3. RESULTS AND DISCUSSION

Atherosclerosis is a chronic inflammatory condition caused by the deposition of lipids in the artery wall and the infiltration of inflammatory cells. It is one of the cardiovascular diseases (CVD) which remains the major cause of mortality in the world, typically claiming a third of all deaths. It is occurring in the modern industrialized countries due to changing life styles and food pattern (Chan et al., 1996; Araujo et al., 2008).

Many therapies have been evolved for the treatment of atherosclerosis. Statins, a class of cholesterol lowering drugs inhibiting cholesterol synthesis, have been most widely prescribed for treating hypercholesterolemia and reducing cardiovascular diseases (Sweetman, 2009). However, adverse effects associated with statin therapy such as liver damage (Parra and Reddy, 2003), myopathy (Kiortsis et al., 2007) and potential drug-drug interaction (Trifiro, 2006) have been reported.

The search for compounds from nutraceutical sources for the prevention of cardiovascular disease has been emphasized (German and Dillard, 2000). Herbal and medicinal plants have also shown significant results in the treatment of hypercholesterolemia. The side effects are lesser by bioactive foods or nutraceuticals because of their wider acceptance in the body but side effects caused by pharmaceutical drugs are grave and serious. Therefore, on the basis of above stated facts, there is an urgent need to have a drug having the dual property of lowering lipid level and antioxidant activities together.
Hence, this present study is designed with an aim to explore the effects of Coenzyme Q₁₀, Riboflavin, Niacin, Selenium and _Emblica officinalis_ (Eo) in controlling the atherogenic disturbances caused by High Cholesterol Diet.

### 3.1 EFFECT OF CoRNS AND Eo SUPPLEMENTATION ON BODY WEIGHT AND ORGAN WEIGHT OF CONTROL AND EXPERIMENTAL ANIMALS

Figure 7 and 8 show the body weight and organ weight (heart and liver) of control and experimental animals. The initial and the final body weight of the animals have been recorded from the day of beginning and completion of the experimental period. There was a significant increase (p<0.05) in the body weight and organ weight of atherogenic animal (Group II) when compared to the control animals. The body weight of Group IV and Group V animals were significantly reduced nearer to that of the control animals. Animals fed with high cholesterol diet may have significant increase in the body weight due to the diet containing cholesterol and bile salt (cholate). Treatment with combinatorial drug and Simvastatin reduced the body weight and organ weight of atherogenic animals when compared with HCD induced untreated animals. No significant alteration in the body weight and organ weight in the drug control groups were observed. This can be attributed to the hypolipidemic effect of CoRNS and Eo which might have reduced the deposition of fats in the organs of these animals.

### 3.2 EFFECT OF CORNS AND EO ON THE LEVELS OF UREA, URIC ACID, CREATININE AND ALBUMIN IN CONTROL AND EXPERIMENTAL ANIMALS

Table 1 summarizes the levels of urea, uric acid, creatinine and albumin in the serum of control and experimental animals. In HCD fed animals (Group II), significant increase in the levels of urea, uric acid and creatinine and decrease in the level of albumin in the serum were observed consistent with other studies.
Elevated levels of urea and creatinine show glomerulopathy. Lipid abnormalities may affect the functions of the kidney (Vazquez-Perez et al., 2001). Increase in the dietary cholesterol in the rats has been shown to increase plasma cholesterol level and induce glomerulosclerosis (Peric-Golia and Peric Golia, 1983). Thus, deterioration of renal function in hypercholesterolemic rats may lead to increased urinary excretion of albumin which could have caused hypoalbuminemia. Microalbuminuria, i.e., slightly elevated albumin excretion in the urine, is considered a novel atheroslerotic risk factor (Jensen, 2000). Several epidemiological studies have shown that increased serum uric acid (UA) level, which is the main end product of purine metabolism, causes endothelial dysfunction which in turn leads to cardiovascular diseases (Tsouli et al., 2006).

Hyperuricemia is an independent predictor of CVD (Ward, 1998). Increased uric acid is due to the accumulation of urate crystals which triggers an inflammatory response in the vascular plaque (Kaya et al., 2010). Treatment with CoRNS and Eo lowered the level of uric acid and increased the level of albumin in Group V animals. The levels were restored to near normal levels. This is may be due to the lipid lowering efficacy of all the drugs which prevent endothelial dysfunction and oxidative metabolism thus, preventing atherosclerosis. The protective effect CoRNS and Eo was more than that of the standard drug, Simvastatin.

3.3 EFFECT OF CoRNS AND Eo ON LEVELS OF HOMOCYSTEINE IN CONTROL AND EXPERIMENTAL ANIMALS

Figure 9 depicts the level of homocysteine in control and experimental animals. The level of homocysteine was elevated in HCD fed animals (Group II) when compared with control animals. The CoRNS and Eo treated animals (Group V) showed decreased levels of homocysteine when compared to the Group II animals. No significant alterations were observed in the drug control animals.
Homocysteine is a non-protein sulphur containing amino acid formed during the metabolism of methionine. Hypercysteinemia is an independent risk factor for atherosclerosis and cardiovascular disease (Nehler et al., 1997). It also induces endothelial dysfunction (Weiss et al., 2002), increases proliferation of vascular smooth muscle and enhances coagulability. The exact mechanism of homocysteine in atherosclerosis is unknown. It appears that it facilitates generation of \( \text{H}_2\text{O}_2 \) which is toxic to vascular endothelium by causing damage to LDL and endothelial cell membrane. It increases collagen production and decreases the availability of nitric oxide. Vitamins B\(_6\) and B\(_{12}\), folate and riboflavin play an important role in homocysteine homeostasis (Clarke, 1998). There are two pathways that govern homocysteine metabolism. They are transsulfuration and remethylation. Transsulfuration is dependent on vitamin B\(_6\) and catabolizes homocysteine to cysteine, while remethylation of homocysteine to methionine is dependent on vitamin B\(_{12}\), folate and riboflavin. FAD\(^+\) is the cofactor necessary to activate folate for homocysteine methylation. Riboflavin and other vitamin fortifications are used in the prevention and treatment of cardiovascular disease (McNulty et al., 2002).

Treatment with CoRNS and Eo would have increased the catabolism of homocysteine and thereby lowering its toxic effects. The drug by means of its potent antioxidant effect might have decreased homocysteine levels and the \( \text{H}_2\text{O}_2 \) generation by it and thereby exhibiting its cardio protective role.

### 3.4 LIPID STATUS IN, PLASMA, HEART, LIVER, KIDNEY AND AORTA OF CONTROL AND EXPERIMENTAL ANIMALS

The levels of lipid profile in plasma, heart, liver and kidney tissues of experimental animals are shown in Tables 2 and 3, respectively. In Table 2, the levels of plasma and heart, total cholesterol (TC) and free cholesterol were increased
significantly \((p<0.001)\) in Group II (atherogenic) animals when compared to control animals. Similarly, the levels of triglycerides (TG), free fatty acids (FFA) and phospholipids (PL) in plasma and heart were also found to be significantly higher when compared to control animals (Group I). On treatment with CoRNS and Eo and Simvastatin to Group IV and V animals, the lipid profiles turned to near normal levels when compared with Group II animals. The drug control animals (Group III) did not show any significant alteration in the lipid profile. Similar results were obtained in liver and kidney tissues also.

Table 4 shows the levels of lipid status in the aorta of experimental animals. The levels of TC, free cholesterol and PL in the aorta in Group II animals were increased in HCD fed animals. The Group IV and V animals treated with Simvastatin and CoRNS and Eo showed significant decrease in the lipid levels.

