Part II

Characterization and Bioactivity
Guided Fractionation of *Tinospora cordifolia* for Dual Inhibition of LOX and COX
Chapter 1

Introduction


2.1.1 Bioactive molecules in medicinal plants

Plants have been a rich source of medicines because they produce a host of bioactive molecules, or secondary metabolites most of which probably evolved as chemical defences against predation or infection (Cox and Balick, 1994). These compounds may not be able to reproduce the same effects when extracted from the plant in a pure form, which is probably due to the interactive effects of the chemical components of the herb or the combination of herbs in ayurvedic preparations (Ross, 2007).

Plant derived natural products such as flavonoids, terpenes and alkaloids (Witherup et al., 1990; Shukla et al., 2010) have received considerable attention due to their diverse pharmacological properties including inflammatory, antipyretic and analgesic activities. Consumption of natural products reduce the risk of developing pathological conditions, including cancer, nervous system disorders, cardiovascular, genetic, and inflammatory diseases (Jurenka, 2009; Newman and Cragg, 2007). Plants contain numerous bioactive molecules that can improve the body’s resistance to cellular stress and prevent the cytotoxicity of various agents.

Natural products and their derivatives have traditionally been the most common sources of drugs, and still represent a fairly large percentage of the pharmaceutical market (Kirkpatrick, 2002). It has long been recognised that natural product structures have the characteristics of high chemical diversity, biochemical specificity and other molecular properties that make them favourable as lead structures for drug discovery (Okoye and Osadebe, 2010). These natural products are used for the treatment of simple rashes (Yadav et al., 2012), fever (Akkol et al., 2012), pain (Wambugu et al., 2011) to the treatment of complex diseases like diabetes (Ocvirk et al., 2013), cancer (Desai et al., 2008), arthritis (Kaur et al., 2012), cardiovascular
disorders (Lozoya, 1980). Plants are a rich source of active ingredients for health care products, with many blockbuster drugs being directly or indirectly derived from plants (Newman et al., 2000). High value plant-derived natural products remain unexplored for their pharmacological activity (Raskin et al., 2002). Due to the known side effects of approved pharmaceuticals, patients often turn to alternative medicine which is considered natural and healthy. Herbal medicines are thus gaining popularity.

2.1.2 Bioactive Fractionation

The scientific community has investigated the biological activities of several plants, microbes and aquatic organisms. The desire and the necessity to investigate the interactions between organisms or between chemical constituents have led to further scientific experiments. The majority of such investigations include interactions between plant or microbial constituents and human or animal pathophysiology (Sargenti and Vichnewski, 2000). These studies provide several fascinating revelations into the possibility of new and improved candidates for drug development (Hostettmann and Marston, 2002). The identification and isolation of the specific compound that is responsible for this biological activity can be possible with effective fractionation of the samples (Gupta et al., 2012). Fractionation is a separation process that helps to differentiate between the important biological components in the sample and others that are not. Current applications of these procedures are in toxicological studies, screening of plant extracts, environmental analysis, analysis of foods, doping analysis and forensics.

The prerequisite for bioactivity guided fractionation is the cooperativity between the analytical separation procedure and the
biomolecular interaction of the separated fraction. Bioactivity guided fractionation is thus defined in simple terms as the fractionation of a complex extract with the simultaneous determination of biological activity of the fractions thereby identifying the bioactive fraction for each purification step (Weller, 2012). This method for the identification and isolation of the bioactive fraction/compound is useful for the detection of any change in the bioactivity of the active component during purification. Significant changes can thus be made for the efficient and effective purification of the bioactive components (Colegate and Molyneux, 1993).

The general outlines for the bioactivity guided fractionation as described by Houghton and Raman (1998a) is as given below:

1. Preparation of plant extract in suitable solvent
2. Bioassay of sample for desired activity
3. Positive sample fractionated into several fractions
4. Bioassay of each fraction for desired activity
5. Repeated steps of fractionation and bioassay as required for sample
6. Elucidating the molecular structure of compounds in active fractions
7. Testing for bioactivity with elucidated structure

2.1.3 Preparation of Plant extract

The procedure for plant extraction involves the collection of plants followed by the thorough cleansing of the plant parts. Quality in the extraction of the plant requires several factors to be considered including the solvent for extraction, the mechanism of extraction, suitability of the plant
part for extraction etc (Chu and Radhakrishnan, 2008). The solvents can either be used in the series of their polarity or as individual solvent ratios. The extraction method is preferred based on the prior knowledge of the compound of interest (Cardellini et al., 1993). If the study is based on a particular compound or on a particular group of compounds (alkaloids, steroids etc.), the extraction needs to be specific for the compound in question (Dinan et al., 2001). A more generalized method is used under conditions where the nature of substance is not predetermined. Other factors involved are the polarity of compound that affects its solubility in a particular solvent, the pH and thermostability of the extract and the reactivity or volatility of the solvent selected (Houghton and Raman, 1998b).

The most commonly used extraction technique is the solvent extraction. Volatile substances and essential oils are extracted with the steam distillation apparatus while the modern techniques include methods like supercritical fluid extraction (SFE) (Blanch et al., 1999). The only disadvantage to such methods like SFE is the highly sophisticated and costly instrumentation that is required. The solvent extraction method requires the solid sample to be in contact with the solvent for a period of time and subsequent separation of the solid debris (Harvey, 2008). Percolation and infusion methods are particularly useful in conditions where heating is not required. Some extractions require continual heat for increased effectiveness of the extract. Decoctions are extractions of plants in heated water while the soxhlet extractor employs other solvents also (Ikan, 1991). The reflux extraction can be more useful for samples with high content of volatile substances.

In the soxhlet apparatus the solvent is in the lower compartment and is heated to vaporize the solvent. This vapour reaches the reflux condenser at
the top and percolates down into the sample in a porous thimble in the middle holder. The extract formed is siphoned off through the side into the lower solvent container. The primary advantage of the soxhlet extraction is the continual recycling of the solvent system leading to an economical use of solvents (Harvey, 2000). Another advantage is the exhaustive extraction of the sample which can be of use in conditions where there is no preconcept to the type of bioactive compound.

The oils and fats can be extracted with the use of non-polar solvents like petroleum ether or hexane (Starmans and Nijhuis, 1996). Defatting of samples is at times required to remove lipid contaminations or interfering substances from the sample. Lipids can also be extracted with solvents like chloroform or methanol which are comparatively more polar (Grabley and Sattler, 2003). Majority of the plant components are found in glycosidic linkages and are effectively extracted in the methanol solvent. The methanol solvent can also extract phenol compounds, oligopeptides, terpenoids and oligosaccharides (Starmans and Nijhuis, 1996). It becomes necessary to take into consideration the possibility of general contaminations in the prepared extract. These contaminations like chlorophyll from leaf samples, proteins and polysaccharides from plant samples can interfere in the further elucidation of the extract (Kinghorn, 2001). Specific techniques have to be employed to remove these contaminations like the passing of the leaf sample through polyvinyl polypyrrolidone (PVPP) (Wall et al., 1996).

2.1.4 Fractionation of the prepared extract

The choice of the fractionation method is based on the type of solvent in which the extract has been prepared (Dias et al., 2012). An extract in methanol provides the information that the sample components are polar while a hexane extract has nonpolar compounds in it. The resulting fraction
should be compatible for use in the bioassay that follows and the choice of solvent that is non toxic to the sample has to be chosen (Williamson et al., 1996). Another observation before fractionation is whether all the components of the sample are required or if certain components need to be eliminated like sugars from seeds.

A compound can be fractionated by precipitation on the addition of solvents or chemicals or by the change in the physical factors like pH, solvent polarity or temperature (Koehn and Carter, 2005). Precipitation can either remove the contaminant from the sample or precipitate the compound of interest. Samples can also be fractionated by mixing in two immiscible liquids where the compounds will preferentially separate out in the two solvents according to their solubility based on partition coefficient. Vigorous mixing of the sample in a separating funnel can reduce the time required for the partitioning of the sample. (Ikan, 1991). Fractional distillation of plant samples can be used for the separation of volatile compounds while dialysis can separate compounds on the basis of molecular size. The separation happens through the small pores on the semipermeable membrane in which the sample is sealed.

2.1.5 Chromatography

The most diverse of the different fractionation methods, chromatography depends on the partitioning of the sample in two phases moving relative to each other. Chromatography is dependent on two basic chemical properties which are adsorption and solubility for the separation of the sample mixture (Snyder et al., 2011). The phenomenon by which the components of the mixture bind to the surface of the chromatographic column is called adsorption. The solubility of the compound in a solvent determines the speed of its movement with the solvent (Wolfender et al.,
2006). Least solubility does not allow movement while the most soluble will migrate easily with the solvent molecules. The differences in the adsorption and solubility properties can be exploited for the efficient separation of the mixture of compounds in the sample (Ikan, 1991). In the chromatographic procedure, the sample is dissolved in the mobile phase which can be a liquid or gas, and forced through the immobile stationary phase that remains immiscible. The mobile and stationary phases are selected on the basis of different solubilities of the sample components in the phases (Houghton and Raman, 1998d). The solubility of a compound in the stationary phase allows it to interact with the stationary phase while the compound with solubility for the mobile phase can flow out with the solvent allowing optimum separation (Jóźwiak and Waksmdzka–Hajnos, 2007).

Biochemical affinity, size exclusion and ion exchange are the additional factors that could also affect the separation of the sample mixture and are employed as specific methods of separation in chromatography (Horváth et al., 1976). Affinity chromatography deals with the affinity of the sample components for chemical groups in the stationary phase like the interaction between an antigen and antibody. The interactions are based on chemical reaction or bond formation between the stationary phase and the sample (Larsson et al., 1983). Gel filtration chromatography uses the size of the sample components and their relative migration in beads of the stationary phase with pores. Small molecules enter the pores of the beads increasing the time of their elution while the larger molecules pass over the beads to be eluted earlier (Giddings, 2002). The third modification to chromatography involves the ionic interactions between the charged sample ions and the charged stationary phase. If the stationary phase is positively charged, then
the negatively charged ions bind to them while the positive ions are eluted out of the column (Kingston, 2011).

The classification of chromatography on the basis of the physical means of contact of stationary and mobile phases gives rise to the planar and column chromatography. In the planar chromatography the stationary phase is planar with support on a flat plate as in thin layer chromatography (Yanagi and Koyama, 1971) or on paper fibers as in paper chromatography. Column chromatography involves the use of a column with packed stationary phase with liquid or gaseous mobile phases (Houghton and Raman, 1998c). The column used can either be simple glass columns for simple column chromatography or metal columns for high performance liquid chromatography (HPLC) (Jones et al., 1980).

Liquid chromatography technique classified on the basis of physical states of the mobile and stationary phases. Liquid-solid column chromatography requires a liquid mobile phase that flows down through the solid stationary phase, separating the components of the sample with it (Skoog et al., 2007). This separation in a column develops through the affinity of each component for the mobile phase (Lehman, 2002). In the liquid chromatography, two phases of separations can be performed based on the polarity of the column and mobile phase (Tolstikov and Fiehn, 2002). Simple Column chromatography is based on normal phase chromatography that requires the stationary phase to be more polar than the mobile phase. This results in the separation of the less polar solutes faster because the more polar components would have a higher adherence to the stationary phase (Glajch et al., 1980). In an HPLC system, the solid column is coated with a polar liquid to achieve normal phase elution (Knox et al., 1978). More recent and modern systems use the reverse phase chromatography where the polarity of the stationary and mobile phases is
reversed as compared to normal phase chromatography (Euerby and Petersson, 2003). A solvent gradient in reverse phase requires the addition of the solvent with the highest polarity initially and that with least polarity last. Common polar solvents mixtures include water, methanol, and acetonitrile (Snyder, 1978).

