INTRODUCTION

Cardiovascular disease (CVD) is the primary cause of mortality in the United States, Europe and much of Asia (Braunwald, 1997). Recently, dietary patterns in many countries have become Westernised after the rapid growth of their economy. Therefore, the number of people with hyperlipidemia, CVD and large intestinal cancer has increased rapidly (Kritchevsky, 1995; Krauss et al., 1998). Similar happenings have also been reported in developing countries and indeed, the burden of CVD is on the alarming rise in India. It is estimated that coronary heart disease (CHD) will be the single most important cause of death in India by the year 2015 (Reddy, 1993). In Indian urban communities, a high prevalence of CHD approaching those in developed countries have been reported (Gupta et al., 1997). The estimated prevalence of CHD is around 3–4% in rural areas and 8–10% in urban areas and among adults older than 20 years, a two fold rise in rural areas and a six-fold rise in urban areas have been recorded over the past four decades. About 29·8 million people were estimated to have CHD in India in 2003 with 14·1 million in urban areas and 15·7 million in rural areas (Ghaffer et al., 2004). The prevalence of stroke is thought to be 203 per 100 000 population among people older than 20 years (Anand et al., 2001). CVD, including CHD, stroke and peripheral vascular disease, is the clinical expression of advanced atherosclerosis (Levy, 1981).

1.1 Atherosclerosis

Atherosclerosis comes from the Greek words ‘athero’ (meaning gruel or paste) and ‘sclerosis’ (hardness). It is the name of the process in which deposits of fatty substances, cholesterol, cellular waste products, calcium and other substances build up in the inner lining of an artery, leading to plaque formation.
It usually affects large and medium-sized arteries. The effects of atherosclerosis differ, depending upon which arteries in the body get narrower and become clogged with plaque. For example, plaque built up in the vessels that supply the heart with oxygen-rich blood may cause chest pain and heart attacks, while plaque built up in the arteries that supply blood to the brain and spinal cord may result in stroke.

In a broad outline, atherosclerosis is a complex multifactorial inflammatory disease, characterized by focal intimal thickening of medium and large sized arteries. These are thought to be initiated by accumulation of lipoproteins within the intima, adhesion of monocytes to the arterial endothelium, emigration of monocytes into the intima, chemotactic stimuli by oxidized lipoproteins and accumulation of cholesterol within macrophages (Ross, 1993). Growth factors, cytokines and other vasoactive substances secreted by macrophages, smooth muscle cells and endothelial cells influence the further progression of atherosclerosis (St Clair, 1997).

1.1.1 Stages of atherosclerosis

The lesions of atherosclerosis can be arbitrarily divided into the following stages: endothelial dysfunction, fatty streak, fibrous plaque and plaque rupture and thrombosis.

Endothelial dysfunction

Numerous pathophysiologic observations in humans and animals led to the formulation of the response-to-injury hypothesis of atherosclerosis, which initially proposed that endothelial denudation was the first step in atherogenesis (Ross and Glomset, 1973). The vascular endothelium plays a central role in
mediating vascular tone and blood flow. Nitric oxide (NO) is the primary vasodilator, and angiotensin II and endothelin-1 are the primary vasoconstrictors secreted by endothelial cells. In addition to regulating vascular tone, the endothelium acts as a barrier that inhibits the inflammatory response, platelet activation, and thrombosis. Most of these effects are mediated by NO, which, in addition to its vasodilatory effect, also reduces leukocyte adhesion and inhibits smooth muscle proliferation and platelet aggregation (de Graaf et al., 1992; Cornwell et al., 1994). An early event in the arterial wall of cholesterol fed animals is the adherence of mononuclear cells to the endothelial cells (Watanabe et al., 1985). Later, it was found that mononuclear cell adherence is triggered by a number of adhesion molecules on endothelial cells such as vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), P-selectin and E-selectin (Cybulsky and Gimbrone, 1991; Kume et al., 1992; Nie et al., 1997). Increased expression of these molecules is considered to be responsible for the adherence of T lymphocyte and monocytes to endothelium (Nagel et al., 1994; De Caterina et al., 1995). These adhesion molecules are up-regulated by elevated levels of atherogenic lipoproteins and cytokines in vitro (Kume et al., 1992). Although, no histopathologic lesion is visible, disruption of the normal function of the vascular endothelium may be the earliest stage of atherosclerosis.

**Fatty streak formation**

The development of "foam cells" (macrophage with massive amounts of cholesterol esters) is a hallmark of both early and late atherosclerotic lesions. Cholesterol accumulation in these cells is thought to be mediated primarily by uptake of modified forms of low density lipoprotein (LDL) via so called scavenger receptors (Yamada et al., 1998). The accumulation of foam cells
represents the bulk of the lesion and occupies two to five or six layers in the intima of the artery, forming the fatty streak which give a yellow discoloration. Fatty streaks are also composed of subendothelial accumulations of macrophages and T-lymphocytes. In the presence of LDL and oxidized LDL (oxLDL) particles, endothelial cells produce several types of adhesion molecules that bind monocytes and T-lymphocytes and promote migration of leukocytes into subendothelial cells (Nagel et al., 1994; De Caterina et al., 1995). The mechanism of formation of fatty streaks is shown in Figure 1.1.

