directed migration of new recruit cells (Gordon \textit{et al.}, 1986). Macrophages are professional phagocytes and remain the first line of defence against pathogens. This process requires the cells to differentiate between native and foreign cells and the processes on macrophage cells allow the recognition of surfaces for unique pathogenic markers (Ginhoux and Jung, 2014).

3.1.5 Dendritic Cells

The classical, non-phagocytic, dendritic cells share the progenitor cells for tissue macrophages and also require the presence of the growth factor, CSF-1. Dendritic cells represent unique cells that are able to activate
T cells by the basic action of being a potent antigen-producing cell (Guilliams et al., 2014). Both subsets of monocytes, the CD14^+CD16^+ monocytes and the CD14^{++}CD16^- monocytes can differentiate into dendritic cells (DCs) using the presence of the granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 in *in vitro* cultures (Sallusto and Lanzavecchia, 1994). These set of cells are both able to internalize the soluble and the particulate antigens, and stimulate proliferation of the T-cells in autologous and allogeneic cultures (Romani et al., 1994). Studies have confirmed the differentiation of monocytes under *in vivo* conditions and the GM-CSF exposed dendritic cells are the most common type of dendritic cells used in studies of humans, and for DC vaccines in immunotherapy (Shortman and Naik, 2007). The importance of dendritic cells lies in the use of these cells in cancer immunotherapy (Thurner et al., 1999) where the self cells are used to produce specific antitumor activity by the generation of effector cells (Leon et al., 2007) targeting the tumour. Such antigen-pulsed dendritic cells have been used successfully in the treatment of various cancers like multiple myeloma, colorectal, prostate, and lung cancer (Amanna and Slifka, 2011). Several studies conducted on the efficacy of this new treatment for cancer reveal the safety of these antigen-loaded dendritic cell vaccinations (Steinman, 2006).

### 3.1.6 Cytokine production by macrophages and dendritic cells

The activation of macrophages or dendritic cells results in the production of cytokines having important roles in the launch of an immune response. Cytokines produced at the onset of an infection are the proinflammatory cytokines and includes tumour necrosis factor-alpha (TNF-\(\alpha\)), interleukin-1 (IL-1) and IL-6. These three cytokines are phenomenal in the initiation of immune response that includes fever and the activation of the
acute-phase response. The body becomes less conductive to a pathogenic onset and initiating sickness (Sallusto and Baggiozini, 2008). This reason has made cytokines attractive targets for therapeutic interventions. Sequential cascades of genetic regulations exist on the exposure to lipopolysaccharide and stimulation of the macrophages. This leads to the early response genes, including a number of classical cytokines such as TNF-alpha, being subjected to regulation while the late response genes are regulated by the autocrine factors, including TNF and type 1 interferon (Taylor et al., 2005).

3.1.7 Objectives of Part III

Researches conducted in animals have played instrumental roles in the understanding of various pathological processes in humans. The differences in interspecies physiology, metabolism, and the mechanism of adaptation to disease are significant and have been ignored. The cytochrome P450 distribution, rate of phase I and phase II reactions vary significantly between canines, hares, rodents and humans even though detoxification by cytochrome P450 is common in all mammals (Smith, 1991). The interspecies differences that mask the toxicity of drug candidates in preclinical studies (Astashkina et al., 2012) have led to the exploitation of in vitro cell culture systems as a valuable tool to study the physiology and pathology of cell interaction with lead candidates, provided they are appropriately validated.

The treatment of macrophage cells with T. cordifolia extract shows activation of the macrophages as evidenced by the enhanced secretion of lysozyme, increased nitrite production and microbicidal activity when compared to lipopolysaccharide (More and Pai, 2011). The cell based studies on the effect of T. cordifolia extracts on the proinflammatory cytokine production of lipopolysaccharide (LPS) induced human monocytes and dendritic cells in primary culture has been studied in this chapter.
The detailed objectives for Part III are as follows:

i. Evaluation of cytotoxicity of *T. cordifolia* methanol extract and bioactive fractions on monocytes

 ii. Culture of monocytes and study of immunomodulatory effect of *T. cordifolia* methanol extract and the bioactive fractions on the production of TNF-α and IL-1β in monocyte cells after LPS stimulation.

 iii. Culture of dendritic cells and study of immunomodulatory effect of *T. cordifolia* methanol extract and bioactive fractions on the production of TNF-α and IL-1β in dendritic cells after LPS stimulation.
Chapter 2

Review of Literature
3.2.1 Introduction to immunotherapy and immunomodulators

The treatment of disease by the induction, enhancement and suppression of immune responses with the help of immunomodulators is better known as immunotherapy (Saroj et al., 2012). The induction or enhancing of immune response is activation immunotherapy while suppressing is suppressive therapy and they are brought into effect by immunomodulators. The use of immunomodulators is preferred due to lower incidence of side effects and resistant microbes (Masih, 2001). Granulocyte colony-stimulating factor (G-CSF) and interferons are examples of licensed immunomodulators whereas IL-2, IL-7, IL-12, various chemokines, and glucans are still in trials for immunotherapy. Cell based immunotherapies have also been found effective for cancer particularly macrophages (Andreesen et al., 1990), lymphocytes (Rosenberg et al., 1988), dendritic cells (Figdor et al., 2004), and other effector cells which target abnormal antigen expression on tumour cells. Monocytes represent precursor cells that are sources of macrophages and dendritic cells. Macrophages that are grown ex-vivo have been reported to recognize tumour cells and destroy them effectively in murine models (Andreesen et al., 1998). Clinical trials in cancer patients have provided positive and safe results as autologous macrophages derived from monocytes and activated by interferon-γ were used (Fridlender et al., 2013). Similarly dendritic cells harvested from patients and grown ex-vivo can effectively present tumour antigens to cytotoxic cells and counter the tumour cells (Figdor et al., 2004).