Figure 10 shows the plasma lipoprotein profile such as high density lipoprotein (HDL)-cholesterol, Very low density lipoprotein (VLDL)-cholesterol and Low density lipoprotein (LDL)-cholesterol of all the five group of animals. The levels of HDL-cholesterol were significantly decreased in Group II animals when compared to control animals whereas the levels of VLDL-cholesterol and LDL-cholesterol were significantly \((p<0.001)\) increased in Group II animals. Treatment with Simvastatin restored the levels of HDL-cholesterol simultaneously while decreasing the levels of VLDL-cholesterol and LDL-cholesterol. In Group V animals, receiving combination of CoRNS and Eo, showed increase in HDL-c when compared to Group II animals, restoring the levels of VLDL-cholesterol and LDL-cholesterol to near normal levels.
Table 5 and 6 show the activity of lipid metabolizing enzymes in plasma, liver and heart of control and experimental animals. The enzymes such as lipoprotein lipase (LPL), cholesterol ester hydrolase (CEH), cholesterol ester synthetase (CES) and lecithin cholesterol acyl transferase (LCAT) are the enzymes that metabolize the lipids in the plasma as well as liver and heart tissues. The activities of all these enzymes were significantly altered in the atherogenic animals (Group II). In atherogenic animals, decreased activity of LPL and CEH and increased activity of CES were observed in plasma and also in heart and liver tissues. The activity of LCAT was also observed to be decreased in plasma and tissues. No significant alterations in the activity of these enzymes were observed in the drug control animals. The Group IV and V animals treated with Simvastatin and CoRNS and Eo showed increased activity of LPL and CEH and decreased activity of CES in plasma as well as in tissues. The activity of LCAT in plasma was increased to near normal levels. The Group V animals treated with CoRNS and Eo showed normalization in the levels of all the enzymes.

Lipids are the structural components of the membrane, involved in maintaining the integrity of the cells and maintaining the fluidity of the cellular membrane components. As membrane lipids play a crucial role in maintaining the integrity, electrolyte balance and also enzyme activity, any modification in the lipid content and quality in the membrane may affect the overall cell function. Increased intake of fats through diet may enhance the development of atherosclerosis. Feeding diet rich in cholesterol has been reported to increase the synthesis of fatty acid and triglyceride in rat liver (Fungwe et al., 1993). Hypercholesterolemia animals showed increase in serum cholesterol, LDL and VLDL along with decreased levels of HDL (Yao et al., 2005).
The observed increase in the levels of total and free cholesterol in Group II animals may be due to the reduced rate of cholesterol esterification. This reduced rate might be due to decreased activity of LCAT in atherosclerosis (Mlekush et al., 1991). The high cholesterol diet influences the deposition of cholesterol in the aorta and other cholesterol in the form of cholesterol ester (Hodis et al., 1991). This may also be the reason for increased cholesterol in the plasma of Group II animals. The deposited cholesterol ester in the tissue needs hydrolysis to release free cholesterol. One of the hydrolyzing factors is HDL and HDL level were found to be decreased in cholesterol fed rats. The insufficient HDL level may lead to the increased free cholesterol level in plasma, enhancing the pathogenesis. The observed increased level of triglycerides in Group II animals may be due to the decreased activity of LPL in HCD fed rats (Mlekush et al., 1991). LPL is an enzyme that is bound to the walls of the blood capillaries hydrolyzing triglycerides to form glycerol and fatty acids. LPL is activated by apo cII and renders a good proportion of lipids to tissue thus, providing shelter to the system from deleterious effects of lipid abnormalities (Eckel, 1989).

Lipoprotein is the vehicle for transporting plasma lipids. The increased levels of VLDL and LDL cholesterol observed in Group II animals may be due to decreased LDL receptor activity that reduces LDL catabolism in cholesterol fed rats (Applebaum-Bowden et al., 1984). Likewise, the reduced level of HDL-c may be due to decreased synthesis of apolipoprotein A-I (Grundy, 1990). HDL promotes the removal of cholesterol from peripheral cells and facilitates its delivery back to the liver. Increased lipid levels may be explained on the basis of the abnormal distribution of cholesterol level in the various lipoprotein fractions. The Group IV animals treated with Simvastatin showed lower levels of lipids and LDL-c and increase in the HDL-c level when compared with Group II animals. In Group V
animals treated with CoRNS and Eo, cholesterol and triglyceride levels in rats were decreased as comparable to those of Simvastatin treated groups. This effect may be due to the increased activity of lecithin: cholesterol acetyl transferase which incorporates free cholesterol, free LDL-c into HDL-c and transferred back to VLDL-c and intermediate density lipoprotein.

A notable observation of this study is the lipid lowering efficacy of the drug. CoQ\textsubscript{10} is the only lipid-soluble antioxidant synthesized endogenously. CoQ\textsubscript{10} inhibits the peroxidation of cell membrane lipids and also that of lipoprotein lipids present in the circulation. CoQ\textsubscript{10} has direct anti-atherogenic effect (Littarru and Tiano, 2007). It decreases the absolute concentration of lipid hydroperoxides in atherosclerotic lesions and of minimizing the size of atherosclerotic lesions in the whole aorta (Witting \textit{et al}., 2000). Niacin (B\textsubscript{3}) can promote the reduction of total cholesterol and an increase in HDL cholesterol and decrease atherogenic small, dense LDL particles. Niacin selectively increases apolipoprotein (apo) A-I (Sakai, 2001; Asztalo, 2010). In this study, the effects of niacin supplementation on the hepatic and serum lipid profiles of Wistar rats were carried out. There is evidence that this vitamin acts on specific receptors and decreases the release of fatty acids from adipose tissue (Karpe and Frayn, 2004) thus, reducing the esterification of triglycerides in the liver and increasing the activity of lipoprotein lipase. It inhibits peripheral lipolysis and hepatic VLDL synthesis, the degradation of shunt apolipoprotein B. Ashen and Blumenthal (2005) reported that therapy with niacin leads to a reduction of free fatty acids (20-40%), triacylglycerols (20-40%), lipoprotein (a) (30-40%) and LDL cholesterol (20-40%) and a significant rise in HDL cholesterol (20-35%). Furthermore, Ganji \textit{et al}., (2009) reported that niacin has antioxidant potential. Administration of selenium is known to suppress the amount of triglyceride, total cholesterol, free fatty acid and low density lipoprotein
cholesterol in the serum of rats fed with high cholesterol diet. Selenium supplementation is responsible for the upregulation of LDL-receptor activity as well as mRNA expression during hypercholesterolemia (Dhiangra and Bansal, 2006). Riboflavin may exert its hypolipidemic activity indirectly by preventing lipid peroxidation with reduced glutathione (Wu, 2004). *Emblica officinalis* is reported to have hypolipidemic activity (Anila and Vijalakshmi, 2002). Flavonoids from *Emblica officinalis* are also known to reduce lipid levels in serum and tissues of atherogenic animals. LCAT showed elevated levels in animals treated with Eo along with CoRNS. The increased level of LCAT causes the transport of cholesterol in the blood and to the liver for further metabolism (Anila and Vijalakshmi, 2002). So combination of all these nutrients given as drug is likely to reduce the lipid profiles to near normal levels in Group V animals. The drug may increase the activity of LPL and LCAT, modules other metabolizing enzymes and hence, keeps the lipid profile in a controlled manner. All the drugs possess hypolipidemic property individually. When given in combination, the additive effect of all these components may be responsible for the highly normalized level of lipid components in the animals treated with the drug. The results indicate that the nutraceuticals possess hypolipidemic effect and hence, can act as an anti-atherogenic agent in combination.

Figure 11 depicts the atherogenic index (AI). Atherogenic indices are powerful indicator of the risk of heart diseases. Higher values represent the higher risk of developing CVD and vice versa (Usorso et al., 2006). There was significant rise in AI in HCD fed animals (Group II). The CoRNS and Eo and Simvastatin treated animals (Group IV and V) showed significant reduction in AI. This indicates the anti-atherogenic property of CoRNS and Eo. No significant alterations in the drug control animals were observed.
3.5 HISTOPATHOLOGICAL CHANGES IN THE AORTA AND LIVER TISSUE

Plate 1 displays the presence of foam cells in the aorta of HCD fed animals (Group II) (H & E, 400x). The control animal showed normal structure of aorta. There was significant decrease in the foam cell formation in the Group V animals compared to Group II animals. The CoRNS and Eo was found to inhibit foam cells formation by inhibiting cholesteryl ester formation by reducing the number of specific binding sites of acetyl LDL (Saito et al., 1992). The anti-atherogenic property of CoRNS and Eo would have enhanced the fatty acid oxidation and thus, by preventing the formation of foam cells. No alteration in the drug control animals was observed.