![Figure 1.1 Development of fatty streak lesion (Glass and Witztum, 2001)](image)

On the surface of endothelial cells, molecules from the selectin family mediate the initial interaction between endothelial cells and leukocytes. Subsequently, firm anchoring of leukocytes to the endothelial surface is mediated by endothelial ICAM-1 and VCAM-1, which is up-regulated by accumulation of modified LDL. (Nagel et al., 1994; De Caterina et al., 1995). Once tightly bound
to the endothelial surface, leukocytes travel into the subendothelial space in response to another set of molecular signals. This subendothelial migration is induced by the presence of bioreactive mediators called chemoattractants in the intima. Chemoattractant such as monocyte chemoattractant protein-1 (MCP-1) encourages transmigration of monocytes and T lymphocytes across the endothelial barrier (Wang et al., 1988; Mach et al., 1999). It has also been shown to be capable of inducing chemotaxis of oxLDL, cytokines and degraded collagens and elastins (Ross, 1997). Lymphocytes within the subendothelial space also interacts with oxLDL, leading to activation and thereby produces a number of cytokines, including CD154, interleukins and tumor necrosis factor (TNF).

Fibrous plaque formation

Endothelial cells, leukocytes and smooth muscle cells were found to secrete inflammatory cytokines and growth factors, as lipid-laden foam cells continue to accumulate and this in turn recruits more leukocytes and stimulates additional smooth muscle cell migration and proliferation. In addition, the macrophage colony stimulating factor (M-CSF) produced by endothelial and smooth muscle cells amplifies the entire process, paving way to further macrophage proliferation in the fatty streak and formation of atherosclerotic lesions (Qiao et al., 1997). As inflammatory cells continue to accumulate, the fatty streak is transformed into a fibrous plaque, which is clearly pasteurized in Figure 1.2. The fibrous plaque is covered by a cap formed by large numbers of smooth muscle cells surrounded by a dense connective tissue matrix and scattered macrophages. Two kinds of common plaques can be morphologically differentiated and are clinically relevant: stable and unstable plaque. Stable plaque is composed of a small lipid core and is covered by a thick fibromuscular
cap with more smooth muscle cells and extracellular matrix and this is the major risk for stenosis or occlusion when it causes a significant reduction of the vascular lumen. Unstable plaque contains a large pool of lipid core, and a thin cap with a large number of inflammatory cells. Morphologically, unstable plaque is fatal and the outcome of its formation is ominous, because regardless of its size, it leads to acute coronary syndrome and is associated with thrombosis (Fan and Watanabe, 2003).

![Diagram of vascular lumen and endothelial cells with labels for fibrinogen, fibrous cap, fibronectin, collagen, macrophages, and Th1 and Th2 cells.]

**Figure 1.2  Formation of fibrous plaque (Glass and Witztum, 2001)**

Intimal hyperplasia occurs as smooth muscle cells transmigrate and proliferate under stimulatory pressure by cytokines (i.e., interleukin-1 (IL-1)), transforming growth factor-β (TGF-β), TNF-alpha (TNF-α) and fibroblast growth factor) produced by activated T cells, foam cells, and dysfunctional endothelium. Some of the recruited smooth muscle cells undergo apoptosis, and the cellular debris attracts even more inflammatory scavenger cells into the developing plaque. Platelet activation is augmented by decreased basal levels of NO and prostacyclin in endothelium and by microfractures in the fibrous cap that
enable the accumulated tissue factor secreted by foam cells to be exposed to circulating cells. In turn, activated platelets further stimulate the production of MCP-1 as well as increase the expression of ICAM-1 on the endothelium via induction of nuclear factor κB (NF-κB), which can also be upregulated by IL-1, TNF, and oxidative and mechanical stressors (Gawaz et al., 1998).

Plaque rupture and thrombosis

As the inflammatory process continues, the atherosclerotic plaque can become larger and histologically more complex. Plaque rupture and thrombosis are the notable complications of advanced lesions that lead to either unstable coronary syndromes or myocardial infarction (Davies, 1990; Ross, 1993; Falk et al., 1996). Figure 1.3 highlights the consequent events as a result of plaque rupture and thrombosis. Small thrombi can become organized and incorporated into the plaque. Atheroma growth can also be mediated by deep fissuring or rupture of the fibrous cap, which exposes the highly thrombogenic lipid core to blood flow. Although plaque rupture is the most common cause of acute coronary syndrome, most ruptures are probably not associated with symptoms but lead to sudden growth and the development of a complex advanced atherosclerotic lesion as a result of incorporation of a relatively large mural thrombus (Yokoya et al., 1999). Finally, plaque size can also increase if small microvessels that have developed within the atherosclerotic plaque become disrupted and bleed. Microvessels form from secretion of fibroblast growth factor and vascular endothelial growth factor from macrophages and other inflammatory cells within the atherosclerotic lesion (Ramos et al., 1998). Regardless of the source, thrombus within the atherosclerotic lesion further amplifies the inflammatory process. Thrombin generation besides stimulating smooth muscle proliferation and migration, also triggers platelet release of
growth factors such as platelet derived growth factor and TGF-β. The latter stimulates smooth muscle cell production of collagen and resorption of the thrombus, which leads to further release of platelet derived growth factor and TGF-β (Selwyn, 2003).

![Figure 1.3 Plaque rupture and thrombosis (Glass and Witztum, 2001)](image)

1.2 Risk factors for development of atherosclerosis

Atherosclerosis correlates with cardiovascular risk factors revealed in epidemiological studies. Numerous epidemiological studies have suggested that there are several genetic and environmental risk factors involved in the development of atherosclerosis (Glass and Witztum, 2001). They are

Factors with a significant genetic component

- Hypercholesterolemia (elevated levels of LDL and VLDL / low levels of HDL)
- Hypertension
- Diabetes Mellitus
- Elevated levels of homocysteine
- Elevated levels of hemostatic factors like fibrinogen
- Obesity
- Family history

**Environmental Factors**

- Smoking
- Lack of exercise
- High fat diet
- Infectious agents

Elevated levels of serum cholesterol referred to as hypercholesterolemia is a dominant risk factor for the development and progression of atherosclerosis and related cardiovascular diseases in humans and experimental animals, even in the absence of other known risk factors (Prasad and Kalra, 1993; Farmer and Gotto, 1997; Glass and Witzum, 2001; Deepa and Varalakshmi, 2005).