2.1.6 Objectives of Part II

Herbal formulations with pharmacological values are gaining importance in the medical field due to the possibility of better and safer drugs. *T. cordifolia* has been known for its numerous therapeutic applications as part of the ayurvedic system of medicines. The interest in this plant is based on the reports of its medicinal properties which include anti-diabetic (Sangeetha *et al.*, 2011), anti-spasmodic (Singh *et al.*, 1984), anti-inflammatory (Utpalendu *et al.*, 1999), anti-arthritic (Abiramasundari *et al.*, 2012), anti-oxidant (Subramanian *et al.*, 2002), anti-allergic (Badar *et al.*, 2005), anti-stress (Shivananjappa and Muralidhara, 2012), hepatoprotective (Sharma and Pandey, 2010), immunomodulatory (Sharma *et al.*, 2012) and anti-neoplastic (Thippeswamy and Salimath, 2007) activities. The fractionation of therapeutically active portions of the plant undergoes suitable methods of extraction to separate out the exact compounds responsible for the effect. The study by Sharma *et al.* (2012) has reported on the immunomodulatory effect of polysaccharide enriched fractions of *T. cordifolia*.

The detailed objectives of Part II are as follows:

1. Preparation of methanolic and aqueous extracts of the stem of *T. cordifolia*.

2. Characterization studies in *T. cordifolia* extracts
   a. Antioxidant Activity
Introduction

i. Effect on DPPH scavenging

ii. Effect on nitric oxide scavenging

iii. Effect on ABTS scavenging

b. Anti-inflammatory Activity: Effect on LOX and COX isoenzymes

3. Fractionation of methanolic extract of *T. cordifolia*

4. Screening of fractions for LOX inhibitory activity

5. Characterization studies of *T. cordifolia* bioactive fraction

a. Antioxidant Activity

   i. Effect on DPPH scavenging

   ii. Effect on nitric oxide scavenging

   iii. Effect on ABTS scavenging

b. Anti-inflammatory Activity

   i. Effect on LOX and COX isoenzymes

   c. Determination of effects of bioactive fractions on kinetic parameters (Km and Vmax)
Chapter 2

Review of Literature
The traditional system of medicine, ayurveda operates on the concept of rasayana which ultimately means substances that promote physical and mental health and that which improves the immunity of the body thereby enhancing the human longevity. Rasayana can also be compared to the modern concept of adaptogens that helps one’s body deal with stress. The original definition for an adaptogen was “the non-specific remedies that increase resistance to a broad spectrum of harmful factors of different physical, chemical and biological natures” (Panossian et al., 1999). Further studies and research into the topic has changed this concept into “a new class of metabolic regulators (of natural origin) which increase the ability of an organism to adapt to environmental factors and to avoid damage from such factors” (Panossian et al., 1999). The Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have accepted this change in concept of adaptogens in theory (EMEA, 2008).

So the focus effectively remains on “regulators of natural origin” which can be characterised into herbal plants and plant products. One of the most known examples include Gynostemma pentaphyllum or the poor man’s ginseng, the basic mechanism of action being stimulating the agonistic function rather than the antagonist function in the body’s innate response. A number of plants used in ayurveda have been selected as probable adaptogenic candidates with T. cordifolia taking up the place as the Indian answer to Ginseng.

2.2.1 Tinospora cordifolia in ayurveda and folklore

Tinospora cordifolia, also known as guduchi was introduced as an adaptogen by the study conducted by Rege et al. (1999), where experimental animals were exposed to biological, physical, and chemical stressors. T. cordifolia was found to exert a normalizing effect against these stressors
together with five other possible adaptogens all used in ayurveda as rasayanas. Guduchi has been a part of ayurvedic medicine for centuries and has been used to treat general weakness, fever, rheumatoid arthritis, diabetes and a host of other varied diseases (Chopra, 1982). It has also been mentioned in the classical texts of ayurveda like astangahridaya, sushrutasamhita, and charaksamhita for Jvara (fever), kamala (jaundice), Svasa (asthma) and vatarakta (gout) (Charka, 1961; Sushruta, 1992; Vaagbhata, 1982).

The medicinal properties of guduchi are attributed in particular to the use of its stem, even though the roots and leaves are also reported with pharmacological uses. The Ayurvedic Pharmacopoeia of India has approved the use of only the stem of *T. cordifolia* as medicine and can be attributed to its comparatively higher alkaloid content (Ayurvedic pharmacopoeia, 2001). The use of dry bark of *T. cordifolia* have been studied in a variety of diseases with its anti-inflammatory (Pendse *et al.*, 1977), anti-allergic (Nayampalli *et al.*, 1986) and anti-pyretic (Ikram *et al.*, 1987) properties. The leaves and roots are particularly used as a part of local tribal and folkloric treatments for fever (VirJee *et al.*, 1984; Shah *et al.*, 1983). The paste of the stem of guduchi and roots of *Solanum surattense* are used as pills against fever by the Baiga tribals of Uttar Pradesh, India (Singh and Maheswari, 1983) while in Mumbai, India the fishermen use guduchi for the treatment of jaundice and dysentery (Shah, 1984). Cancer was also treated with the powdered root and stem bark of guduchi with milk by the Khedbrahma tribals of Gujarat, India who consider it as a part of their daily food (Bhatt and Sabnis, 1987).

### 2.2.2 Ethnobotanical concepts of *T. cordifolia*

The plant can be found spread throughout the length of India especially in Kerala as part of foliage. It is a climber with slender green stems initially and the stems becoming thicker with time. These older stems
are succulent with long fleshy aerial roots from the branches and tastes bitter. The bark is characterised by large rosette-like lenticels and a grayish colour. The leaves are large, cordate and membraneous in type. The flowers are arranged as axillary and terminal racemes with the male flowers in clusters and female flowers solitary. These flowers are actually small in size with yellow or greenish yellow colours and found in summer. Ovoid, red drupes that are fleshy and single seeded are usually found in winter. The phytochemistry of the plant has been extensively studied and includes the presence of large quantities to alkaloids and terpenoids. The plant also contains glycosides, steroids, aliphatic compounds and more bioactive molecules which will be dealt with in detail in Chapter 5.

2.2.3 Bioactivity studies on *T. cordifolia*

The studies that have been conducted on the plant extracts have shown the light on the justification of *T. cordifolia* being a part of the adaptogens with varied and far reaching effects on the human system. Several studies based on the bioactivity and bioactivity guided fractionation of *T. cordifolia* have been reported in the scientific literature. The studies reviewed here have been grouped based on the bioactivity assay conducted.

*a. Standardization studies of *T. cordifolia*

The study by Sivakumar and Rajan (2011) has attempted to standardize the extracts of stems of *T. cordifolia* for berberine content in different solvent fractions of petroleum ether, methanol, aqueous, and chloroform to develop a HPLC method for the quantification of berberine (Sivakumar *et al.*, 2014). In both the wild and micropropagated extracts the methanolic extract contained the highest concentration of berberine. These studies have not conducted bioactivity guided fractionation but have
attempted for the characterization and standardization of chemical constituents in *T. cordifolia* plant extract.

b. **Effect on diabetes and carbohydrate metabolism**

The use of different extracts of *T. cordifolia*, the details of which are given below, as an effective diabetes management system has been extensively studied, but with contradictory results. The study conducted in alloxan induced diabetic rats with the aqueous extract of the roots of *T. cordifolia* have shown an increase in glutathione and vitamin C levels after oral administration for 6 weeks (Stanley *et al.*, 1999). A corresponding decrease has been observed in the levels of thiobarbituric acid reactive substances, ceruloplasmin and alpha-tocopherol levels in plasma.

A similar study on the effectiveness of the root extract of *T. cordifolia* on the induction of hypoglycaemia and hypolipidemia in alloxan induced diabetic rats have been reported with an associated increase in body weight, total haemoglobin concentrations and hepatic hexokinase activity (Stanley *et al.*, 2000). The study by Grover *et al.* (2000) compares the hypoglycaemic effect of *T. cordifolia* with *Eugenia jambolana* in diabetic animals where *T. cordifolia* was found effective only in mild diabetes and had no effect in severe diabetes. The extract demonstrated an inability to restore enzyme levels of hexokinase, the glucokinase and the phosphofructokinase enzymes. The contradictory findings in both cases are further complicated by another study on both alloxan and streptozotocin induced diabetes in rats where the stem extract of *T. cordifolia* exhibits significant anti-hyperglycaemic comparable to the effect of glibenclamide standard (Dhulia *et al.*, 2011).
A possible explanation to the mechanism of action of the plant extract in alloxan induced hyperglycaemic rats and rabbits have been explored with the help of histological studies. There was no evidence in the study to a regeneration of the β cells in the islets of Langerhans of pancreas projecting the involvement of regulation in carbohydrate metabolism (Rawal et al., 2004a). Another study by Rawal et al. (2004b), have suggested that the mechanism of action to be due to inhibition of peripheral glucose release and favourable effects on the endogenous insulin secretion and glucose uptake. The presence of alkaloids, palmatine, jatrorrhizine and magnoflorine significantly reduced the fasting blood glucose levels and suppressed any increase of blood glucose after 2g/kg glucose load (Patel and Mishra, 2011). These alkaloids also raise serum insulin levels in vivo and have led to the hypothesis of mechanisms of insulin releasing and insulin-mimicking activity. Further studies by Patel and Mishra (2012) in rats have shown that magnoflorine was more potent as alpha-glucoside inhibitor.

The extract of *T. cordifolia* was found to significantly reduce the onset of diabetic retinopathy as seen by Agarwal et al. (2012) evidenced by the reduced blood glucose and glycated haemoglobin, prevention of cataract formation and reduction in levels of angiogenic markers, VEGF and PKC in streptozotocin induced rats. The treatment which lasted 24 weeks also demonstrated reduced levels of TNF-α and IL-1β, protection against depletion of antioxidant enzymes- glutathione and catalase. The histopathological studies further reveal the destruction of pancreatic islet structure and a thickening of basement membrane of the retinal and glomerular vasculature of diabetic rat which was not seen in extract treated rats. The ayurvedic formulation Transina used for the management of Alzheimer’s disease contains *T. cordifolia* with other plant drugs. This
herbal formulation shows evidence that the increase in islet superoxide dismutase, which results in the accumulation of degenerative free radicals in the beta-cells of islets, are prevented. This observation could be the cause of the hypoglycaemic effect earlier demonstrated in streptozotocin induced rats (Subramonian et al., 2002). Daily oral feeding of the T. cordifolia extract in these streptozotocin induced rats for 40 days could prevent polyuria but had no effect on renal hypertrophy (Singh et al., 2006).

Very few studies have been conducted on the bioactivity guided fractionation to further evaluate the anti-diabetic activity of T. cordifolia. The leaf of T. cordifolia was extracted successively in petroleum ether, chloroform, ethyl acetate, and methanol and studied for α-Amylase inhibition by zymography in the study conducted by Shareef et al. (2014). The results indicate the potent amylase inhibition by the ethyl acetate and methanol fractions of T. cordifolia. Independent studies conducted on streptozotocin induced diabetes in wistar rats, Selvaraj et al. (2012a) have reported the fractionation of T. cordifolia stem methanolic extract in methanol: water (4:1), ethyl acetate, acid base extraction and neutral extractions forming five fractions. The ethyl acetate soluble fraction (F1) contains steroids, ethyl acetate insoluble fraction (F2) contains fats and oils, the acidic (F3), basic (F4) and neutral (F5) fractions contains glycosides, flavones and phenolic compounds with the additional steroids in the F3 and alkaloids, saponins in F5. F4 was found to be a better anti-diabetic fraction as compared to F1 due to the enhanced insulin secretion. A parallel study (Selvaraj et al., 2012b) on the F3 and F5 fractions have revealed that both are effective for antidiabetic activity but comparatively F5 can enhance the secretion of insulin. The satva or the residue of the aqueous extract has been reported with mild hypoglycaemic activity (Sharma et al., 2013).
c. Effect of *T. cordifolia* on liver and hepatic systems

The hepatoprotective activity of *T. cordifolia* ayurvedic formulation on CCl₄ induced hepatotoxicity was established in male albino rats (Latha *et al.*, 1999). This experimental finding could not be confirmed in another study by Reddy *et al.* (1993) which reports on the failure of the chloroform extract of *T. cordifolia* in providing hepatoprotection to CCl₄ induced rats. The latter study also demonstrates that the alcoholic extracts were ineffective on elevated levels of the serum enzymes, serum alkaline phosphatise, serum amino transferases and serum bilirubin but was able to decrease liver weight. Other clinical studies conducted by Prakash and Rai (1996) in patients diagnosed with infective hepatitis treated with Guduchi tablets showed a significant correction in altered liver function and relief to symptoms.