Plate 2 shows the histopathological changes in the liver tissue (H & E, 400x) of atherogenic rats and the effect of CoRNS and Eo on the high cholesterol fed rats. The control animal shows normal liver architecture. High cholesterol diet fed rats showed hypertrophic changes in the liver tissue, while CoRNS and Eo treatment might have significantly prevented these changes in the treated group of animals. The animals treated with CoRNS and Eo showed more restoration as evidenced by normal architecture of liver tissue. This shows the anti-atherosclerotic property of CoRNS and Eo which has induced marked changes in the liver tissue and aorta. Drug control animals showed no changes in the liver tissue.

3.6 EFFECT OF CORNS AND EO ON MARKER ENZYMES IN SERUM, HEART, LIVER AND KIDNEY OF CONTROL AND EXPERIMENTAL ANIMALS

Figure 12 and Table 7 show the activity of marker enzymes in serum, heart, liver and kidney, respectively in control and experimental animals. The
marker enzymes lactate dehydrogenase (LDH), alkaline phosphatase (ALP), alanine amino transferase (AST) and aspartate amino transferase (ALT) were increased in HCD fed animals (Group II) when compared with control animals (Group I). Treatment with Simvastatin and CoRNS and Eo to Group IV and V animals showed decreased activity of the marker enzymes when compared to Group II animals. No significant alteration was observed in drug control animals.

The amount of cellular enzymes in the blood reflects the alteration in plasma membrane integrity. Moreover, the AST and ALT activities have been used as an indicator of liver function. The increase in ALT activity is due to hepatocellular damage. LDH, AST and ALT serve as diagnostic markers that leak out from damaged tissue to blood stream when cell membrane become permeable or rupture (Ebenezar et al., 2003). Feeding of fat-cholesterol enriched diet to the animals lead to increased activities of AST, ALT, ALP and LDH in serum while supplementation of CoRNS and Eo leads to decreased activity of these enzymes. This observation indicates that fatty infiltration and degeneration of liver cells caused by fat-cholesterol feeding were significantly reduced by CoRNS and Eo.

Riboflavin administration was found to increase the total flavin that has the capacity to capture reactive metabolite. Recent experimental studies have shown that prevention of hepatotoxicity and decrease in aminotransferase to near normal levels are due to exogenous supplementation of antioxidants. Riboflavin and Niacin neutralize hydroxyl and superoxide radicals (Powers, 2003). CoQ_{10} quenches singlet oxygen and polyunsaturated fatty acid radicals (Crane, 2007). CoQ_{10} also helps in regeneration of vitamin C and vitamin E in conjunction with Niacin and Riboflavin. Treatment with Selenium was found to reduce hepatotoxicity (Nagamatsu and Haegawa, 1993). Eo has flavonoids which act as antioxidant agents in the removal of oxidants formed by the induction of HCD to the animals. All these drugs when
given in combination to Group V animals act as potent antioxidants which prevent hepatocellular damage and may repair the membrane thus, preventing leakage of the aminotransferases.

The activity of serum and cardiac CPK has been depicted in Fig13 and 14. There was an increase in the activity of serum CPK in atherogenic animals (Group II) with a parallel drop in the activity in the heart tissue. The animals treated with CoRNS and Eo showed a significant increase of CPK in the cardiac tissue and drop in the activity in serum.

The findings reveal that oxidative stress caused due to increased formation of superoxide anion could contribute to the inflammatory condition in the vessel walls, leading to the formation of endothelial dysfunction, which is one of phenomenon of atherosclerosis. Treatment with CoRNS and Eo to Group V animals would have reduced the leakage of cytosolic CPK from myocardial cells. These animals showed a decrease in the accumulation of lipids in serum thus, preventing oxidative damage to cardiac cells, protecting the animals from the formation of atherosclerosis.

3.7 EFFECT OF CoRNS AND Eo ON REACTIVE OXYGEN SPECIES (ROS) LEVELS IN THE TISSUES OF CONTROL AND EXPERIMENTAL ANIMALS

Figure 15 shows the levels of ROS in the heart and liver tissues of control and experimental animals. There were significant increase (p<0.05) in the levels of ROS in the heart and liver of Group II animals when compared to control animals. There were significant decrease in the levels of ROS in the heart and liver tissue of Simvastatin and CoRNS and Eo treated animals (Group IV and V) when compared to Group II animals. No significant alteration was observed in drug control animals.
In cellular and sub cellular membranes, due to the increase in the lipids, may cause oxidative damage leading to undesirable consequences by ROS. Endogenous sources of ROS in aerobic mammalian cells are the mitochondrial electron carriers, enzymes, LDL and membrane lipids. In biological systems, free radicals are generated in the form of ROS, such as superoxide anion and hydroxyl radicals, hydrogen peroxide, singlet oxygen, nitric oxide and peroxynitrate. These ROS causes destructive and irreversible damage to cellular components such as lipids, protein and DNA (Catala, 2009). Lipid peroxidation is a form of oxidative damage that occurs in cell membranes when unsaturated fatty acids react with excess ROS (e.g., OH radical or a transition metal/oxidant complex) to form both fatty acid radicals and lipid peroxides (Burton and Traber, 1990). Hypercholesterolemic atherosclerosis is associated with increased ROS production. Increased levels of oxygen radicals are known to produce endothelial cell injury, which represents a critical initiating event in the development of atherosclerosis.

Recent interest has been focused on strategies to enhance the removal of ROS by using antioxidants to enhance endogenous antioxidant responses. Natural antioxidants protect dietary lipids from oxidation, but may also provide health benefits associated with preventing damage due to biological degeneration. The CoRNS and Eo treated animals showed reduction in ROS level when compared with Group II animals. This may be due to the potent free radical quenching property of the drug. The drug control animals did not show any increase in the ROS level.

CoQ_{10} can act as a generator or a quencher of ROS (Crane, 2007) and because it is widely consumed by humans as a dietary supplement, it has acquired increasing attention with regard to its function in the reduced form as an antioxidant. It protects membrane phospholipids and plasma low-density lipoprotein from lipid peroxidation and as recent data indicate, also protects mitochondrial membrane
proteins and DNA from free-radical induced oxidative damage (Ernster and Dallner, 1995). In herbal products, phenolic compounds have been shown to be effective antioxidant constituents. Many polyphenolic components are known to exert more powerful antioxidant inhibiting lipid peroxidation through chain-breaking peroxyl-radical scavenging. They can also directly scavenge ROS such as, hydroxyl; superoxide and peroxynitrite radicals (Tsao and Akhtar, 2005). Many selenoproteins have the capacity to scavenge ROS. Glutathione peroxidase requiring Se protects the membranes from ROS attack. CoQ\textsubscript{10}, Riboflavin and Niacin are proved to be powerful antioxidants and reduces reduces oxidants, preventing the cell from damage (Yuvarj \textit{et al.}, 2009). Thus, CoRNS and Eo act as powerful antioxidant in reducing ROS. All these additive effects may be responsible for the decreased levels of ROS in the drug treated animals. The effect of CoRNS and Eo was significantly greater than that of standard drug, Simvastatin.