### 1.3 Hypercholesterolemia

Hypercholesterolemia is a common clinical disorder that may begin early in life in humans, and it subsequently promotes atherogenesis by injuring the vascular wall. The underlying mechanisms for these deleterious effects involve a local inflammatory response and release of cytokines and growth factors in the vascular wall. Consequent activation of oxidation-sensitive mechanisms in the arterial wall, modulation of intracellular signaling pathways, increased oxidation of LDL and quenching of NO can all impair the functions
controlled by the vascular wall and lead to the development of atherosclerosis (Napoli et al., 2001). Hypercholesterolemia induced microvascular alterations can be demonstrated in animal models within a few days after the placement of diet enriched with cholesterol, i.e., long before the appearance of fatty streak lesions in large arteries (Scalia et al., 1998; Stokes et al., 2001).

Hypercholesterolemia has systemic effects in various tissues. It increases the incidence of ischemic disease in tissues such as heart and brain by virtue of its ability to restrict blood flow in lesion-prone arteries and also promote ischemic tissue damage by enhancing vulnerability of the microcirculation to the deleterious effects of ischemia and other inflammatory stimuli (Stokes et al., 2002). Feeding of cholesterol diet produces severe hypercholesterolemic and vascular atherosclerotic lesion and increased oxidative stress in several tissues (Balkan et al., 2002a,b). Hexeberg et al. (1993) observed that there is intracellular lipid accumulation in cardiomyocytes and several alterations in the structure and functional properties of the myocardium in high cholesterol fed rats. Occlusion of a coronary artery by lipid deposits can cause severe local oxygen starvation and ultimately the degeneration of a localized portion of the heart muscle.

Hypercholesterolemia generated by a diet with high levels of cholesterol also causes fat deposition in the liver, hepatic steatosis or hypertrophy of the liver and depletion of hepatocyte population. It also causes the malfunction of liver, which is apparently presented through microvesicularstenosis due to the intracellular accumulation of lipids (Gupta et al., 1976; Assy et al., 2000).

Recently, lipids have been reported to play a potential role in the pathogenesis of progressive glomerulosclerosis (Atarashi et al., 1999; Ishiyama
et al., 1999). It has been demonstrated that the short term experimental hypercholesterolemia caused by cholesterol feeding leads to spontaneous arterial constriction and/or increased reactivity to various vasoconstrictors, which leads to renal vasoconstriction and associated decrease in glomerular filtration rate in normal rats, (Rossitch et al., 1991; Chowienczyk et al., 1992; Casino et al., 1993) thereby leading to ischemic renal failure. Further, it has been shown that hypercholesterolemia induces vasoconstriction by altering the metabolism of NO (Creager et al., 1990; Ohara et al., 1993). Alterations in membrane lipid composition influences membrane fluidity, cation transport and Na⁺, K⁺-ATPase activity in all cells of the organism, which can also predispose renal tubular cells to the injury of hypoxia. This may be an additional mechanism explaining the possible deleterious role of hypercholesterolemia in ischemic renal failure (Broderick et al., 1989; Lijnen et al., 1994).

1.3.1 Hypercholesterolemia and oxidative stress

A growing body of evidence suggests that numerous pathological conditions are associated with an increased vascular production of reactive oxygen species (ROS) (Harrison and Ohara, 1995; Kojda and Harrison, 1999). This form of pro-oxidant shift in vascular redox status, commonly known as oxidative stress, represents a common pathological mechanism activated by many cardiovascular risk factors (Kojda and Harrison, 1999). Cholesterol, one of the major risk factors in the genesis of atherosclerosis, is suggested to exert a pro-oxidant effect. The overproduction of ROS has been implicated in the pathology of atherosclerosis. According to "response-to-injury" theory of the genesis of atherosclerosis, endothelial cell injury is the basic mechanism for initiation and maintenance of atherosclerosis (Ross, 1986). Hypercholesterolemia
has been reported to cause endothelial injury (Ross and Harker, 1976) by its increased production of ROS (Prasad and Kalra, 1993).