d. Anti-Stress or Antioxidant Activity of *T. cordifolia*

A comparative study on the methanol, ethanol and aqueous extracts of *T. cordifolia* have revealed the significance of the methanol extract on antioxidant activity which was demonstrated by scavenging of DPPH, superoxide radical and hydroxyl radicals. The study also sheds light on the iron metal chelating capacity of *T. cordifolia* resulting in the reduction of the ferrous form of iron (Bhawya and Anilakumar, 2010) which prevents iron mediated free radical formation. The toxic effect of lead was neutralized with the treatment by aqueous extract of *T. cordifolia* stem and leaves in a study conducted on albino mice for 30 days. The decrease in haematological values by lead exposure was significantly improved on treatment and daily administration of the extract (Sharma and Pandey, 2010). *T. cordifolia* was also used successfully as an adjuvant drug with chloroquine for the treatment of malaria caused splenomegaly. The use of an adjuvant drug was for three
patients showing reduced response to traditional chloroquine for a time period of six months (Singh, 2005)

e. Antimicrobial activity of *T. cordifolia*

Lunavath *et al.* (2012) have studied the antimicrobial effect of different fractions of *T. cordifolia* leaf in hexane, carbon tetrachloride, chloroform and aqueous fraction with a moderate activity against *E. coli*, *P. aeruginosa*, and *S. aureus* in the chloroform and aqueous extracts. A similar study has reported that the fractionation of the rectified spirit extract of *T. cordifolia* stem has resulted in the fraction TC-1 with antibacterial, antifungal and brine shrimp cytotoxic activities. FTIR, $^1$H NMR, $^{13}$C NMR spectral analyses on the fraction has revealed the presence of an alkaloid (Molla *et al.*, 2014). The study by Uddin *et al.* (2011) has also reported on the antimicrobial and cytotoxic activity of *T. cordifolia* methanol extract which was fractionated into petroleum ether, carbon tetrachloride (CCl$_4$), chloroform and aqueous soluble fractions. The CCl$_4$ soluble fraction was found to have moderate anti-microbial action against gram negative and gram positive bacteria and high cytotoxic activity against brine shrimp. The chloroform fraction was also highly cytotoxic on brine shrimp.

f. Gout Relieving activity of *T. cordifolia*

The validation of the use of *T. cordifolia* leaves in gout was correlated to the xanthine oxidase inhibitory activity of the petroleum ether, chloroform, ethyl acetate and residual fractions of the methanol extract of the plant (Chaudhari *et al.*, 2009). The ethyl acetate fraction demonstrates highest potency and is followed by the petroleum ether, chloroform and residual fractions respectively in the order of potency as compared to allopurinol standard.
Part II: Chapter 2

**g. Allelopathic activity of *T. cordifolia***

The allelopathic effect of *T. cordifolia* was studied in detail by Raoof and Siddiqui (2013) with the fractionation of the plant leaves in two different ways. The aqueous leachate of the leaf of *T. cordifolia* was subjected to acid hydrolysis and precipitated to form the organic component. While a second set of extraction was performed in petroleum ether followed by methanol, chloroform and water. Allelopathic activity was high in the aqueous leachate as evidenced in the increase of carbohydrate content and decrease of protein and chlorophyll content.

**h. Antifeedant Activity of *T. cordifolia***

Sivasubramanian *et al.* have reported on the fractionation of *T. cordifolia* stem chloroform extract in a 70-325 mesh silica gel column eluted with 1-100% combinations of chloroform and methanol resulted in 16 fractions yielding compound 2 and 3. The second fractionation in 230-400 mesh silica gel column yielded compounds 1, 4, 5 and 6. Initial chloroform fractions yielded sitosterol, cycloartanol and stigmasterol. The structures of the compounds were elucidated and confirmed by 1D and 2D NMR analysis as compound 1: tincordin, compound 2: tinosporide, compound 3: 8-hydroxytinosporide, compound 4: columbin, compound 5: 8-hydroxycolumbin and compound 6: 10-hydroxycolumbin. The efficacy of the identified compounds in antifeedant activity was studied on *E. vitella*, *P. xylostella* and *S. litura* and compared with azadirachtin-A as standard revealing the highest potency by compound 3 equivalent to the standard.

**i. Effect of *T. cordifolia* on miscellaneous target systems***

A drug formulation containing *T. cordifolia* was studied for effectiveness against the *Entamoeba histolytica* induced amoebiasis and was
found to be effective against various enzymes like α-amylase, protease, alkaline phosphatase, acid phosphatase, aldolase, DNase and RNase (Sohni et al., 1995). Another study conducted by Nandaa (1997) on the effect of \textit{T. cordifolia} containing herbal formulation, Minofil have claimed an absence of side effects when compared to estriol treatment for postmenopausal syndromes in women. Clinical studies on the effectiveness of an adjuvant therapy with a multidrug herbal formulation containing \textit{T. cordifolia} on tuberculosis patients have demonstrated the benefits on subjective parameters like appetite, general sense of well-being and on the objective parameters like weight and serum proteins (Phadtare et al., 1995).

\textbf{j. Anti-Inflammatory Activity of \textit{T. cordifolia}}

Sharma and Singh (1980) have reported the anti-inflammatory effect of the decoction of \textit{T. cordifolia} on carageenan induced hind paw oedema in rats. The effect of \textit{T. cordifolia} were studied among two other plants on the carageenan induced hind paw oedema and cotton pellet granuloma in rats with the anti-inflammatory activity comparable to the standard acetylsalicylic acid (Utpalendu et al., 1999). A recent study (Siddalingappa et al., 2011) in mice and rats on the analgesic and anti-inflammatory activity of \textit{T. cordifolia} aqueous extracts have also demonstrated the efficacy of the plant extract over standard anti-inflammatory drug, diclofenac. The satva of the \textit{T. cordifolia} consists of the whitish solid powder obtained on the extraction of the fresh stem of the plant in aqueous solvent and it has been found to be useful for fever, burning sensation and for the pitta disorders (Mishra and Vaishya, 2002). A study based on the comparative properties of the satva of \textit{T. cordifolia} grown together with other plants particularly \textit{Azadirachta indica} was reported to have incorporated the beneficial properties of the host plant (Sinha et al., 2004) which greatly increases its
medicinal properties. Onkar et al. (2012) has studied the antioxidant effects of the *Tinospora satva* as one of its therapeutic property.

**k. Anticancer activity of *T. cordifolia***

The anticancer potential of the ethanol extract of *T. cordifolia* stems was studied in C6 glioma cells with the fractionation of the ethanol extract in hexane, chloroform, ethyl acetate and butanol (Mishra and Kaur, 2013). The hexane and chloroform fractions exhibit inhibition of proliferation of the C6 glioma cells and cytotoxicity studies in MTT assay shows a 6-10% reduction of the effective IC$_{50}$ value of ethanol extract in the chloroform and hexane bioactive fractions.

**l. Immunomodulatory activity of *T. cordifolia***

The immunomodulatory functions of the *T. cordifolia* plant were independently studied where one study used the stem and the other used the root of the plant. Immunomodulation of the plant was thus reported by both the studies to be present in the polysaccharide fractions having arabinogalactan content. Sharma et al. (2012) has employed a three complex set of fractionation methods for the separation of polysaccharide enriched fractions from the stem of *T. cordifolia*, while the study by Verma et al. (2006) pursued a specific isolation of polysaccharide on a Sephacryl S-400 gel permeation column. In the study by Sharma et al. (2012) the methanol extract undergoes a series of precipitation steps to remove other contaminants and the final extract in pure water undergoes ethanol precipitation for polysaccharides forming the first fraction, while the second fraction employs hot water extraction of polysaccharide. The third approach to fractionation involves the ethanol extract of the stem followed by acetone precipitation of polysaccharide completed with TCA precipitated removal of
proteins. The results of this study suggest that even though all the fractions demonstrate immunomodulation, the highest activity is shown by the fraction in hot water with lowest sugar content (41%) and the lowest activity is shown by the acetone precipitated fraction with highest sugar content (86%). The fraction with the highest activity of immunomodulation was also found to contain columbin, tinoside, jatrorrhizine and furanoditerpene as revealed by QTof-MS-MS analysis. The study by Verma et al. (2006) has reported that the presence of arabinogalactan in the active fraction contributed to the observed in vitro mitogenic stimulation of mice spleen lymphocytes.
Chapter 3

Materials and Methods
2.3.1 Materials

2.3.1.1 Plant Material

Fresh stems of *T. cordifolia* were collected from local regions of Kottayam, Kerala. The plant material was washed repeatedly, cleaned thoroughly and shade dried. The plant is as shown in Plate 1 and Plate 2. The dried stems were then powdered and stored in airtight containers until further use. The plant material was identified and authenticated by the Taxonomist at Department of Botany, St. Thomas College, Palai, Kottayam. The voucher specimens of the plant were deposited at the Kerala Forest Research Institute Herbarium (voucher no: JA 17151) and at the Calicut University Herbarium (voucher no: JA 17168) for future reference.

2.3.1.2 Other Materials and Instruments

HPLC grade solvents (hexane, methanol and chloroform), Silica gel (60-120 Mesh), Linoleic acid and other chemicals for the assay were purchased from Merck Specialities Pvt. Ltd., India. 5-LOX screening enzyme, 15-LOX, Arachidonic acid, Nordihydroguaiaretic acid (NDGA), Indomethacin, COX-1 and COX-2 were obtained from Cayman Chemicals, USA. Linoleic acid, Xylenol orange, Tetra methyl-p-phenylenediamine (TMPD), Diphenylpicrylhydrazyl (DPPH), 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Naphthylethylendiaminedihydrochloride (NEDD), Hematin, Ascorbic acid, Butylated Hydroxy Toluene (BHT) were purchased from HiMedia Laboratories, India. All other reagents and solvents used were of analytical grade either from Merck Limited, India or from HiMedia Laboratories, India. Ultra-pure water was prepared by an ultra-pure water purification system (PURELAB Option-Q, ELGA Lab Waters, India). 96 well plates were read on the iMark Microplate Absorbance Reader and washed with
Plate 1: *Tinospora cordifolia* Plant

Plate 2: Stem of *Tinospora cordifolia*
the Model 1575 Immunowash Microplate Washer from BioRad, India. Spectrophotometric assays were conducted on UV1800 Spectrophotometer from Shimadzu.

2.3.2 Extraction of *T. cordifolia*

2.3.2.1 Preparation of methanol extract of *T. cordifolia*

The dried powder of the stems of *T. cordifolia* were weighed and 30 g was extracted exhaustively first with hexane in a Soxhlet apparatus to defat the sample. The supernatant obtained was removed and any traces of the solvent were removed from the sample. The residual plant material was then again extracted with methanol in a Soxhlet apparatus at temperatures of 40°C. The extraction was considered complete when the exudate becomes colourless. The methanol extract (TCM) was subsequently concentrated to dryness and stored desiccated at 4°C till further use. The yield was recorded. The extract was resuspended in appropriate solvent, checked for interference and subsequently diluted as per protocol given earlier in chapter 1.

2.3.2.2 Preparation of Aqueous extract of *T. cordifolia*

The stems of *T. cordifolia* were collected fresh and cleaned thoroughly to remove any traces of dirt. The coarsely ground fresh plant material was allowed to soak in ultrapure water for 24 hours at room temperature. The suspension was collected, centrifuged and the supernatant (WSF) was concentrated in a rotary evaporator at 40°C. The extract was then lyophilised for complete removal of water and stored at 4°C. The extract was resuspended in appropriate solvent for further experiments, as required. The extracts were also checked for interference and subsequently diluted as per protocol described earlier.
2.3.3 Antioxidant Assay (Prior et al., 2005)

2.3.3.1 DPPH Scavenging Assay (Sharma and Bhat, 2009)

The DPPH is a stable free radical that gives a deep violet colour with a characteristic absorption band at 520nm. When in reaction with a hydrogen donor, the DPPH acts as a scavenger and is reduced to the corresponding hydrazine. The deep violet colour of the DPPH radical becomes colourless or pale yellow when neutralized. The pale yellow colour remains due to the presence of the picryl group (Prakash, 2001). DPPH can react with weak antioxidants and both hydrophilic and lipophilic antioxidants. The method is highly reproducible and requires use of ambient temperatures that significantly reduce the risk of thermal degradation of antioxidant samples.