3.8 EFFECT OF CoRNS AND Eo ON THE LIPID PeroxidATION (LPO) IN PLASMA, TISSUE AND ERYTHROCYTE MEMBRANE OF CONTROL AND EXPERIMENTAL ANIMALS

Oxidative stress is one of the causative factors that link hypercholesterolemia with atherogenesis. Hypercholesterolemia increases the over production of free radicals, increases mitochondrial respiration and lowers the antioxidant status (Thiruchenduran \textit{et al.}, 2011). Table 8 and Fig 16 indicate the levels of lipid peroxides in plasma, heart, liver and erythrocyte membrane of control and experimental animals. An elevated level of LPO was observed in the Group II animals when compared to the control rats. The Group IV and V animals showed significant reduction in the LPO levels when compared to Group II animals. No significant alteration in drug control animals (Group III) when compared to Group I animals was observed.
Recently, the antioxidants have attracted many researchers and these are used as potent lipid lowering agents. The enhanced level of lipid peroxides observed in the Group II animals may be due to HCD feeding, which induce free radical production in rats. Lipid peroxidation is a chain event that enhances malondialdehyde (MDA) production (Lee et al., 2007). Peroxides cause membrane damage. The animals treated with CoRNS and Eo (Group V) showed marked reduction in LPO. This may be due to the free radical quenching property of these nutraceuticals.

3.9 EFFECT OF CoRNS AND Eo ON THE ACTIVITIES OF MEMBRANE BOUND ATPASES ENZYMES IN THE ERYTHROCYTE MEMBRANE OF CONTROL AND EXPERIMENTAL ANIMALS.

Figure 17 depicts the activity of ATPases enzymes in the erythrocyte membrane of control and experimental animals. The activity of Na⁺/K⁺, Mg²⁺ and Ca²⁺ ATPases were significantly decreased in Group II atherogenic animals when compared to control animals. A significant increase in the activities of the ATPases was observed in Group IV and V animals treated with Simvastatin and CoRNS and Eo when compared with Group II animals. No significant alteration was found in drug control animals.

The simplicity, availability and ease of isolation make erythrocyte as an excellent model for membrane studies. ATPases are a class of enzymes that catalyze the decomposition of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and a free phosphate ion. This dephosphorylation reaction releases energy. This process is widely used in all known forms of life. Such enzymes are integral membrane proteins (anchored within biological membranes) and move solutes across the membrane, typically against their concentration gradient. These are called “transmembrane ATPases”. 

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Transmembrane ATPases import many of the metabolites necessary for cell metabolism and export toxins, waste and solutes that can affect cellular processes. An important example is the sodium-potassium exchanger (Na⁺/K⁺-ATPase), which establishes the ionic concentration balance that maintains the cell potential. ROS production can damage arterial walls including an impairment of the endothelium dependent vasodilatation or endothelial dysfunction (John and Schmieder, 2003). The interaction of ROS with biological membranes produces a variety of functional modifications due to either direct interaction with the molecular mechanisms or to oxidative modification of the environment of biological macromolecules. Na⁺/K⁺-ATPase is an antioxidant plasma membrane-associated protein complex whose activity has been found to be altered in various cell types under hyperlipidemic conditions. Na⁺/K⁺-ATPase couples the energy released from the intracellular hydrolysis of ATP to the transport of cellular ions, a major pathway for the controlled translocation of sodium and potassium ions across the cell membrane. Moreover, alterations of the antioxidant status and increased lipoperoxidation have been also proposed as a cause of Na⁺/K⁺-ATPase reduction in erythrocyte membranes (Gruia et al., 2009). The negative correlation between Na⁺/K⁺-ATPase activity and lipoperoxidation suggests that increasing free radicals production may play a role in the currently reported reduction of erythrocyte Na⁺/K⁺-ATPase activity and consequent increase in the blood pressure (Carlos, 2012). Na⁺/K⁺-ATPase may be considered as an index of cardiovascular complications induced by diabetes (Dhalla et al., 1998).

The Ca²⁺-ATPase is present in the plasma membrane and is the major active calcium transport protein responsible for the maintenance of normal intracellular calcium levels in a variety of cell types. Abnormal Ca²⁺-ATPase activity and intracellular calcium levels were reported as important mechanisms
responsible for the cardiac dysfunction in diabetes (Dhalla et al., 1998). Ca$^{2+}$-ATPase inhibition may be due to decreased level of GSH in the atherogenic condition. Many of the ATPase contains sulphhydryl groups in their active site. Inhibited activity of Ca$^{2+}$-ATPase in the Group II animals may be due to activated oxygen and depletion of thiols status. Reactive oxygen species formed may attack the membranes of intracellular organelles and lead to a decrease in cardiac Ca$^{2+}$-ATPase activity (Ziegelhoff et al., 1997). Decreased membrane fluidity induced by increased oxidative stress has been linked to the abnormalities in calcium metabolism (Lehotsky et al., 1999).

Mg$^{2+}$-ATPase present in the plasma membrane, actively transport of magnesium across cell membrane. The reduced activity of Mg$^{2+}$-ATPase may be due to the LPO caused due to high fat diet, which might have reduced the antioxidant status, thereby damaging the membrane. Membrane fluidity has a strong influence on important membrane functions such as the conformation and thus, the activity of membrane associated enzymes (Rizvi and Zaid, 2005). The role of oxidative damage of membrane in atherosclerosis contributes to the alteration in the activities of membrane-bound enzymes. ATPases of the cardiac cells play a significant role in the contraction and relaxation cycles of the cardiac muscle by maintaining normal ion levels (Ca$^{2+}$, Na$^+$, K$^+$, Mg$^{2+}$) within the myocytes. Changes in the properties of these ion pumps affect the cardiac function.

The treatment of the Group V animals with CoRNS and Eo might have stabilized the membrane property. It is thought that CoQ$_{10}$ stabilizes myocardial calcium-dependent ion channels and prevent the depletion of metabolites essential for ATP synthesis (Greenberg and Frishman, 1990). CoQ$_{10}$ also decreases blood viscosity and improves blood flow to cardiac muscle in patients with ischemic heart disease (Kato et al., 1990). The Selenate is actively absorbed by a mechanism
common in sulphate, depending on Na$^+$ gradient and maintained by the Na$^+$/K$^+$-ATPase (Mataix verdu and Llopis, 2002). Selenium increases the concentration of reduced glutathione (Pascae et al., 1987) and it prevents the peroxidative damage (Butler et al., 1987). All these cluster of properties may be responsible for the improvement of ATPases activities in CoRNS and Eo treated animals and its preference over sole Simvastatin.

In the present study, the decrease in the activity of ATPases may be due to LPO caused by atherogenesis in the Group II animals. The combined effect of CoRNS and Eo treatment could have significantly increased the activity of all the ATPases by means of its antioxidant property.

3.10 ANTIOXIDANT STATUS IN PLASMA, HEART, LIVER, AORTA AND ERYTHROCYTE OF CONTROL AND EXPERIMENTAL ANIMALS

The activity of enzymatic and non-enzymatic antioxidants in blood and tissues in control and experimental animals have been depicted in Table 9, 10, 11 12 and 13, respectively. A significant reduction in the activity of the enzymatic antioxidants such as SOD, CAT and GPx and non-enzymatic anti-oxidants such as vitamin C and E were observed in the Group II animals when compared to the Group I animals. The Group IV and V animals treated with Simvastatin and CoRNS and Eo, respectively have shown a significant increase in the activity of these antioxidants. The drug control animals showed no abnormal alteration in the activity of these enzymes.

Excess oxidative stress is caused by an imbalance between pro- and anti-oxidant enzymes, leading to an overproduction of free radicals, including superoxide, hydroxyl radicals and lipid radicals, which may damage cellular
components, interfering with normal function. Lipid peroxidation does appear to play a major role in the pathology of atherosclerosis (Halliwell and Gutteridge, 1990). During the formation and development of atherosclerosis, the intensity of lipid peroxidation and the activity of the antioxidant defense system significantly change. It is known that erythrocytes contain powerful preventative and chain-breaking antioxidants and so limit lipid peroxidation. Hypercholesterolemia leads to increased cholesterol accumulation in the erythrocytes and endothelial cells thereby activating them to produce oxygen free radicals. Thus, erythrocytes are extremely vulnerable to these oxidative challenges and hypercholesterolemia (Vijayakumar and Nalini, 2006).