3.2.2 Plants in immunotherapy

Plant based immunotherapy currently consists of the generation of specific antibodies in plants and administration as edible vaccines (Ma and Hein, 1995). Another approach is the use of grass pollen as an
immunotherapeutic drug (Durham et al., 1999) with very few side effects. *T. cordifolia* has been reported to have an immunomodulatory function in traditional ayurveda and several validation studies have been conducted which points to a possible candidate for immunotherapy.

### 3.2.3 Immunomodulation by *T. cordifolia*

A formulation containing *T. cordifolia* was reported with immunomodulatory activity by the reduction of the average degree of infection in experimental amoebiasis of golden hamsters. Humoral and cell mediated immune responses were stimulated as shown by haemagglutination and inhibition of leukocyte migration (Sohni and Bhatt, 1996). Syringin and cardiol isolated from *T. cordifolia* was reported to possess immunomodulatory action as per the reduction of immuohaemolysis. The effect was attributed to the inhibition of the C3 convertase, increased serum IgG and macrophage activation (Kapil and Sharma, 1997). Eosinophils are reported to be reduced in persons with HIV (Kalikar et al., 2008; Akhtar, 2010).

### 3.2.4 Immunosuppression by *T. cordifolia*

Rege et al (1989; 1993) has reported on the immunosuppressive activity of *T. cordifolia* in the clinical study with patients having obstructive jaundice. Immunosuppression was also evidenced by the studies on the water and ethanol extracts of the stem of *T. cordifolia* which inhibits cyclophosphamide-induced anemia with a higher potency of the water extract (Manjreker et al., 2000). Another study on the ethanol extract of *T. cordifolia* reported on different parameters like the skin allograft rejections, delayed type hypersensitivity shows that it improves phagocytosis with no effects on the cell mediated and humoral immune system (Atal et al., 1986). Polyclonal mitogenic activity in B-cells was expressed by the
arabinogalactan isolated from the stems of *T. cordifolia* and does not require the involvement of macrophages (Chintalwar *et al.*, 1999). A detailed study by Mathew and Kuttan (1999) on the methanol extract of *Tinospora* in mice has reported increases in total white blood cell count and bone marrow cellularity that signifies an increase in the stimulated bone marrow stem cells. Other results in this study include increased circulating antibody, plaque forming cells in spleen and activated macrophages with a synergistic approach to cyclophosphamide for the reduction of the growth of solid tumours in the animals.

### 3.2.5 *T. cordifolia* studies in macrophages

The effect of *T. cordifolia* on ochratoxin A induced carcinogenicity in mice leads to increase in chemotaxis suppressed by the ochratoxin and a decreased production of TNF-α and IL-1β from activated macrophages in the mice (Dhuley, 1997). A compound named as NII-70 was isolated from *T. cordifolia* and found to activate macrophages causing an increase in the production of TNF-α, IL-12 and nitric oxide. The compound also exhibits a reduction in Leishmania infection and tumour implants in experimental animals. Cordifolioside A, N-methyl-2-pyrrolidine, 11-hydroxymustakone, magnoflorine and tinocordioside are isolated from *Tinospora* and appear to induce macrophage activation (Sharma *et al.*, 2012). The polysaccharide G1-4A also stimulates macrophages and has been confirmed to be an agonist of the TLR4 receptor on macrophages (Raghu *et al.*, 2009) and also suggests that G1-4A is a selective TLR4 receptor modulator and protects mice from endotoxicity caused by lipopolysaccharide induction (Desai *et al.*, 2007). The (1,4)-α-D-glucan, also from *Tinospora* is a reported TLR6 receptor agonist (Nair *et al.*, 2006) while a protein isolated is known to stimulate macrophage activity (Aranha *et al.*, 2012). Increased phagocytosis and the
phagocytotic index is being reported in activated macrophages (Sengupta et al., 2011) in mice. In toxicity induced by carbon tetrachloride and in surgery, *T. cordifolia* increases intracellular killing capacity with confirmed alteration of macrophage morphology suggesting protective effects (Bishayi et al., 2002).

In rheumatoid arthritis patients, paired combinations of *Tinospora* and Ginger have been studied and shows a reduction of symptoms by 44% in subjects which was comparable in potency to the reference, hydroxychloroquine sulfate (Chopra et al., 2012). This has been similar in another study where *T. cordifolia* in Ghee with ginger exhibits improvement in symptoms of rheumatoid arthritis with treatment (Lekurwale et al., 2010). G1-4A directly stimulates dendritic cells as shown by increase in the expression of MHC-II, CD40, CD86 and CD80 appearing to be additive with lipopolysaccharide exposure (Pandey et al., 2012).