The plant extracts, TCM and WSF were mixed with 0.135mM DPPH in methanol solution. The mixture was shaken vigorously and incubated at room temperature for 30 minutes in dark. The absorbance was read at 520 nm and compared to positive control butylated hydroxyl toluene (BHT). The solvent was taken as the blank. A control was also prepared following the same protocol except for the addition of plant extract. The percentage of DPPH scavenging of the plant extracts were calculated as per the following equation:

Percent DPPH Scavenging

\[
\text{Percent DPPH Scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of plant extract}}{\text{Absorbance of control}} \times 100.
\]

and EC_{50} was calculated. The EC_{50} value can be defined as the amount of antioxidant necessary to decrease the absorbance of DPPH by 50% of the initial absorbance (Mishra et al., 2012).
2.3.3.2 NO Scavenging Assay (Garrat, 1964)

The original NO scavenging activity described by Greiss has been modified significantly by Garrat (1964). The presence of sodium nitroprusside in an aqueous solution at physiological pH can spontaneously generate the synthesis of nitric oxide. The nitric oxide so produced can further interact with oxygen and produce nitric ions that can be estimated by use of Griess reagent. The sulfanilamide and NEDD under acidic conditions in Greiss reagent competes for nitrite in the reaction and forms a diazonium salt which in presence of the azo dye NEDD gives a pink colour. Scavenger of nitric oxide competes with oxygen leading to reduced production of nitric oxide. A variety of biological and experimental samples can be used with a limit of detection 2.5 uM. This diamine is a simple polar molecule and hence a much more soluble dye in acidic aqueous medium.

Sodium nitroprusside (5 mM) in phosphate-buffered saline was mixed with different concentrations of the T. cordifolia methanol and aqueous extracts and incubated at 25 °C for 60 min. The aliquots from this reaction mixture were treated with Greiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% NEDD). The absorbance of the chromophore formed was read at 546 nm and referred to the absorbance of standard solutions of Ascorbic acid treated in the same way with Griess reagent. A control was also prepared following the same protocol except for the addition of plant extract. The solvent was taken as the blank. The percentage of NO scavenging of the plant extracts were calculated as per the following equation:

\[
\text{Percent NO Scavenging} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of plant extract}}{\text{Absorbance of control}} \right) \times 100.
\]
and EC$_{50}$ was calculated. The EC$_{50}$ value can be defined as the amount of antioxidant necessary to decrease the absorbance of NO by 50% of the initial absorbance (Mishra et al., 2012).

2.3.3.3 ABTS Scavenging Assay (Re et al., 1996)

In this assay, the colourless ABTS is converted to its blue-green coloured radical cation, ABTS•+ by addition of potassium persulphate and one-electron oxidation. The one-electron oxidation commences immediately but becomes stable after 12-16 hours. Addition of the antioxidant samples reduces the absorbance of the preformed radical cation and is directly proportional to the number of ABTS•+ radicals converted to its colourless neutral form.

ABTS was dissolved in phosphate buffer (pH7.0, 7.4mM) and activated to ABTS•+ radical by addition of 2.6mM potassium persulphate in a 1:1 ratio with occasional stirring and 16-18 hours for activation. Then the solution was centrifuged (5min, 7000g), filtered and diluted with methanol (1:60). The plant extracts were added with different concentrations to the preformed radical cation with 20 minutes of incubation at dark. Absorbance of the solution was measured at a wavelength of 734 nm and BHT was used as positive standard. A control was also prepared following the same protocol except for the addition of plant extract. The solvent was taken as the blank. The percentage of ABTS scavenging of the plant extracts were calculated as per the following equation:

\[
\text{Percent ABTS Scavenging} = \frac{[\text{Absorbance of control} - \text{Absorbance of plant extract}] \times 100}{\text{Absorbance of control}}
\]
and EC$_{50}$ was calculated. The EC$_{50}$ value can be defined as the amount of antioxidant necessary to decrease the absorbance of ABTS by 50% of the initial absorbance (Mishra et al., 2012).

2.3.4 Lipoxygenase Inhibition Assay (Gay et al., 2003)

Reagents:

0.1M Tris-HCl Buffer, pH 7.4

150 µM Sodium Linoleate

FOX Reagent (30 mM sulphuric acid, 100 µM xylenol orange, 100 µM iron (II) sulphate in 9:1 ratio of methanol/ water)

Enzyme (5-LOX, 12-LOX, or 15-LOX)

Protocol:

FOX assay involves the conversion of ferrous ion (Fe$^{2+}$) to ferric ions (Fe$^{3+}$) at the acidic pH provided by sulphuric acid. This oxidation of ferrous ions is brought about by lipid hydroperoxides formed in the reaction. The ferric ions form a complex with the yellow coloured acidified xylenol orange dye to yield a blue product with maximum absorbance at 560nm. The reaction product is not sensitive to the surrounding environment, particularly oxygen content and light, and can rapidly generate peroxide measurements. The FOX assay becomes significant when it measures the initial fatty acid oxidation as compared to later stages of oxidation and hence can detect early membrane associated stress events in tissue.

The enzyme was preincubated with the plant extracts for 5 minutes at 37°C. Typical reaction mixture contains 0.1M Tris-HCl buffer, pH 7.4, 150µM sodium linoleate (substrate) and the enzyme-plant extract preincubated mixture in a total volume of 300µl with 20 minutes of
incubation at room temperature in the dark. The reaction was terminated by
the addition of FOX reagent followed by 20 minutes of incubation at room
temperature in the dark. The colour developed was measured at 560 nm. A
blank with methanol and a control without plant extract was also prepared.
Nordihydroguaiaretic acid (NDGA) was used as positive control and the
experiment was conducted in three independent experiments. The solvent
was taken as the blank. The LOX activity can be calculated using the
following formula. The reaction rate at 560 nm was determined using the
chromogen extinction coefficient of 9.47 mM$^{-1}$. The extinction coefficient
has been adjusted for the path-length of the solution in the well. One unit of
enzyme utilizes one µmol of linoleic acid per minute at 25°C.

Lipoxygenase Activity (µmol/min/ml)

\[
= \text{Absorbance}_{560} \text{ (Sample)} - \text{Absorbance}_{560} \text{ (Blank)} \times \text{sample dilution}
\]

\[
9.47 \text{ mM}^{-1} \times \text{Volume of enzyme} \times \text{time of incubation}
\]

The percent inhibition for each inhibitor can be calculated using the
following equation:

Percent of Inhibition (%)

\[
= \left( \text{EA of control} - \text{EA of blank} \right) - \left( \text{EA of Plant Extract} - \text{EA of Blank} \right) \times 100
\]

\[
\left( \text{EA of Control} - \text{EA of Blank} \right)
\]

Where EA stands for Enzyme Activity.

A graph was drawn with the Percent Inhibition as a function of the inhibitor
concentration to determine the IC$_{50}$ value (concentration at which there was
50% inhibition) and the results were expressed in terms of IC$_{50}$ values.
2.3.5 Cyclooxygenase Inhibition Assay (Copeland et al., 1994)

Reagents:

100mM Tris-HCl, pH 8.0

Hemin, 15µM

EDTA, 3µM

Protocol:

The COX-TMPD assay particularly measures the peroxidase activity of COX enzyme. This peroxidase activity can be assayed colorimetrically by monitoring the appearance of oxidized TMPD with both crude (cell lysates/tissue homogenates) and purified enzyme preparations (Copeland et al., 1994). The TMPD assay for measuring the enzyme activity of COX is a chromogenic assay that involves the oxidation of TMPD and the simultaneous reduction of PGG$_2$ to PGH$_2$. The TMPD is a phenylenediamine that is easily oxidised with the loss of two electrons in a one electron oxidation forming its radical cation (Micaelis et al., 1939).

The assay mixture contained Tris-HCl buffer (100mM, pH 8.0), hemin (15µM), EDTA (3µM), enzyme and test compound. The mixture was preincubated at 25°C for 15 min and then the reaction was initiated by the addition of arachidonic acid (100 µM) and TMPD in total volume of 1ml. The enzyme activity was measured by estimating the initial velocity of TMPD oxidation for the first 25s of the reaction, following the increase in absorbance at 603 nm. The COX activity was calculated from the reaction rate at 603 nm using the TMPD extinction coefficient of 0.00826µM$^{-1}$ adjusted to the path length. One unit of enzyme activity was defined as the amount of enzyme that causes the oxidation of 1.0 nmol of TMPD per minute at 25°C. PGG$_2$ reduction to PGH$_2$ requires the oxidation of two
molecules of TMPD. Indomethacin was used as positive control. The solvent was taken as the blank.

Cyclooxygenase Activity (nmol/min/ml)

\[
\text{Cyclooxygenase Activity} = \left( \frac{\text{Absorbance}_{603} \text{ (Sample)} - \text{Absorbance}_{603} \text{ (Blank)} \times \text{sample dilution}}{0.0086 \mu M^{-1} \times \text{Volume of enzyme} \times \text{time of incubation}} \right) \div 2
\]

The percent inhibition for each inhibitor can be calculated using the following equation:

Percent of Inhibition (%)

\[
= \left( \frac{\text{EA of control} - \text{EA of blank} - \text{(EA of Plant Extract} - \text{EA of Blank}) \times 100}{\text{EA of Control} - \text{EA of Blank}} \right)
\]

Where EA stands for Enzyme Activity.

A graph was drawn with the Percent Inhibition as a function of the inhibitor concentration to determine the IC\textsubscript{50} value (concentration at which there was 50% inhibition of enzyme activity) and the inhibition results were expressed in terms of IC\textsubscript{50} values.

2.3.6 Bioactivity Guided Fractionation of TCM

The crude methanol extract of \textit{T. cordifolia} was subjected to fractionation on a glass column with silica gel (60 – 120 mesh) packing. The column was packed as per the wet packing system for columns. The plant extract (312.75mg) was adsorbed on 1 gram of silica gel and gently layered on top of the column. The flow-rate was set as 2ml per minute and the column was run with solvents in the order as given in figure 3.1. The eluted fractions were collected in aliquots of 10 ml and were then pooled on the basis of UV- Visible spectral analysis data (190-1100nm) and the pooled
fractions were concentrated to dryness, lyophilized and yield noted. The fractions were reconstituted and were analysed for lipoxygenase inhibition to confirm biological activity as per protocol described earlier. Fractions that exhibited maximal activity in the bioassay were considered for further structure elucidation studies.

2.3.7 Screening of Fractions for LOX Inhibition

The final 24 fractions were reconstituted in DMSO/methanol according to the nature of eluting solvent used (the fractions that were eluted in 100% chloroform was reconstituted in DMSO due to non-solubility in methanol). These fractions were then checked for inhibition of LOX activity. The 15-LOX enzyme was selected for screening as per earlier use. The protocol for LOX assay and inhibition was as per protocol described earlier in Chapter 2. The fraction with maximal inhibitory activity and with minimal inhibitory activity in the bioassay was considered for further characterisation studies and for structure elucidation studies. Since the objective was to identify inhibitor molecules that inhibit both LOX and COX, the fraction which inhibited LOX enzyme was also used for analysis of COX inhibition.
2.3.8 Antioxidant activity of *T. cordifolia* bioactive fractions

The fractions were characterized for antioxidant capacity by free radical scavenging effect on DPPH, on Nitric oxide, and on ABTS radicals as per the protocol described in detail earlier (2.3.5). The EC$_{50}$ values were calculated and results compared with the positive control for each set of experiments.
2.3.9 Effect of bioactive fractions of *T. cordifolia* on 5-LOX and 12-LOX activity

The fractions were characterized for effect on 5-LOX and 12-LOX enzymes as per the Ferrous Oxidation- Xylenol orange assay as described in detail earlier (2.3.4). The IC$_{50}$ values were calculated and results compared with positive control, NDGA.

