Chapter-6A

Antiinflammatory activity of *M. terminale Dalz*
6A.1 Introduction

Inflammation is an adaptive response that is triggered by noxious stimuli and condition such as chemical or physical injury. It causes the activation of cellular and systemic component of the immune system. The initial response involves the innate immune system where by cells including macrophages, mast cells and dendritic and natural killer cells converge at the site of injury. Chemical mediators released by the innate immune cells, such as cytokines, chemokines and reactive oxygen species, allow recruitment of leukocytes to the area of injury or infection and lead to the elimination of pathogens and tissue repair with dendritic and natural killer cells initiating the adaptive immune response (Moore et al., 2010). Generation of inflammatory mediators can then activate various downstream signaling pathways to modulate cell proliferation, cell death and differentiation and amplify the response to the initial offence. The response to tissue injury or infection requires a well-organized interaction of immune and inflammatory cells and their products. On the other hand, chronic inflammation is prolonged, dis-regulatory and maladaptive response that involves persistent active inflammation, tissue destruction and failed attempts at tissue repair. Such inexorable inflammation is associated with a host of chronic human condition and diseases, including atherosclerosis, ischemic heart disease, cancer obesity, inflammatory bowel diseases, crohn’s disease, diabetes and auto-immune diseases (Weiss, 2008; Ferguson and Laing, 2010; Grivennikov et al., 2010; Libby, 2002; Nathan, 2008; Drouet, 2012). There is also increasing evidence that inflammatory mechanisms are involved in both the development and progression of atherosclerosis and its clinical manifestations (Ross, 1999). Inflammation is certainly important in the pathophysiology of cerebral ischemia in the setting of stroke (Barone and Feuerstein, 1999). It also appears that in cerebral ischemia occurring after subarachnoid hemorrhage, head injury, or cardiac arrest, inflammatory mechanism plays an important role in pathophysiology (Fassbender et al., 2001; Mussack et al., 2001). Excess body fat, particularly central adiposity, is associated with concomitant and persistent increase in low grade inflammation. Consumption of meals typical of the Western diet, that is, meals that are energy dense and provide a surplus of readily available carbohydrates and fat, induces an acute inflammatory stress in both healthy weight and overweight individuals (Jellema et al., 2004; Ghanim et al., 2009; Manning et al., 2008; Calder et al., 2011; Edirisinghe et al., 2011; Burton-Freeman et
al., 2012). In contrast, population studies indicate that diet rich in fruits and vegetables are inversely associated with inflammatory stress (Calder et al., 2011). Likewise, higher intake of fruits and vegetables are associated with lower prevalence of cancer, type-2 diabetes and Alzheimer's disease (Dauchet et al., 2009).

6A.1.1 General overview

Inflammation is the normal, protective and temporary response of the innate immune system to pathogens and injury. However, with recurrent stimuli or inefficient regulation, chronic inflammation follows. Quantifiable inflammatory responses are characterized by the production of pro-inflammatory molecules and anti-inflammatory cytokines acting as signals between immune cells to coordinate the inflammatory response. Cytokines are immune-modulatory molecules that control the inflammatory response. Nuclear factor kappa B (NF-κB), a redox-sensitive transcription factor, is a central orchestrator of the inflammatory response. Once NF-κB is activated, it stimulates the expression of a number of genes including those responsible for the production of cytokines (i.e., tumor necrosis factor (TNF)-α, interleukin (IL-6, IL-1β), chemokines (i.e., monocyte chemoattractant protein (MCP-1), adipokines (i.e., leptin, adiponectin), cell adhesion molecules and soluble intercellular adhesion molecule-1 and acute phase proteins (i.e., (hs) C-reactive protein (CRP), fibrinogen) (Pahl, 1999). Other important mediators of inflammation include pattern recognition receptors (PRR) such as Toll-like receptors (TLRs) and kinases such as mitogen-activated protein kinase (MAPK) and C-jun N-terminal kinases (JNK). The inflammatory response can be triggered by stimuli such as endotoxin (lipopolysaccharide from bacteria, viruses) and changes in levels of reactive oxygen species (ROS), cellular redox status, fatty acids, cytokines, growth factors and carcinogens among others.

Localized acute inflammation is part of host's normal protective response to tissue injury and infection by invading microbial pathogens (Cotran et al., 1999). Although this inflammatory response to a range of harmful stimuli is protective to the host, if kept uncontrolled it can result in a wide range of acute, chronic and systemic inflammatory disorders. Indeed, some of the most common and difficult to treat diseases are linked to excessive, uncontrolled it can result in a wide range of acute, chronic and systemic inflammatory disorders. Indeed, some of the most common and difficult to treat diseases are linked to excessive, uncontrollable, or chronic
inflammation. The involvement of inflammatory pathways in the initiation of all of these diseases is well established; the specific role by which inflammation contributes to their pathogenesis is not fully understood. The recent findings that the resolution of inflammation is an active process (Serhan et al., 2000; Serhan et al., 2002; Lawrence et al., 2002) have provided new insights and created new paradigms for understanding and treating these conditions. The key role of a number of lipid mediators in the initiation of the inflammatory response and the subsequent progression toward resolution is given in Fig. 6A.1.

Among the first signaling events following microbial infection or tissue injury is the release of pro-inflammatory lipid mediators, such as leukotrienes and prostaglandins that launch a series of signaling cascades with the ultimate goal of destroying the invading pathogens and repairing the damaged tissue (Smuelsson et al., 1987; Vane, 1982). Thus, the biosynthesis and release of the potent chemotactic agent leukotriene B4 (LTB4) promotes the recruitment of neutrophils (PMNs) to the inflamed tissue, while the formation of prostaglandins E2 and D2 further accelerates the inflammatory process, ultimately resulting in a condition of acute inflammation (Lawrence et al., 2002). Despite its critical host-protective function, acute inflammation is not sustainable over a prolonged period of time giving rise to disruptive conditions of chronic inflammation that may be responsible for the pathogenesis of a wide range of diseases that can be attributed to a failure of resolution (Serhan et al., 2007). Typically, the therapeutic treatment of such condition involves the inhibition of pro-inflammatory mediators, but in many cases such approaches are often not very effective. The recognition of the proactive nature of the resolution of inflammation has revealed alternative therapeutic paradigms based on resolving acute inflammation and preventing the onset of chronic inflammation (Serhan et al., 2007). Indeed, a number of endogenous lipid mediators identified are able to act in this manner, suggesting a lipid mediator i.e., leukotriene and prostaglandins, to the anti-inflammatory and pro-resolving actions of lipoxins, resolvins, protectins and maresins. Each family of these pro-resolving mediators exert specialized actions, including blocking neutrophil recruitment, promoting the recruitment and activation of monocytes and mediating the nonphlogistic phagocytosis and lymphatic clearance of apoptotic neutrophils by activated macrophages. Then the combined actions of these mediators, the resolution of
inflammation is completed and homeostasis is reached (Serhan et al., 2007; Bannenberg et al., 2005; Schwab et al., 2007).

Figure 6A.1. From initiation of acute inflammation to resolution: Inflammatory response to microbial infection and tissue injury, and the role of selected cell types and specialized pro-resolving lipid mediators (Charles and Nicos, 2011).

6A.1.2.1 Inflammation and Atherosclerosis

Cardiovascular disease (CVD) continues to be the leading cause of death in developed countries. Atherosclerosis is one of the most common causes of CVD. It is a chronic disease that begins in fetal life, slowly progresses during childhood and adolescence and then accelerates in fits and spurts in adult life. Recent studies have indicated that atherosclerosis is an inflammatory disease (Libby, 2002; Hansson et al., 2006; Mizuno et al., 2011) and it has been widely accepted that inflammation plays a critical role in the pathogenesis of atherosclerosis. The recruitment and activation of macrophages is considered to be the most important early event in the development of atherosclerotic lesions. Activated macrophages release various pro-inflammatory cytokines that amplify the local inflammatory response in the lesion (Libby, 2002). The atherosclerotic process is initiated when cholesterol containing low-density lipoproteins accumulate in the intima and activate the endothelium. Leukocyte adhesion molecules and chemokines promote recruitment of monocytes and T-cells. Monocytes differentiate into macrophages and up-regulate pattern recognition receptors, including scavenger receptors and toll-like receptors. Scavenger receptors mediate lipoprotein internalization, which further leads to foam-cell formation.
6a.1.2.2 Inflammation and Cancer

An association between cancer and inflammation was made more than a century ago form the identification of leukocytes in tumor tissue (Balkwill, 2001). Since then, inflammation has been implicated in tumor development, invasion and metastasis and in the development of clinical features such as fever and cachexia. More recently, inflammation has also been implicated as affecting the patient’s ability to tolerate cytotoxic drugs (Moore, 2010). The presence of an inflammatory infiltrate in tumor tissue could represent its role as a contributor to either the development of cancer or the host response to the tumor. The link between the chronic inflammatory diseases and cancer has been well documented. It is believed that 15-20% of deaths from cancers are attributable to underlying infection or inflammation (Moore, 2010). The mechanism for cancer development in the presence of chronic inflammation involves the continuous presence of cytokines, chemokines, reactive oxygen and nitrogen species and activation of key transcription factors such as nuclear factor-κB and the signal transducer of transcription. It is believed that these factors result in genetic instability and subsequent mutations in oncogenic and tumor suppressor pathways (Moore, 2010).

6a.1.2.3 Chronic Inflammation and Diabetes

Obesity is now a leading public health concern throughout the world. Obesity is associated with a chronic, systemic low grade state of inflammation. The link between inflammation and obesity was first observed in 1993, when the inflammatory cytokine TNF-α was shown to arise from adipose tissue in obese rodents and contribute to their insulin resistance (Hotamisligil et al., 1993). It was later found that adipose tissue was infiltrated by macrophages in obese children and adults and mice, in proportion to how far they exceed normal body weight. These macrophages express TNF-α, inducible nitric oxide synthase (iNOS), and other inflammatory substance (Nathan, 2008) and the calcium sensing receptor (CaSR) is expressed in human adipose cells and plays a role in obesity-associated pro-inflammatory cytokine expression while contributing to the abundance of differentiated adipocytes (Cifuentes et al., 2010). Obesity results from chronic positive energy balance. However, adipose tissue is not merely a store of excess fatty acids or a heat insulator but also an endocrine organ. It secretes a variety of cytokines, as well as adiponectin, initelectin, macrophage migration inhibitory factor (MIF), leptin, resistin, serpin, vascular
endothelial growth factor (VEGF) and visfatin, all of which regulate immune function through endocrine, paracrine and autocrine pathways. The medical complications of obesity, including diabetes, hypertension and atherosclerosis are characterized by increases in pro-inflammatory cytokines and markers of inflammation such as an elevated leukocyte count and increased circulating IL-6 and C-reactive protein (CRP) levels (Lee and Pratley, 2005). Obesity-induced inflammation also plays an important role in the development of insulin resistance and type-2 diabetes. Insulin resistance is defined as an inadequate response by insulin target tissues, such as skeletal muscle, liver and adipose tissue, to the physiological effects of circulating insulin (Schenk et al., 2008). Many lines of evidence have shown that chronic activation of proinflammatory pathways within insulin target cells can lead to obesity-related insulin resistance. Adipocytes are the unique source of secreted adipokines such as leptin and adiponectin, which can promote insulin sensitivity, as well as resistin and retinol-binding protein 4 (RBP 4), which can impair insulin sensitivity. Thus, the mixture of adipokines secreted by adipose tissue in a given pathophysiologic state can have important effects on systemic insulin sensitivity (Schenk et al., 2008).