### 3.2.6 Effect of *T. cordifolia* on cancer cells

Exposure of the HeLa cells to *T. cordifolia* extracts of methanol, water and methylene chloride results in dose-dependent and significant increase in cell death as compared to non-drug-treated controls groups. The results call attention to guduchi as an antineoplastic agent (Jagetia et al., 1998). Tumour associated macrophages (TAMs) have an increased activity with oral supplementation of this herb to rats and mice evidenced by reduced tumour size (Singh et al., 2004; and the activation of dendritic cells from activated macrophages (Vanderheyde et al., 2001) appears to be true in tumour bearing rats transplanted with dendritic cells from *T. cordifolia* fed rats (Singh et al., 2005).
3.2.7 Toxicity or Side effects of the administration of *T. cordifolia*

Significant information on side effects is not available. The Indian council of medical research (ICMR) has reported the plant is traditionally considered to be safe in the dosage mentioned. The genotoxic risk of the aqueous extract of *T. cordifolia* was studied by different genotoxicity tests and the results confirm the non-mutagenic effect (5000 µg/plate) in *Salmonella typhimurium*, non-clastogenic effect (3000 µg/ml) in human peripheral blood lymphocytes (Chandrasekaran *et al.*, 2009). The extract also shows non-clastogenic and non-DNA damaging effect in bone marrow erythrocytes and peripheral blood lymphocytes of male Balb/c mice at doses of 150, 200 and 250 mg/kg body weight.
Chapter 3

Materials and Methods
3.3.1 Materials

RPMI-1640 supplemented with 2 mM L-glutamine, 100µg/ml streptomycin, 100U/mL penicillin and 10% fetalbovine serum (HiMedia Chemicals India) was used as culture medium. The LPS (Escherichia coli), LPS (*Pseudomonas aeruginosa*) and Rolipram were purchased from Sigma Chemicals, USA. Quantikine Human TNF-alpha Immunoassay kit from R&D systems, USA. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), EZ count™ MTT Cell Assay Kit were purchased from HiMedia Chemicals, India. Detoxi-Gel™ Endotoxin Removing Columns (Thermo Fisher Scientific Inc., USA) were used for endotoxin removal. Human Interleukin 1β ELISA MAX™ Deluxe kit and Human TNF-alpha ELISA MAX™ Deluxe kit from BioLegend, USA were used for the quantitation of IL-1beta and TNF-alpha. Haier HR40-IIA2 biosafety cabinet, Revco UXF600 Ultra low Temperature Freezers from Thermo Scientific, USA, Tali Image Cytometer from Invitrogen Life Technologies, USA, New Brunswick Galaxy S CO2 incubator from Eppendorf, USA.

3.3.2 Preparation of cells for culture

3.3.2.1 Isolation of Monocyte cells from Blood

Fresh blood was collected from the blood bank of Medical College, Kottayam. The monocytes were separated with the use of HiSep LSM 1077 (HiMedia, Laboratories, India) which consists of polysucrose (5.7g/dl) and sodium diatrizoate (9g/dl) with an adjusted density of 1.0770 ± 0.0010 g/ml. HiSep LSM is based on an adaptation to the method by Boyum (1968) where differential migration of blood layered on polysucrose-sodium diatrizoate solution into three layers of separated RBC and granulocytes pellet, a middle interphase of mononuclear cells and platelets and an upper
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layer of plasma. 2.5ml of HiSep LSM was aseptically transferred to a tube containing 7.5ml diluted blood and centrifuged at 400 x g for 15-35 minutes at room temperature. The upper supernatant contains plasma and platelets which can be removed as much as possible without disturbing the other layers. The interface containing monocytes was then carefully aspirated into a clean tube. The separated layer was washed with equal volumes of isotonic phosphate buffered saline by centrifugation at 160-200 x g for 10 minutes at room temperature. The wash was repeated again and pellet resuspended in appropriate medium. The cell count and cell viability was checked.

3.3.2.2 Generation of Dendritic cells from PBMC

Fresh blood was collected from the blood bank of Medical College, Kottayam. The monocytes were separated with the use of HiSep LSM 1077 as described earlier (4.3.2). The isolated cells were plated with required amount of PromoCell DC Generation Medium DXF w/o cytokines and incubated for 1 hour at 5% CO\textsubscript{2} and 37\textdegree C in the CO\textsubscript{2} incubator. The adherent cells were washed three times with warm PromoCell DC Generation Medium DXF w/o cytokines. A required amount of Promocell DC generation medium DXF supplemented with 1x Componenet A of the Cytokine Pack moDC DXF was added to the cells and incubated for 3 days at 37\textdegree C and 5% CO\textsubscript{2}. The medium was changed on the 3\textsuperscript{rd} day and the immature moDCs were incubated for 3 days at 37\textdegree C and 5% CO\textsubscript{2}. The stimulated process was completed by supplementing 1x of Component B of the Cytokine Pack moDC DXF on the 6\textsuperscript{th} day without medium change and incubated at 37\textdegree C and 5% CO\textsubscript{2} for an additional 24-48 hours. Loosely attached cells were dislodged and the contents were transferred to a tube to harvest the moDC cells removing the supernatant. The cells were resuspended in medium and the cells were counted.
3.3.3 Endotoxin removal from *T. cordifolia* extracts