2.3.10 Effect of bioactive fractions of *T. cordifolia* on COX-1 and COX-2 activity

The fractions were characterized for effect on COX-1 and COX-2 enzymes as per the Tetramethyl-p-Phenylenediamine (TMPD)-COX assay as described in detail earlier (2.3.5). The IC$_{50}$ values were calculated and results compared with the positive control, indomethacin.

2.3.11 Evaluation of effect of bioactive fractions on Kinetic parameter of Km and Vmax of LOX enzymes

The mode of inhibition of the LOX enzyme by the bioactive fractions was determined by using Lineweaver–Burk equations. The LOX assay was carried out as per protocol given earlier. Different substrate concentrations (based on earlier analysis) 5, 10, 25, 50, 150, 200 and 250µM of sodium linoleate was prepared for the study of kinetic data in presence and absence of plant extract. The values of kinetic parameters of the enzymes (Km and Vmax) in the presence and absence of bioactive fractions were determined by analysis of Lineweaver-Burk plots and the apparent inhibition constant (Ki) values were calculated as per the equation by Garrett and Grisham (2010) using the SigmaPlot version 12.5.

\[
V_i = \frac{V_{MAX}[S]}{K_M (1 + \frac{I}{K_i}) + S}
\]
2.3.12 Evaluation of effect of bioactive fractions on Kinetic parameter of Km and Vmax of COX enzymes

The mode of inhibition of the COX enzyme by the bioactive fractions was determined by using the Lineweaver–Burk equations. The COX assay was carried out as per protocol given earlier in 2.3.5. Different substrate concentrations (based on earlier analysis) 10, 50, 100 and 200µM of arachidonic acid was prepared for the study of kinetic data in presence and absence of bioactive fractions, F11 and F9. The values of kinetic parameters of the enzymes (Km and Vmax) in presence and absence of the bioactive fractions were determined by analysis of Lineweaver-Burk plots and the apparent inhibition constant (Ki) values were calculated as per the equation by Garrett and Grisham (2010) using the SigmaPlot version 12.5.

\[ V_I = \frac{V_{max}[S]}{K_m(1 + \frac{[I]}{K_i}) + S} \]

2.3.13 Statistical analyses

In all the studies, the values of independent experiments are expressed as mean ± standard deviation (S.D) for n determinations where n=3 unless otherwise stated. All the assays used solvent control for the blank and were deducted from the absorbance values. 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 Sigma Plot (Systat Software, San Jose, CA). Significance of differences with respect to control was calculated by the Sigma Plot software.
Chapter 4

Results
2.4.1 Extraction of *T. cordifolia*

The shade dried and powdered plant materials were used for the extraction process. Thirty grams of the dried powder was first defatted with hexane and then extracted with methanol by using Soxhlet apparatus. The solvent was evaporated to dryness and the dried crude extract was stored dessicated at 4°C. The percentage yield of methanol extract of *T. cordifolia* (TCM) was 7.3% and the extract was further reconstituted in appropriate solvent. The extracts were checked for interference as per protocol given in chapter 1 and diluted (6.25x10^{-4} ag/µl for LOX assays and 0.0625 ug/µl for COX assays).

For the preparation of aqueous extract, thirty grams of freshly ground stems of *T. cordifolia* was soaked in ultrapure water for 24 hours and supernatant was collected. The supernatant was centrifuged, concentrated, lyophilised and stored dessicated at 4°C. The percentage yield of water extract of *T. cordifolia* was 13.5% and the extract was further reconstituted in appropriate solvent. The extracts were checked for interference as per protocol given in Part I and diluted (0.1364 pg/µl for LOX assays and 13.64 ng/µl for COX assays).

2.4.2 Antioxidant activity of *T. cordifolia* bioactive fractions

2.4.2.1 Effect of *T. cordifolia* extracts on DPPH Scavenging

The strong absorption maximum of the DPPH scavenging assay at 520 nm is characterized by the presence of a deep violet colour of the DPPH radical which on neutralization changes to a colourless or a pale yellow colour of the picryl group. This change in absorbance at 520 nm was monitored during the course of the reaction to find out the antioxidant power of the samples. The EC_{50} value can be defined as the amount of antioxidant necessary to decrease the absorbance of DPPH by 50% of the initial
absorbance (Mishra et al., 2012). The results for the EC$_{50}$ values are as given in Table 2.1. The plant extracts exhibit DPPH radical scavenging activity but is not significantly better than control.

Table 2.1: Effect of *T. cordifolia* extracts on DPPH Radical Scavenging

<table>
<thead>
<tr>
<th>Name of Extract</th>
<th>EC$_{50}$ ($\mu$g/$\mu$l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butylated hydroxy toluene</td>
<td>2.07 ± 0.1</td>
</tr>
<tr>
<td>TCM</td>
<td>20.76 ± 0.25$^*$</td>
</tr>
<tr>
<td>WSF</td>
<td>6.01 ± 0.07$^*$</td>
</tr>
</tbody>
</table>

The effect of the TCM extract and the WSF extracts on the DPPH scavenging activity was compared to the butylated hydroxyl toluene (BHT) standard. Results expressed as Mean ± SD of three independent experiments. *P<0.001, EC$_{50}$ values are significantly higher than control and hence the extract does not have better scavenging activity than control.

2.4.2.2 Effect of *T. cordifolia* extracts on Nitric Oxide Scavenging

The presence of scavengers or antioxidants can compete with oxygen thereby reducing the production of nitric oxide and was measured at the absorption maxima of 546nm for the Greiss assay. The efficient concentration 50 (EC$_{50}$) values were calculated from the absorbance at 546 nm. The results for the EC$_{50}$ values are as given in Table 2.2. The plant extract TCM exhibits NO radical scavenging activity and is significantly better than control.

Table 2.2: Effect of *T. cordifolia* extracts on NO Scavenging

<table>
<thead>
<tr>
<th>Name of Extract</th>
<th>EC$_{50}$ ($\mu$g/$\mu$l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic Acid</td>
<td>5.53 ± 0.36</td>
</tr>
<tr>
<td>TCM</td>
<td>4.24 ± 0.27$^*$</td>
</tr>
<tr>
<td>WSF</td>
<td>No Action</td>
</tr>
</tbody>
</table>

The effect of the TCM extract and the WSF extracts on the nitric oxide (NO) scavenging activity was compared to the ascorbic acid standard. Results expressed as Mean ± SD of three independent experiments. *P<0.001, EC$_{50}$ values of TCM is significantly lower than control and hence the extract has better scavenging activity than control.
2.4.2.3 Effect of *T. cordifolia* extracts on ABTS Scavenging

The assay protocol involves the decolourisation of the previously formed blue-green coloured ABTS radical with absorption maxima at 415nm into colourless ABTS molecule. The assay measures the hydrogen-donating capacity of antioxidants to scavenge the ABTS radical generation in aqueous phase. The EC$_{50}$ value can be defined as the amount of antioxidant necessary to decrease the absorbance of ABTS by 50% of the initial absorbance. The results for the EC$_{50}$ values are as given in Table 2.3. The plant extracts exhibit ABTS radical scavenging activity and TCM is significantly better than control while WSF is not significantly better than control.

Table 2.3: Effect of *T. cordifolia* extracts on ABTS Radical Scavenging

<table>
<thead>
<tr>
<th>Name of Extract</th>
<th>EC$_{50}$ (µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butylated hydroxy toluene</td>
<td>0.856 ± 0.02</td>
</tr>
<tr>
<td>TCM</td>
<td>0.049 ±0.33*</td>
</tr>
<tr>
<td>WSF</td>
<td>21.13 ± 0.01#</td>
</tr>
</tbody>
</table>

The effect of the TCM extract and the WSF extracts on the ABTS scavenging activity was compared to the butylated hydroxyl toluene (BHT) standard. Results expressed as Mean ± SD of three independent experiments. * P<0.001, EC$_{50}$ values are significantly lower than control and hence the extract has better scavenging activity than control. # P<0.001, EC$_{50}$ values are significantly higher than control and hence the extract does not have better scavenging activity than control.

2.4.3 Effect of *T. cordifolia* extracts on 5-LOX activity

2.4.3.1 Effect on 5-LOX activity

The inhibition of 5-LOX enzyme was carried out using the Ferrous Oxidation-Xylenol orange assay (FOX). The protocol measures the oxidation of the ferrous ion by the hydroperoxide product of 5-LOX enzyme and substrate, linoleate, and is visualised by the formation of the ferric ion-xylenol
orange blue complex. The plant extracts TCM and WSF reconstituted in methanol were used as per protocol for study of effect on the activity of LOX. The IC$_{50}$ values for the enzyme are as given in Table 2.4. The plant extracts exhibit 5-LOX enzyme inhibition and is significantly better than control.

**Table 2.4: IC$_{50}$ values for inhibition of 5-LOX activity: Comparison with NDGA standard**

<table>
<thead>
<tr>
<th>Name of Extract</th>
<th>5-LOX (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDGA</td>
<td>2.75 ± 0.05</td>
</tr>
<tr>
<td>TCM</td>
<td>9.34 x 10$^{-14}$ ± 0.0004$^*$</td>
</tr>
<tr>
<td>WSF</td>
<td>2.1x10$^{-5}$ ± 0.003$^#$</td>
</tr>
</tbody>
</table>

The effect of the TCM extract and the WSF extracts on the 5-LOX activity was compared to the NDGA standard. Results expressed as Mean ± SD of three independent experiments. *P*<0.001, IC$_{50}$ values are significantly lower than control and hence the extracts have better inhibition of enzyme than control. $^#P$<0.05, IC$_{50}$ values are significantly lower than control and hence the extracts have better inhibition of enzyme than control.

**2.4.3.2 Effect on 12-LOX activity**

The inhibition of 12-LOX enzyme was carried out using the Ferrous Oxidation-Xylenol orange assay (FOX). The protocol measures the oxidation of the ferrous ion by the hydroperoxide product of 12-LOX enzyme and substrate, linoleate, and is visualised by the formation of the ferric ion-xylenol orange blue complex. The plant extracts TCM and WSF reconstituted in methanol were used as per protocol for study of effect on the activity of LOX. The IC$_{50}$ values for the enzyme are as given in Table 2.5. The plant extracts exhibit 12-LOX enzyme inhibition and is significantly better than control.
Table 2.5: IC$_{50}$ values for inhibition of 12-LOX activity: Comparison with NDGA standard

<table>
<thead>
<tr>
<th>Name of Extract</th>
<th>12-LOX (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDGA</td>
<td>0.302 ± 0.68</td>
</tr>
<tr>
<td>TCM</td>
<td>9.01x10$^{-14}$ ± 0.007$^*$</td>
</tr>
<tr>
<td>WSF</td>
<td>3.4x10$^{-5}$ ± 0.002$^*$</td>
</tr>
</tbody>
</table>

The effect of the TCM extract and the WSF extracts on the 12-LOX activity was compared to the NDGA standard. Results expressed as Mean ± SD of three independent experiments. $^*$ P<0.001, IC$_{50}$ values are significantly lower than control and hence the extracts have better inhibition of enzyme than control.

2.4.3.3 Effect on 15-LOX activity

The inhibition of 15-LOX enzyme was carried out using the Ferrous Oxidation-Xylenol orange assay (FOX). The protocol measures the oxidation of the ferrous ion by the hydroperoxide product of 15-LOX enzyme and substrate, linoleate, and is visualised by the formation of the ferric ion-xylenol orange blue complex. The plant extracts TCM and WSF reconstituted in methanol were used as per protocol for study of effect on the activity of LOX. The IC$_{50}$ values for the enzymes are as given in Table 2.6. The plant extracts exhibit 15-LOX enzyme inhibition and is significantly better than control.