6A.1.3 Medicines for Inflammation

The anti-inflammatory agents, including nonsteroidal anti-inflammatory drugs (NSAID) and disease-modifying antirheumatic drugs (DMARDs), are widely used in treating inflammation. However, many of them have dose-dependent side effects and none of them are suitable for primary prevention, which significantly limit their use. On the other hand, it has been recognized that lifestyle and environment play an important role in inflammatory responses. As a major aspect of the environment, diet can be a key element in managing inflammatory process (Montero et al., 2013).

Polyphenols are a large group of phytochemicals found ubiquitously in the plant kingdom. Flavonoids are a major subclass of polyphenols and can be found in a variety of foods such as fruits (Montero et al., 2013), vegetables (Li et al., 2010), nuts (Vinson and Cai, 2012), wine (Li et al., 2011), cocoa (Gu et al., 2006), soybeans (Ho et al., 2002) and olive oil (Mateos et al., 2001). Medicinal plant contain appreciable amount of flavonoids, particularly anthocyanins. A diet rich in plant flavonoids is associated with a lower risk of chronic disease development and specifically CVD mortality in men and women (Mink, 2007; Liu et al., 2000). Moreover, a higher intake of diet-derived anthocyanins is associated with lower risk of hypertension.
(Cassidy et al., 2011; Jennings et al., 2012), myocardial infarction (Cassidy et al., 2013), type 2 diabetes (Wedick et al., 2012) and cancer (Cutler et al., 2008; Mursu et al., 2008). Over the past decade or more, the “antioxidant” hypothesis has been the prevailing wisdom of how polyphenols/flavonoids/anthocyanins impart their health benefits; however, this view has been under considerable scrutiny in recent years as research has revealed numerous other biological activities of these important plant components, among them is anti-inflammatory activity (Seeram et al., 2001).

Sterols are a subgroup of steroids and occur naturally in animals, plants and fungi. The most familiar type of animal sterol is cholesterol. Cholesterol is vital to cellular function and a precursor to bile acids and steroid hormones (e.g. estrogen). The peripheral cholesterol metabolism is a complex homeostasis regulating uptake, synthesis, distribution, metabolizing and efflux of cholesterol. The cholesterol normally presents in stable molecular structural conditions. Plant sterols have high structural and functional similarity to cholesterol. The main plant sterols differ chemically only from cholesterol in their side chain by an additional ethyl or a methyl group at C24 and a double bond at C22 (Salen et al., 1985). Animal cells are unable to synthesize plant sterols form any precursor (Salen et al., 1970). Both cholesterol and plant sterols can be oxidized into respectively cholesterol oxidation products (COPs) and plant sterol oxidation products (POPs). In contrast to cholesterol, COPs and POPs, the sole source of plant sterols is dietary intake. Although depending on the nature of the diet, most commonly available plant sterols are sitosterols, campesterol and stigmasterol. Hence, most predominantly available POPs are derived from these plant sterols. Several hypotheses exist on the mechanism by which plant sterols reduce plasma cholesterol concentration - 1. Micellar competition in the intestine, 2. Competition for the intestinal esterase activity, 3. Competition for cholesterol transporters the brush border of enterocytes, 4. Competition for esterifying enzymes, 5. Competition for chylomicron incorporation (Smet et al., 2012).

In the past decade, understanding of the role of diet in promoting health by regulating inflammation, especially via certain nutrients or dietary components, has grown substantially. Dietary habits that encourage increased consumption of fruits, vegetables, whole grains and nuts may lead to reduced inflammation (Bakker et al., 2010; Galland, 2010; Masters et al., 2010). Dietary factors, including adequate omega-3 fatty acids intake and increased consumption of fruits, vegetables, nuts, medicinal plants and whole grain are associated with a lower incidence of chronic
diseases. These dietary factors provide a variety of nutrients as well as non-nutritive bioactive constituents that could modulate immunomodulatory and inflammatory processes (Giugliano et al., 2006, Watzl, 2008). The attention to increasing fruit and vegetable consumption is a practical and important way to optimize good health (Van and Pivonka, 2000). Numerous studies have shown an inverse correlation between fruit and vegetable consumption and inflammation status. The greater the variety of fruits and vegetables consumed in the diet, but not quantity, the greater the benefit in terms of risk for diseases associated with chronic inflammation such as cardiovascular disease (Bhupathiraju and Tucker, 2011). Higher intakes of fruits and vegetables result in lower CRP (Esmailzadeh et al., 2006).

Tea drinking, particularly of green tea has been inversely related to the risk of cardiovascular disease, owing to its catechin content, a compound belonging to the flavonoid family (Arts et al., 2001). Black tea, particularly those that are fermented, are virtually devoid of catechins yet have been shown in vivo, to demonstrate cardiovascular benefits similar to those of green tea. During processing to produce black tea, the catechins are converted to theaflavins and thearubigins, both of which have been shown to exhibit the anti-inflammation property (Lorenz et al., 2009).

*Mecycylon* species, one of the important medicinal plants, is being widely used in southern part of Karnataka state in India for treating many diseases. It is an important folk medicine and found to be effective in treating dysentery, fever, diabetes, diarrhea, piles and haemoptysis. Keeping in view of the need for a potent antiinflammatory drug, in this study, an attempt has been made to validate the plant extracts for its inflammatory activity using experimental animals.
6A.2 Materials and methods

6A.2.1 Chemical and reagents

Carrageenan was obtained from Sigma Chemical Co. (St. Louis, USA). All other chemicals and reagents were of analytical grade procured from Himedia labs, Mumbai.

6A.2.1.2 Animals

Wistar rats weighing 140±20 g of either sex was procured from the S.S.I medical college, Davangere, Karnataka, India. The animals were housed at controlled conventional condition (temperature 22±2°C, relative humidity 50±10%, 12 h light-dark cycle) and fed with the standard pellet and drinking water ad libitum throughout the experiment. The animals were kept under starvation for 24 h before starting the experiment. All the studies were performed in accordance with the guidelines for the care and use of laboratory animals, as adopted and promulgated by the Institutional Animal Care Committee, CPCSEA, India (Reg.no.No-628/02/c/CPCSEA).

6A.2.3 Carrageenan-Induced Paw Edema

The carrageenan-induced hind paw edema test was conducted according to the method previously described by Winter et al, (1962). Acute inflammation was produced by subplantar injection of 0.1 mL of 1% suspension of carrageenan with 2% gum acacia in normal saline, in the right hind paw of the rats, 1 h after the oral administration of the test sample as well as the controls. The paw volume was measured plethysmometrically at 0.5, 1, 2, 3 and 4h after the injection. Standard 100 mg/kg, p.o. suspended in 2% gum acacia was used as control. Percent inhibition of the inflammation was determined by applying statistics on raw data followed by the calculation of percent inhibition for each group by comparing with control group. The inflammation potential of the plant extracts was expressed in percentage inhibition using the following equation:

\[ \% \text{Inhibition} = 1 - \frac{dt}{dc} \times 100, \]

\( dt \) = Difference in paw volume in the drug treated group

\( dc \) = Difference in paw volume in control group.
6A.3 Results

We have evaluated anti-inflammatory activities to clarify the traditional belief in the pain and inflammation relieving effect of different extracts of *M. terminale Dalz* plant. Carrageenan induced hind paw edema models were used to evaluate the anti-inflammatory activity and the results are reported in Figure. 5.2 and Table 5.1. Compared to all the extracts of *M. terminale Dalz*, methanolic extract reduced the inflammation in the paw and exhibited a dose dependent anti-inflammatory activity. The paw edema inhibition ratios of petroleum ether, chloroform and methanolic extracts were 20.5, 55.1 and 66.6% at a dose of 300 mg/kg body weight at 4th hour. The indomethacin, as a standard could prevent the paw edema with the inhibition ratios of 82% at 100 mg/kg bw at 4th hour. The time-dependent cure showed that the paw swelling ratio will rise till 4 hours after carrageenan injection. Compare to methanolic extract and standard drug, petroleum ether extract of *M. terminale Dalz* plant failed to show any anti-inflammatory effect on carrageenan induced paw edema in mice at higher dose of 300 mg/kg bw. On the other hand, the chloroform extract of plant showed moderate anti-inflammatory activity of 41 and 55.1% at 100 and 300 mg/kg bw at 4th hour. The comprehensive analysis of Table 6A.1 demonstrated that the anti-inflammation activity of the three different extracts of *M. terminale Dalz* decreased in the order: Methanolic extract > Chloroform extract > Petroleum ether extract.

6A.4 Discussion

In the present study, different extracts of *M. terminale Dalz* exerted anti-inflammatory effects. In the early stage of inflammation responses, the anti-inflammatory activity is investigated by the inhibition on vascular permeability, a prominent feature of inflammatory pathological process. On the other hand, carrageenan induced rat paw oedema is a widely used method to determine anti-inflammatory activity of many natural and newly synthesized organic compounds. This constitutes a simple, routine animal model for evaluation of pain at the site of inflammation without any injury of damage to the inflamed paw (Paschapur et al., 2009; Petersson et al., 2001; Sini et al., 2010). Rats paw oedema had been increasingly used to test new anti-inflammatory drugs and natural product, as well as to study the mechanisms involved in inflammation. The development of oedema in
the rat hind paw following the injection of carrageenan has been described as a biphasic, age weight dependent event in which various mediators operate in sequence to produce the inflammatory response.