Selenium is known as an essential component of glutathione. Lipid peroxides have been implicated in the initiation of atherosclerosis and in order for the GPx to be able to counteract the adverse effects of peroxides, sufficient amounts of selenium must be available for the formation of the enzyme. Glutathione peroxidase assumes a critical importance in the protection of the red cell against oxidative destruction (Lassen and Horder, 1994). The mechanism of action of this enzyme requires the presence of selenocysteine in the active site. Group II animals showed decrease in the activity of GPx, CAT and SOD levels in erythrocytes, plasma, heart and liver tissues. It has been reported that selenium-dependent GPx activities in liver, aorta, heart muscle, erythrocyte and platelet in atherosclerotic rabbits were decreased significantly and that these tissues suffered from lipoperoxidative damage (Chen et al., 1993). Supplementation with CoRNS and Eo to the Group V animals showed enhanced levels of GPx, CAT and SOD. The increased level of GPx may be due to the Se induction in these animals which has increased the activity of GPx since it requires Se for its activity.
The present work shows that the changes in lipid peroxidation are accompanied by decrease in the activities of antioxidant enzymes namely SOD, CAT and GPx as well as the levels of GSH, vitamin C and vitamin E. The decreased activity of these enzymatic and non-enzymatic antioxidants in tissue and plasma in Group II might be due to the supersaturation of the enzyme SOD with a high concentration of ROS formed. Due to increase in the level of lipid peroxides, the levels of free radicals overcome the saturation level which in turn inhibits the activity of these antioxidant enzymes. This decline in SOD activity leads to the down regulation of H$_2$O$_2$. Since H$_2$O$_2$ is the substrate for the enzymes CAT and GPx, these were also found to be decreased. Antioxidants are substances that protect against oxidative damage even when present in significantly smaller amounts than the target molecule. Decrease in antioxidant system results in oxidative stress which in turn deregulates cellular functions.

Cells constantly produce O$_2\cdot$ as a by-product of normal aerobic metabolism. SOD is the main defense against superfluous O$_2\cdot$ catalyzing the conversion of disproportionate H$_2$O$_2$ and O$_2$. Excess oxidants causing cellular damage in tissues are captured by SOD and further removed by CAT and GPx. First, SOD converts superoxide anion to hydrogen peroxide in a cellular antioxidant reaction (Liu and Mori, 1993). Catalase is found predominantly in peroxisomes and converts H$_2$O$_2$ to H$_2$O with a high catalytic rate. Thereafter, GPx, a Se-containing enzyme, independently detoxifies the hydrogen peroxide produced into H$_2$O. Glutathione-S-transferase is a family of multifunctional enzymes that catalyze the conjugation of glutathione with a large number of compounds with an electrophilic center and render the products more water soluble (Egaas et al., 1995). Reduced glutathione (GSH) is a small water-soluble tripeptide present in millimolar concentrations and one of the most important non-proteinaceous antioxidants in
cells. GSH protects by both directly scavenging oxidants and serving as the reductant for GSH-dependent antioxidant enzymes, such as GPx. In the antioxidant defense mechanism, therefore, dietary supplementation of Se is an important method to increase Se content and Se containing GPx in tissues, which are closely correlated with dietary level of Se (Schrauzer, 2000). α-Tocopherol (vitamin E) is the most studied antioxidant in experimental and human atherosclerosis (Upston et al., 2003). It can act along with vitamin C as antioxidant in reducing the oxidants formed during lipid peroxidation (Palmer et al., 1997).

CoQ₁₀ is recognized as a non-enzymatic antioxidant which prevents atherosclerosis and LDL oxidation (Lönn et al., 2012), where it scavenges radicals directly and regenerates α-tocopherol from reduced tocopheroxyl radical (Lass, 1998). Flavin mononucleotide (FMN⁺) and flavin adenine dinucleotide (FAD⁺), the active coenzymic forms of riboflavin (B₂) participate in non-redox reactions such as photo-repair of thymidine dimers in photo-damaged DNA (Imada, 2003). The coenzymic forms of niacin such as NAD⁺ and NADH participate in oxido-reduction reactions. FAD⁺ serves as a coenzyme for glutathione reductase and other enzymes. Glutathione reductase mediates the regeneration of reduced glutathione, which plays an important role in scavenging free radicals and reactive oxygen species (Rivlin, 2001). Riboflavin may exert its hypolipidemic activity indirectly by preventing lipid peroxidation with reduced glutathione (Wu et al., 2004). Riboflavin is also essential for the synthesis of other B vitamins and coenzymes especially niacin and pyridoxine from tryptophan. Tannoids such as emblicanin A, emblicanin B, punigluconin and pedunculagin present in Eo (Zhang et al., 2003) have the ability to quench the peroxides formed with the induction HCD diet (Treadway and Linda, 1994). Selenium plays a central role in enzymatic defense pathways against oxidative damage in tissues. The effects of Se are mediated through antioxidant
metabolism by GSH-Px in which Se is incorporated in the core of selenocysteine (Yeh et al., 1997). Numerous studies have demonstrated the beneficial effect of dietary Se on the prevention of biological membrane peroxidation as well as several diseases of animals (Pallares et al., 2002). Supplementation with Se along with CoRNS and Eo may increase the activity of GPx which further strengthens the antioxidant status in the Group V animals. Hence, the Group V animals showed marked elevation of the antioxidants in the plasma, liver, aorta, cardiac tissue and erythrocytes when compared to Group II animals. CoRNS and Eo prevents macromolecular damage and also increases tissue defense system against free radical generation and attack. The combination of CoRNS and Eo showed much elevated levels of antioxidants since the nutritive drug are potent natural antioxidants.

3.11 EFFECT OF CoRNS AND Eo ON THE ACTIVITY OF GLUTATHIONE METABOLIZING ENZYMES AND THIOLS IN CONTROL AND EXPERIMENTAL ANIMALS

Table 14 and 15 represent the activity of glutathione metabolizing enzymes and thiols in cardiac, kidney and hepatic tissues of control and experimental animals. The activity of $\gamma$-GT, GR and GST were decreased with a drop in TSH and NPSH levels in Group II animals when compared to control animals. Enhanced activities of these enzymes were observed in Group IV and V animals treated with Simvastatin and CoRNS and Eo. No significant alteration was observed in the drug control animals.

Hepatic GST and GPx have been reported to decrease with the decrease in GSH levels in the plasma and tissues. The changes in the activity of these enzymes may be due to lipid peroxide levels induced by HCD to the animals in
Group II animals. \( \gamma \)-GT, is a biochemical marker of inflammation (Emdin and Pompella, 2005). \( \gamma \)-GT is independently associated with cardiovascular mortality (Ruttmann et al., 2005). It has been reported that \( \alpha \) GST protect cells from ROS induced Lipid peroxidation during oxidative stress caused by various physico-chemical agents (Sharma et al., 2011). GST and GR were found to be increased on treatment with Simvastatin (Group IV) and CoRNS and Eo (Group V). Increased oxidative stress and decreased antioxidant levels might have decreased the activity of glutathione metabolizing enzymes. The CoRNS and Eo treated animals showed better results as the nutrients are potent antioxidant which strengthens the defense system and hence increase the activity of glutathione metabolizing enzymes.

\( \gamma \)-GT, GR and GST play an important role in maintaining thiol content of the cell (Paolicch et al., 2006). Increased oxidative stress with decreased enzymatic and non-enzymatic antioxidant status in HCD fed rats results in the decreased activity of glutathione metabolizing enzymes and fall in thiol content. Supplementation with the nutraceuticals such as CoRNS and Eo which are potent antioxidants scavenges free radicals, restores the activity of glutathione metabolizing enzymes and prevents the decline of thiols. Hence, the drug can be assumed to act as potent anti-atherosclerotic agent and show better results than the standard drug, Simvastatin.