The Detoxi-Gel Endotoxin Removal Column contains immobilized polymixin B, an antibiotic as a stable affinity matrix that can remove endotoxins and resist recontamination of the sample. The presence of endotoxins (part of outer membrane of cell-wall of Gram-negative bacteria) contaminations in plant samples can severely influence immunomodulatory measurements in cell based assays (Gusenleitner *et al.*, 2006). This leads to mandatory removal of endotoxins from plant extracts prior to cell base assays. The Detoxi-Gel Column was initially regenerated by washing with five resin-bed volumes of 1% sodium deoxycholate, and has to be followed by a wash with 3-5 resin-bed volumes of pyrogen-free water to remove the detergent. The column has to be equilibrating with 3-5 resin-bed volumes of pyrogen-free water. The sample was then applied to the column, allowed to flow through the gravity-flow column and finally collected. The solutions must be stored frozen after endotoxin removal.

3.3.4 Effect of *T. cordifolia* extracts on cytotoxicity

The MTT assay utilizes tetrazolium dyes to assess the cytotoxicity of probable medicinal drugs. The MTT is converted to the purple coloured formazan which has an absorption maximum at 570nm by the viable cells with active metabolism. On death of these cells, the ability to convert MTT is lost and can serve as an important marker for viable cells. The reduction of MTT into its formazan form requires NADH dependent enzymes that can transfer electrons to the MTT molecule (Marshall *et al.*, 1995). This probable involvement of NADH dependent enzymes has led to assumptions of the involvement of the mitochondria of viable cells (Berridge and Tan, 1993). The insoluble precipitate of the formazan that accumulates inside cells and on the
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Cell surface requires to be solubilised before measuring the absorbance (Denizot and Lang, 1986).

Colorimetric MTT assay was performed to assess the basal cytotoxicity of *T. cordifolia* extract. Monocytes at a density of 1x10⁶ cells/ ml were seeded in a 96-well flat bottom culture plates and incubated for 1 hour. The cells were treated with different fractions of endotoxin removed extracts diluted in 0.1% DMSO and incubated for 2 hours. MTT was then added to each well and incubated for a further 2 hours. The formazan dye formed, which upon dissolution into solubilization solution exhibits purple color, the intensity of which is proportional to the number of viable cells. The absorbance was measured spectrophotometrically at 570 nm. A graph was plotted with absorbance on y axis and concentration of extract on x-axis and the result analysed.

3.3.5 Evaluation of *T. cordifolia* methanol extract for Immunomodulatory Effect on TNF – α production in whole blood

Fresh blood was collected from the blood bank of Medical College, Kottayam and was diluted with RPMI medium as required. The endotoxin removed plant extract or standard was prepared in DMSO was added to the blood sample and incubated at 37°C in a CO₂ incubator for 1 hour. To this mixture lipopolysaccharide (LPS) was added at a final concentration of 1ng/ml and incubated for 5 hours in a CO₂ incubator at 37°C. The reaction mixture was then centrifuged at 3000 rpm for 5 mins. Supernatant solution was collected and measured for TNF-a using a solid phase ELISA kit from R&D Systems, USA. Rolipram was used as positive standard.

The Quantikine Human TNF-alpha Immunoassay kit from R&D systems, USA is a solid phase ELISA that can measure human TNF-alpha concentrations from samples like cell culture supernatants, serum, plasma
and whole blood. The microplate was pre-coated with capture antibody. Samples or standards were added to the wells for binding with antibody and the wells were washed. A second HRP (Horseradish peroxide)-labeled antibody was added to the wells. Any detection antibody that remains unbound was washed away and followed by the addition of tetramethylbenzidine (TMB) substrate solution. The diimine formed after the reduction of hydrogen peroxide to water by HRP results in a blue color signifies the presence of the TNF-alpha analyte in the sample and is proportional to the concentration of the analyte. A stop reagent like sulphuric acid changes this blue color to yellow and the absorbance can be measured at 450 nm (Martin et al., 1984). The data was plotted as log absorbance versus log concentration using a 4-parameter logistic curve fit. The concentration of each sample was determined from standard curve with appropriate dilutions and used to calculate the percent inhibition as per the formula given below:

\[
\text{Percent Inhibition (\%)} = \left(\frac{\text{Activity of Control} - \text{Activity of Test}}{\text{Activity of Control}}\right) \times 100
\]

Inhibition results were expressed in terms of IC\textsubscript{50} values.