There are several mediators involved in inflammation, histamine, serotonin and bradykinin are the first detectable mediators in the early phase of carrageenan-induced inflammation; prostaglandins (PGs) are involved in the increased vascular permeability and are detectable in the late phase of inflammation. Local systemic inflammation is associated with enhanced levels of the pro-inflammatory cytokines TNF-α, IL-1 and IL-6 (Cuzzocrea et al., 1999). The initial phase or oedema, which is not inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, has been attributed to the release of histamine, 5-hydroxytryptamine (5-HT) and bradykinin. The second accelerating phase of swelling has not only been correlated with the elevated production of prostaglandins, but more recently has been attributed to the induction of inducible cyclooxygenase (COX-2) in the hind paw (Nantel et al., 1999). It can be blocked by the NSAIDs (Handy and Moore, 1998). Local neutrophil infiltration and activation also contribute to this inflammatory response by producing, among other mediators, oxygen derived free radicals such as superoxide anion (O$_2^-$) and hydroxyl radicals (Salvemini et al., 1996; Posadas et al., 2004). Another important mediator in acute inflammation is nitric oxide (NO) which is produced in pathological conditions by three distinct isoforms of nitric oxide synthase (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). Carrageenan causes the production and release of NO at the injured site. Perfusion of a non-selective NOS inhibitor, NG monomethyl-L-arginine acetate (L-NMMA), which exhibits some selectivity for inhibition of neuronal and endothelial isoforms, suppressed the release of NO following carrageenan injection. Perfusion of an inducible NOS inhibitor, aminoguanidine hemisulfate (AG), suppressed the release of NO 2.5-8 h after carrageenan injection. Neurectomy completely suppressed NO release for up to 3 h and partially suppressed NO release 4.5- 8 h after carrageenan injection. These findings indicate that nNOS contributes to the NO production in both the early and late phase and that iNOS only contributes to the late phase. The production and release of NO by these NOSs are thought to contribute to tissue injury and inflammation-induced oedema and hyperalgesia (Handy and Moore, 1998; Omote et al., 2001).
The reduction of carrageenan-induced paw edema by different extract of *M. terminale Dalz* plant extracts and indomethacin was measured for 4 h (Table 6A.1). Rats edema by carrageenan reached the highest volume at 3 h after injection of carrageenan. The methanolic extract reduced the carrageenan induced paw edema durable at 0.5, 1, 2, 3 and 4 h after oral administration. Standard Indomethacin showed the significant reduction of paw edema. Paw edema experimental model exhibits a high degree of reproducibility. Carrageenan induced edema is a biphasic response and the cellular and molecular mechanism of the carrageenan induced inflammation is well characterized. It is known that the third phase of the edema-induced by carrageenan, in which the edema reaches its highest volume, is characterized by the presence of prostaglandins and other compounds of slow reaction (Spector and Willoughb, 1963). It is suggested that the action mechanism of methanolic extract of *M. terminale Dalz* plant may be related to prostaglandin synthesis inhibition, as described above for the anti-inflammatory mechanism in the inhibition of the inflammatory process induced by carrageenan (DiRosa *et al.*, 1971). Although many plant extracts have been clinically used as anti-inflammatory remedies in the treatment of rheumatoid arthritis, their modes of action remain unclear. It is reported that the plant extracts of *M. terminale Dalz* extract contains multiple components including diterpenoids, alkaloids, triterpenoids and flycosides (Peethambar *et al.*, 2013). These compounds could have contributed to the anti-inflammatory properties of the plant extracts. Suppression of NO production is believed to be closely linked with an anti-inflammatory action. Many studies have also demonstrated that flavonoids and phenolic acid compounds produced significant anti-inflammatory activities, such as arbutin, catechin, rutin, quercetin and luteolin (Deliorman *et al.*, 2007; Arslan *et al.*, 2010). The *in vivo* anti-inflammatory activity of *M. terminale Dalz* plant extracts has not been clearly assessed so far. Therefore, this work was designed to determine anti-inflammatory activity of different extracts of *M. terminale Dalz* plant using the carrageenan induced paw edema model. The result implies that an inflammatory activity of plant partly arises from its prevention from the release of inflammatory mediators at the first stage. The methanolic extracts of *M. terminale Dalz* further proved its significant anti-inflammatory potential in *in vivo* study by controlling biphasic inflammatory events induced by carrageenan. The early phase (0.5 to 2 h) of the inflammation is due to the release of histamine, serotonin and similar substances. The later phase (3 to 4) is associated with the kinin like substances.
i.e. prostaglandin promptly controlled both the phases of inflammation. On the other hand petroleum ether extract failed to show good activity, due to the absence of alkaloids and phenolic contents. Normally, petroleum ether extract contains oils and fats. These oils and fats are unable to inhibit the prostaglandin, NO radicals, COX-2 and it is also fails to inhibit the cytokines. The chloroform extract contains the phenolic and alkaloids which may responsible for the moderate anti-inflammatory activity.
### 6A.5 Tables and Graphs

**Table 6A.1:** Anti-inflammatory activity of *M. terminale Dalz* extracts

<table>
<thead>
<tr>
<th>Group Time in hour</th>
<th>Control (100 mg/kg) indomethacin</th>
<th>Petroleum ether extract (in mg/kg)</th>
<th>Chloroform extract (in mg/kg)</th>
<th>Methanolic extract (in mg/kg)</th>
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<tr>
<td></td>
<td>0.5</td>
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<td>0.21±0.08</td>
<td>0.48±0.07</td>
<td>0.41±0.06</td>
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<td>0.53±0.02</td>
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Effects of the different extracts of *M. terminale Dalz* 100 and 300 mg/kg and indomethacin 100 mg/kg on carrageenan induced rats paw edema. Results are presented as the mean ± S.E.M from six rats. (n=6).
Figure 6A.2: Effect of different extracts of *M. terminale Dalz* plant at 300 mg/kg of indomethacin on carrageenan induced paw edema in rats. Results are presented as the mean ± S.E.M from six mice. A (0.5<sup>th</sup> h), B (1<sup>st</sup> h), C (2<sup>nd</sup> h), D (3<sup>rd</sup> h) and E (4<sup>th</sup> h).
Chapter-6B

Anticancer activity of *M. terminale* Dalz
6B.1 Introduction

Cancer, known medically as malignant neoplasia, is a broad group of diseases involving unregulated cell growth. There is no perfect definition that describes all cancers. They are a large family of diseases which form a subset of neoplasms, which show features suggestive of malignancy. A neoplasm or tumor is a group of cells that have undergone unregulated growth, and will often form a mass or lump, but may be distributed diffusely (Anand et al., 2008). In cancer, cells divide and grow uncontrollably, forming malignant tumors which may invade nearby parts of the body. Cancer may also spread to more distant parts of the body through the lymphatic system or blood stream, but some tumors do not invade neighboring tissues and do not spread through the body. There are over 200 different known cancers that affect humans (Anand et al., 2008). Some of the most common are breast cancer, brain cancer, leukemia (blood cancer), testicular cancer, mesothelioma and lung cancer. The causes of cancer are diverse, complex and only partially understood. Many things are known to increase the risk of cancer, including tobacco use, dietary factors, certain infections, exposure to radiation, lack of physical activity, obesity and environmental pollutants (Anand et al., 2008). These factors can directly damage genes or combine with existing genetic faults within cells to cause cancerous mutation (Kinzler et al., 2002). Approximately 5-10% of cancers can be traced directly to inherited genetic defects (Kushi et al., 2012). Many cancers could be prevented by avoid smoking, taking more vegetables, fruits and whole grains in diet, consumption of less meat and refined carbohydrates, maintaining a healthy weight, exercising, minimizing sunlight exposure and being vaccinated against some infectious diseases (Anand et al., 2008). The chances of surviving the disease vary importantly by the type and location of the cancer and the extent of disease at the start of treatment. While cancer can affect people of all ages, and a few types of cancer are more common in children, the risk of developing cancer generally increases with age. In 2007, cancer caused about 13% of all human deaths worldwide (7.9 million). The number of death are increasing as more people live to an old age and as mass lifestyle changes occur in the developing world (Jemal et al., 2011).
6B.1.1 Causes of cancer

Cancer is primarily an environmental disease with 90-95% of cases attributed to environmental factors and 5-10% due to genetics (Anand et al., 2008). Environmental, as used by cancer researchers, means any cause that is not inherited genetically, such as lifestyle, economic and behavioral factors, and not merely pollution (Biesalski et al., 1998). The tobacco (25-30%), diet and obesity (30-35%), infections (15-20%), radiation (10%), stress, lack of physical activity and environmental pollutants are the common environmental factors that cause cancer in human beings (Anand et al., 2008).

6B.1.2 Cancer caused by chemicals

Mutation caused by agents that damage DNA are known as induced mutation that mutate DNA are called mutagens and are of three main types: mutagenic chemicals, radiation and heat. Even if there are no dangerous chemicals or radiation around, mutations still occur, though less frequently (Biesalski et al., 1998). These are spontaneous mutations that are due to errors in DNA replication. The enzymes of DNA replication are not perfect and occasionally make mistakes. In addition, DNA undergoes certain spontaneous chemical reactions at a low but detectable rate, and this rate goes up with increasing temperature (Biesalski et al., 1998). The chemical substance which directly involved in causing cancer is termed as carcinogen. The most common mutagens are toxic chemicals that react with DNA and alter the chemical structure of the bases. These chemical compounds have the ability to damage the genome or disrupt cellular metabolic processes (Sasco et al., 2004). Several radioactive substances are considered as carcinogens, but their carcinogenic activity is attributed to radiation. Common examples of non-radioactive carcinogens are inhaled asbestos, certain dioxins and tobacco smoke (Sasco et al., 2004).

There are several substances that have been linked to specific types of cancer. Tobacco smoking is associated with many forms of cancer and causes 90% of lung cancer (Sasco et al., 2004; Biesalski et al., 1998). Many mutagens are also carcinogens, but some carcinogens are not mutagens. Alcohol is an example of a chemical carcinogen that is not a mutagen (Seitz et al., 1998). In Western Europe 10% of cancers in males and 3% of cancers in females are attributed to alcohol (Schutze et al., 2011). The chemical compounds present in tobacco causes cancer in the lung, larynx, head, neck, stomach, bladder, kidney, esophagus and pancreas in tobacco
CHAPTER 6B

consuming persons (Kuper et al., 2002). Tobacco smoke contains over fifty known carcinogens, including nitrosamines and polycyclic aromatic hydrocarbons (Kuper et al., 2002). Tobacco is responsible for about one in three of all cancer deaths in the developed world and about one in five worldwide (Sasco et al., 2004; Kuper et al., 2002). Lung cancer death rates in the United States have mirrored smoking patterns, with increases in smoking followed by dramatic increases in lung cancer death rates and, more recently, decreases in smoking rates since the 1950s followed by decreases in lung cancer death rates in men since 1990 (Thun and Jemal, 2006; Dubey and Powell, 2008).

Cancer related to one’s occupations is believed to represent between 2-20% of all cases (Irigaray et al., 2007). Every year, at least 200,000 people die worldwide from cancer related to their workplace (WHO, 2007). Most cancer deaths caused by occupational risk factors occur in the developed world (WHO, 2007). It is estimated that approximately 20,000 cancer deaths and 40,000 new cases of cancer each year in the U.S are attributable to occupation (NIOSH, 2007). Millions run the risk of developing cancers such as lung cancer and mesothelioma from inhaling asbestos fibers and tobacco smoke, or leukemia from exposure to benzene at their workplaces (WHO, 2007).

6B.1.3 Diet and exercise

Diet, physical inactivity and obesity are related to approximately 30-35% of cancer deaths (Kushi et al., 2006). In United States excess of body weight is associated with the development of many types of cancer and is a factor in 14-20% of all cancer deaths (Kushi et al., 2006). Physical inactivity is believed to contribute to cancer risk not only through its effect on body weight but also through negative effects on immune system and endocrine system (Kushi et al., 2006). Diets that are low in vegetables, fruits and whole grains and high in processed or red meats are linked with a number of cancers (Kushi et al., 2006). A high-salt diet is linked to gastric cancer, aflatoxin B1, a frequent food contaminate, with liver cancer and betel nut chewing with oral cancer (Park et al., 2008). This may partly explain difference in cancer incidence in different countries. The gastric cancer is more common in Japan due to its high-salt diet and colon cancer is more common in United States (Brenner et al., 2009). Immigrants develop the risk of their new country, often within one
CHAPTER 6B

generation, suggesting a substantial link between diet and cancer (Buell and Dunn, 1965).

6B.1.4 Infections

The infectious diseases are also one of the reasons for 18% of cancer deaths worldwide (Anand et al., 2008). This percentage of deaths varies in different regions of the world from a high of 25% in Africa to less than 10% in the developed world (Anand et al., 2008). The viruses are major infections causing agents causes cancer and bacteria and parasites may also take part in causing cancer. A virus that can cause cancer is called an oncovirus. These include human papillomavirus (cervical carcinoma), Epstein-Barr virus (B-cell lymphoproliferative disease and nasopharyngeal carcinoma), Kaposi’s sarcoma herpes virus (Kaposi’s sarcoma and primary effusion lymphomas), hepatitis B and hepatitis C viruses (hepatocellular carcinoma), and Human T-cell leukemia virus-1 (T-cell leukemias). Bacterial infection may also increase the risk of cancer, as seen in Helicobacter pylori-induced gastric carcinoma (Pagano et al., 2004). Parasitic infections strongly associated with cancer include Schistosoma haematobium (squamous cell carcinoma of the bladder) and the liver flukes, Opisthorchis viverrini and Clonorchis sinensis (chlongiocarcinoma) (Samaras et al., 2010).