Table 2.6: IC$_{50}$ values for inhibition of 15-LOX activity: Comparison with NDGA standard

<table>
<thead>
<tr>
<th>Name of Extract</th>
<th>15-LOX (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDGA</td>
<td>8.47 ± 0.15</td>
</tr>
<tr>
<td>TCM</td>
<td>2.5x10$^{-11}$ ± 0.089$^*$</td>
</tr>
<tr>
<td>WSF</td>
<td>1.4x10$^{-5}$ ± 0.0007$^*$</td>
</tr>
</tbody>
</table>

The effect of the TCM extract and the WSF extracts on the 15-LOX activity was compared to the NDGA standard. Results expressed as Mean ± SD of three independent experiments. $^*$ P<0.001, IC$_{50}$ values are significantly lower than control and hence the extracts have better inhibition of enzyme than control.
2.4.4 Effect of *T. cordifolia* extracts on activity of COX enzymes

The inhibition of the COX-1 and COX-2 enzymes was carried out using the COX-TMPD assay and was considered appropriate as the major assay for inhibition of COX enzymes. The COX assay involves the conversion of the substrate arachidonic acid to PGG$_2$ and requires the cyclooxygenase part of the enzyme. The peroxidase part of the COX enzyme simultaneously converts PGG$_2$ to PGH$_2$ and TMPD to its oxidised form which can be measured. The extracts TCM and WSF were used as per protocol for study of effect on the activity of COX. The enzymes COX-1 and COX-2 were used separately to determine the effect of *T. cordifolia* extracts. The IC$_{50}$ values of TCM and WSF extracts for effect on COX activity was as given in Table 2.7. WSF had no effect on COX-1 while COX-2 as inhibited by both TCM and WSF.

**Table 2.7: Comparative IC$_{50}$ values for inhibition of COX-1 and COX-2 with Indomethacin (IM) Standard**

<table>
<thead>
<tr>
<th>Name of Extract</th>
<th>COX-1 (ng/µl)</th>
<th>COX-2 (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin</td>
<td>$3.705 \times 10^{-3} \pm 0.06$</td>
<td>$3.01 \pm 0.007$</td>
</tr>
<tr>
<td>TCM</td>
<td>$7.57 \times 10^{-4} \pm 0.055^#$</td>
<td>$5.73 \times 10^{-2} \pm 0.012^*$</td>
</tr>
<tr>
<td>WSF</td>
<td>$1.59 \times 10^3 \pm 0.003^§$</td>
<td>$1.63 \pm 0.004^*$</td>
</tr>
</tbody>
</table>

The effect of the TCM extract and the WSF extracts on the COX-1 and COX-2 activity was compared to the indomethacin (IM) standard. Results expressed as Mean ± SD of three independent experiments. $^*P<0.05$, IC$_{50}$ values are significantly lower than control and hence the extract has better inhibition of enzyme than control. $^#P<0.001$, IC$_{50}$ values are significantly lower than control and hence the extracts have better inhibition of enzyme than control. $^§P<0.001$, IC$_{50}$ values are significantly higher than control and hence the extracts do not have better inhibition of enzyme than control.

2.4.5 Bioactivity Guided Fractionation of TCM

The details of the 24 fractions collected and the percentage of yields are as shown in Table 2.8. The fractions F1, F2, F3 were eluted in
chloroform and were subsequently reconstituted in dimethyl sulfoxide (DMSO) while the fractions F4 – F24 were reconstituted in methanol. The reconstituted fractions were analysed for interference as per standard protocol for FOX assay as described earlier. Fractions or samples showing interference were diluted till the interference was removed.

Table 2.8: Name, Yield of fractions and Choice of Reconstituting Solvent

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Collected Fractions (% yield)</th>
<th>Eluting Solvent (%)</th>
<th>Reconstitution Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F1 (0.4), F2 (0.03), F3 (0.02)</td>
<td>CHCl\textsubscript{3} 100</td>
<td>MeOH 0</td>
</tr>
<tr>
<td>2</td>
<td>F4(0.02), F5(0.02), F6(0.94), F7(0.81), F8(0.95)</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>F9(1.95), F10(0.4), F11(0.01)</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>F12(1.3), F13(0.2)</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>F14(0.5), F15(0.8), F16(0.8), F17(0.5)</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>F18(0.9), F19(1.7), F20(0.2), F21(0.03)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>F22(0.04), F23(0.4)</td>
<td>MeOH 50</td>
<td>Water 50</td>
</tr>
<tr>
<td>8</td>
<td>F24(1.6)</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

F stands for the Fraction and the numbers are from 1 – 24. MeOH stands for methanol and CHCl\textsubscript{3} stands for chloroform. Yield as percentage is given in brackets.

2.4.6 Screening for Bioactivity of LOX Inhibition

The fractions were screened for inhibition of the activity of the 15-LOX enzyme as per earlier protocol and the results are as shown in Fig 2.2.
Fraction 11 (F11) showed maximal inhibitory activity against 15 LOX with the lowest IC$_{50}$ value and fraction 9 (F9) showed minimal inhibition against 15-LOX with highest IC$_{50}$ value as shown in Table 2.9.

The fractions were screened for LOX inhibitory action with the 15-LOX enzyme and the values were compared to find F11 with the most inhibitory action and F9 with the least. Values are expressed as mean ± sd for three independent experiments.
Table 2.9: IC\textsubscript{50} Values of Fractions from Screening studies for LOX inhibition

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Fraction Name</th>
<th>IC\textsubscript{50} Values for 15 LOX Activity (µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NDGA</td>
<td>0.0085 ± 0.15</td>
</tr>
<tr>
<td>2</td>
<td>F11</td>
<td>0.016 ± 0.009*</td>
</tr>
<tr>
<td>3</td>
<td>F9</td>
<td>3.05 ± 0.002#</td>
</tr>
</tbody>
</table>

The effect of the F11 fraction and the F9 fraction on the 15-LOX activity was compared to the NDGA standard and used as the screening method. Results expressed as Mean ± SD of three independent experiments. \*P<0.05, IC\textsubscript{50} values are significantly higher than control and hence the extract does not have better inhibition of enzyme than control. \#P<0.05, IC\textsubscript{50} values are significantly higher than control and hence the extract does not have better inhibition of enzyme than control.

2.4.7 Antioxidant activity of \textit{T. cordifolia} bioactive fractions

2.4.7.1 Effect of \textit{T. cordifolia} fractions on DPPH scavenging

The DPPH assay protocol measures the change in the absorption maximum at 520 nm characterized by the neutralization of deep violet DPPH. The EC\textsubscript{50} value was calculated as described earlier. The results for the EC\textsubscript{50} values are as given in Table 2.10. The fractions show minimal inhibition of DPPH radical scavenging as compared with effect of butylated hydroxyl toluene (BHT) as evidenced by a high EC\textsubscript{50} value.

Table 2.10: Effect of \textit{T. cordifolia} extracts on DPPH Radical Scavenging

<table>
<thead>
<tr>
<th>Name of Extract</th>
<th>EC\textsubscript{50} (µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butylated Hydroxy Toluene</td>
<td>2.07 ± 0.1</td>
</tr>
<tr>
<td>Fraction 11</td>
<td>514 ± 0.021*</td>
</tr>
<tr>
<td>Fraction 9</td>
<td>77.06 ± 1.36*</td>
</tr>
</tbody>
</table>

The effect of the F11 fraction and the F9 fraction on the DPPH radical scavenging activity was compared to the butylated hydroxyl toluene standard and used as the screening method. Results expressed as Mean ± SD of three independent experiments. \*P<0.001, EC\textsubscript{50} values are significantly higher than control and hence the extract does not have better scavenging activity than control.
2.4.7.2 Effect of *T. cordifolia* fractions on Nitric Oxide scavenging

The NO scavenging assay protocol measures the ability of the antioxidant in inhibiting the production of nitric oxide and subsequent measurement of nitrite produced from NO. The EC$_{50}$ value is as given in Table 2.11. The fractions show minimal inhibition of NO scavenging as compared with effect of ascorbic acid evidenced by a high EC$_{50}$ value.

**Table 2.11: Effect of *T. cordifolia* extracts on NO Scavenging**

<table>
<thead>
<tr>
<th>Name of Extract</th>
<th>EC$_{50}$ (µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>5.53 ± 0.36</td>
</tr>
<tr>
<td>Fraction 11</td>
<td>1066 ± 0.08*</td>
</tr>
<tr>
<td>Fraction 9</td>
<td>No Effect</td>
</tr>
</tbody>
</table>

The effect of the F11 fraction and the F9 fraction on the nitric oxide scavenging activity was compared to the ascorbic acid standard and used as the screening method. Results expressed as Mean ± SD of three independent experiments. *P<0.001, EC$_{50}$ values are significantly higher than control and hence the extract does not have better scavenging activity than control.

2.4.7.3 Effect of *T. cordifolia* fractions on ABTS scavenging

The ABTS Radical Scavenging assay protocol involves the measurement of the change in absorbance characterized by the decolourisation of the blue-green ABTS radical. The EC$_{50}$ value was calculated as described earlier. The results for the EC$_{50}$ values are as given in Table 2.12. The fractions show minimal ABTS Radical Scavenging activity as compared with effect of butylated hydroxyl toluene (BHT) evidenced by high EC$_{50}$ value.
Table 2.12: Effect of *T. cordifolia* fractions on ABTS Radical Scavenging

<table>
<thead>
<tr>
<th>Name of Extract</th>
<th>EC$_{50}$ (µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butylated Hydroxy Toluene</td>
<td>0.856 ± 0.02</td>
</tr>
<tr>
<td>Fraction 11</td>
<td>364 ± 0.02*</td>
</tr>
<tr>
<td>Fraction 9</td>
<td>306 ± 0.007*</td>
</tr>
</tbody>
</table>

The effect of the F11 fraction and the F9 fraction on the ABTS scavenging activity was compared to the butylated hydroxy toluene standard and used as the screening method. Results expressed as Mean ± SD of three independent experiments. *P<0.001, EC$_{50}$ values are significantly higher than control and hence the extract does not have better scavenging activity than control.

**2.4.8 Effect of Bioactive fraction on LOX activity**

The inhibition of 5-LOX and 12-LOX enzymes was carried out using the Ferrous Oxidation-Xylenol orange assay (FOX). The oxidation of the ferrous ion was measured and the IC$_{50}$ values were calculated. The fractions F11 and F9 reconstituted in methanol were used as per protocol for study of effect on the activity of LOX after interference assay and subsequent dilution (4.2 ng/µl for F11 and 0.4985 µg/µl for F9). The IC$_{50}$ values for the enzymes are as given in Table 2.13. The fractions show a potent significant (P<0.05) inhibition of the 5-LOX and 12-LOX enzymes with higher significance for F11 than F9 fractions which are comparable to NDGA. The plant extracts exhibit LOX enzyme inhibition and Fraction 11 is significantly better than control for 5-LOX and 12-LOX and not better than control for 15-LOX. Fraction 9 is not significantly better than control for 5-LOX, 12-LOX and 15-LOX.
### Results

#### 2.4.9 Effect of Bioactive fraction on COX activity

The inhibition of COX-1 and COX-2 enzymes was carried out using the TMPD-COX assay. The oxidation of TMPD was measured and the IC$_{50}$ values were calculated. The fractions F11 and F9 reconstituted in methanol were used as per protocol for study of effect on the activity of COX after interference assay and subsequent dilution (42 µg/µl for F11 and 49.85 µg/µl for F9). The IC$_{50}$ values for the enzymes are as given in Table 2.14. The plant extracts exhibit COX enzyme inhibition and is not significantly better than control for COX-1 and COX-2. However F9 exhibits a COX-2 inhibition at levels comparative to that of positive control.

#### Table 2.13: Comparative IC$_{50}$ values of fractions for LOX enzymes

<table>
<thead>
<tr>
<th>Name of Extract</th>
<th>5-LOX (ng/µl)</th>
<th>12-LOX (ng/µl)</th>
<th>15-LOX (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDGA</td>
<td>2.75 ± 0.05</td>
<td>0.302 ± 0.68</td>
<td>8.47 ± 0.15</td>
</tr>
<tr>
<td>Fraction 11</td>
<td>0.041 ± 0.0003$^$</td>
<td>0.058 ± 0.003$^#$</td>
<td>15.94 ± 0.009$^*$</td>
</tr>
<tr>
<td>Fraction 9</td>
<td>7.91 ± 0.02$^*$</td>
<td>14.8 ± 0.32$^*$</td>
<td>3049 ± 0.002$^*$</td>
</tr>
</tbody>
</table>

The effect of the F11 fraction and the F9 fraction on the LOX activity was compared to the NDGA standard and used as the screening method. Results expressed as Mean ± SD of three independent experiments. $^*$ P<0.001, IC$_{50}$ values are significantly higher than control and hence the extracts do not have better inhibition of enzyme than control. $^\$ P<0.001, IC$_{50}$ values are significantly lower than control and hence the extracts have better inhibition of enzyme than control. $^#$ P<0.05, IC$_{50}$ values are significantly lower than control and hence the extracts have better inhibition of enzyme than control.