3.3.6 Effect of bioactive fractions of \textit{T. cordifolia} extracts on lipopolysaccharide stimulated monocytes

Fresh blood was collected from the blood bank at Medical College, Kottayam and monocytes were isolated as per earlier protocol (4.3.4). The isolated and washed monocytes were cultured in RPMI 1640 medium containing 10% FBS. The cells were placed in a CO\textsubscript{2} incubator at 37°C and 5% CO\textsubscript{2} for overnight incubation. The adherent cells were removed by centrifugation at 180 x g for 15 minutes and resuspended in fresh media with a maximum cell count of 5x10\textsuperscript{5} cells/ml after dilution. The monocyte cell suspension (5 x 10\textsuperscript{5} cells/ml) was incubated in 24 well plates with and
without different concentrations of endotoxin removed *T. cordifolia* extracts TCM, F11 and F9 in the CO$_2$ incubator at 37°C and 5% CO$_2$ for 2 hours. The monocytic cells were stimulated by exposure and incubation with 50 ng/ml concentrations of lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* (Sigma Chemicals, USA) for 16 hours under the same conditions as above. Untreated cells were considered as control and medium was considered as blank. Supernatants collected and assayed for TNF-$\alpha$ and Interleukin 1$\beta$ by ELISA (BioLegend, San Diego). The data was plotted as absorbance versus log concentration using a 4-parameter logistic curve fit. The concentration of each sample was determined from standard curve with appropriate dilutions and used to calculate the percent inhibition as per the formula given below:

$$\text{Percent Inhibition (\%)} = \left( \frac{\text{Activity of Control} - \text{Activity of Test}}{\text{Activity of Control}} \right) \times 100$$

Inhibition results were expressed in terms of IC$_{50}$ values.

### 3.3.7 Effect of Bioactive fraction of *T. cordifolia* extracts on lipopolysaccharide stimulated Dendritic cells

Fresh blood was collected from the blood bank at Medical College, Kottayam and monocytes were isolated and dendritic cells were generated as per protocol described earlier (4.3.4). The dendritic cell suspension ($5 \times 10^5$cells/ml) was incubated in 24 well plates with and without different concentrations of endotoxin filtered *T. cordifolia* extracts and bioactive fractions F11 and F9 in the CO$_2$ incubator at 37°C and 5% CO$_2$ for 2 hours. The dendritic cells were stimulated by exposure and incubation with 20 ng/ml concentrations of lipopolysaccharide (LPS) from *Escherichia coli* (Sigma Chemicals, USA) for 16 hours under the same conditions as above. Untreated cells were considered as control and medium was considered as blank. Supernatants collected and assayed for TNF-$\alpha$ and IL-1$\beta$ proteins by
ELISA (BioLegend, San Diego). The data was plotted as absorbance versus log concentration using a 4-parameter logistic curve fit. The concentration of each sample was determined from standard curve with appropriate dilutions and used to calculate the percent inhibition as per the formula given below:

\[
\text{Percent Inhibition (\%)} = \left( \frac{\text{Activity of Control} - \text{Activity of Test}}{\text{Activity of Control}} \right) \times 100
\]

Inhibition results were expressed in terms of IC$_{50}$ values for IL-1β. In the case of TNF-α, as percentage inhibition at different concentrations.

### 3.3.8 Statistical analyses

In all the studies, the values of separate sets of experiments are expressed as mean ± standard deviation (S.D.) for n determinations where n=3 unless otherwise stated. Data analyses were performed using SigmaPlot version 12.5. All statistical analyses were performed by using One Way ANOVA followed by the post hoc Dunnett’s test, which is a multiple comparison procedure to compare each of a number of treatments with a single control. These calculations were done using SigmaPlot (Systat Software, San Jose, CA). Differences were considered to be significant when the $p$ value was calculated to be $<0.05$. 
Chapter 4

Results
3.4.1 Dendritic cell (DC) Production from Blood Monocytes

The isolated monocytes were cultured with PromoCell DC Generation Medium for 8 days with supplementation of cytokines and growth factors for differentiation. The final loosely attached cells were harvested, resuspended and the cells checked for viability. The cells were photographed on the 3rd, 6th and the 8th day for confirmation of change in monocyte morphology into dendritic cells. The cells have numerous long processes as evident for normal dendritic cells.

Fig 3.1: Morphology of Dendritic cells on the Third Day of the culture

Cells of isolated PBMC in culture for generation of dendritic cells with supplementation of cytokines. Third day of culture changes in morphology of rounded mononuclear cells can be seen.

Fig 3.2: Morphology of Dendritic cells on the Sixth Day of the culture

Cells of isolated PBMC in culture for generation of dendritic cells with supplementation of cytokines. Sixth day of culture, significant changes in morphology and formation of immature moDC’s.
Results

Fig 3.3: Morphology of Dendritic cells on the Eighth Day of the culture

Cells of isolated PBMC in culture for generation of dendritic cells with supplementation of cytokines. Eighth day of culture with formation of mature moDC’s having characteristic long processes of dendritic cells can be seen.

3.4.2 Cytotoxicity Assay

The extracts of *T. cordifolia* were screened for cytotoxic activity against normal blood monocytes by the MTT assay as described earlier and the results are as shown in Fig 3.4. All the extracts were non-cytotoxic to blood monocytes with the fraction 11 showing least cytotoxicity and the values are as shown in Table 3.1. F9 with least bioactivity for the LOX and COX enzyme inhibition shows the highest cytotoxicity of 17%.