6B.1.5 Radiations

Some types of radiation cause mutations. Up to 10% of invasive cancers are related to radiation exposure, including both ionizing radiation and non-ionizing ultraviolet radiation (Anand et al., 2008). Additionally, the vast majority of non-invasive cancers are non-melanoma skin cancers caused by non-ionizing ultraviolet radiation. The main sources of ionizing radiation are medical imaging and radon gas. Radiation can cause cancer in most parts of the body, at any age, although radiation-induced solid tumors usually take 10-15 years and can take up to 40 years to become clinically manifest and radiation induced leukemia typically requires 2-10 years to appear (Little, 2000). Some people, such as those with nevoid basal cell carcinoma syndrome or retinoblastoma are more susceptible than average to developing cancer from radiation exposure (Little, 2000). The radiation causes double effect in children and adolescents as developing cancer from radiation exposure to adults; radiation
exposure before birth has ten times the effect (Little, 2000). Residential exposure to radon gas, has similar cancer risks as passive smoking (Little, 2000).

Low-dose exposures, such as living near a nuclear power plant, are generally believed to have no or very little effect on cancer development (Little, 2000). Radiation is a more potent source of cancer when it is combined with other cancer-causing agents, such as radon gas exposure plus smoking tobacco (Little, 2000). Unlike chemical or physical triggers for cancer, ionizing radiation hits molecules within cells randomly. If it happens to strike a chromosome, it can break the chromosome, result in an abnormal number of chromosomes, and inactivate one or more genes in the part of the chromosome that it hit, delete parts of the DNA sequences, cause chromosome translocations or cause other types of chromosome abnormalities. Major damage normally results in the cell dying, but smaller damage may leave a stable, partly functional cell that may be capable of developing cancer (Little, 2000). Three independent stages appear to be involved in the creation of cancer with ionizing radiation: morphological changes to the cell, acquiring cellular immortality (losing normal, life-limiting cell regulatory processes), and adaptations that favor formation of a tumor. Even if the radiation particle does not strike the DNA directly, it triggers responses from cells that indirectly increase the likelihood of mutations.

Medical use of ionizing radiation is a growing source of radiation-induced cancers. Ionizing radiation may be used to treat other cancers, but this may, in some cases, induce a second form of cancer (Little, 2000). It is also used in some kinds of medical imaging. It is estimated that 0.4% of cancers in 2007 in the United States are due to CTs performed in the past and that this may increase to as high as 1.5-2% with rates of CT usage during this same time period (Brenner and Hall, 2007). On the other hand prolonged exposure to ultraviolet radiation from sun can lead to melanoma and other skin malignancies (Cleaver and Mitchell, 2000). Clear evidence establishes ultraviolet radiation, especially the non-ionizing medium wave UVB, as the cause of most non-melanoma skin cancers, which are the most common forms of cancer in the world (Cleaver and Mitchell, 2000).
6B.1.6 Heredity

Majority of cancers are non-hereditary and these are primarily caused by an inherited genetic defect. Less than 0.3% of the populations are carriers of a genetic mutation which has a large effect on cancer risk and this cause less than 3-10% of all cancer (Roukos, 2009). Some of these syndromes include: certain inherited mutations in the genes BRCA1 and BRCA2 with a more than 75% risk of breast cancer and ovarian cancer (Roukos, 2009) and hereditary nonpolyposis colorectal cancer which is present in about 3% of people with colorectal cancer among others (Cunningham et al., 2010).

6B.1.7 Physical agents

Some substances cause cancer primarily through their physical, rather than chemical effects on cells (Maltoni and Holland, 2000). A prominent example of this is prolonged exposure to asbestos, naturally occurring mineral fibers which are a major cause of mesothelioma, which is a cancer of the serous membrane, usually the serous membrane surrounding the lungs (Maltoni and Holland, 2000). Other substances in this category, including both naturally occurring and synthetic asbestos-like fibers such as wollastonite, attapulgite, glass wool and rock wool are believed to have similar effects (Maltoni and Holland, 2000). Non-fibrous particulate materials that cause cancer include powdered metallic cobalt and nickel and crystalline silica. Usually, physical carcinogens must get inside the body and require years of exposure to develop cancer (Maltoni and Holland, 2000).

6B.1.8 Hormones

Hormones are the regulatory biochemical that is produced in all multicellular organisms by glands and transported by the circulatory system to a distant target organ to coordinate its physiology and behavior. Hormones serve as a major form of communication between different organs and tissue. Hormones regulate a variety of physiological and behavioral activities, including digestion, metabolism, respiration, tissue function, sensory perception, sleep, excretion, lactation, stress, growth and development, movement, reproduction and mood (Claire, 2010). Some hormones play a role in the development of cancer by promoting cell proliferation (Henderson et al., 2000). Insulin like growth factors and their binding proteins play a key role in cancer cell proliferation, differentiation and apoptosis. This suggests possible involvement of
insulin in carcinogenesis (Rowlands et al., 2009). Hormones are important agents in sex-related cancers such as cancer of the breast, endometrium, prostate, ovary, testis, thyroid cancer and bone cancer (Henderson et al., 2000).

6B.1.9 Treatment

Cancer has not permanent cure so far. It can only be cured if all of the cancerous cells are cut out or killed in place. If cancer cells treated during earlier stages, there are more chances of cure. There are few different types of treatments like radiotherapy or radiation therapy (which uses radiation), chemotherapy (which uses strong medications) and immunotherapy or biological therapy (Antibodies) are used to inhibit the growth of cancer cells (Rowlands et al., 2009). After the above said treatment, patients may need radiotherapy or chemotherapy to keep the tumor from growing again. During the cancer treatment the normal or healthy cells are also get affected due to toxic effect of treatment (hair fall, causing nausea and vomiting etc) (Rowlands et al., 2009).

Chemotherapy is one the cheapest and oldest method used to inhibit the cancer cells growth. Chemotherapy is the treatment of cancer with one or more cytotoxic anti-neoplastic drugs (chemotherapeutic agents) as part of a standardized regimen. Many varieties of anticancer drugs are available in the market, which are divided into broad categories such as alkylating agents and antimetabolites (Rowlands et al., 2009). Traditional chemotherapeutic agents act by killing cells that divide rapidly, one of the main properties of most cancer cells. Targeted therapy is a form of chemotherapy which target specific molecular difference between cancer and normal cells. The first targeted therapies to be developed blocked the estrogen receptor molecule, inhibiting the growth of breast cancer. Another common example is the class of Bcr-Abl inhibitors, which are used to treat chronic myelogenous leukemia (CML) (Lind, 2014). Currently there are targeted therapies for breast cancer, multiple myeloma, lymphoma, prostate cancer, melanoma and other cancers. The effectiveness and efficacy of the chemotherapy depends on the type of cancer and the stage. In combination with surgery, chemotherapy has proven to be useful in a number of different cancer types. The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body. Even when it is impossible for chemotherapy to provide a permanent cure, chemotherapy may be useful to reduce symptoms like pain or to reduce the size of an inoperable tumor in the hope that surgery will be possible in the
future. The important organic compound groups like Alkylating agent (Cyclophosphamide), Anthrocyclines (Daunorubicin), Taxanes (Paclitaxel), Histone Deacetylase Inhibitors (Vorinostat), Inhibitors of Topoisomerase I (Irinotecan), Inhibitors of Topoisomerase II (Etoposide), Kinase inhibitors (Bortezomib), Nucleotide analogs and precursor analogs (Methotrexate and Flurouracil) and Vinca alkaloids and derivatives are mainly used in chemotherapy (Lind, 2014).

Many tremendous efforts have been made over the past decades to improve the available therapeutic options and a large number of potent chemotherapeutic anticancer agents have been identified and successfully used in clinical practice, cancer still remains a major cause of disease and death in most of the countries. New therapeutic options to treat cancer are a high priority for most of the pharmaceutical companies and independent research organizations worldwide. Considerable research activity is devoted to the discovery of more potent treatments, while minimizing their toxic side effects. However, most anticancer agents display a narrow therapeutic window due to their lack of selectivity against cancer cells (Shengquan and Sze Ngong, 2013; Kratz et al., 2008). The ultimate goal of cancer chemotherapy is the development of selective drugs that can kill malignant tumors cells or render them benign without affecting normal cells. Thus, there is an overwhelming need to develop new chemo-preventative agents that are both effective and safe (Spom and Liby, 2005).

One practical approach to this problem is the use of plants as a platform for drug development. Plants have played a dominant role in the development of sophisticated traditional medicine systems. The WHO estimates that approximately 80% of the populations in Asian and African countries depend on traditional medicine for primary health care. Plant products, however, also play an important secondary role in the health care sectors of developed countries, with 70-80% populations of developed countries having used some form of alternative or complementary medicine (e.g. acupuncture). Herbal treatments are the most popular form of traditional medicine, and are highly lucrative in the international market place. The global market for herbal products is expected to reach $5 trillion by 2050 (Anand and Neetu, 2011). Plants products have a long history of use in the treatment of cancer. More than 3000 plant species were used as anticancer herbal medicine worldwide (Kaur et al., 2011). Plant based drug discovery has resulted in the development of many anticancer drugs currently in clinical use. Besides, this also provides a platform
for design of novel and safe drugs through proper understanding of the complex synergistic interaction of various constituents of anticancer herbs (Larkin., 1983; Saxe, 1987). There are four major structural classifications of plant-derived anticancerous compounds are their namely vinca alkaloids, epipodophyllotoxin lignans, taxane diterpenoids and camptothecin quinolone alkaloid derivatives. These substances embrace some of the most exciting new chemotherapeutic agents currently available for use in a clinical treatment.

6B.1.9.1 Vinca alkaloids

For the first time Madagascar periwinkle isolated Vinblastine and Vincristine alkaloids from plant *Catharanthus roseus G.(Apocynaceae)* which start new era of using plant material as anticancer agents (Verma and Singh, 2010). These two drugs have been used in clinical oncology for almost 50 years and work by blocking polymerization of tubulin molecules into microtubules, preventing the formation of the mitotic spindle which results in metaphase arrest and apoptosis (Jordan *et al.*, 1991). Using two alkaloids as parent molecules, many semisynthetic analogues have been developed. Videsine was the first semisynthetic drug enters into human clinical trials in which the carbon 23 acetyl group in vinblastine was changed to an amido group (Jordan and Wilson, 2004). This drug is primarily used to treat acute lymphocytic leukaemia. Vinorelbine is another semisynthetic derivative of vinblastine. The drug was approved in France in 1989 and it gained approval to treat metastatic breast cancer (MBC) in 1991.

6B.1.9.2 Epipodophyllotoxin lignans

Paclitaxel is one of the major and important plant anticancer agent that has made a bigger impact in chemotherapy. Paclitaxel was isolated from the bark of *Pacific Yew, Taxus brevifolia Nutt (Taxaceae)* which provides further evidence for the success of natural product drug discovery. Paclitaxel was the first compound discovered to promote microtubule formation and has been used in the treatment of several types of cancers particularly ovarian and breast cancer (Kinghorn and Seo, 1996). Docetaxel is the first semisynthetic drug shows significant clinical activity in a wide range of tumors and a different toxicity pattern than the parent molecule. Docetaxel was prepared from paclitaxel (Bissery *et al.*, 1995)
6B.1.9.3 Taxane diterpenoids

Odophyllotoxin obtained from *Podophyllum peltatum*, is another important anti-cancer compound. This compound can reversibly bind to tubulin and therefore had potential as an anticancer agent. Etoposide and teniposide are the two key analogues of podophyllotoxin. These drugs exert their anticancer activity by acting as inhibitors of the enzyme topoisomerase-II and useful in the treatment of various cancers (Srivastava *et al.*., 2005).