3.12 EFFECT OF CoRNS AND Eo ON THE ACTIVITIES OF LYSOSOMAL ENZYMES IN LIVER, PLASMA AND HEART TISSUE OF CONTROL AND EXPERIMENTAL ANIMALS

Table 16 and 17 depict the activities of lysosomal enzymes namely acid phosphatase (ACP), \( \beta \)-glucuronidase(\( \beta \)-Glu), \( \beta \)-galactosidase(\( \beta \)-gal), N-acetyl-\( \beta \)-D glucosaminidase-(NAG) and Cathepsin-D(Cat-D) in plasma, heart and liver of
control and experimental animals. The activity of these enzymes were significantly increased in the HCD animals (Group II) when compared to the control rats in the plasma and the tissues such as liver and heart. The Group IV and V animals treated with the standard drug and CoRNS and Eo have shown significant decrease in the activities of these enzymes when compared to Group II animals. There was no significant change observed in the drug control animals.

Lysosomes are the group of cytoplasmic organelles present in numerous animal tissues, characterized by their content of acid hydrolases. These cytoplasmic vesicles contain hydrolytic enzymes that are capable of digesting the macromolecules like polysaccharides, nucleic acids and lipid. Lysosomes are involved in the intracellular digestion of a variety of substances into simple low molecular weight compounds that can be utilized by metabolic pathways of the cell. Lysosomal enzymes take part in biotransformation of drugs and toxins and catalyze hydrolytic cleavage of intra- and extracellular substances that must be removed from a cell. Disruption of lysosomal membranes can result in the release of lysosomal enzymes causing cellular digestion (Maciejewski et al., 2001). These events produce a reduction in membrane integrity (Bertram et al., 1988). The activity of pharmacological substances may bring about a disturbance of lysosomal space which is manifested by alteration in activities of lysosomal enzymes. Lysosomal enzymes have been studied extensively in atherosclerosis.

Lysosomal damage is well established as a biomarker of stress in a diverse range of animals. Lysosomal membrane damage causes the release of hydrolytic enzymes which in turn damage the cells and tissues. The atherosclerotic condition may elevate the activity of these enzymes. Lysosomal enzymes play an important role in the inflammatory process. The damage caused by the enzymes of lysosomal and mitochondrial origin and the modification of tissue constituents by
these enzymes play an important role in myocardial ischemia. Lysosomal enzymes are important mediators of acute myocardial infarction and its release into cytoplasm stimulate the inflammatory mediators like oxygen radicals, prostaglandins, etc. (Ravichandran et al., 1991). Elevated lysosomal enzymes in the extra cellular fluid occur as a result of decreased lysosomal membrane stability. This eventually affects the metabolism of different connective tissue constituents viz glycosaminoglycans, glycoprotein, collagen and results in irreversible cardiac damage. It has been suggested that oxygen free radicals generated during ischemia in addition to the direct myocardial damaging effect may also be responsible for the cardiac damage through the release of lysosomal enzymes (Kalra and Prasad, 1994). The membrane deterioration of lysosomes by HCD induced LPO could have resulted in the leakage of enzymes from the enclosed sacs. The increased activities of glycohydrolases and cathepsin-D observed in this study indicate the possible infiltration of inflammatory cells at the site of infarction.

β-glucuronidase is a sensitive marker of lysosomal integrity and released due to the presence of free radicals (Michihara et al. 2005). Cathepsin-D is a ubiquitous aspartyl endoprotease sorted to the lysosomes and found intracellularly. However, in some physiological and pathological conditions, Cat-D is secreted by various cell types. Indeed, procathepsin D was found in human, bovine and rat milk (Baechle, et al., 2006).

The elevated activity of these enzymes may be due to the leakage of these enzymes due to cellular damage by the free radicals generated due to HCD supplemented to the Group II animals. The animals treated with CoRNS and Eo have decreased activity of these enzymes due to their antioxidant property which quench the free radicals. The reduced oxidants maintain the cell integrity and thus, maintain the normal levels of these enzymes.
3.13 LEVELS OF PROTEIN BOUND CARBOHYDRATES (PBC) IN PLASMA, HEART, LIVER AND KIDNEY TISSUES OF CONTROL AND EXPERIMENTAL ANIMALS

Table 18 and 19 show the levels of glycoprotein components such as hexose, hexosamine, hexuronic acid and sialic acid in plasma, heart, liver and kidney of control and experimental animals. Group II animals fed with HCD have shown significant increased levels of hexose, hexosamine, hexuronic acid and sialic acid when compared to the control animals. The Group IV and Group V animals treated with Simvastatin and CoRNS and Eo showed significant reduction in the glycoprotein component levels. Drug control animals did not show any significant changes.

Glycoproteins play a key role in mediating cell surface functions such as cell-cell recognition, cellular adhesion, antigenecity, intracellular processing of proteins and cell activation. Changes in collagen, elastin and glycosaminoglycans of arterial wall have been reported in humans and experimental atherosclerosis (Srinivasan et al., 1979). The glycosaminoglycans of the arterial wall are in the protein bound form and these proteo glycosaminoglycans are reported to be involved in altered permeability, lipid metabolism, homeostasis, thrombosis and vascular cell proliferation in the aorta of diet induced atherosclerotic monkey (Radha krishnamurthy et al., 1982).

The role of sialic acid in the pathogenesis of atherosclerosis and as a predictor of cardiovascular events has attracted much attention in recent years. Sialic acids are a family of \(N\)- and \(O\)-substituted derivatives of neuraminic acid, an amino sugar. These are generally found as terminal sugar residues on oligosaccharides of both glycoprotein and glycolipid. The functions of sialic acids in biological systems
include conformational stabilization, protease resistance, charge, enhancement of water binding capacity, cellular recognition, protein targeting and developmental regulation. Increased sialic acid in blood has been reported as one of the markers for atherosclerosis, cancer, etc. One possible explanation could be secretion (or) shedding of glycoproteins from cell membrane into the circulation due to peroxidative damage of membrane proteins for repairing the damage cause by free radical by feeding the rats with HCD (Kaviarasana et al., 2005).

Increased inflammations with concomitant elevation in plasma and tissue hexose, hexosamine, hexuronic acid and sialic acid have been reported in animals fed with cholesterol. On treatment with Coenzyme Q$_{10}$, Riboflavin Niacin, Selenium and Eo, glycoprotein components were reverted back to near normal levels. This could be due to the cytostabilizing property of this drug. This drug might have altered cell membrane glycoprotein synthesis and structure. The drug also have anti-inflammatory property. They preserve endothelial function during myocardial infarction. This has attributed to the reduced levels of protein bound carbohydrates in Group V animals.

3.14 EFFECT OF CoRNS AND Eo ON ACTIVITY OF ELECTRON TRANSPORT CHAIN (ETC) COMPLEX IN CONTROL AND EXPERIMENTAL ANIMALS

Figure 18 and 19 show the activities of Electron Transport Chain (ETC) complexes in liver and heart of control and experimental animals. The activities of all ETC complexes were significantly decreased (p<0.001) in Group II animals when compared with control animals. There was a significant increase in the activity of complexes in the animals treated with Simvastatin and CoRNS and Eo. No significant changes were seen in the drug control animals.
Mitochondria exist in multiple copies in cell and contain highly folded inner-membrane structures or cristae that serve primarily to localize the respiratory chain components that generate the proton gradient needed to drive ATP synthesis. Mitochondria is unique in that they contain their own DNA (mt DNA) of the genes that comprises about 13 mt DNA, that are translated into proteins, all of which are localized in the inner mitochondrial membrane as components of the complex I,II,III and IV(oxidative phosphorylation enzymes) (Gibson,2005). The primary physiological function of the mitochondrion is to generate adenosine triphosphate (ATP) through oxidative phosphorylation via the ETC. The ETC is one of the major sources of cellular ROS production. Respiratory chain dysfunction leads to increased production of superoxide, which in turn leads to ageing and inflammatory diseases (Madamanchi, 2007). The deleterious effects resulting from the formation of ROS in mitochondria are prevented to a large extent by various antioxidant systems (Ballinger, 2005).