Table 3.1: Comparative values of Cytotoxicity assay for *T. cordifolia* extract and bioactive fraction

<table>
<thead>
<tr>
<th>Samples</th>
<th>Survival Rate Index (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>F 11</td>
<td>88.05 ± 0.79*</td>
</tr>
<tr>
<td>F 9</td>
<td>82.72 ± 0.95*</td>
</tr>
<tr>
<td>TCM</td>
<td>87.77 ± 1.2*</td>
</tr>
</tbody>
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Comparative cytotoxicity values for monocytes by the extracts of *T. cordifolia* were assayed by the MTT assay explained earlier. Data are given as Means ± SD from three independent experiments. *P*<0.05, values are significantly lower than control (cells without extract).
Cytotoxic effect of *T. cordifolia* extracts on monocytes in culture. Culture wells were treated with MTT and formazan crystals formed were measured at 570nm. Data are given as Means ± SD from three sets of independent experiments. Control were the cells without extract treatment.

### 3.4.3 Screening of Immunomodulatory Assay

The TCM extract of *T. cordifolia* was screened for immunomodulatory activity by the effect on TNF-α protein from whole blood under in vitro conditions as per the protocol described by the Quantikine Human TNF-alpha Immunoassay kit from R&D systems, USA and the results are as shown in Fig 4.1. *T. cordifolia* TCM extract showed significant inhibitory activity against TNF-α as compared with Rolipram standard and the IC\textsubscript{50} values are as shown in Table 3.2.
Table 3.2: Comparative values for Inhibition of TNF-α Activity in Whole Blood

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC_{50} (ng/µl)</th>
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<tbody>
<tr>
<td>Rolipram</td>
<td>10.027 ± 0.115</td>
</tr>
<tr>
<td>TCM extract</td>
<td>0.544 ± 0.12*</td>
</tr>
</tbody>
</table>

Inhibition of proinflammatory cytokine TNF-α production by LPS induced whole blood cells. Cell supernatants from treated cells were immunoassayed for TNF-α production by ELISA. Data are given as Means ± SD from three independent experiments. *P<0.001, the value is significantly lower than control hence the plant extract is better than control.

3.4.4 Effects of bioactive fraction F11 and F9 on Lipopolysaccharide stimulated Monocyte Cells

Monocytes have a significant role in the initiation and progression of immune response through regulation of cytokine production. The study on the effect of *T. cordifolia* fractions on release of cytokines by monocytes has been conducted after LPS induced stimulated for 16 hours. The extracts were preincubated with the cells for 2 hours before LPS stimulation and Rolipram was used as positive control. The results for TNF-α and for IL-1β are as shown in Table 3.3 and Table 3.4. TCM was found to be the most active in the inhibition of TNF-α and IL-1β production by LPS stimulation monocytes while F9 has the least. F9 has no effect on the production of TNF-α in these cells while F11 showed an IC_{50} value (as shown in Table 3.3) which was higher than Rolipram. However the concentration of extract at ng levels indicate the possibility of identifying molecules which could be developed as inhibitors of TNF-α production.
Table 3.3: IC<sub>50</sub> Values of Bioactive fraction for production of TNF-α in LPS stimulated monocytes

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Sample Name</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; Values for TNF-α (ng/ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rolipram</td>
<td>0.218 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>F11</td>
<td>14.41 ± 0.23*</td>
</tr>
<tr>
<td>3</td>
<td>F9</td>
<td>No Inhibitory Activity</td>
</tr>
<tr>
<td>4</td>
<td>TCM</td>
<td>7.8x10&lt;sup&gt;-6&lt;/sup&gt;±0.0005#</td>
</tr>
</tbody>
</table>

The inhibition of proinflammatory cytokine production by LPS induced monocytes. Culture supernatants from treated cells were immunoassayed for TNF-alpha production by ELISA. Data are given as Means ± SD from three sets of independent experiments. *P<0.001, values are significantly higher than control, hence extract is not better than control. #P<0.001, values are significantly lower than control, hence extract is better than control, F11

Table 3.4: IC<sub>50</sub> Values of bioactive fraction for production of IL-1β in LPS stimulated monocytes

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Sample Name</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; Values for IL-1β (ng/ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F11</td>
<td>14.68 ± 0.87</td>
</tr>
<tr>
<td>2</td>
<td>F9</td>
<td>258.58 ± 36.05*</td>
</tr>
<tr>
<td>3</td>
<td>TCM</td>
<td>5.52x10&lt;sup&gt;-6&lt;/sup&gt;±0.0004#</td>
</tr>
</tbody>
</table>

Inhibition of proinflammatory cytokine production by LPS induced monocytes. Culture supernatants from treated cells were immunoassayed for IL-1β production by ELISA. Data are given as Means ± SD from three sets of independent experiments. *P<0.001, values are significantly higher than control, hence extract is not better than control, F11. #P<0.001, values are significantly lower than control, hence extract is better than control, F11
3.4.5 Effect of bioactive fraction on Lipopolysaccharide stimulated Dendritic cells

DCs have a crucial role in the innate and adaptive immune responses through cytokine regulation. The study on the effect of *T. cordifolia* fractions on the release of cytokines by dendritic cells has been conducted after LPS induced stimulated for 16 hours. The fractions were preincubated with the cells for 2 hours before LPS stimulation and Rolipram was used as positive control. The results for TNF-α and for IL-1β are as shown in Table 3.5 and Table 3.6. TCM was found to be the most active in the inhibition of IL-1β production by LPS stimulated dendritic cells while F9 has the least for IL-1β. The F11, and TCM extracts show high levels of inhibition (>90%) for the production of TNF-α at the concentrations used in the assay due to which the IC_{50} values could not be calculated.