6B.1.9.4 Camptothecin quinolone alkaloid derivatives

Combretastatins have been isolated from the bark of the South African tree *Combretum caffrum* Kuntze (*Combretaceae*). This compound is active against colon, lung and leukemia cancers. The combretatin A series is historically known for its remarkable biological activity in terms of inhibition of tubulin assembly and in *vitro* cytotoxicity against human cancer cell lines (Cirla and Mann, 2003). Since the pharmacological investigation of plants used in folk medicine could lead to the discovery of new anticancer agents, these are the proposed new therapeutic alternatives for inhibition of cancer cells. In this regard, the objectives of present study were to ascertain if *M. terminale* Dalz has anticancer activity and to identify molecules which inhibit the growth of cancer cells effectively.
6B.2 Materials and Methods

6B.2.1 Chemicals and Reagents

RPMI-1640, FBS, Penicillin, Streptomycin, MTT (3-4, 5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) was obtained from Sigma Chemical Co. (St. Louis, USA). All other chemicals and reagents were of analytical grade procured from Himedia labs, Mumbai.

6B.2.2 Tumor cell lines and incubation of cell lines

All tumor cell lines used in this investigation were originated from human and mice and procured from American Type Culture Collection (ATCC; Mumbai; India). The cancer cell lines in the experiment were Dalton’s lymphoma ascites cells (DLA) (CRL-1647), Ehrlich ascites carcinoma Cells (EAC) (CCL-77), Human neuroblastoma cell (IMR 32) (CCL-127) and HeLa cells (CCL-17). For incubation of each cell line in the experiment, different mediums were used such as DMEM (Dulbeco’s Modified Eagle’s Medium), RPMI-1640, MEM (Minimum Essential Medium) after adding 10% FBS and 1% penicillin/streptomycin (100 U/ml). Cell lines were adapted and sub-cultured in mediums and incubated at 37° C and 5% CO₂ atmosphere (Heracll 150i, Thermoscientific, India).

6B.2.3 Inhibition of cancer cell proliferation

Inhibitory effects on the cancer cells were assessed using MTT assay (Mosmann, 1983) to examine the survival rates of cancer cells. The tumor cells were adjusted at a concentration of 3x10⁴ cells/ml, 90 µl/well was added in 96 well micro plates, cultivated in an incubator at 37°C, 5% CO₂ for 12 h to attach the cells and added 10 µl each to adjust extracts at a concentration of 50, 100, 200, 400 and 800 mg/ml. Distilled water with an equal amount of the sample was added to the control group and then cultured for 72 h. A 10 µl of MTT (3-4, 5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) solution at a concentration of 5 mg/ml was added to each well and then cultured in an incubator for 4 h. Culture medium with MTT solution was eliminated and 150 µl of DMSO was added and stirred for 30 min to dissolve each cell. Absorbance was measured at 540 nm using a microplate reader and then the obtained values were converted to relative cell growth rates by taking each cell of the non-sample group as 100% as shown below (Mosmann, 1983).
Inhibition of cancer cell proliferation (%) = \{(\text{Absorbance of control group} - \text{Absorbance of sample treated group})/ \text{absorbance of control group}\} \times 100

6B.3 Results

The effect of *M. terminale Dalz* plant extracts was identified by examining the survival rate of carcinoma cells. The methanolic extract was the most effective compared to other extracts and showed very good activity against EAC with survival rates of 77.4% at 50 µg/ml, 47.3% at 200 µg/ml and 19.3% at 800 µg/ml of the extract (Table 6B.1). On the other hand, the methanolic extract was less effective against IMR 32 cell line (71.4% at 800 µg/ml), moderately against DLA cell line (31.4% at 800 µg/ml) and HeLa cell line (35.3% at 800 µg/ml). The chloroform extract showed good anticancer activity against DLA (55.3% at 800 µg/ml) and EAC (56.9% at 800 µg/ml). Further, chloroform extract showed moderate anticancer activity against HeLa cell line (67.4% at 800 µg/ml) and showed impotent anticancer activity against IMR 32 cancer cell lines. In contrast, the petroleum ether extract of *M. terminale Dalz* possessed very weak anticancer activity against all cancer cell lines.

The inhibitory effect of purified compounds from chloroform and methanolic extracts were determined by examining the survival rate of carcinoma cells. Compound MTD5 was the most effective purified compound to display good anticancer activity (Table 6B.2). The survival rates of MTD5 for EAC and DLA cells was 55.8% and 60.2% at 50 µg/ml, 35.2% and 36.7% at 400 µg/ml, 13.9% and 16.4% at 800 µg/ml concentration (Table 6B.2). On the other hand, MTD5 failed to show good activity against HeLa and IMR 32 cells line with survival rate of 31.7% and 39.8% at 800 µg/ml concentration. The inhibitory effect of Compound MTD2 from chloroform extract also showed good activity against DLA and EAC cell line with a survival rate of 19.5% and 18.4% at a concentration of 800 µg/ml. MTD2 was unable to show anticancer activity against HeLa and IMR 32 cancer lines.

Compound MTD1 from chloroform extract of *M. terminale Dalz* plant showed moderate anticancer activity and it is unsuccessful to show good anticancer activity against the IMR 32 cancer cell line (Survival rate of 105.3% at 800 µg/ml). On the other hand, the MTD3 and MTD4 were unable to exhibit anticancer activity against all the four cancer cell lines.
6B.4 Discussion

There are two types of cell death in nature, namely necrosis and apoptosis. It is well documented that apoptosis is a programmed and physiological mode of cell death. The characters of apoptosis include a lot of key morphological features, such as membrane bleb, chromatin condensation, shrinks, deforms and generation of apoptotic bodies (Hacker, 2000). After detaching from its neighbors, it undergoes chromatin condensation and inter-nucleosomal cleavage of the DNA before fragmenting into compact membrane enclosed structures termed “apoptotic bodies”. Cancer cell growth is dependent on the balance between proliferation and apoptosis. In addition, apoptosis is a type of cell death process regulated in an orderly way by a series of signal cascades under certain situations (Fan et al., 2005). It is well known that apoptosis and cell cycle deregulation are closely related events and disruption of cell cycle progression may ultimately lead to apoptotic death. The progression (metastatic spread) of tumors from one organ to another part of body is reported to be a major cause of poor clinical outcome in cancer patients (Son et al., 2011; Prince and Thompson, 2002). High percentage of cancer mortality is associated with metastatic spread of tumor cells from the original site to the different part of body importantly brain, lung, kidney and bone marrow. Extensive efforts have been made to define the mechanism of metastasis and its inhibition (Figueira et al., 2009; Tsubaki et al., 2012). Here, the reported mechanistic aspect of inhibition of cancer cells growth by naturally occurring compounds that may be relevant to the theme of the present study is briefly discussed. The present study is designed to define the mechanism of inhibition of cancer cell by two ways. The first one through the antioxidant property of M. terminale Dalz plant extracts and secondly, synergic activity of the compounds present in the plant extracts.

Medicinal plants have a good free radical scavenging activity and also contain a very good percentage of phenolic and flavonoids (Kris et al., 2002). This antioxidant property is mainly due to phenolic and flavonoids content present in the plants. Due to its good antioxidant properties, some of the medicinal plants are used traditionally to cure many disorders (Kris et al., 2002). The association between a higher intake of fruit and vegetables and a decreased risk of developing some types of cancer has been suggested to be attributable to the content of antioxidants and other secondary metabolites present in plants (Kris et al., 2002). However, because of a
number of secondary metabolites in plants have been estimated to be in thousands, it is difficult to evaluate which compound or combinations of compounds exert health promoting effects (Mullick and Gasser, 2004). The anticancer effects of fruit, berries, vegetables and medicinal plants might be exerted in several ways such as suppressing mutagenesis, inhibiting cell proliferation, or causing induction of apoptosis (Steinmetz and Potter, 1996).

In this present investigation, the effects of different extracts of *M. terminale Dalz* on cell proliferation in four cancer cell lines (HeLa, IMR 32, DLA and EAC) were investigated. The anticancer activity was correlated between the contents of different antioxidant molecules of the plant and inhibition of cancer cell proliferation. Our present investigation has showed highest antioxidant activity of different extracts of *M. terminale Dalz* plant. The methanolic extract of the plant showed good phenolic and flavonoids content and showed good free radical scavenging activity, metal chelating activity, total reductive ability, lipid peroxidation inhibition and H₂O₂ scavenging activity. The methanolic extract of plant also proved to be nontoxic in the animal model experiment. The present anticancer activity results indicates the strong antioxidant activity of phenolic and flavonoid contents present in the methanolic and chloroform extracts may contribute to the anticancer activity and thus prevent the cancer cell lines growth (Joy and Kuttan, 1995). The mechanism of this activity is not completely understood but may be attributed to their free radical scavenging activity (Bagchi et al., 2000). The phenolic compounds such quercetin, tannins and ellagitannins have been showed to have anticarcinogenic activity (Rajesh kumar et al., 2002). Some of the hydrolysable tannins were shown to be potent inhibitors of wheat embryo Ca²⁺ dependent protein kinase (CDPK), rat brain Ca²⁺ protein kinase and phospholipid dependent protein kinase (PKC) and Ca²⁺ calmodulin dependent myosine light chain kinase (Foo, 1993). Tannins and related compounds were reported to have a potent antioxidant activity (Liu et al., 2008) and thus possessing anticancer activity also. The growth inhibition of four cancer cell lines was concentration dependent (Table 6B.1).

Similar work was carried out using 52 traditionally used species of Thai medicinal plants for their in vitro cytotoxic, antioxidant, lipase inhibitory and antimicrobial activities (Kaewpiboon et al., 2012). The hexane, dichloromethane, ethanol and water extracts were applied to panel of human cancer cell lines using MTT cytotoxicity assay (Kaewpiboon et al., 2012). The Thai medicinal plant
Bauhiniastry chnifolia exerted strong in vitro cytotoxic activities against human cancer cell lines. The above data had a positive linear correlation between antioxidant capacities and total phenolic contents implying that phenolic compounds in M. terminale Dalz plant extracts could be main components contributing to the anticancer activity. Similar type of work was also carried out using methanolic leaf extract of Indigofera cassiodes against transplantable tumors and human cancer cell lines (kumar et al., 2011). The extract was investigated for its in vitro cytotoxicity using a panel of cancer cell lines. The extract exhibited potent in vitro cytotoxicity against all the tested cancer cell lines, but it was found to be safe on normal cells (kumar et al., 2011).

The compound present in the methanolic and chloroform extracts of plant showed a synergic activity for the enhancement of cancer cell line inhibition. On the other hand, it cannot be excluded that compounds other than phenolic compounds like the flavonoids and other glycosides are also involved in the anticancer activity of the plant extracts. It was found to have a direct relationship between the antioxidant and synergic activity of the plant extracts. Furthermore, the petroleum ether extract of M. terminale Dalz which lacks phenolic and flavonoid compounds did not show inhibition activity towards cancer cell lines. The petroleum ether extract contains only oils and fats which are unable to inhibit the enzyme and cause any harmful effects on cancer cell line growth. The petroleum ether extract showed weak antioxidant activity as compared to other extracts which could be the reason for reduced anticancer activity of petroleum ether extract.

It is important to find out whether the purified phytochemical has the same health benefit as does the whole mixture of plant extracts in which the phytochemicals are present (Sasaki, 1989). It is hypothesized that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent antioxidant and anticancer activities and that the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole extract (Sasaki, 1989). It is estimated that about 8000 phytochemicals are present in whole foods, and there are quite possibly many more.