In the mitochondrial electron transport chain, complex IV retains all partially reduced intermediates until full reduction of oxygen is achieved. Other complexes may leak electrons to oxygen, partially reducing this molecule to $O_2$. Complex I and III are the primary source of $O_2$ production in mitochondria. Superoxide is released into the matrix from complex I, whereas it is released into both the matrix and inner membranous space by complex III. ROS production depends on the metabolic state of mitochondria (Camello-Almaraz et al., 2006).

Both oxLDL and free cholesterol can alter mitochondrial damage and function. High-fat diets had reduced the expression of genes involved in free radical scavenging of SOD and GPx (Ballinger, 2005). Mitochondrial respiratory chain complex I (NADH: Ubiquinone oxidoreductase) catalyzes electron transfer from NADH to Ubiquinone, coupled to translocation of proton through the inner
mitochondrial membrane. The electrons are transferred from NADH to the primary electron, non-covalently bound flavin mononucleotide and through a series of iron sulphur clusters to Ubiquinone (Preston et al., 2001). Complex III (cytochrome bc\(_1\)) that transfers electron from Ubiquinone to cytochrome c can then react with molecular oxygen to form O\(_2^+\) (Sarasta, 1999). Complex IV (cytochrome c oxidase) is an intrinsic membrane protein spanning the mitochondrial inner membrane. The peroxides produced during lipid peroxidation may easily damage this membrane leading to reduction of cytochrome c oxidase (Wen and Garg, 2004). Hence, decreased activity of all these enzymes might be due to the increase of peroxides, which in turn damage mitochondria.

If the mitochondrion is altered, release of cytochrome c, the sole water soluble component of the electron transport chain, can potentially halt the electron transfer, leading to failure in maintaining the mitochondrial membrane potential and ATP synthesis. Furthermore, for the reason that cytochrome c carries electrons from cytochrome c reductase (Complex III ) to cytochrome c oxidase (Complex IV), by which oxygen molecules are reduced to water, a blockade at this step would increase the production of ROS with subsequent lipid peroxidation (Cai et al., 2000).

Coenzyme Q\(_{10}\) is a naturally occurring hydrophobic compound found in humans was first discovered in the mitochondrial respiratory chain, where it plays an essential role in oxidative phosphorylation. CoQ\(_{10}\) is recognized as an antioxidant in the mitochondrial membrane, where it scavenges radicals directly and regenerates \(\alpha\)-tocopherol from the tocopheroxyl radical (Lass, 1998). CoQ\(_{10}\) suppresses the generation of reactive oxygen species by blunting the expression of NADPH oxidase (Sohet et al., 2009) and scavenges lipid peroxidation products during free radical reactions (Tsuneki et al., 2007). Ubiquinone has a strong influence on at least three mitochondrial enzymes (complexes I, II and III) as well as enzymes in other parts of
the cell. The antioxidant activity confers protection against lipid peroxidation and works together with vitamin E in prevention of damage to lipid membranes and plasma lipids (Singh et al., 2003).

Flavin mononucleotide (FMN⁺) and flavin adenine dinucleotide (FAD⁺), the active coenzymic forms of Riboflavin (B₂) participate in non-redox reactions. The coenzymic forms of niacin such as NAD⁺ and NADH also participate in oxido-reduction reaction. FAD serves as a coenzyme for glutathione reductase and other enzymes. Glutathione reductase mediates the regeneration of reduced glutathione, which plays an important role in scavenging free radicals and reactive oxygen species (Rivlin, 2001). Selenium, as an essential component of GPx, plays a critical role in protecting aerobic organisms from oxygen radical-initiated cell injury (Yeh et al., 1997). The flavonoids and tannoids present in the Eo quench the free radicals formed and protects from cell damage (Treadway and Linda, 1994). Hence, these nutraceuticals reduce the ROS formed and protect the mitochondrial complex enzymes and enhance their activities in the Group V animals.

3.15 EFFECT OF CoRNS AND Eo ON THE LEVELS OF PLASMA C-REACTIVE PROTEIN (CRP) AND FIBRINOGEN OF CONTROL AND EXPERIMENTAL ANIMALS

Figure 20 and 21 show the levels of C-reactive protein and fibrinogen. The levels of CRP and fibrinogen were significantly increased in the Group II animals when compared to control animals. It has been reduced significantly (p<0.05) in the CoRNS and Eo treated animals (Group V) than the Group IV animals treated with Simvastatin. No significant changes were observed in drug control animals.
C-reactive protein is the prototype acute phase protein in humans and numerous animals. CRP directly participates in the process of atherogenesis by modulating endothelial function and its concentration known to predict cardiovascular events (Ridker et al., 1998). CRP is a sensitive marker of inflammation (Sudano et al., 2006). Inflammation-related events lead directly or indirectly to pro-oxidative conditions for plasma lipoproteins.

Fibrinogen plays a significant role in homeostasis and thrombosis and is able to promote the formation of atherosclerotic plaques through various mechanisms (Papageorgiou et al., 2010). Fibrinogen-fibrin composition is able to promote the formation of atherosclerotic plaques in atherosclerosis by enhancing the deposition of lipids into the vessel walls, thereby attracting macrophagocytes which swallow the lipid material and form foam cells. Secondly, fibrinogen plays a key role in thrombosis, which causes plaque instability in atherosclerosis. Thirdly, it is able to contribute to atherogenesis through interactions with the endothelial cells, smooth muscle cells and macrophages (Kannel, 2005).

CRP and fibrinogen have been increased in Group II animals fed with HCD compared with the control animals. High cholesterol levels favour oxidative stress leading to the modification of LDL. Under conditions of oxidative stress, the lipoprotein phospholipids become progressively oxidized (Madamanchi et al., 2005). Free radicals play an important role in the dysfunction of the endothelium. Endothelial dysfunction is characterized by a functional disruption of the protective endothelium, unleashing not only the internalization of cholesterol, but also the recruitment of inflammatory cells into the vessel wall, initiating the atherosclerotic process. Oxidative stress may be a determinant of increased CRP levels and promote proatherosclerotic inflammatory processes at the earliest stages of coronary heart disease. Elevated cholesterol has also been shown to trigger the release of the
inflammatory mediator CRP, a useful clinical marker of CVD (Sudano et al., 2006). It is reported that supplementation with antioxidants in humans showed anti-inflammatory effects and significantly reduced the CRP level (Block et al., 2004). Thus, the CoRNS and Eo treated animals might have reduced CRP and fibrinogen by the antioxidant property and showed better results in modulating the CRP and fibrinogen levels in the drug treated animals.

3.16 EFFECT OF CoRNS AND Eo ON THE LEVELS OF TUMOR NECROSIS FACTOR ALPHA (TNF-α) OF CONTROL AND EXPERIMENTAL ANIMALS

Figure 22 shows the levels of TNF-α in the cardiac tissue of control and experimental animals. The levels of TNF-α in Group II were increased significantly than the control animals. The animals treated with Simvastatin (Group IV) and CoRNS and Eo (Group V) animals showed significant reduction in the levels of TNF-α. The drug control animals showed no significant changes.