Hypercholesterolemia may enhance formation of lipid peroxidative compounds which are formed when ROS react with increased levels of plasma and tissue lipids. The precipitating events in radical generation during the evolution of atherosclerosis, appear to involve early injury to the vascular endothelial layer (Hennig and Chow, 1988). Penetration and accumulation in arterial wall of triglycerides-rich lipoproteins, including VLDL and chylomicrons, activates the synthesis of factors like plasminogen activator factor 1, protein kinase C and NF-κB that then can initiate the inflammatory responses (Stemerman, 2000). Release of cytokines and growth factors (Ross, 1999) may in turn stimulate ROS producing enzymes like nicotinamide adenine dinucleotide phosphate-reduced (NADPH) oxidase, the major source of superoxide anion in vascular cells and myocytes (Griendling et al., 2000), xanthine oxidase (Cardillo et al., 1997), cyclooxygenase (COX), NO synthase, myeloperoxidase (Berliner and Heinecke, 1996) and lipoxygenase (Kunsch and Medford, 1999). This cascade may also involve local activation of renin-angiotensin system which can promote oxidation via angiotensin II-induced stimulation of NADPH oxidase (Rajagopalan et al., 1996). In hypercholesterolemia, an important role for increased production of ROS is also ascribed to xanthine oxidase, which due to an increase in plasma cholesterol, is released into the circulation where it binds to endothelial cells and sustains production of superoxide radicals (White et al., 1996). LDL, the major carrier of blood cholesterol, becomes atherogenic as a result of oxidation (Heinecke, 1998). Oxidised LDL then possesses greater efficacy in initiation of superoxide production and endothelial dysfunction (Hein and Kuo, 1998)
In hypercholesterolemia, platelets, polymorphonuclear leukocytes (PMNLs), endothelial cells, monocyte etc., were found to act as cellular sources for the production of ROS. Hypercholesterolemia increases cholesterol content of platelets and endothelial cells (Stuart et al., 1980; Gorog and Kakkar, 1987). Cholesterol enhances platelet function and cholesterol rich platelets released substances, which include thrombin, histamine and adenosine diphosphate (ADP) (Shattil et al., 1975; Henry, 1977). Histamine and ADP activate phospholipase A$_2$ (Ruzicka and Printz, 1984), which acts on phospholipids to release arachidonic acid. Increase in phospholipase A$_2$ activity may also arise from increase in intracellular calcium (van den Bosch, 1980), which accompanies hypercholesterolemia (Le Quan - Sang et al., 1987). Activation of phospholipase would result in increased synthesis of prostaglandins and leukotrienes in various cells along with the release of arachidonic acid. Increased production of thromboxane A$_2$ and prostacyclin in aorta in experimental atherosclerosis has been reported (Henriksson et al., 1987). The intermediate steps (conversion of prostaglandin G$_2$ to prostaglandin H$_2$) in the biosynthesis of prostaglandins from arachidonic acid produce ROS (Panganamala et al., 1976). This in turn leads to tissue injury by initiating lipid, protein and DNA oxidative modifications. According to the evidences presented above, it can be hypothesized that hypercholesterolemia may increase production of ROS and lead to endothelial cell injury, which sets the stage for atherosclerosis (Prasad and Kalra, 1993).

Oxysterols are oxygenated derivatives of cholesterol, and were found to be increased in the plasma of cholesterol diet fed rabbits (Mahfouz et al., 1997). This may be formed exogenously by autoxidation of cholesterol and endogenously by free-radical attack upon cholesterol or by enzymatic processes (Lyons and Brown, 2001). Oxysterols represent one of the primary pro-atherogenic components of cholesterol-rich diet (Staprans et al., 1998). The
biological effects attributable to oxysterols include inhibition of the enzyme hydroxymethylglutaryl-coenzyme A reductase (HMG-CoA reductase) leading to reduced endogenous cholesterol synthesis, alteration in cellular membrane properties and the induction of cell death in a number of in vitro models (Guardiola et al., 1996). Of the biological effects attributable to oxysterols, apoptosis is the main factor, which is evident in a variety of cell lines (Ryan et al., 2004).

1.3.2 Hypercholesterolemia and inflammation

There are numerous evidences showing that inflammatory processes play a central role in the pathogenesis of atherosclerosis (Ross, 1986). Arterial inflammation may be initiated and sustained by several proinflammatory risk factors and it causes endothelial dysfunction which leads to compensatory responses that alter the normal properties of the endothelium (Alexander, 1994). Hypercholesterolemia has long been appreciated, that large arteries assume an inflammatory phenotype during the development of atherosclerotic lesions and only recently it has been understood that hypercholesterolemia also induces phenotypic changes in microcirculation that are consistent with oxidative and nitrosative stresses and inflammatory response (Stokes et al., 2002).

In the arterial wall, LDL particles may be modified and taken up by macrophage scavenging receptors, which leads to the foam cell formation and initiation of an inflammatory response (Steinberg, 1997; Ross, 1999). Atherosclerotic plaques exhibit significant infiltration by activated macrophages and T-cells (van der Wal et al., 1989; Kaartinen et al., 1996). These inflammatory cells in atherosclerotic plaques release matrix-degrading enzymes and thrombogenic substances that may provoke plaque disruption and local
thrombosis (Henney et al., 1991; Galis et al., 1994; Moreno et al., 1996). Thus, the local inflammatory process may be critically responsible for plaque destabilisation, manifesting clinically as acute ischemic stroke (Moreno et al., 1996; DeGraba et al., 1998). The process of atherosclerosis involves a number of cell types like monocytes, macrophages, endothelial cells, platelets, lymphocytes and smooth muscle cells, as well as intercellular signalling that is mediated by cytokines, growth factors, vasomotor molecules and adhesion molecules (Ross, 1993 and 1999). The proinflammaory risk factors leads to local (vascular) and systemic (extravascular) inflammation that triggers the production of inflammatory cytokines (IL-1β, TNF-α and IL-6), adhesion molecules (ICAM, VCAM and selectins) and acute phase proteins (CRP, fibrinogen and serum amyloid A) from the cells involved in atherosclerotic process (Saadeddin et al., 2002). Further, it has also been demonstrated in our laboratory that the proinflammatory markers have systemic effects in atherogenesis (Deepa and Varalakshmi, 2006).

It has been suggested that NF-κB is involved in early as well as in later stages of the inflammatory-proliferative process of atherogenesis (Berliner et al., 1995; Brand et al., 1996; Zhou et al., 2003). NF-κB is a well-known transcription factor, activated by oxidative stress. NF-κB is composed of two DNA-binding subunits, p50 and p65, and it is associated with the regulation of numerous genes encoding proteins in immune function, inflammation and cellular growth control (Grimm and Baueuerle, 1993). Dysregulation of the NF-κB system is likely to play an important role in acute or chronic inflammatory diseases, such as sepsis, Crohn's disease and rheumatoid arthritis (Baueuerle and Henkel, 1994). Under stressed conditions in endothelial cells, activation of NF-κB leads to the expression of cell adhesion factors such as
VCAM-1 and ICAM-1 (Sen and Packer, 1996; Geng et al., 1997). These events are known to further accelerate the formation of atherogenic lesions and cell death.