In this study, we isolated five pure compounds from the chloroform and methanolic extracts and were tested for their anticancer activity (MTD1, MTD2, MTD3, MTD4 and MTD5). From the results obtained, the anticancer effects of the plant related compounds may be mainly ruled by their conformational preferences,
which strongly determine their anticancer activity. Many experimental studies indicated that the size, shape and planarity of the purified compounds and derivatives are important factors for their mechanism of cytotoxicity and antitumor activity (Mauffret, 1991). Further, acidic and basic properties of purified compounds are also important for the anticancer activity. It is known that the pharmacological activities of drugs depend on their interaction. They are expected to be significantly different for the charged and uncharged drugs (Cirino et al., 2005). For analyzing structure activity relationships, three structural components were considered: 1. the presence of substituents on ring, 2. the nature of the ring, 3. position of the ring.

It has been established that ellipticine, a drug isolated from Ochrosia elliptica (Apocinaceae), has powerful anti-proliferative properties. Its chemical structure consists in a tetracyclic skeleton composed of pyridine fused with the carbazole system (Sengupta, 1995). Ellipticine and related compounds exert their cytotoxic and antineoplastic effects by a multimodal mechanism of biological action (inhibition of DNA-topoisomerase II, DNA intercalation, covalent binding to DNA and redox generation of cytotoxic free radicals) (Jurayi et al., 1994). Several quaternary ellipticinium salts (datelliptum, pazcellipticine and others) were previously reported to possess high cytotoxic activity (Pierson et al., 1998). Datelliptium and pazcellipticine bearing hydrophilic substituents on a pyridine ring which increase water solubility (Pierson et al., 1998). The compound MTD1 contains furan ring which may be responsible for good anticancer activity. Thus, in general compounds with the alkyl chain confers greater activity for the inhibition of cell proliferation (MTD1). The groups present (hydroxyl group, methyl group and carboxylic acid group) the ring play a very important role in the anticancer activity of MTD1. The substitution of a methyl group for the hydrogen increases the activity. MTD5 contains naphthyridine and the ring hydroxyl groups are directly attached at positions 3 and 7 of the ring and a derivatized carboxylate group is also present in the naphthyridine ring and these kinds of structures are known to possess anticancer activity (Pierson et al., 1998). Plant phenolic acids appears to be highly reactive inside the cell and an accurate evaluation of the exact amount of agent necessary to reach a certain tissue, enter the cell wall and perform an effective tumor inhibitory function needs to be assessed in detail (Newmark, 1992).

In this study, out of five purified compounds (MTD1, MTD2 and MTD5), three compounds (MTD2 and MTD5) were found to possess significant activity at
micromolar concentration. Thus, our findings have important implications for combinations of phenolics and flavonoids in the inhibition cancer cells. However, further studies are needed to elucidate the underlying mechanisms of combination effects of bioactive components in the inhibition of cancer cell lines by methanolic and chloroform extracts of *M. terminale Dalz* plant.
6B.5 Tables and Graphs

Table 6B.1: Survival rate of cancer cells as affected by the concentration of \textit{M. terminale Dalz} plant extracts

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Cancer cell</th>
<th>Concentration of Plant extracts in (\mu g/ml)</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
<th>800</th>
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<tbody>
<tr>
<td>Petroleum ether</td>
<td>HeLa</td>
<td>140.4±18.66</td>
<td>121.3±13.31</td>
<td>99.2±17.26</td>
<td>94.5±14.40</td>
<td>90.3±11.00</td>
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</tr>
<tr>
<td></td>
<td>IMR 32</td>
<td>133.2±11.4</td>
<td>118.4±13.3</td>
<td>110.4±10.8</td>
<td>101.3±9.5</td>
<td>98.4±7.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DLA</td>
<td>104.3±2.8</td>
<td>100.4±6.8</td>
<td>98.3±17.2</td>
<td>94.8±8.9</td>
<td>91.3±8.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EAC</td>
<td>112.4±14.5</td>
<td>105.2±8.3</td>
<td>99.2±8.4</td>
<td>96.2±9.5</td>
<td>92.1±1.1</td>
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<tr>
<td>Chloroform</td>
<td>HeLa</td>
<td>103.2±21.0</td>
<td>96.3±4.0</td>
<td>88.4±9.4</td>
<td>78.3±21.3</td>
<td>67.4±6.8</td>
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<tr>
<td></td>
<td>IMR 32</td>
<td>114.7±16.8</td>
<td>109.4±8.5</td>
<td>101.7±3.0</td>
<td>95.3±8.4</td>
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<tr>
<td></td>
<td>DLA</td>
<td>85.3±13.2</td>
<td>79.8±15.4</td>
<td>71.2±6.1</td>
<td>64.7±3.2</td>
<td>55.3±4.8</td>
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<tr>
<td></td>
<td>EAC</td>
<td>91.8±13.9</td>
<td>84.2±4.5</td>
<td>72.1±3.7</td>
<td>63.1±11.2</td>
<td>56.9±2.6</td>
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<tr>
<td>Methanolic</td>
<td>HeLa</td>
<td>90.1±5.9</td>
<td>84.4±9.4</td>
<td>72.3±13.4</td>
<td>51.4±12.1</td>
<td>35.3±10.4</td>
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<tr>
<td></td>
<td>IMR 32</td>
<td>101.3±5.0</td>
<td>96.4±3.0</td>
<td>90.2±20.2</td>
<td>82.1±1.3</td>
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<tr>
<td></td>
<td>DLA</td>
<td>80.3±5.8</td>
<td>75.3±1211</td>
<td>66.7±15.43</td>
<td>51.3±7.5</td>
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<tr>
<td></td>
<td>EAC</td>
<td>77.4±3.4</td>
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<td>68.2±20.2</td>
<td>47.3±4.5</td>
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</table>

**Abbreviations:** Cell lines: Cervical carcinoma, HeLa; Human neuroblastoma cell, IMR 32; Dalton's lymphoma ascites cells, DLA; Ehrlich ascites carcinoma, EAC. All the values are mean ± SD of three independent measurements.
Table 6B.2: Survival rate of cancer cells as affected by the concentration of purified compounds of *M. terminale* Dalz plant extracts

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Cancer cell</th>
<th>Concentration of Plant extracts in μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>MTD1</td>
<td>HeLa</td>
<td>113.5±18.6</td>
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<tr>
<td></td>
<td>IMR 32</td>
<td>121.5±11.4</td>
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<tr>
<td></td>
<td>DLA</td>
<td>102.4±2.8</td>
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<tr>
<td></td>
<td>EAC</td>
<td>109.5±14.5</td>
</tr>
<tr>
<td>MTD2</td>
<td>HeLa</td>
<td>80.9±21.0</td>
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<td></td>
<td>IMR 32</td>
<td>112.5±16.8</td>
</tr>
<tr>
<td></td>
<td>DLA</td>
<td>75.8±13.2</td>
</tr>
<tr>
<td></td>
<td>EAC</td>
<td>70.5±13.9</td>
</tr>
<tr>
<td>MTD5</td>
<td>HeLa</td>
<td>74.3±5.9</td>
</tr>
<tr>
<td></td>
<td>IMR 32</td>
<td>94.5±5.0</td>
</tr>
<tr>
<td></td>
<td>DLA</td>
<td>60.2±5.8</td>
</tr>
<tr>
<td></td>
<td>EAC</td>
<td>55.8±3.7</td>
</tr>
</tbody>
</table>

**Abbreviations:** Cell lines: Cervical carcinoma, HeLa; Human neuroblastoma cell, IMR 32; Dalton’s lymphoma ascites cells, DLA; Ehrlich ascites carcinoma, EAC. All the values are mean ± SD of three independent measurements.
Chapter-6C

Antimalarial activity of *M. terminale* Dalz
6C.1 Introduction

Malaria is the world’s most devastating parasitic disease, accounting for an estimated 207 million cases and 6.27 million deaths annually, mainly among children less than 5 years of age in Africa (WHO, 2014). The magnitude of malaria in terms of morbidity and mortality in humans makes it a major public health problem in tropical and subtropical countries. Despite the impressive initial results of the National Malaria Control and Eradication Programs initiated in 1950s, there was a complete failure to eradicate malaria in many countries due to technical, operational and socio-economic difficulties, which led to resurgence of malaria in many parts of the world. The control program has been hampered by the spread of drug resistance in parasite and insecticide resistance in mosquito vectors (Ballou et al., 1987). Sub-Saharan Africa and countries in tropical Africa account for more than 90% of total malaria incidence and great majority of deaths due to malaria. Malaria is caused by five species of the genus *Plasmodium* namely, *Plasmodium vivax* (*Pv*), *P. falciparum* (*Pf*), *P. malariae* (*Pm*), *P. ovale* (*Po*) and *P. knowlesi* (*Pk*). Common clinical presentations of infection with all four *Plasmodium* species are periodic paroxysm, chills, rigors, sweating, body aches, headache, nausea, general weakness and prostration (Ballou et al., 1987).

Severe life-threatening complications such as cerebral malaria (CM), severe anemia, acidosis, respiratory distress, jaundice, acute renal failure (ARF), acute respiratory distress syndrome (ARDS), etc., occur mostly with *Pf* infection. A few reports have appeared indicating association of severe complications of malaria with *Pv* infection. Recently, life threatening complications with *P. knowlesi* (*Pk*) infection has been reported in human (Ballou et al., 1987). Renal involvement has been reported in *Pf, Pm* and recently in *Pv* infections. *Pm* associated nephropathy was reported mainly from Africa, that too before 1980. The malaria caused by *Pf* is responsible for the most deaths that occur primarily in young children and pregnant women in sub-saharan Africa. In the last few decades there has been a deterioration in malaria control in many endemic areas because of increases in parasite resistance to antimalarials and insecticide-resistant vectors, declining economic conditions, and ecologic and climate change. Superimposed on this resurgence of cases, there has been an increase in international travel to and immigration from malaria-endemic areas. It has been estimated that approximately one billion individuals cross
international boundaries in the year 2012 and in future this number is expected to double within a decade (UNWTO, 2013). The combination of increased travel and escalating drug resistance has resulted in an increasing number of travelers being exposed to resistant malaria. In present situation, 10,000 to 30,000 travelers from industrialized countries contact malaria each year (Handszuh and Waters, 1997).

The risk for acquiring malaria for travelers depends primarily on their specific risk behaviors (rural travel, night time exposure, unscreened accommodation) and whether they are traveling to areas with drug-resistance malaria. Information on the geographic risk of malaria is available from the WHO, which compiles available data on malaria incidence worldwide (WHO, 1996). Although these data under report the true incidence in many countries by at least fivefold and lag several years behind current drug susceptibility patterns, they provide useful information on malaria risk for the traveler by identifying trends or changes in transmission. The latest compilation highlights the following important points: seven areas account for two thirds of the reported malaria cases worldwide (India, Africa, Vietnam, Sri Lanka, Soloman island and Colombia), Belize, Nicaragua and Guatemala have the highest incidence rates in Central America; *Pf* malaria has become a significant problem in the Amazon regions of Brazil, Guyana, Peru and Bolivia. The malaria cases more severe in the India subcontinent with an increasing proportion of drug-resistance *Pf* found in major travel destinations such as Delhi, Bombay and Calcutta: drug resistant *Pf* malaria continues to evolve worldwide, led by Thailand in which high levels of resistance to mefloquine and halofantrine are well documented, and sensitivity to quinine is decreasing; and finally chloroquine and primaquine resistant or tolerant strains of *Pv* are well established in Oceania and seem to be spreading to other geographic areas, such as Guyana and Southeast Asia.