**Table 3.5: IC_{50} Values of *T. cordifolia* extracts for TNF-α in LPS stimulated dendritic cells**

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Sample Name</th>
<th>IC_{50} Values for TNF-α (ng/ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rolipram</td>
<td>0.218 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>TCM</td>
<td>1.13x10^{-8} ± 0.0001</td>
</tr>
</tbody>
</table>

The inhibition of proinflammatory cytokine production by LPS induced dendritic cells. Culture supernatants from treated cells were immunoassayed for TNF-alpha production by ELISA. Data are given as Means ± SD from three sets of independent experiments. P<0.05 represent significant difference compared with cells treated with rolipram.
Inhibition of TNF-α production in LPS induced dendritic cells by F11. Culture supernatants from treated cells were immunoassayed for TNF-α production by ELISA. Data are given as Means ± SD from three independent experiments.

Table 3.6: IC₅₀ Values of T. cordifolia extracts for IL-1β in LPS stimulated dendritic cells

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Sample Name</th>
<th>IC₅₀ Values for IL-1β (ng/ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F11</td>
<td>9.46 ± 0.67</td>
</tr>
<tr>
<td>2</td>
<td>F9</td>
<td>473.57 ± 13.12</td>
</tr>
<tr>
<td>3</td>
<td>TCM</td>
<td>1.8x10⁻⁷ ± 0.0009</td>
</tr>
</tbody>
</table>

Inhibition of proinflammatory cytokine production by LPS induced dendritic cells. Culture supernatants from treated cells were immunoassayed for IL-1β production by ELISA. Data are given as Means ± SD from three independent experiments.
Chapter 5
Discussion
Cell based studies are considered a major part of the present day scientific approach to the effect of an extract or a compound in a biological system with respect to its environment. These in vitro cell studies reflect the environment of human cells and include either cancer cell lines or primary cell cultures. The primary cells are difficult to maintain in cultures but studies signify the differences in the proteomes of primary cells and cancer cell lines. The study by Alge et al. (2006) have reported on the differential protein expression between primary and immortalized human retinal pigment epithelial cells which can bring about significant changes to the cell response to external stimuli.

In this study we have used monocytic and monocyte derived dendritic cells to study the effect of bioactive fraction of T. cordifolia on proinflammatory cytokine production by these cells after lipopolysaccharide (LPS) stimulation. Monocytes, monocyte derived dendritic cells and other primary cells provide an insight into the response of cells to stimuli in in vivo conditions. Preliminary to any studies on role of the extracts on production of proinflammatory cytokines by monocytes and monocyte derived dendritic cells there is a necessity to confirm two important factors. The first is the absence of endotoxin in plant extracts being studied since they could be a source of immunomodulants. The second factor is to check for cytotoxic effects of extracts on the cells being used for the study. Accordingly the extracts were passed through endotoxin removal columns prior to the use in experimental study. MTT assay of monocytes treated with the plant extract showed that the fraction had no cytotoxic effect on monocytes as shown by the high survival rate of 82-88%.

Chronic inflammation is mediated by the presence and active involvement of monocytes. The migration of monocytes into inflamed
tissues and differentiation into macrophages is the initial stage of chronic inflammation. Of the two subsets of human monocytes only the classical monocytes with CD14$^{++}$ CD16$^{-}$ expressions migrate to the injured tissue with the help of neutrophils (Swirski and Robbins, 2013). After phagocytosis, these cells differentiate into cells resembling non-classical monocytes (CD14$^{+}$CD16$^{+}$) and mediate different tissue repair mechanisms (Arnold et al., 2007). The non-classical monocytes circulate in the blood vessels and migrate to non-inflamed tissues while the classical monocytes prefer inflamed tissue. In injury after myocardial infarction, the monocytes and macrophages recruited to the heart release proteolytic enzymes and reactive oxygen species resulting in the exacerbation of the injury to surviving myocytes (Nahrendorf et al., 2010).

Monocyte cells differentiate into dendritic cells (DC) or macrophage cells in the inflamed tissues. The presence of dendritic cells in atherosclerosis has been reported (Choi et al., 2009) while monocytes are direct implicated in the initiation of atherosclerosis (Rajavashisth et al., 1998). Adaptive immune responses that operate in immunological memory are regulated by the actions of dendritic cells. DC can also activate antigen-specific CD4$^{+}$ T-cell responses (Domínguez and Ardavín, 2010). Dendritic cells have also been reported to be central to the development of chronic eosinophilic airway inflammation as a trigger of asthma (Lambrecht et al., 1998). The significance of these cells in chronic inflammation has been the factor behind the selection of these cells for the study.