1.3.3 Hypercholesterolemia and nitrosative stress

Vascular function is compromised under conditions of inflammation and atherogenesis (Tsao and Cooke, 1998; Bogle et al., 2000). Defects in lipoprotein metabolism and vascular reactivity are the fundamental pathological responses to hypercholesterolemia, with extensive evidences suggesting that reactive nitrogen species (RNS) play an important role in the initiation and progression of these lesions (Panasenko et al., 1991; Berliner et al., 1995). Hypercholesterolemia is a central pathogenic factor of endothelial dysfunction caused in part by an impairment of endothelial NO. NO exhibits a wide spectrum of functions in both normal and pathological states. Physiological amounts of NO synthesized by constitutive nitric oxide synthase (NOS) isoforms (endothelial NOS and neuronal NOS) participates in neurotransmission and cardiovascular signaling (Goldstein et al., 1996), while excessive amounts of NO released by inducible NOS (iNOS) may preserve endothelial function in the setting of hypercholesterolemia, whereas increased superoxide generation may result in abnormal endothelium-dependent relaxation with the generation of powerful pro-oxidant peroxynitrite (Beckman et al., 1990), which is detected in atherosclerotic lesions (White et al., 1994; Buttery et al., 1996). Peroxynitrite may promote atherogenesis by reducing the beneficial physiological actions of NO and oxidizing lipoproteins (Darley-Usmar et al., 1992; White et al., 1994) and cytotoxic molecule that cause cell damage and injury (Freeman, 1994). The iNOS expression is induced by the activated NF-κB, since iNOS gene contains functional NF-κB binding sites in its promoter (Xie et al., 1993; Kim et al.,
1997). iNOS-derived NO overproduction appears to be a ubiquitous mediator of vascular inflammatory conditions, including atherogenesis (Gross and Wolin, 1995). Aliev et al. (2000) have reported an increased iNOS immunoreactivity in cholesterol enriched diet fed rats.

**HYPERCHOLESTEROLEMIA**

- Low - density lipoproteins
  - ROS
  - Oxidised LDL ➔ Endothelial dysfunction
  - Monocyte uptake of oxidised LDL ➔ Monocytes and platelet adherence
  - **Foam cells formation** ➔ Release of growth factor and cytokines
  - Plaque remodeling ➔ Smooth muscle cell activation
  - Plaque rupture ➔ Atherosclerotic plaque
  - Thrombosis ➔ Atherosclerosis

**Figure 1.4**  **Key events in the development of atherosclerosis**

(adapted from Vogel, 1999)
1.4 Cholesterol Lowering Therapy

Traditional approaches in treating CVD have largely failed. Too often, physicians treat only ischemia and ignore the underlying disease process itself—atherosclerosis (Fanorow, 2003). The National Cholesterol Education Program (NCEP) guidelines encourage a trial of lifestyle modification (diet and exercise) before initiation of drug treatment. Although, there are dietary measures in reducing fat intake, they fall short in helping most patients (Stefanick et al., 1998). Numerous studies correlate increased exercise with decreased cardiovascular mortality (Leon and Connett, 1991) and it was found that though aerobic exercise may increase high-density lipoprotein (HDL) levels, its effect in lowering LDL is often minimal to approximately 4 to 6% (Kokkinos et al., 1995). Although, diet and exercise are found to be helpful in meeting LDL goals, these steps are seldom successful without the addition of a pharmacologic agent.

The pharmacological agents such as HMG-CoA reductase inhibitors (statins), ezetimibe, bile acid sequestrants, fibrates and nicotinic acids are widely used for cholesterol lowering. The characteristics and adverse effects of these drugs (Table 1.1) have been reviewed earlier (Knopp, 1999; Schmitz and Langmann, 2006).
Table 1.1 Lipid lowering drugs with its side effects

<table>
<thead>
<tr>
<th>Drug</th>
<th>Lipid effect</th>
<th>Adverse effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statins</td>
<td>• LDL ↓ 30-55%</td>
<td>• Hepatitis</td>
</tr>
<tr>
<td></td>
<td>• HDL ↑ 5-10%</td>
<td>• Weight loss</td>
</tr>
<tr>
<td></td>
<td>• TG ↓ 5-30%</td>
<td>• Increase serum aminotransferases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Myopathy with serum creatine kinase (&gt;1000U/l)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Rhabdomyolysis with renal failure</td>
</tr>
<tr>
<td>Ezitimibe</td>
<td>• Cholesterol absorption ↓ 54%</td>
<td>• Myopathy</td>
</tr>
<tr>
<td></td>
<td>• LDL ↓ 17 – 20%</td>
<td>• Increases serum creatine kinase</td>
</tr>
<tr>
<td>Bile acid</td>
<td>• HDL ↑ 1-4%</td>
<td>• Rhabdomyolysis</td>
</tr>
<tr>
<td>sequestrants</td>
<td>• TG ↓ 2-5%</td>
<td>• Pancreatitis</td>
</tr>
<tr>
<td></td>
<td>• LDL ↓ 10-20%</td>
<td>• Diminish absorption of lipophilic vitamins</td>
</tr>
<tr>
<td></td>
<td>• HDL ↑ 2-5%</td>
<td>• Constipation</td>
</tr>
<tr>
<td></td>
<td>• TG ↓ 5-10%</td>
<td></td>
</tr>
<tr>
<td>Fibrates</td>
<td>• LDL ↓ 5-20%</td>
<td>• Myopathy with impaired renal function</td>
</tr>
<tr>
<td></td>
<td>• HDL ↑ 10–20%</td>
<td>• Erectile dysfunction</td>
</tr>
<tr>
<td></td>
<td>• TG ↓ 20 - 50%</td>
<td>• Gall stones</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>• LDL ↓ 5 - 20%</td>
<td>• Flushing</td>
</tr>
<tr>
<td></td>
<td>• HDL ↑ 15 – 35%</td>
<td>• Myopathy</td>
</tr>
<tr>
<td></td>
<td>• TG ↓ 20 - 50%</td>
<td>• Hyperglycemia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Hepatitis</td>
</tr>
</tbody>
</table>