India has the largest population in the world at risk of malaria, with 85% living in malarious zones (Sharma, 1996). The combination of *Pf* and *Pv*, six primary malaria and vectors, several ecotypes including urban malaria, and various transmission intensities ranging from unstable to hyper-endemic create a challenging epidemiological scenario in India (Sharma, 1996). At the time of Indian independence in 1947, there were about 75 million cases and 800,000 deaths a year (Akhtar *et al.*, 1977). After independence, health care was prioritized, and the control of malaria was one of India’s key aims (Sharma, 1996). In 1953, the National Malaria Control Program was launched and protected a population of about 165 million with
dichlorodiphenyltrichloroethane (DDT) spraying (Sharma et al., 1996). The control program developed into the National Malaria Eradication Program in 1958. Reliable surveillance gradually developed during the eradication period, and the program seemed to be highly effective with only 99,667 malaria cases and no deaths reported in 1965 (Sharma et al., 1996). However, long-term success of malaria control could not be sustained. Increasing insecticide resistance in mosquitoes, urbanization, development projects, population migration, integration with the general health services, financial difficulties, and other operational challenges laid the foundation for a resurgence of malaria. In 1976, malaria cases reached a post-eradication peak of 6.47 million cases (Pattanayak and Roy, 1980).

The chloroquine resistant Pf was first documented in the northeast karbi-Anglong district of Assam in 1973 (Sehgal et al., 1973). Routine monitoring of antimalarial resistance using in vivo efficacy trials was initiated in 1978 by 13 regional teams. Although several protocols for drug-resistance monitoring have been used in the past three decades, the test system generally includes patients with defined criteria, supervised treatment and follow-up for clinical and parasitological outcomes. Initial reports of sulfapirimethamine resistance emerged in 1979, again in Karbi-Anglong, Assam (Das et al., 1981). A national antimalarial-drug policy was introduced in 1982 to improve malaria case management and established sulfapirimethamine as the treatment for chloroquine-resistant areas (Das et al., 1981). Drug effectiveness monitoring by the national program and others has provided data to guide treatment strategy and update policy. Artesunate plus sulfadoxine-pirimethamine replaced the latter alone as the second-line drug in 2005 for use of chloroquine treatment failures, and as the first-line antimalarial treatment in areas with documented drug resistance (NVBDCP guidelines). In 2007, artemesinin plus sulfadoxine-pirimethamine was selected as the first-line treatment in high-risk districts and areas with identified resistance, with the goal of covering most of the nation’s Pf burden. In 2010, this treatment became the first-line treatment throughout India (NVBDCP guidelines).

Few efficacy trials exist for other antimalarial compounds in India and none for routine monitoring. Resistance to mefloquine and quinine is reported but seems to be rare (Dua et al., 2003) and cases are not well documented. Trials of artemisinin combination treatments in India have consistently showed successive treatment above 95% (Valecha et al., 2009; Arora et al., 2008). Only a few case reports from Mumbai,
Uttar Pradesh and Bihar of which, chloroquine resistant *Pv* malaria exist (Valecha *et al.*, 2009). Contrary to these reports, systematic trails from across the country have reported 100% efficacy of standard dose chloroquine (25 mg/kg over 3 days) (Valecha *et al.*, 2006). Chloroquine resistant *Pv* is not a serious concern in India.

Chemotherapy has traditionally played an important role in the treatment and control of malaria. Quinoline containing antimalarial drug are the most effective drugs for malaria chemotherapy. This group of compounds has evolved from the structural modification of quinine and include 4-aminoquinoline compounds such as chloroquine and mefloquine of which former is more effective, cheap, safe and commonly available drug. The dihydrofolate reductase inhibitors include proguanil chloropropguanil, pyrimethamine and trimethoprim and sufa drugs like dapsone, sulfalene, sulfamethoxazole and sulfadoxine. These drugs are used in combinations. The classically such combination sulphadoxine and pyrimethamine used as first line drug in Thailand and other parts of the world. Tetracycline and its derivatives such as doxycycline are very potent antimalarial and are used for both treatment and prophylaxis. In areas where response to quinine has deteriorated, tetracyclines are often used in combination with quinine to improve cure rates.

The other useful antimalarials are artemisinin compounds synthesized from the plant *Artemisia annua*. These compounds (artesunate, artemether, arteether) are most effective antimalarials and seem to have effects on protein synthesis by the malaria parasite. These are used for the treatment of severe malaria and have showed very rapid parasite clearance in comparison to quinine compounds. Artemisinin and mefloquine combination is being used in some south east Asian countries, for the treatment of uncomplicated malaria, where the multidrug resistant strains of *Pf* are prevalent (Pattanayak *et al.*, 1980).

### 6C.1.2 Mechanisms of resistance to antimalarial drugs like chloroquine

Chloroquine is the drug that has been most studied but its mechanism of action still remains to be fully elucidated. The mechanism of antimalarial action of quinolone containing drugs (like chloroquine) has been investigated by many workers and several therapeutic targets have been suggested. Most of the drug targets are localized in the acid food vacuole of the parasite (Krogstad *et al.*, 1987; Geary *et al.*, 1986). It is believed that resistance of *Pf* to chloroquine is due to increased capacity for the parasite to expel chloroquine at a rate that does not allow chloroquine to reach
levels required for inhibition of heme-polymerization (Foley and Tilley, 1997). This chloroquine efflux occurs at a rate of 40 to 50 fold faster among resistant parasites than that in sensitive ones (Krogstad et al., 1987). Further, evidence supporting this mechanism is provided by the fact that chloroquine resistance would be reversed by drugs which interfere with this efflux system (Martin et al., 1987) but the biochemical basis of this efflux is a matter of debate. The efflux of chloroquine and in fact the entire chloroquine resistance phenotype can be reversed with Ca\textsuperscript{2+} ion channel blocker, such as verapamil and dilitazem (Krogstad et al., 1987; Geary et al., 1986; Foley and Tilley, 1997; Martin et al., 1987).

Current molecular studies of \textit{Pf} isolates suggest that few gene loci are associated with chloroquine resistance to \textit{Pf}. These genes have been named as \textit{pfmdr}-1 and 2, \textit{pfcrt}. The \textit{pfmdr}-1 gene located on chromosomes-5 and coding for P-glycoprotein homologue-1 (\textit{pgh}-1) has generated interest in resistance to chloroquine and other antimalarials. Studies conducted in different geographical areas of the world suggest that the point mutation of aspartic acid to tyrosine in codon 86 (A-86 to T-86) is associated with chloroquine resistance (Djimde et al., 2001; Von., 1997). Several other \textit{pfmdr}-1 ploymorphisms-Phe 184, Cys 1034, Asp 1042 and Tyr 1246 have been implicated to varying degrees in chloroquine resistance has been identified on chromosome 7 and encodes a transmembrane protein in a digestive vacuole of malaria parasites (Fidock et al., 2000). Sets of point mutations in \textit{pfcrt} gene have been found to be associated with \textit{in vitro} chloroquine resistance in \textit{Pf} from Africa, South America and South-East Asia (Durand et al., 2001). Recently researchers found that the substitution of threonine (T76) for lysine (K76) at codon 76 was present in all chloroquine resistant isolates and absent in all sensitive isolates (Djimde et al., 2001).

\textbf{6C.1.3 Importance of plants and its derivative as antimalarial drugs}

Despite great effort and resources for the eradication of malaria, it still remains as a grave public health problem involving hundreds of thousands of deaths annually (WHO, 2010). While research on vaccines is at an advanced stage, drug therapy is still the principle tool for the control and eradication of the disease. The emergence of strains of \textit{Pf} and \textit{Pv} which are resistant to first and second line antimalarials (multidrug resistant or MDR) have motivated the search for new drugs representing new and distinct chemical classes and mechanisms of action than those of the antimalarial drugs currently in use (Kaur et al. 2009). Chemical compounds of
novel structure and of natural origin represent a major source for the discovery and development of new drugs for diseases especially malaria (Schmidt et al. 2012). Historically, plants used in traditional medicine as antimalarials have provided substances which have proved to be useful as antimalarials or have served chemists as structural models for the development of semi-synthetic drugs of purely synthetic analogs (Schmidt et al., 2012). This is true of the most important antimalarial natural products revealed to date: quinine (isolated from the bark of Cinchona spp.) and artemisinin (isolated from Artemisia annua leaves).

The therapeutic efficacy and complex molecular structure of quinine lead to the development of purely synthetic analogs chloroquine, primaquine, mefloquine, among others in the last century (Plowe, 2009). More recently, semi-synthetic derivatives (e.g. sodium artesunate, artemether, arteether and dihydroartemisinin) prepared in one or more steps from isolated artemisinin have become key pharmaceutical components in formulations used in what is commonly called artemisinin combination therapy (ACT) for the treatment of resistant and MDR Pf infections (Plowe, 2009; Willcox, 2011). The extracts of a large number of plant species including many that are used in traditional medicine have been evaluated for in vitro antiplasmodial activities and some have also been tested in in vivo models, usually in mice infected with Plasmodium berghei (Pb), Plasmodium yoelii (Py) or Plasmodium chabaudi (Pc). In some cases, the constituent responsible for their activities have been isolated but relatively few have been studied further to assess their potential as lead compounds for the development of new antimalarial drugs (Wright, 2005). In recent years, the monoterpene indole alkaloid ellipticine has been the subject of a number of pharmacological studies and its derivatives have been studied in clinical trials against different forms of cancer. Ellipticine has been isolated from the alkaline ethanolic extract of the bark of the Amazonian tree Aspidosperma vargasii (Apocynaceae) (Andrade-Neto et al., 2007; Henrique et al., 2010) which is used in traditional medicine as an antimalarial also (Oliveira et al., 2003). In vitro antiplasmodial activity of ellipticine was first reported by Andrade-Neto et al (2007). Recently, the antimalarial activity of ellipticine was independently confirmed and the comparable or superior activity of four derivatives of ellipticine against Pf in vitro was described (Pasemar et al., 2011; Pohlit et al., 2012).

The roots of the West African climbing shrub Crypolepis sanguinolenta have proven efficacy according to clinical trials (Willcox, 2011). The in-depth literature
survey has confirmed that no data is available regarding the antimalarial activity of
different extracts of *M. terminale Dalz* and its purified compounds. Herein, we report
the antimalarial activities of five isolated and purified compounds from the leaves.
The overall aim of this work was to provide comparative *in vitro* antimalarial efficacy
data for all the three crude extracts and purified compounds from these extracts.

6C.2 Material and Methods

6C.2.1 Chemicals and Reagents

RPMI 1640, HEPES, D-sorbitol, SYBR green, Triton X-100 and chloroquine
were obtained from Sigma Chemical Co. (St. Louis, USA). All other chemicals and
reagents were of analytical grade procured from Himedia labs, Mumbai.

6C.2.2 Parasite strains and parasite culturing

The *Pf* strain isolates were taken by venipuncture on ACD (acid citrate-
dextrose) from outpatients with uncomplicated malaria before starting the treatment.
The chloroquine sensitive 3D7 strain was maintained in continuous culture in group
O⁺ve human erythrocytes and suspended at a 4% hematocrit in RPMI 1640
supplemented with 25 mM HEPES, 25 mM NaHCO₃ and complemented with 10%
O⁺ve human serum (Achur et al., 2003). The cultures were incubated at 37°C in an
atmosphere of 90% nitrogen, 5% oxygen and 5% carbon dioxide.

6C.2.3 Assessment of SYBR green fluorescence linearity

Experimental conditions and plate reader settings were verified and/or
adjusted by examining the SYBR green I fluorescence linearity of parasitemia values
between 0 and 5%, as determined by microscopic examination of Giemsa-stained
parasites. This method was previously described by Smilkstein et al., (2004) and
briefly is as follows. Triplicate wells of 3D7-parasitized erythrocytes (in early ring or
schizont stages) were serially diluted with non-infected erythrocytes at a constant 2%
 hematocrit in culture medium (100 μL final volumes). Next, 100 μL of lysis buffer
(20mM Tris (pH 7.5), 5 mM EDTA, 0.008% (wt/vol) saponin, and 0.08% (vol/vol)
Triton X-100 containing SYBR green I (1X final concentration) were added directly
to the plates and gently mixed. The plates were then incubated for another hour at
room temperature in the dark and examined for the relative fluorescence units (RFU) per well.

