ANTIHYPERLIPIDEMIC STUDY OF SELECTED NATURAL ANTIOXIDANTS AND DEVELOPMENT OF SYNERGISTIC SEQUEL BASED FORMULATION

Ph.D. Thesis

By

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SUMMARY

Hyperlipidemia is among the most common chronic pathological condition, associated with atherogenic events such as blood vessels occlusion, hypertension and plaque formation. The excessive release of cellular reactive oxygen species (ROS) in hyperlipidemia leads towards oxidative stress by depleting blood endogenous antioxidants. The polyunsaturated fats and cholesterol are more prone to be attacked by these ROS because of the double bond in chain lengths such as in LDL-C and infects others initiating a series of reactions. These radicals when engulfed by immune cells results in foam cell formation and endothelial cell injury. Various treatment protocols of synthetic and natural compounds were developed and applied. Among which the combination therapies were found more effective than individual therapies by providing synergistic effects. Natural antioxidants have well reported efficiency in scavenging ROS, preventing the lipid peroxidation and blood antioxidants depletion. Based on different mechanism of actions, availability in local food items and cost, we applied selected dietary antioxidants (AOX) in various combination dose ratios to determine the extent of synergism in hyperlipidemic state.

The AOX are mostly used in bulk doses individually and in combinations but the pro-oxidant effects at high doses prevents their own effects. However applying in low dose combinations is well suited for getting an optimized dose rationale with maximum benefits and minimized dose dumping, enzyme saturation and side effects. In current study natural antioxidants treatments were applied in various ratios in hyperlipidemic animal model and compared with statin and one another.
SUMMARY

Hyperlipidemia was induced in adult male albino rabbits and alteration in blood lipid profiles and endogenous antioxidants such as glutathione, methionine, N-acetylcysteine, ascorbic acid and α-tocopherol were observed. Further treatments with blends of selected three antioxidants (Oligomeric proanthocyanidins, niacin and pterostilbene) in various ratios were evaluated to get maximum effects. The therapies were designed in individual, two drug and three drug combinations and applied for 12 weeks using atorvastatin for comparison. The total dose of AOX was maintained at 100mg/kg in each therapy. Blood samples were analyzed periodically to observe change in lipid profiles, and blood endogenous antioxidants were evaluated at the end of therapy period with HPLC-ECD and UV based methods. New method was developed and optimized for determination of glutathione, its precursors, ascorbic acid and lipid peroxidation (malondialdehyde). The fat soluble antioxidants all-trans retinol and α-tocopherol were observed with reported method. The effectiveness of natural antioxidants blends was evident in treatments and validated the superiority of low dose blends over individual high dose therapies. The two drug combinations (OPC with NA, OPC with PT and NA with PT) were able to reduce LDL/HDL ratio and AI down to levels from -43.2% to -50.30% $p <0.001$ max and -15.24% to -23.63% $p <0.001$ max respectively. Compared to this in individual therapies maximum results were -37.08% $p <0.001$ and -18.52 $p <0.001$ respectively. The three drug combinations showed more pronounced effects and retarded LDL/HDL and AI by -59.30% and -25.09% $p <0.001$ max respectively, observed in 50:30:20 blend of OPC, NA and PT. The results were also comparable with the statin therapy while more significant in sparing endogenous antioxidants.
Evaluating blood antioxidants it was observed that initial rise in various blood antioxidants levels after two weeks of cholesterol dosing, might be due to the over activation of antioxidant enzymes; catalase, glutathione peroxidase, superoxide dismutase and others; accelerating the production of GSH against peroxides radicals. Compared to individual therapies, significant up-regulation was observed in methionine, GSH and NAC up to $35.08 \pm 1.60 \, \mu\text{mol/l}$, $3.82 \pm 0.47 \, \mu\text{mol/l}$ and $4.21 \pm 0.425 \, \mu\text{mol/l}$ ($p < 0.001$ each) respectively in combination therapies against the disease control $19.30 \pm 1.18 \, \mu\text{mol/l}$, $3.01 \pm 0.28 \, \mu\text{mol/l}$ and $3.73 \pm 0.412 \, \mu\text{mol/l}$. The all-trans retinoic acid showed opposite results and increased parallel with the lipid levels from $1.80 \pm 0.10 \, \mu\text{mol/l}$ (blank control) to $4.48 \pm 0.24 \, \mu\text{mol/l}$ (disease control). The treatment retarded its elevation down to $3.26 \pm 0.12 \, \mu\text{mol/l}$ ($p < 0.001$) max; observed on 70:30 ratio of OPC and PT. Homocysteine level was significantly controlled down to $10.09 \pm 1.39 \, \mu\text{mol/l}$ ($p < 0.01$), although differ non significantly among treatments. The maximum effect up to $5.29 \pm 0.51 \, \mu\text{mol/l}$ ($p < 0.001$) on AA was observed in 50:30:20 blends of OPC, NA and PT compared to disease control $4.90 \pm 0.59 \, \mu\text{mol/l}$.