Although these drugs were found to be highly beneficial in lowering cholesterol levels (hyperlipidemic condition), their potentials are greatly hampered by adverse side effects. On the other hand, numerous medicinal plants are known for their potent biological and pharmacological applications with lesser/no side effects. Hence, paying greater attention in rediscovering biomolecules with hypolipidemic property from medicinal plants would definitely be a good approach in treating hyperlipidemia.
A novel drug candidate combining a triterpene (lupeol) and \( \omega-6 \) fatty acid (linoleic acid)

Medicinal plants have a long history of use and their use is widespread in both developing and developed countries. In herbal medicine, the term *herbs* is used to refer not only to herbaceous plants but also to bark; roots; leaves; seeds; flowers and fruit of trees and extracts of the same that have valued medicinal qualities (Craig, 1999). Herbal medicines provide rational means for the treatment of many diseases that are inflexible and incurable in other systems of medicine. Medicinal plants are widely used because of their fewer side effects, better patient tolerance, relatively less expenditure and acceptance due to long history of use. Medicinal plants are renewable in nature unlike the synthetic drugs that are obtained from non-renewable sources of basic raw materials such as fossil sources and petrochemicals (Samanta *et al.*, 2000). Cultivation and processing of plants often is environment friendly unlike the pollution by chemical industry. Cultivation of medicinal plants can also be a source of income for poor families. Also, plants are often less prone to the emergence of drug resistance. Due to all these advantages, medicinal plants are gaining popularity and continue to be a major source of new lead compounds.

Traditional plant-derived medicines are being increasingly utilized to treat and prevent many diseases. A variety of herbs and herbal extracts contain different phytochemicals with biological activity that can provide therapeutic effects. Research interest has been focused on various herbs that possess hypolipidemic, antiplatelet and anti-inflammatory properties, which may be useful adjuncts in helping to reduce the risk of cardiovascular disease. In different herbs, a wide variety of active phytochemicals, including the flavonoids, terpenes, lignans, sulfides, polyphenolics, carotenoids, coumarins,
saponins, plant sterols, curcuminoids, and phthalides have been identified (Craig, 1999). Keeping this in consideration, the present study has been designed to appraise the antiatherosclerotic role of pentacyclic triterpene, lupeol isolated from stem bark of *Crataeva nurvala*.

1.5.1 Triterpenes

Triterpenes are a wide-spread group of natural compounds with considerable practical significance. In our everyday life we all encounter either directly or indirectly various terpenes, such as mono- and sesqui-terpene components of essential oils, which contribute to the aroma of plants, triterpenes of different types found in all higher plants, tetraterpene carotenoids that are abundant in our daily food and polyterpenes, of which the most important is latex, used in the manufacture of rubber and other rubber components. Almost all terpenes have some biological activities in animals including man and also play a meaningful role in human medicine (Patocka, 2003).

Most important group of terpenes are triterpenes and triterpene glycosides, (also known as saponins), representing one of the numerous classes of natural compounds. There is a growing interest in natural triterpenes caused as much by the scientific aspects of extraction and structural analysis of these compounds. Triterpenes represent a very large class of plant secondary metabolites, which have been demonstrated to exhibit a variety of biological activities. Triterpenes may be divided into more important chemical structure families. The main groups of triterpenes and their glycosides are represented by tetracyclic derivatives and pentacyclic derivatives.
1.5.2 Pentacyclic triterpenes

Pentacyclic triterpenes have a basic skeleton of 30 carbon atoms. They are biosynthetic derivatives of isoprene, via squalene, and they are found in relatively high proportions in plant waxes (Moyna et al., 1983). They are also found in many vegetarian foods and have been used in the traditional medicine of many Asian countries because of their minimal toxicity (Liu, 1995; Novotny et al., 2001; Shishodia et al., 2003; Cichewicz and Kouzi, 2004). The naturally occurring pentacyclic triterpenes are amyrin, betulin, betulinic acid, oleanolic acid, ursolic acid, 18-α-glycyrrhetinic acid and lupeol. The structures of medicinally important triterpenes are given in figure 1.5. Ursolic, betulinic and oleanolic acids have been subject of interest in oncology because they exhibit antineoplastic activity on several kinds of cancers, including inhibition of tumorogenesis, antimitogenic, antiangiogenic and antiviral properties (Huang et al., 1994; Li et al., 2002). Moreover, it has also been demonstrated that these natural products exert anti-inflammatory effects, such as the inhibition of the key enzymes lipoxygenase, cyclooxygenase 2 (COX-2) and iNOS (Suh et al., 1998; Subbaramaiah et al., 2000), as well as hepatoprotection in humans (Liu, 1995). Triterpenoids are also found to suppress the transcription nuclear factor NF-κB (Takada and Aggarwal, 2003). Studies conducted in many scientific centers have made use of many compounds of this category- lupeol, oleanolic acid and ursolic acid (Abreu and Branco, 2003; Patocka, 2003).
Figure 1.5 Structure of pentacyclic triterpenes
1.6 *Crataeva nurvala*

*Crataeva nurvala* Buch Ham (Capparidaceae) is one of the medicinal plants recorded in the Indian system of Medicine (Nadkarni, 1976). The pentacyclic triterpene lupeol was isolated earlier in our laboratory from stem bark of *Crataeva nurvala* (Baskar *et al*., 1996). It has been found to possess diverse pharmacological properties (Della-Loggia *et al*., 1994; Kweifio-Ogio *et al*., 1995; Akihisa *et al*., 1996; Guevara *et al*., 1996; Geetha *et al*., 1998).