#### Table 2.14: Comparative IC$_{50}$ values of fractions for COX enzymes

<table>
<thead>
<tr>
<th>Name of Extract</th>
<th>COX-1 (ng/µl)</th>
<th>COX-2 (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin</td>
<td>$3.705 \times 10^{-3} \pm 0.06$</td>
<td>$3.01 \pm 0.007$</td>
</tr>
<tr>
<td>Fraction 11</td>
<td>$19.87 \times 10^{6} \pm 0.25^*$</td>
<td>$59.26 \pm 0.42^*$</td>
</tr>
<tr>
<td>Fraction 9</td>
<td>$216.25 \times 10^{6} \pm 20.02^*$</td>
<td>$4.83 \pm 0.001^#$</td>
</tr>
</tbody>
</table>

The effect of the F11 fraction and the F9 fraction on the COX-1 and COX-2 activity was compared to the indomethacin standard and used as the screening method. Results expressed as Mean ± SD of three independent experiments. $^*$ P<0.001, IC$_{50}$ values are significantly higher than control and hence the extracts do not have better inhibition of enzyme than control. $^\$ P<0.05, IC$_{50}$ values are significantly higher than control and hence the extracts do not have better inhibition of enzyme than control.
2.4.10 Effect of F11 and F9 on the Kinetic parameters (Km and Vmax) of the LOX isoenzymes

The kinetic type and parameters of inhibition of *T. cordifolia* by F11 and F9 was evaluated using the Lineweaver-Burk plot and the results are as shown in Fig 2.3, 2.4 and 2.5 for the 5-, 12-, and 15-LOX enzymes respectively. The Lineweaver-Burk plot for analysis of kinetic parameters performed in the presence of *T. cordifolia* bioactive fractions on the activity of 5-LOX, 12-LOX and 15-LOX, has revealed a mixed type of inhibition. The apparent Vmax was decreased in all cases and apparent Km values increased compared to uninhibited enzyme. The results are as indicated in Table 2.15, 2.16 and 2.17 for 5-, 12-, 15-LOX enzymes respectively. The Ki values were calculated and compared using the enzyme kinetics module of SigmaPlot software and are as given in Table 2.18.

a) Values for 5-LOX

<table>
<thead>
<tr>
<th>Name of extract</th>
<th>K_M (uM)</th>
<th>V_MAX(uMol/min/mg protein)</th>
<th>Type of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 LOX</td>
<td>19.86±1.8</td>
<td>11.5±0.13</td>
<td></td>
</tr>
<tr>
<td>F11</td>
<td>65.28±1.66*</td>
<td>10.08±0.35#</td>
<td>Mixed</td>
</tr>
<tr>
<td>F9</td>
<td>122.02±0.34*</td>
<td>10.35±0.42#</td>
<td>Mixed</td>
</tr>
</tbody>
</table>

The Km and Vmax values of the F11 and F9 fractions for 5-LOX activity at different substrate concentrations were compared with the pure enzyme not treated with plant extract. Results expressed as Mean ± SD of three independent experiments. *P<0.001, values are significantly higher than uninhibited enzyme. # P<0.001, values are significantly lower than uninhibited enzyme.
The 5-LOX activity was measured with different substrate concentrations and the results plotted as reciprocals in the Lineweaver-Burk plot. The km and Vmax values were calculated from the graph and compared. Results expressed as Mean ± SD of three independent experiments.

b) 12-LOX

**Table 2.16: K_M and V_MAX values for 12-LOX**

<table>
<thead>
<tr>
<th>Name of extract</th>
<th>K_M (µM)</th>
<th>V_MAX (µMol/min/mg protein)</th>
<th>Type of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 LOX</td>
<td>11.06±0.01</td>
<td>1.47±0.002</td>
<td></td>
</tr>
<tr>
<td>F11</td>
<td>29.97±1.19*</td>
<td>1.009±0.041*</td>
<td>Mixed</td>
</tr>
<tr>
<td>F9</td>
<td>29.61±2.9*</td>
<td>0.659±0.006§</td>
<td>Mixed</td>
</tr>
</tbody>
</table>

The Km and Vmax values of the F11 and F9 fractions for 12-LOX activity at different substrate concentrations were compared with the pure enzyme not treated with plant extract. Results expressed as Mean ± SD of three independent experiments. * P<0.001, values are significantly higher than uninhibited enzyme. * P<0.05, values are significantly lower than uninhibited enzyme. § P<0.001, values are significantly lower than uninhibited enzyme.
The 12-LOX activity was measured with different substrate concentrations and the results plotted as reciprocals in the Lineweaver-Burk plot. The km and Vmax values were calculated from the graph and compared. Results expressed as Mean ± SD of three independent experiments.

c) 15-LOX

Table 2.17: $K_M$ and $V_{MAX}$ values for 15-LOX

<table>
<thead>
<tr>
<th>Name of extract</th>
<th>$K_M$ (µM)</th>
<th>$V_{MAX}$ (µMol/min/mg protein)</th>
<th>Type of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 LOX</td>
<td>4.08±0.05</td>
<td>0.5±0.004</td>
<td></td>
</tr>
<tr>
<td>F11</td>
<td>109.47±6.2*</td>
<td>0.195±0.01*</td>
<td>Mixed</td>
</tr>
<tr>
<td>F9</td>
<td>128.44±2.9*</td>
<td>0.44±0.004*</td>
<td>Mixed</td>
</tr>
</tbody>
</table>

The $K_M$ and $V_{MAX}$ values of the F11 and F9 fractions for 15-LOX activity at different substrate concentrations were compared with the pure enzyme not treated with plant extract. Results expressed as Mean ± SD of three independent experiments. *P<0.01, values are significantly higher than uninhibited enzyme. P<0.001, values are significantly lower than uninhibited enzyme.
The 15-LOX activity was measured with different substrate concentrations and the results plotted as reciprocals in the Lineweaver-Burk plot. The km and Vmax values were calculated from the graph and compared. Results expressed as Mean ± SD of three independent experiments.

**Table 2.18: Ki values observed for fractions of T. cordifolia**

<table>
<thead>
<tr>
<th>Name of extract</th>
<th>Kᵢ (ng/ul)</th>
<th>5-LOX</th>
<th>12-LOX</th>
<th>15-LOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>F11</td>
<td>0.0118±0.004</td>
<td>0.092±0.18</td>
<td>0.011±0.006</td>
<td></td>
</tr>
<tr>
<td>F9</td>
<td>0.659±0.25°</td>
<td>7.08±9.8°</td>
<td>0.705±0.49°</td>
<td></td>
</tr>
</tbody>
</table>

The Ki values were calculated for fractions 11 and 9 using the SigmaPlot version 12. The results are expressed as mean ± sd for three independent experiments. ° P<0.001, F11 shows better binding than F9.
2.4.11 Effect of *T. cordifolia* extracts on the kinetics of COX-2 enzyme

The kinetic type and parameters of inhibition of COX-2 enzyme by bioactive fractions of *T. cordifolia* was evaluated using the Lineweaver-Burk plot and the results are as shown in Fig 2.6 for COX-2 enzyme. The Lineweaver-Burk plot, for analysis of kinetic parameters performed in the presence of *T. cordifolia* extracts on the activity of COX-2, has revealed a mixed type of inhibition. The apparent Vmax was decreased in all cases and apparent Km values increased compared to uninhibited enzyme. The results are indicated in Table 2.19 and Figure 2.6. The Ki values were calculated and compared using the enzyme kinetics module of Sigma Plot software and are as given in Table 2.19.

<table>
<thead>
<tr>
<th>Name of extract</th>
<th>$K_M$ (uM)</th>
<th>$V_{MAX}$ (uMol/min/mg protein)</th>
<th>$K_i$ (ng/ul)</th>
<th>Type of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td>14.7±0.43</td>
<td>0.0151±0.002</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F11</td>
<td>23.11±2.7*</td>
<td>0.0105±0.0006*</td>
<td>0.012±0.003</td>
<td>Mixed</td>
</tr>
<tr>
<td>F9</td>
<td>16.33±0.35*</td>
<td>0.0127±0.0003*</td>
<td>0.037±0.02*</td>
<td>Mixed</td>
</tr>
</tbody>
</table>

The $K_M$ and $V_{MAX}$ values of the F11 and F9 fractions for COX-2 activity at different substrate concentrations were compared with the pure enzyme not treated with plant extract. Results expressed as Mean ± SD of three independent experiments. * $P<0.001$, values are significantly higher than uninhibited enzyme. # $P<0.05$, values are significantly lower than uninhibited enzyme. § $P<0.05$, fraction 9 values are significantly higher than fraction 11 and hence the fraction has better activity than control.
The 15-LOX activity was measured with different substrate concentrations and the results plotted as reciprocals in the Lineweaver-Burk plot. The km and Vmax values were calculated from the graph and compared. Results expressed as Mean ± SD of three independent experiments.
Chapter 5

Discussion
Dual inhibition of the LOX and COX enzymes by medicinal plants opens new avenues for drug development research. Medicinal plants have the added advantage of being a safer alternative to synthetic counterparts. *T. cordifolia* was selected for further characterization of dual inhibition. The methanol extract of the dried stems of *T. cordifolia* was prepared in the context of the methanol being a better solvent for plant secondary metabolite extraction (Parekh *et al.*, 2005). The aqueous extract was prepared based on the use of this plant in ayurveda where the fresh plant stems are used for extractions. The methanol and aqueous extracts were analysed for inhibition of 5-LOX, 12-LOX, 15-LOX, COX-1 and COX-2 enzymes. Positive controls are necessary to correlate the results of the experiment (Johnson and Besselsen, 2002) and for comparative evaluation of bioactive molecules in samples. Hence, NDGA was used as the positive control for LOX assay while indomethacin was used for the COX assay. IC$_{50}$ values of different extracts for inhibition of 5-LOX, 12-LOX, 15-LOX, COX-1 and COX-2 enzymes were calculated and compared with IC$_{50}$ values of NDGA and indomethacin. The comparison of the methanol and aqueous extracts indicate a higher potency of the methanolic extracts in enzyme inhibition as is evidenced by other similar studies in other plant extracts (Hammuel *et al.*, 2011; Sreejamole *et al.*, 2011). The aqueous extracts show an IC$_{50}$ value of $2.1 \times 10^{-5}$, $3.4 \times 10^{-5}$ and $1.4 \times 10^{-5}$ ng/µl for LOX enzymes and concentrations while the methanol extracts have an IC$_{50}$ of $9.34 \times 10^{-14}$, $9.01 \times 10^{-14}$ and $2.5 \times 10^{-11}$ ng/µl. This could be possibly because alcoholic solvents tend give a better extraction of secondary metabolites as compared to aqueous extracts (Robards, 2003). The IC$_{50}$ values indicate the effective concentration of plant extracts that exert a 50% inhibition of the enzyme and reflects on the
efficacy of the extract on the bioactivity being evaluated, in this case, inhibition of LOX and COX isoenzymes. A low IC\textsubscript{50} value indicates high affinity of the inhibitor binding to the enzyme while a high value shows low affinity. This observation indicates the superior efficiency of the methanol extracts as compared to aqueous extracts. NDGA has IC\textsubscript{50} values of 2.75, 0.302, 8.47 ng/µl concentrations for 5-, 12- and 15- LOX enzymes respectively and indomethacin has IC\textsubscript{50} values of 3.705x10\textsuperscript{-3}, 3.01 ng/ul for COX-1 and COX-2 enzymes respectively. The methanol extract of \textit{T. cordifolia} (TCM) with IC\textsubscript{50} values of 9.34x10\textsuperscript{-14}, 9.01 x 10\textsuperscript{-14}, and 2.5 x10\textsuperscript{-11} respectively for 5-, 12- and 15- LOX enzymes are more potent in the inhibition of the LOX enzymes particularly 5- and 12-LOXs as compared to 15-LOX. 5-LOX enzyme is found to be induced in chronic inflammation (Gheorghe \textit{et al.}, 2009) and the inhibition of 12-LOX enzyme has led to induction of apoptosis (Wong \textit{et al.}, 2001). The dual role of 15-LOX in inflammation has been reported earlier by His \textit{et al.} (2002) where 15-LOX isoforms have opposing stimulatory and inhibitory effects on cancer cell proliferation. The low inhibition of the 15-LOX enzyme is beneficial as the 15-LOX enzyme is involved in both proinflammatory and anti-inflammatory activity (His \textit{et al.}, 2002). In the case of the COX enzymes the IC\textsubscript{50} values for methanol extract are7.57x10\textsuperscript{-4}, 5.73x10\textsuperscript{-2} ng/µl for COX-1 and COX-2 respectively and that for the aqueous extracts is 1.59 x10\textsuperscript{3}, 1.63 x10\textsuperscript{6} ng/µl for COX-1 and COX-2 respectively. The IC\textsubscript{50} values indicate that in the case of COX inhibition also the methanol extracts were better than aqueous extracts. COX-1 enzyme is present constitutively in the body while COX-2 enzyme is induced under conditions of inflammation in certain tissues and hence it becomes quite important that when inhibition of COX enzymes are evaluated, the inhibitors be more specific for COX-2 (Parente, 2001). Experimental data shown in results (chapter 4 of this part) are in agreement
with this reported observation. The expression of COX-2 enzyme is particularly increased in rheumatoid arthritis leading to a constitutive expression of COX-2 enzyme and increased resistance to apoptosis and production of angiogenic factors (Crofford, 1999). It is to be noted that the data as shown in results (chapter 4) of this part of work indicate the high potency of the methanol extract in inhibiting the activity of LOX isoenzymes and in the case of COX isoenzymes a more specific inhibition with COX-2 as shown by IC$_{50}$ values.