6C.2.4 Preparation of predosed microtiter drug plates

Sterile 96-well tissue culture plates containing 9 serial dilutions of each antimalarial drug and different plant extracts of *M. terminale Dalz* and its purified compounds, originally suspended in dimethyl sulfoxide or 70% ethanol at various stock concentrations, in test culture medium were made freshly on the day of the assay. No difference was seen in IC$_{50}$ determination between previously frozen of fresh drug assay plates (data not shown).

6C.2.5 Assay of antimalarial activity

The intrinsic antimalarial activity of the extract was evaluated on synchronized *Pf* 3D7 strain. The infected blood cells were washed three times in RPMI 1640, and *in vitro* culture was performed in triplicate in 96 well plates using the non isotopic semi-micro test of LeBras and Deleron (1983). Each well contained 700 µL of infected blood cells, RPMI 1640 supplemented with 25 mM HEPES, 25 µL of variously concentrated either the plant extracts or plant purified compounds or chloroquine was added in triplicate. In each plate, three wells free from drugs, purified compounds and extracts were considered as controls. The plates were incubated as mentioned above for 24 and 74 h at 37°C (hematocrit 4%, parasitemia 0.1%). After the exposure time, the supernatant was removed from every well and SYBR green I fluorescence technique is used to count the cell layer to determine the number of schizonts. These values were then expressed as a percentage, relative to the control cultures without extract. IC$_{50}$ values were graphically determined (percent inhibition versus concentration).

6C.3 Results

*In vitro* activity of *M. terminale Dalz* plant extracts against CQ-sensitive *Pf* strain (3D7) are summarized in the Fig. 6C.1. Out of three extracts tested, the methanolic extract of plant showed significant anti-plasmodial activity at a concentration of 185 µg/mL (IC$_{50}$). The chloroform extract showed moderate anti-plasmodial activity against 3D7 strain (IC$_{50}$=370 µg/mL). The petroleum ether extract of *M. terminale Dalz* plant showed weak or no activity (IC$_{50}$=740 µg/mL).
The five isolated pure compounds from chloroform and methanolic total extract of *M. terminale Dalz* were assayed for *in vitro* activity against *Pf* 3D7 strains. The results of the study indicated that *in vitro* activity of purified compounds of plant displayed very good anti-plasmodial activity. From the IC\(_{50}\) values for each substance against malaria parasite, it was possible to determine selective indices (Table 6C.1). The compound MTD1 was the least active compound, however, it did significantly inhibit *Pf* growth (IC\(_{50}\)=24 µg/mL) compared with the crude extracts (Table 6C.2). The potent *in vitro* activity of the newly isolated compounds (MTD2, MTD3, MTD4 and MTD5) has been evaluated against the 3D7 strain (IC\(_{50}\) values of 6, 4, 5 and 5 µg/ml, respectively). Among these compounds, the MTD3 exhibited a low IC\(_{50}\) value of 4 µg/ml and it is the most active compound as compared to MTD4 and MTD5. Further, a moderate anti-plasmodial activity was exhibited by MTD2 with an IC\(_{50}\) value of 6 µg/mL.

6C.4 Discussion

Herbs and medicinal plants are complex mixtures of different compounds which have been used as different crude extracts in various preliminary studies as commonly used in Indian traditional medicine. In this preliminary study, we have used the crude extract as well as five isolated compounds of *M. terminale Dalz* as promising and selective antimalarial agents against chloroquine sensitive 3D7 strains. The relative potencies of the three plant crude extracts and isolated pure compounds against 3D7 *Pf* in descending order is as follows: MTD3 > MTD4 > MTD5 > MTD2 > MTD1 > Methanolic extract > Chloroform extract > Petroleum ether extract. The IC\(_{50}\) values for these compounds are compound MTD5 (5 µg/mL), MTD4 (5 µg/mL), MTD3 (4 µg/mL) and MTD2 (6 µg/mL) which indicates very potent and showed promising antimalarial activity against the 3D7 chloroquine sensitive strains of *Pf*. The methanolic extract of *M. terminale Dalz* showed good anti-parasitic activity, compared to other crude extracts. The low potency of antimalarial activity of the crude extract of *M. terminale Dalz* plant is partly explained by the low yield of active ingredient in chloroform and petroleum ether extracts as compared with methanolic extract.

Potential new targets for treatment of malaria include parasite enzymes that degrade hemoglobin in host blood (Semenov et al., 1998). Several sulfones have been
known as inhibitors of erythrocytic malaria parasites on cysteine and aspartic proteases (Rosenthal et al., 1996). A synergic effect has been noticed when mixture of cysteine and aspartic protease inhibitors were treated to control malarial parasite, Pf (Semenov et al., 1998). The methanolic extract of plant showed potent antimalarial activity when compared with remaining extracts. These results are similar to the previous study on the evaluation of sulfone inhibitors (Semenov et al., 1998). Therefore, these phenolics and flavonoids may partially inhibit the cysteine and aspartic proteases of malarial parasites. Further studies may provide the mode of inhibitory action of phenolics and flavonoids on the protease activity. Some plant extracts also revealed their ability to reverse chloroquine resistance as in the case of the malagashanine, an alkaloid extracted from Malagasy strychnos used in traditional medicine in Madagascar (Rafatro et al., 2000).

Flavonoids and phenolics have been frequently identified in phytochemical analysis of plants used for treating malaria in various malaria endemic areas (Nundkumar and Ojewole, 2002). However, over 6500 flavonoids have been characterized so far and controversial data have been obtained regarding their anti-plasmodial activity due to diversity of compounds (Phillipson and Wright, 1991; Brandao et al., 1997; Bickii et al., 2000). More recently, several flavonoids have been isolated from Artemisia afra, related to Artemisia annua, the famous traditional Chinese medicinal plant very efficient to treat multidrug resistant malaria (Kraft et al., 2003).

It is important to know if there is any correlation between antimalarial activity and structure of the five isolated compounds. The analysis indicates that MTD1 containing furan ring displayed a moderate anti-plasmodial activity. The results indicate that the hydroxyl group at 2\textsuperscript{nd} position and methyl group at 5\textsuperscript{th} position of the furan ring influence the activity, but it unable to show good activity when compared standard chloroquine. The presence of carboxylic acid group at 3\textsuperscript{rd} position could have a role in the activity and the oxygen atom of furan ring also may contribute for the antimalarial activity of the MTD1. MTD2 contains one methyl group and it has isochromene ring system. The oxygen atom present in the ring contains lone pair of electrons and has the ability to donate electrons. The methyl group could interact hydrophobically with plasma membrane of the parasite. These hydrophobic and hydrophilic interactions could possibly lead to growth inhibition of Pf parasites.
Among all the purified compounds, MTD3 showed very good activity compared other purified compounds and plant extracts. MTD3 contains long chain of carbon, which could help in the hydrophobic interaction of membrane lipids of 3D7 Pf strains. On the other side of the chain it contains carboxylic acid group and it could also play very important role in the inhibition of the parasite growth. The MTD4 also showed good activity compared to the crude extracts of M. terminale Dalz plant. MTD4 (molecular formula C₁₆H₃₂O₂) has a molecular weight of 296.4, it has hydroxyl and methyl group at 4\textsuperscript{th} position. The methyl and hydroxyl groups could lead to hydrophobic and hydrophilic interaction with the enzymes. This interaction may block the enzymes which is very important for the growth of the parasite.

Among all the purified compounds, MTD5 showed good activity when compared to MTD1. MTD5 has two methyl groups in the naphthyridine ring and two hydroxyl groups are present at 3\textsuperscript{rd} and 7\textsuperscript{th} position of naphthyridine ring. It also contains two carboxylic acid groups at 2\textsuperscript{nd} and 8\textsuperscript{th} position of the ring. Naphthyridine ring contains nitrogen atom, which is very similar to chloroquine molecule. Further, the nitrogen atoms of MTD5 are hydrophilic and able to undergo protonation at lower pH value such as that occurring in the parasitic digestive vacuole (pH 5.5), and thus accumulate in higher concentration by pH-trapping mechanism. The hydroxyl group, methyl and carboxylic acid groups may help in interaction of MTD 5 with Pf parasites.

The mode of action of pure compounds on Pf remains unknown, but it is almost the same as other antimalarial drugs. It could be stated that, if therapeutically effective concentrations of candidate flavonoids and phenolic compounds could be reached and well tolerated in humans, the effect of these compounds as complementary agents in association with other antimalarial drugs should be tested in the near future. Many attempts have been made to use molecules devoted to reverse resistance by interfering with the ATP-binding site and the cytosolic nucleotide-binding domain (NBD) of P-glycoprotein (Pgp)-like transporter, with little success due to the high concentration required, leading to major adverse effect. Chalcone, flavones and other flavonoids substructures have been demonstrated to bind to the C-terminal nucleotide binding domain (NBD2) of Pgp and to possess MDR reversing activity (Conseil et al., 1998; Maitrejean et al., 2000; Hadjeri et al., 2003). The compound 3, 5, 7 – trihydroxy-4- methoxy flavone, has been showed to antagonize nucleotide binding and this is probably related to the reverting effect on the MDR
phenotype in cancer cells (Boumendjel et al., 2002). It has also been demonstrated that flavone binding to Pgp-like transporter of Leishmania tropica is responsible for reversal of daunomycin resistance of this parasite (Perez-Victoria et al., 2001; Perez-Victoria et al., 2002). Therefore, the activity of MTD1, MTD2, MTD3, MTD4 and MTD5 may be attributed to partial isomerization to other forms under the experimental (physiological) conditions.

In summary, here we have demonstrated that five purified compounds (MTD1, MTD2, MTD3, MTD4 and MTD5) and two crude extracts (methanol and chloroform) of M. terminale Dalz have a direct antimalarial activity on 3D7 strains of Pf adapted culture. It could be speculated that the efficacy of traditional medicine could partly be due to the wide distribution of flavonoids and phenolic compounds in these plants. New safe and inexpensive drugs against Plasmodium species are badly needed and natural products isolated from plants, used in association with current drugs, which could help to delay the spread of resistance.
6C.5 Tables and Graphs

Figure 6C.1 Anti-plasmodial activity of different extracts of *M. terminale* Dalz

![Graph showing anti-plasmodial activity of different extracts]

Effect of increasing concentration of different plant extracts of *M. terminale* Dalz and standard chloroquine on the *in vitro* growth of chloroquine-sensitive (3D7) *Plasmodium falciparum*. Parasitized red blood cells were exposed for 48 h to increasing concentrations of plant extracts – A (11.5 μg/mL), B (23 μg/mL), C (46.3 μg/mL), D (92.5 μg/mL), E (185 μg/mL) and F (370 μg/mL). Results are expressed as % survival as compared to control. The value represents the mean of experiments done three times at different concentrations of the plant extracts in triplicate.
Table 6C.1 The IC$_{50}$ values of crude plant extracts, standard drug and purified compounds.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Compounds</th>
<th>IC$_{50}$ values in µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Petroleum ether extract</td>
<td>740</td>
</tr>
<tr>
<td>2</td>
<td>Chloroform extract</td>
<td>370</td>
</tr>
<tr>
<td>3</td>
<td>Methanolic extract</td>
<td>92</td>
</tr>
<tr>
<td>4</td>
<td>MTD1</td>
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</tr>
<tr>
<td>8</td>
<td>MTD5</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>Standard Drug (Chloroquine)</td>
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</tr>
</tbody>
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