**General Characteristics of *C. nurvala* Buch Ham**

**Family** : Capparidaceae

**Synonyms** : *C. religiosa* Forst; *C. roxburghii* Br; *C. Odora*; *C. unilocularis* Ham; *Capparis trifoliate*; *C. Magma* (Lour) DC.

**English Name** : Three leaved caper

**Sanskrit Name** : Varuna, Asmarighna

**Hindi Name** : Varuna, Baruna, Barun, Bilasi, Bila

**Tamil Name** : Mavilangam, Marvilangam, Maralingam

The plant is found throughout India, growing wild as well as cultivated, especially along river banks. It has been recorded as indigenous to Chennai, Kerala and Karnataka (Aiyer and Kolammal, 1964).

* C. nurvala* is a medium sized deciduous and much branched tree (Figure 1.6). The parts used for medicinal uses are bark and leaves. The
Figure 1.6 *Crataeva nurvala* leaves and flowers

Figure 1.7 *Crataeva nurvala* stem bark
macroscopic feature of the bark shows that it is about 6-15 cm in length and 5-12 mm in thickness (Figure 1.7). The thinner bark pieces are channelled, but the thicker pieces are flat and slightly curved. Microscopically, the mature bark is characterized by a sclerosed cork, followed by a zone of phelloderm, composed of stone cells and parenchyma. The secondary phloem consists of sieve tubes, companion cells, phloem parenchyma, stone cells and a very few phloem fibres, traversed by medullary rays. In the outer and middle region, sieve tubes get crushed forming keratenchyma. Some are moderately thick-walled and possess simple pits; others are highly thick-walled with radiating canals and are circular, elliptical or elongated like fibres.

1.6.1 Chemical constituents of plant

Ceryl alcohol, friedelin, betulinic acid, diosgenin and lupeol from the bark; cetyl alcohol, ceryl alcohol, tricontane, tricontanol, β-sitosterol and glucocapparin have been isolated from the fruit. The root bark of C. nurvala was found to possess lupeol, lupeol acetate, α-spinasterol acetate, ψ-taraxasterol, 3-epilupeol and β-sitosterol as the major constituents, while lupenone, β-sitosterol acetate, sugars, tannins and flavonoids as the minor constituents (Lakshmi and Chauhan, 1975).

1.7 Lupeol

Lupeol [lup-20(29)-en-3β-ol] is a naturally occurring triterpene found in various fruits and vegetables of many medicinal plants (Proitti et al., 1981; Calle-Alvarez et al., 1983; Sturm et al., 1996). A significant quantity of the compound is present in the Crataeva nurvala stem bark. The compound has been shown to possess various pharmacological properties. It has been shown to act as a strong anti-inflammatory (Della loggia et al., 1994; Akihisa et al., 1996),
antiarthritic (Kweifio-Okio et al., 1995; Geetha et al., 1998) antimutagenic and antitumor agent in various animal model systems (Kingston and Munjal, 1978; Guevara et al., 1996).

1.7.1 Pharmacological action of lupeol

Lupeol is known to possess many properties of pharmacological importance. Oleanolic acid and ursolic acid which are structurally similar to lupeol, have been reported to possess hypoglycemic, antihyperlipidemic (antiatherosclerotic) and antioxidant activities (Somova et al., 2003). Oleanolic acid (50mg/kg, p.o. for 9 days) also decreased the elevated blood cholesterol and LDL levels by more than 40% in experimental rats (Liu et al., 1987). Ursolic acid prevented experimental atherosclerosis by lowering blood cholesterol (44%) and β-lipoprotein levels (50%) (Parafenteva, 1979). Lupeol has been shown to possess LDL protective activity during LDL oxidation studies performed in in vitro condition (Andrikopoulos et al., 2003). Lupeol is also known to exhibit hypotensive activity (Saleem et al., 2003). Lupeol administered orally at a dose of 2 g/kg body weight produced no adverse effects in rats and mice, and after 96 h of observation no mortality was recorded, which indicated its very low toxicity (Malini et al., 1995).

Antioxidant therapy may be useful to counteract the effects of oxidative stress on blood vessels, arterial pressure and low-density lipoproteins (Digiesi et al., 2001). Lupeol suppressed N-formyl-methionyl-leucyl-phenylalanine and arachidonic acid induced superoxide generation in human neutrophils. This effect might be due to the –CH₃ group present in the C-17 position of the lupeol structure (Yamashita et al., 2002). Earlier in our laboratory, Nagaraj et al. (2000) showed the protective effect of lupeol on lipid peroxidation (LPO) and
antioxidant status in rat kidney after chronic cadmium exposure. Shirwaikar et al. (2004) also demonstrated the protective role of lupeol against free radical induced nephrotoxicity in rats. Lupeol also reversed the inhibition of the activities of several antioxidant enzymes such as catalase, glutathione peroxidase, glucose-6-phosphate dehydrogenase, glutathione reductase and glutathione-S-transferase. In addition, lupeol ameliorated increased generation of LPO and hydrogen peroxide and depleted levels of glutathione. This further suggests the antioxidant role of lupeol against oxidative stress, during the early stages of tumor promotion (Saleem et al., 2001; Saleem et al., 2004).