For optimized AOX blend, stable formulations will least excipients number and quantities were developed. The compatibility tests via FTIR, drug content and physical consistency of the admixtures were conducted. The total quantity of AOX dose was formulated in half quantities.

For drug determination a novel HPLC-UV method for niacin and pterostilbene was developed using cetrimide as amphiphilic ion pairing agent. Method was successfully applied to plasma and dissolution samples as both drugs can’t be determined on spectrophotometrically simultaneously. The mobile phase was composed of 5mM
cetrimide aqueous and organic phase (80:20 acetonitrile: methanol) in ratio of 30:70 (v/v), adjusted to pH 2.5 with formic acid. Rosuvastatin was used as internal standard while OPC was analyzed using reported muriatic acid method.

Due to the wetting problem and poor flowability, granulation technique was followed using dry granulation, wet granulation with deionized water and with 2% PVP-K30. Mixtures were compressed into oblong shallow concave punches at the adjusted weight of 880 mg/tab. Prior to compression the parameters such as bulk and tapped densities, Hausner ratio, angle of repose, Carr’s index and powder/granule flowabilities were also evaluated. Tablets were subjected to in-vitro studies including mechanical strength, disintegration and dissolution for drug release.

Lab formulations produced fair to good results regarding precompresion properties, disintegration and in-vitro release. Using cross carmellose sodium individually (F6) in high quantity or in low ratio combination with sodium starch glycolate (F7) produced good disintegration and drug release profiles. Compared to this using high quantity of tablettose-80, micro crystalline cellulose and sodium starch glycolate individually showed low release profiles. However all the formulations released all three drugs within the standard limits of uncoated units.

The present study supports and signifies the use of natural antioxidants besides conventional therapies for better controlled results. This will ultimately improve therapy and reduce the side effects of conventional drugs in chronic treatments.
CHAPTER 1

INTRODUCTION
1. INTRODUCTION

1.1. Blood Lipids

Lipids are naturally hydrophobic occurring molecules involved in various functions including signaling, as stored energy backup, immunity, formation and maintenance of cell membrane integrity. Besides their recognition as harmful to cardiovascular and general health, lipids perform vital roles in synthesis of various endogenous compounds such as vitamin-D and hormones including steroids, adrenal gland hormones, aldosterone and sex hormones. They are also responsible for the solubility, absorption and transport of fat soluble vitamins (Vitamin A, D, E and K) from gastrointestinal tract (GIT) to general circulation. They are broadly classified in to various groups including fatty acids, glycerolipids e.g. triglycerides (TGs), sterol lipids e.g. cholesterol or total cholesterol (TC), and sphingolipids and are made up of ketoacyl and isoprene units.

Cholesterol and other lipids don’t get dissolved in blood and requires carriers for transportation called lipoprotein. Lipoproteins are composed of outer protein layer and inner core of cholesterol and triglycerides. They are classified into chylomicrons, very low density lipoproteins (VLDL-C), intermediate density lipoproteins (IDL-C), low density lipoproteins (LDL-C) and high density lipoproteins (HDL-C). HDL-C contain apolipoprotein-A (Apo-A) and LDL-C has apolipoprotein-B (Apo-B), which serves as major structural binding proteins to receptors for the delivery of lipids. Blood normal lipid profile is provided in table 1.1. 

5-7
### Table 1.1: Normal values of lipid profile

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Normal ranges</th>
<th>Considered risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>&lt; 200 mg/dl is desirable</td>
<td>&gt; 240 mg/dl</td>
</tr>
<tr>
<td>Low density lipoproteins</td>
<td>&lt; 100 mg/dl</td>
<td>160-189 mg/dl</td>
</tr>
<tr>
<td>High density lipoproteins</td>
<td>&gt; 60 mg/dl</td>
<td>&lt; 40 mg/dl</td>
</tr>
<tr>
<td>Very low density lipoproteins</td>
<td>&lt; 40 mg/dl</td>
<td>&lt; 60 mg/dl</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>&lt; 150 mg/dl</td>
<td>200-500 mg/dl</td>
</tr>
</tbody>
</table>