Generation of reactive species or prooxidants in the body occur as the result of metabolic reactions causing cellular and tissue damage (Irshad and Chaudhuri, 2002). These reactive species can be oxygen derived (ROS species) or nitrogen derived (NO, N$_2$O$_3$ etc). These oxidants can cause damage to cells either by chemical chain reactions like lipid peroxidation, or by oxidation of DNA or proteins (Irshad and Chaudhuri, 2002). In the biological system, free radical and metabolite generation by macrophages and neutrophils occur in the presence of stimulus resulting in increased oxygen consumption and is known as oxygen burst (Gaddi et al., 2004). LTB4 produced by LOX enzymes can cause increased gastrointestinal damage. Increase in leukocyte infiltration by LTB4 (Fantone and Ward, 1982) result in ulceration by the occlusion of microvessels, reduced blood flow in mucosa and release of free radicals, proteases and inflammatory mediators thereby causing tissue necrosis (Kern and Kehrer, 2005). Resultant oxidative stress has been reported to be the cause of several diseases such as inflammatory (Ramakrishna et al., 1997), cardiovascular (Singh and Jialal, 2006), neurological (Sas et al., 2007) and other malignancies (Kinnula and Crapo, 2004). Antioxidants from endogenous or exogenous sources serve to counter this effect. Therefore evaluation of free radical scavenging activity
of the plant extract in study (T. cordifolia) becomes important as these scavengers can remove the free radicals that are generated at site of injury and prevent progression of inflammation in chronic inflammatory conditions.

The ABTS radical scavenging assays are applicable to both hydrophilic and lipophilic antioxidants because of suitability of aqueous and nonpolar organic solvents (Prior et al. 2005). The ABTS technique is not affected by the ionic strength of the reaction system and is characterized by higher detection of analyte levels (Floegel et al., 2011). The DPPH assay uses a radical dissolved in organic media applicable to hydrophobic systems (Kim et al., 2002). The ABTS assay was hence considered to be a better reflection of the antioxidant capacity than DPPH (Martysiak-Żurowska and Wenta, 2012). Increased levels of nitric oxide and its oxidised derivatives can be toxic (Szabo & Thiemermann, 1994) in a biological system causing cell and tissue injury at relatively high concentrations (Szabo & Thiemermann, 1994). The nitric oxide intermediates are genotoxic (Marcocci et al., 1994a,b) and deaminate DNA and affect activity of several DNA repair proteins (Wink et al., 1991). Nitric oxide inhibition can increase mortality (Broderick et al., 2006) and the alternative approach becomes scavenging of the radical allowing free nitric oxide for normal physiological functions (Sueishi et al., 2011). Taking the above reports into consideration DPPH, ABTS and Nitric oxide scavenging properties of the T. cordifolia extracts have been studied in detail in this study. The $IC_{50}$ values indicate the efficiency of the extracts on free radical scavenging activity. The free radical scavenging activity of the extracts of T. cordifolia (TCM and WSF) was compared with standard BHT (for DPPH and ABTS) and ascorbic acid (for NO scavenging).
LOX are sensitive to antioxidants due to the inhibition of formation of lipid hydroperoxides. The antioxidants can scavenge the lipid hydroperoxides that act as substrates for the enzyme leading to a non-availability of the substrates for LOX catalysis (Rackova et al., 2007). The study conducted by Czapski et al. (2012) have reported on the antioxidant effects of LOX inhibitors which produces resultant effects in LOX-independent oxidative stress.

The inhibition of the COX and LOX enzymes result in increased intracellular levels of unbound fatty acids which has been proposed to stimulate apoptosis and is correlated by studies in COX-2 (Cao et al., 2000) and LOX inhibitors (La et al., 2003). The scavenging of the DPPH and ABTS radicals are indicative of the general antioxidation potential of the extracts while NO scavenging is more relevant in chronic inflammatory conditions in biological systems. The methanol extract was found to be relatively better as a general free radical scavenger shown by its ability to scavenge ABTS free radical and a better scavenger of NO as compared to the aqueous extract. On the basis of the LOX/COX inhibition data obtained and antioxidant activity of the two extracts, the methanol extract was found to be more potent as compared to the aqueous extract for LOX/COX inhibition and NO scavenging and hence the methanol extract of T. cordifolia was selected for further fractionation studies.

Bioactivity guided fractionation allows the identification of the chemical nature of the bioactive compound and ascertains the type of secondary metabolites present. Fractionations of plant extracts have an important role in the determination of bioactive compounds. Michel et al. (2013) have conducted a bioactivity-guided study of the ethanol extract of stem bark of Pterocarpus dalbergioides and identified the butanol fraction
Discussion

with anti-hyperglycaemic and anti-inflammatory activities. Further fractionation of the butanol fraction has led to the isolation of gentisic acid, gallic acid and genistin for the first time from the plant studied. Similarly the fractionation of a plant extract ultimately leads to the identification of the lead compounds in bioactivity but should proceed with minimal damage to the constituents.

The methanol extract of *T. cordifoila* was subjected to column chromatography as discussed in Materials and Methods (Chapter 3 of this part). The fractions obtained after column chromatography was collected and pooled on the basis of spectral analysis resulting in 24 fractions. Bioactivity was screened in these 24 fractions by the LOX inhibitory assay. Since the major objective was identification of dual inhibitors for LOX/COX, the strategy was to identify inhibitors for LOX as experimental data with respect to IC$_{50}$ values for inhibition, shown in results (Chapter 4 of this part), indicate that the extracts had better inhibition for LOX than COX enzymes and hence it would be better to screen for LOX inhibition of the extracts and then identify for COX inhibition in the same bioactive fraction. This strategy is further supported by studies which suggest that the LTB$_4$ can induce the generation of free radicals under conditions of inflammation thereby resulting in necrosis of the cells (Fantone and Ward, 1982), that the inhibition of COX enzymes lead to the flux of arachidonic acid to the LOX pathway resulting in the formation of the more damaging leukotrienes. In addition, this observation was not repeated in the inhibition of the LOX enzymes because the shift in arachidonic acid flux towards the COX pathway was minimal (Yang *et al.*, 2008).

The importance of LOX inhibition led to the screening for potent LOX inhibition as the criteria for the selection (Wei *et al.*, 2008) and the
fraction which exhibited inhibition for LOX was checked for COX inhibition. Fraction 11 shows the lowest IC$_{50}$ value for LOX inhibition while fraction 9 has the highest. The IC$_{50}$ values indicate the higher potency of the fraction 11 as compared to standards and fraction 9 for 5-, 12-, 15-LOX, COX-1 and -2 enzymes. The fractions were then characterized further by the antioxidant studies, and kinetic studies in LOX and COX. Antioxidant activity of the plant extracts are important in the pathophysiology of the biological system where unchecked free radicals can result in tissue and cell damage (Irshad and Chaudhuri, 2002). It is to be noted that the free radical scavenging property of the bioactive fractions (F11 which showed most inhibition of LOX/COX isoenzymes and F9 which showed least inhibition of LOX/COX isoenzymes) had no significant scavenging property for free radicals in general or for nitric oxide radical in particular (in fact F9 showed no effect on NO scavenging). It is quite possible that free radical scavenging activity of TCM would be present in some other fraction (other than F11 and F9). This observation points to the fact that inhibition of LOX/COX as shown in results is by a mechanism which is independent of free radical scavenging. The high free radical scavenging activity particularly NO scavenging property of TCM present in other fractions was not looked into since the focus of the study was identifying dual LOX/COX inhibition rather than on free radical scavenging. Free radical scavenging studies in this study finds relevance only to extent that inhibition of LOX/COX is independent of free radical scavenging activity.

Analysis of inhibition of COX isoenzymes by the fractions show that neither F11 nor F9 are better than indomethacin. However F9 showed an inhibition similar to (though not significantly better) indomethacin for COX-2. Hence it could be concluded that F11 showed better activity for LOX
isoenzymes and F9 showed better inhibition for COX-2. It is to be noted that neither of these inhibited COX-1 isoenzymes. Therefore these fractions F11 for LOX isoenzymes and F9 for COX-2 isoenzyme activity.

Evaluation of kinetic parameters Km and Vmax was done for LOX isoenzymes and COX-2. Results presented in chapter 4 of this part show that the methanol extract had a better inhibition for COX-2 as per IC\textsubscript{50} values and hence kinetic parameters for inhibition of COX-1 enzyme was not analysed further. The results of kinetic studies in LOX isoenzymes and COX-2 enzyme indicate mixed inhibition of the LOX and COX enzymes characterized by the increase in Km and a corresponding decrease in Vmax. The increased Km values can negatively affect the binding affinity of the enzyme to the substrate and cause the reduction in rate of reaction. Inhibition by the mixed mechanism can help further evaluation of the mechanism of action of the bioactive compound. The mixed inhibitor can directly bind to the free enzyme and to the enzyme substrate complex which is advantageous in the removal of substrate or the non-accumulation of the substrate for change in flux of arachidonic acid during inflammatory conditions into the COX pathway (Törnquist \textit{et al.}, 1994). Experimental data from kinetic studies have shown very low Ki values for fraction 11 as compared to fraction 9 which also indicate the better binding efficiency of the inhibitor to the enzyme (Sharma, 2012). The mixed inhibition also indicates the possibility of an alternative binding site for the inhibitor that modulates the enzyme activity by decreasing the concentration of free enzyme available for the reaction to proceed (Price and Stevens, 1999). The Km values are found increased, causing a decreased affinity of enzyme for the substrate while the Vmax values were found decreased and reduce the reaction rate of the enzyme (Cleland, 1979).
Bioactivity guided fractionation of the methanol extract of *T. cordifolia* have resulted in 24 fractions which were screened for LOX inhibition. Fraction 11 was found to be the most potent inhibitor for LOX enzyme with Fraction 9 having the lowest potency on LOX but better inhibition on COX-2 and no effect on COX-1. Hence further structure elucidation studies were conducted with both the bioactive fractions of *T. cordifolia*. 