Beneficial effects of pentacyclic triterpenes in atherosclerosis extend probably beyond their cholesterol lowering and antioxidant actions which may be particularly important in protecting vessel wall and preventing acute vascular episodes. Many studies have been reported indicating that triterpenes possess anti-inflammatory properties (Kapil and Sharma, 1995; Safayhi and Sailer, 1997; Cantrell et al., 1999). Inhibition of the iNOS and COX-2 enzyme activities have been observed with some triterpenes (Suh et al., 1998). Lupeol was found to inhibit cytokine production (TNF-α and IL - 1β) from lipopolysaccharide treated macrophages performed in in vitro studies. These reports indicate that lupeol may prevent the production of some proinflammatory mediators, which are likely to contribute to its anti-inflammatory activity (Fernandez et al., 2001).

Lupeol possesses a structural resemblance to many triterpenes reported to hold antitumor activity. Lupeol has been used to treat skin ailments. Topical application of lupeol prior to benzoyl peroxide (BPO) treatment, resulted in a significant inhibition of BPO induced cutaneous ornithine decarboxylase activity and a significant reduction in BPO-enhanced [³H] thymidine uptake in cutaneous DNA in a dose-dependent manner (Saleem et al., 2001; Sultana et al., 2003).
Hata et al. (2002) suggested that the carbonyl group at C-17 was essential for the apoptotic effects of lupeol on B16 2F2 melanoma cells. Aratanechemuge et al. (2004) studied the oligonucleosomal-sized DNA fragmentation which resulted from the continuous exposure of HL-60 cells to lupeol. In contrast, they observed no induction of apoptosis by lupeol in normal lymphocytes prepared from healthy volunteers. They concluded lupeol triggered apoptosis in the HL-60 leukemia cells. A strategy to selectively induce apoptosis of leukemia cells without altering healthy cells is a major goal for the development of new therapeutic techniques.

1.7.2 Ester derivative of lupeol

Linoleic acid is an essential ω-6 fatty acid with 18 carbon atoms and two double bonds. Vegetable oils are the primary dietary source of linoleic acid. It is a nutraceutical, which has cardioprotective effect (Foulon et al., 1999). It lowers cholesterol and triglyceride levels and thereby reduces the risk of heart disease and also lowers blood pressure (Chen et al., 1996). Polyunsaturated fatty acids have been shown to lower serum cholesterol levels when replacing saturated fatty acids in the diet (Mensink and Katan, 1989). Earlier studies showed that dietary intake of n-6 polyunsaturated fatty acids are inversely related to the incidence of cardiovascular disease. Dietary linoleic acid has long been accepted as having hypocholesterolemic effect (Keys et al., 1957).

Esterification of lupeol enhanced the efficiency of the parent drug by increasing its bioavailability, penetration and retention ability into the cell membrane (Nikiema et al., 2001). On the same lines, lupeol linoleate has been synthesized, which may be more effective than unmodified lupeol. Lupeol linoleate was synthesized by the method used in this lab earlier (Geetha and
Varalakshmi, 1998). Lupeol linoleate, whose spectrum of anti-inflammatory activity appears to be different from classical NSAIDs, with distinct advantage of its freedom from gastric ulcerogenic effects, is likely to have excellent therapeutic potential (Geetha and Varalakshmi, 2001) in inflammatory conditions.

In our laboratory, the anti-inflammatory and antiarthritic effects of lupeol and its derivative lupeol linoleate were studied (Geetha and Varalakshmi, 1998) and they also have antilithic effect (Vidya et al., 2002). The hepatoprotective effect of lupeol and lupeol linoleate against cadmium toxicity has also been evaluated in our laboratory and the oral administration of triterpene changed the tissue redox system, by scavenging the free radicals and by improving the antioxidant status of the liver (Sunitha et al., 2001). Lupeol and its ester derivative also rendered cardioprotection in cyclophosphamide induced toxicity (Sudharsan et al., 2005 and 2006).
Scope of the Present Study
1.8 SCOPE OF THE PRESENT STUDY

Cardiovascular disease is the primary cause of mortality worldwide (Braunwald, 1997). High cholesterol diet has been reported to adversely affect the health of humans and animal models (Prasad and Kalra, 1993; Ma et al., 1997; Aliev et al., 2000; Han et al., 2002). Hypercholesterolemia is a common clinical disorder that may begin early in life in humans and subsequently leads to the development of atherogenic plaque. It is characterized as a systemic disease which can restrict blood flow in numerous tissues resulting in ischemia. The underlying mechanisms responsible for these abnormalities may emanate from high lipid levels and activation of oxidative and inflammatory mechanisms.

The drugs that encompass hypolipidemic, antioxidative and anti-inflammatory effects can protect from the abnormalities associated with hypercholesterolemia. Dietary changes and the proper use of natural ingredients can have a dramatic effect on plasma cholesterol levels besides the other beneficial effects that improve the patient's overall health. The drugs available in the modern medicinal system are good enough to provide symptomatic relief, but are associated with undesirable side effects. Evaluating the therapeutic potential of medicinal plants on a scientific basis would be very beneficial in treating atherosclerosis. Lupeol, a pentacyclic triterpene and its ester derivative have been reported to possess lipid lowering, cardioprotective, antioxidant, hepatoprotective and anti-inflammatory effects. This encouraged us to evaluate the effect of triterpenes on lipemic-oxidative stress and inflammatory aberrations in hypercholesterolemia.
The present study is aimed to investigate the following:

- Evaluation of the efficacy of triterpenes on lipemic – oxidative stress and inflammatory disorders associated with hypercholesterolemia on the basis of biochemical and molecular assays.

- To study the effects of triterpenes in ameliorating 25-hydroxycholesterol induced toxicity under *in vitro* condition.