1.2. Regulation of lipids in body

1.2.1. Cholesterol regulation

From intestine cholesterol and fatty acids are taken by chylomicrons into muscle cells for energy production or fat synthesis, the excess are taken to the liver for storage. Almost 30% cholesterol is absorbed from diet and the rest is synthesized in body, this ratio may change depending on genetic constitution and dietary cholesterol. Cholesterol is synthesized almost in every cell endoplasmic reticulum controlled by a sensing protein called sterol regulatory element-binding protein (SREBP). Initiated through mevalonate pathway using Acetyl-CoA. Lanosterol a hydrophilic intermediate is formed which is then converted into cholesterol. The rate limiting step is hydroxyl methyl glutaryl coenzyme A (HMG-CoA) reductase enzyme that converts acetate to lanosterol as summarized in Figure 1.1.
1.2.2. Triglyceride regulation

Triglycerides (TG) constitutes 40-55% of total body fat and are involved in glucose homeostasis in blood and as major constituent of human skin oil. TGs are broadly classified in saturated and unsaturated fats. In saturated fats all carbon bonds are saturated with hydrogen, while unsaturated has double bonds in its chains and are most
vulnerable sites for easy invasion \(^1\). The endogenous production of TGs occurs through acetyl Co-A mechanism, for which the genes are activated by SREBP-1c. Initially saturated fatty acids are synthesized from malonyl CoA, which converts into monoacylglycerol 3-phosphate in the presence of glycerol 3-phosphate acyltransferase (GPAT) and finally to triglycerides and phospholipids \(^1\) as shown in figure 1.2.

\[\text{Acetyl-CoA} \rightarrow \text{Malonyl CoA} \rightarrow \text{Saturated fatty acids} \rightarrow \text{Fatty acyl CoA}\]

\[\text{Acetyl CoA carboxylase} \rightarrow \text{NADPH} \rightarrow \text{Mono unsaturated fatty acids} \rightarrow \text{GPAT}\]

\[\text{Triglycerides + Phospholipids} \leftarrow \text{Mono acylglycerol 3-phosphate}\]

**Figure 1.2:** Mechanism of endogenous triglycerides synthesis.

**1.2.3. Metabolism of lipids**

Among various enzymes; lipoprotein lipase enzyme (LPL), peroxisome proliferated activated receptors (PPARs) and cholesterol 7-\(\alpha\)-hydroxylase enzyme are the most prominently involved in lipid metabolism. The LPL is expressed in adipose, macrophages and in endothelial cells and causes the hydrolytic breakdown of TGs in circulating chylomicrons, VLDL-C and other tissues to release free fatty acids (FFAs). It also enhances uptake of circulating cholesterol esters rich LDL-C particles \(^1\). PPARs are a family of nuclear receptors composed of three members \(\alpha\), \(\gamma\) and \(\delta\). Upon activation by ligands such as retinoic acid; heterodimerize with the retinoid X receptors (RXR \(\alpha\), \(\beta\) and \(\gamma\)) and promote homeostasis of lipids and glucose in the body \(^1\). PPAR\(\alpha\) breaks FFA
chains and presents to mitochondrial β-oxidation for energy and also promote hepatic TGs hydrolysis. PPAR γ delivers opposite function and its stimulation by ligands like prostaglandin-E2 (PG-E2) and leukotrienes (LT) leads to accumulation of lipids in adipose tissues. It also plays roles in adipocytes cells differentiation and glucose- insulin homeostasis. The PPAR δ has roles in skin repairing, wound healing and adipose tissue massing. Lipids from gut are converted to bile acids via cholesterol 7-α-hydroxylase enzyme, stored in bile and further utilized in micelles formation, absorption and metabolism of fats.

1.2.4. Functions of lipids

Lipids are essential part of mammalian cells and plays major role in maintaining its structure and functions. They also modulate proteins in transmembrane signaling and trafficking such as G-protein coupled receptor signaling. Lipids in macrophages and leukocytes act in inflammation and immunity, by promoting phagocytosis. Cholesteryl esters rich lipid droplets in adrenal cortex, testes and ovaries are utilized in the synthesis of steroid hormones, pulmonary surfactant during maturation of lungs, as signaling molecule and ligands for transcription factors.

In heart cells TGs rich lipid droplets metabolize by PPARα and mitochondrial function for energy. They attach with proteins at hydrophobic sites and prevent their aggregation. They also act as lubricants in synovial fluids, as carriers for hydrophobic drugs and maintain the cutaneous permeability barriers and body temperature.
Besides their vital functions, they are also responsible for the initiation and progression of coronary artery disease, diabetes, obesity, Parkinson’s, dementias and hyperlipidemias.  