Hyperlipidemia could cause proinflammatory cytokines up-regulated and lead to liver tissue damage. Tumour Necrosis Factor alpha is a pluripotent activator of inflammation and acts by inducing a proinflammatory cytokine cascade. TNF-α induces activation of biochemical markers of inflammation in the atherosclerotic plaque such as nonspecific serum amyloid. CRP and TNF-α are important factors for evaluation of risk factors of atherosclerosis. Inflammatory mediators such as TNF-α have been associated in vitro with mitochondrial dysfunction and increased ROS generation (Moe et al., 2004). In a model for congestive heart failure, application of TNF-α to culture cardiac myocytes increased ROS generation and myocyte hypertrophy (Nakamura et al., 1998). TNF-α causes mitochondrial dysfunction by reducing complex III activity in the ETC, increasing ROS production and causing damage to mtDNA (Suematsu et al., 2003).
Diets rich in antioxidants are known to increase glutathione and other antioxidants. Antioxidant therapies hold promise for improving mitochondrial performance (Pieczenik and Neustadt, 2007). Treatment with CoQ\textsubscript{10} may inhibit inflammation by decreasing oxidative damage and increasing ATP generation, resulting in the decrease in the extent of atherosclerosis as well as improvement in the chemical composition and biology of atheroma (Jan Fedacko et al., 2011). CoQ\textsubscript{10} exhibits anti-inflammatory properties reducing the release of proinflammatory cytokines during inflammatory injury (Schmelzer et al., 2008). It was reported that CoQ\textsubscript{10} administration caused NF-kB activation with subsequent inflammatory reactions responsible for renal injury (Kang et al., 2009). Elevated TNF-\alpha is known as an important step for activation of the NF-kB signaling pathway (Li and Verma, 2002).

In this context, niacin has been shown to have potent antioxidant and anti-inflammatory properties (Ganji, et al., 2009). As a precursor for synthesis of NAD\textsuperscript{+}, niacin increases cellular concentration of NAD\textsuperscript{+} and regulates expression of glucose-6-phosphate dehydrogenase, which is the rate-limiting enzyme in the pentose phosphate pathway and a major source of cellular reduced NAD(P)H (Yan et al., 1999). Repletion of cellular NAD(P)H contents can lower ROS production by raising cellular redox capacity, maintaining antioxidant enzymes (catalase and glutathione reductase) in their active forms and inhibiting ROS-generating oxidases (Ganji et al., 2009). Selenium plays a central role in enzymatic defense pathways against oxidative damage in tissues. The effects of Se are mediated through antioxidant metabolism by GSH-Px (Yeh et al., 1997). Riboflavin and Eo reduce oxidative stress, thereby reducing ROS and hence reduces the level of TNF-\alpha. Hence, the combination of CoRNS and Eo shows better result in reducing TNF-\alpha level in the cardiac tissue.
In conclusion, the present study demonstrated that TNF-α was significantly down-regulated in animals treated with CoRNS and Eo in comparison with the hyperlipidemia animals, which demonstrated that CoRNS and Eo could release anti-inflammatory cytokine and decrease TNF-α expression by some effective drug component. The current study provides an effective approach for studying the mechanism underlying the pathogenesis of hyperlipidemia and treating hyperlipidemia complications.

3.17 MOLECULAR ANALYSIS

3.17.1 DNA damage and fragmentation studies in the control and experimental animals

Cellular targets for oxidative modification through ROS include lipid, protein and particularly DNA (Evans et al., 2004). To combat the harmful effects of ROS, living cells have acquired a number of defenses. However, when ROS production is dramatically increased under conditions such as hypercholesterolemia, these repair systems do not mitigate all damages, ultimately leading to DNA fragmentation.

Agarose gel electrophoresis of cellular DNA ladder pattern (streak) of DNA fragmentation is shown in the Plate 3. Present study shows that there was a marked increase in the levels of DNA fragmentation in Group II animals (lane 3) when compared to control animals (lane 2). Due to increased ROS production which alters the macromolecules such as lipid, protein and especially nucleic acid –DNA, this might be due to increased endogenous DNAase activity by ROS which cut the internucleosomal region of liver cell (hepatocytes) into many fragments. The CoRNS and Eo (drug control) (lane 4) itself had no fragmentation of DNA when compared with control animals. CoRNS and Eo treatment restores the HCD induced
DNA fragmentation to near control due to its free radical scavenging activity. This shows the free radical scavenging property of CoRNS and Eo. The reduction in ROS by treatment with CoRNS and Eo has reduced the DNA fragmentation, which shows that these nutraceuticals are anti-atherosclerotic agents in the treatment of atherosclerosis.

3.17.2 Effect of CoRNS and Eo on the mRNA expression of TNF-α in cardiac tissue of control and experimental animals

TNF-α is the major pro-inflammatory cytokine present in an elevated level in the CVD. This cytokine is one of the inflammatory markers for the pathogenesis of atherosclerosis. Plate 4 and 5 show the gene expression pattern of TNF-α. The gene expression of TNF-α was significantly increased in Group II animals when compared to Group I control animals. On treatment with the combined therapy of CoRNS and Eo to Group V animals, the TNF-α expression was down-regulated.

CoQ₁₀ exhibits anti-inflammatory properties reducing the release of proinflammatory cytokines during inflammatory injury (Schmelzer et al., 2007). Niacin inhibits inflammation (Ganji et al., 2009). Niacin inhibits vascular inflammation by lowering endothelial ROS production and LDL oxidation. Selenium, a cofactor of many selenoproteins, reduces ROS, which is one of the factors for the production of TNF-α. Emblica officinalis possess flavanoids and tannoids which have anti-inflammatory function (Chen et al., 2011; Yokozawa et al., 2007). All these nutrients when given in combination have additive effect and thus, act as potent anti-inflammatory agents.
3.17.3 Effect of CoRNS and Eo on the mRNA expression of IL-10 in cardiac tissue of control and experimental animals

Plate 6 depicts the expression of IL-10, an anti-inflammatory marker. There was lowered level of IL-10 in Group II animals when compared to control animals. The treatment of animals with Simvastatin and CoRNS and Eo have elevated the levels of IL-10 when compared to Group II animals. The drug control animals showed no significant changes.

Inflammation plays a major role in the pathogenesis of atherosclerotic lesions of vascular walls. Inflammatory system was found in the atheromatous plaque, a relation between lipid metabolism, endothelial damage and inflammation. Atherosclerotic plaque contains activated macrophages and T lymphocytes, adhesion molecules, chemokines and cytokines, matrix-degrading enzymes and prothrombotic factors. Circulating inflammatory markers such as C-reactive protein (CRP), fibrinogen and interleukins are found in patients (Banach et al., 2004).

Interleukin (IL)-10 is one of the most important mediators that physiologically limits and down-regulates inflammation. Indeed, IL-10 has been proved to have several protective features acting against atherosclerotic disease. IL-10 down-regulates inflammatory activation of monocytes and macrophages by transcriptional and post-transcriptional inhibition of the entire range of pro-inflammatory cytokines. Cytokines are responsible for the modulation of immunological and inflammatory processes and play a significant role in the pathogenesis of coronary artery disease (CAD). Tumour necrosis factor is the major pro-inflammatory cytokine while Interleukin-10 (IL-10) is the major anti-inflammatory cytokine in patients with CAD (De Waal Malefyt et al., 1991). Both act as markers for CAD (Rajappa et al., 2009). IL-10 is produced
predominantly by macrophages within the local atherosclerotic lesion where it could play a significant role in the modulation of the local inflammatory response for both macrophages and T cells. Attenuation of atherogenesis by IL-10 was attributed to its anti-inflammatory effects, most notably its ability to inhibit the release of several pro-inflammatory cytokines (including IL-1, TNF-α and IL-8) from monocytic cells, and to induce the production of IL-1 receptor antagonist (Terkeltaub, 1999). IL-10 also has anti-apoptotic feature. Anti-apoptotic properties have been reported in cultured macrophages (Han et al., 2010). It can reduce the level of ROS. IL-10 may down-regulate over expression of lowered plasma VLDL and LDL cholesterol levels in LDL-R/- mice (Von Der Thusen et al., 2001). The role of IL-10 is to inhibit inflammatory molecules (e.g. TNF-α iCAM-1, and MMP9) and reducing apoptosis (Han et al., 2010).

It is reported that supplementation with antioxidants in humans showed anti-inflammatory effects (Block et al., 2004). The anti-inflammatory function of CoRNS and Eo has elevated the IL-10, as the drug is the potent anti-inflammatory agent and thus, acts as a potent anti-atherogenic agent.