Immune cells respond to inflammatory stimulation and produce a wide range of cytokines, chemokines and growth factors, such as TNF-α, interleukins (IL-1, IL-6, IL-8) and vascular endothelial growth factor (VEGF) (Mantovani et al., 2008). Typical proinflammatory cytokines
produced by monocytes on exposure to inflammatory stimuli are TNF-α, IL-1, and IL-12 and the TNF-alpha can then induce the production of IL-1α, IL-1β, IL-1Ra and IL-6 by monocytic cells (Danis et al., 1995). The dendritic cells exposed to stimuli are induced for the production IL-1, IL-6, IL-12, TNF and interferons (Blanco et al., 2008). Downen et al. (1999) reports that the production of proinflammatory cytokines TNF-α and IL-1β results in the neurotoxicity of human astrocyte cells of central nervous system.

Hughes et al. (1996) have reported that the inhibition of TNF-α can increase the survival rate in acute pancreatitis as evidenced in experimental models. Plasma levels of TNF-α and IL-1β are found elevated in undialyzed chronic renal failure indicating increased production and low clearance (Pereira et al., 1994). The drugs for IL1 beta inhibition are being considered for licensing and are undergoing trials. The use of anti-TNF drugs or TNF inhibitors are currently in clinical use which allows to suppress abnormal B-cell activity and is widely used in rheumatoid arthritis. Most of the available drugs are monoclonal antibodies like infliximab, adalimumab, certolizumab, and golimumab (Scallon et al., 2002). Other approaches for TNF inhibition include circulating receptor fusion protein like etanercept, xanthine derivatives like pentoxifylline (Marques et al., 1999) and Bupropion (Brustolim et al., 2006). The side effects and complications of TNF-α are huge with reports of rare cancer Hepatosplenic T-Cell Lymphoma in adolescents and young adults treated for Crohn’s disease with TNF blockers (Ochenrider et al., 2010). There is increased risk of TNF inhibitor patients to severe bacterial, mycobacterial, fungal, viral, parasitic, and other opportunistic infections (Crum et al., 2005). Latent tuberculosis
infection was found to be activated with the use of infliximab (Keane et al., 2001).

The inhibition of TNF by natural compounds has been studied with curcumin, and catechins (Siddiqui et al., 2006). The evaluation of TNF-α production by LPS stimulated whole blood exhibited marked inhibition of production in presence of methanol extract and was more efficient than Rolipram, a standard inhibitor. This study was repeated in monocyte and dendritic cultures using bioactive fraction (which were inhibiting LOX/COX enzymes) of T. cordifolia as immunomodulant. The IC₅₀ values for TNF-α by bioactive fraction indicates F9 had no significant activity. Though F11 inhibited TNF-α production in LPS stimulated monocyte it was not as effective as Rolipram. F9 had no effect on TNF-α production by LPS stimulated monocytes. It is quite possible that other fractions could exhibit a better activity. This was not carried out since the attempt was to isolate bioactive molecule from F11 as it showed maximum LOX isoenzyme inhibition and F9 as it showed better COX-2 inhibition.

Experiments were repeated with monocyte derived dendritic cells. The response of moDC to TCM and to bioactive fraction 11 and 9 are as shown in results of this part. It is to be noted that TCM and both fractions gave a very high percentage inhibition of TNF-α production by LPS stimulated dendritic cell. It was not possible to get dose dependent inhibition curve at dilution of the plant extract that was used for the study. IL-1β production in LPS stimulated monocytes and dendritic cells shows that the extract and fraction could inhibit IL-1β production as shown by IC₅₀ values, but it is not possible at this moment to comment as this observation due to lack of a positive control (a known drug). It is to be noted that the highest percent inhibition of TNF-α production by LPS stimulated dendritic cell is
not a false positive observation (due to cell death) comes from the fact that IL-1β production in the same culture wells showed a dose dependent inhibition.

Unstimulated resting macrophages have characteristically low levels of TNF mRNA which are increased 50-fold on stimulation of these cells by LPS resulting in increased TNF-α level (Beutler et al., 1986). The analysis of the synovial fluid of rheumatoid arthritis patients indicate elevated levels of TNF-α (Saxne et al., 1988), IL-1 (Nouri et al., 1984) and IL-6 (Hirano et al., 1988). Other functions of these proinflammatory cytokines include destruction of cartilage(Dayer et al., 1985), bone resorption (Dayer et al., 1986) and the inhibition of proteoglycan synthesis in particular chondrocytes (Saklatvala, 1986). Drugs which inhibit TNF-α demonstrate an efficacy in the treatment of RA. Keffer et al. (1991) have demonstrated that the inhibition of TNF-α by inhibitor drugs can disrupt the germinal centers in the lymphatic system by the inhibition of the functioning of follicular dendritic cells. These cells are focal cells in the germinal centers that hold the B cells in place.

The mechanism of inhibition of TNF-α production by LPS stimulated monocytes or dendritic cells could be due to the suppression of DNA binding activity of NFκB and its resultant activation (Chong et al., 2002). The stimulation of the cells results in increased levels of cAMP which then increases the synthesis or stability of the NFκB by decreasing the degradation of NFκB inhibitor (Aosasa et al., 2001).