1.3. **Hyperlipidemia**

1.3.1. **General introduction**

Hyperlipidemia also called dyslipidemia and hyperlipoproteinemia refers to the rise of cholesterol content, TGs or a class of lipoproteins in the blood circulation above normal levels. This pathologic condition describes different clinical manifestations that are involved in lipoprotein metabolism. According to American survey among major causes of cardiovascular diseases, hyperlipidemia is the most common factor with 53.4% adults with abnormal TC level and 32% elevated LDL-C level. Lipids circulate in blood attached with proteins, so rise in lipid level is also called hyperlipoproteinemia. It is also a major risk factor for developing cardiovascular diseases since there is abnormal rise in circulating lipids due to disorders in lipid metabolism. Elevated lipid levels are also involved in development of atherosclerosis. Studies have reported that main cause of hyperlipidemia is the over production of excessive VLDL-C particles which imparts drastic effects on lipoproteins resulting in increased level of LDL-C remnants and decreased HDL-C. In elevated triglyceride condition the LDL-C contains high amount of triglycerides and almost depleted of Cholesteryl esters, liver LPL hydrolyze these triglycerides to FFA increasing the risk of atherogenecity.

Hyperlipidemia is often associated with metabolic syndromes that include adipose tissues accumulation, elevated triglyceride level, diabetes mellitus, obesity and high
blood pressure. According to Canadian guidelines patients of 40 and above age having any of these clinical conditions like hypertension, diabetes, familial coronary artery disease obesity, HIV infection, chronic renal diseases and other pathologic conditions their blood lipid profile must be checked for any abnormalities.

1.3.2. Types of hyperlipidemia

According to Fredrickson classification which was also adopted by World Health Organization (WHO), hyperlipidemia or hypercholesterolemia is classified into five classes summarized as follows.

Familial Hyperlipidemias are the clinical conditions caused by specific gene disorders while the secondary causes include metabolic and other syndromes.

Type I: Elevated serum cholesterol, triglyceride and chylomicron level primary due to deficiency of lipoprotein lipase enzyme or apolipoprotein-C2. May be also cause by systemic lupus erythematosus. Main symptoms include acute pancreatitis, lipemia, eruptive skin xanthomas.

Type IIa: There is elevated total cholesterol and LDL-C either due to familial hypercholesterolemia or hypothyroidism. Symptoms include tendon xanthomas.

Type IIb: It is also called familial combined hyperlipidemia. There is high level of plasma TC, TG, LDL-C and VLDL-C. Secondary causes includes nephritic syndrome, diabetes etc.

Type III: Elevated TC and TG level, excessive chylomicron remnants and intermediate density lipoproteins. Secondary causes include hypothyroidism, diabetes and obesity.
Type IV: Elevated TC, TG and VLDL-C level. It is primarily caused by familial hypercholesterolemia and familial triglyceridemia. Secondary causes include diabetes and chronic renal diseases.

Type V: Elevated TC, TG, chylomicrons and VLDL-C level. Primary cause is familial triglyceridemia and lack of lipoprotein lipase enzyme level or apolipoprotein-C2. Secondary causes include alcohol, diuretics, β blockers, oral estrogen.

During normal metabolic and/or pathologic processes various byproducts are get released or leaked. They in normal levels perform physiological functions but excess release results in serious complications. Among them reactive oxygen species or free oxygen radicals are the most important byproducts possessing multi roles in both physiologic and pathologic conditions. They are discussed in the following section.

1.4. Reactive oxygen species

Reactive oxygen species (ROS) or free radicals are the molecules or atoms capable of existence independently having one or more unpaired electron. They also have the capability to extract hydrogen ion from other stable molecules making them oxidized and unstable. Example includes superoxide (O$_2^-$), hydroxyl (OH), S (sulphur containing radical), carbon tetra chloride (CCl$_4$), induced nitric oxide (NO$^-$) in which electron is delocalized between the two atoms, stable radicals includes hydrogen peroxide H$_2$O$_2$ and hypochlorous acid (HOCl) $^{36}$. 
1.4.1. Factors responsible for the release and removal of reactive oxygen species

Free radicals are released in various metabolic mechanisms as byproduct in almost all organs. Factors that are responsible for the release of oxygen free radicals (O$_2^-$, H$_2$O$_2$, OH) includes nicotinamide adenine dinucleotide (NADH) oxidase, xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) - cytochrome P-450 reductase, acetyl Co-A reductase, mono amine oxidase (MAO), glycolate oxidase, macrophages, leukocytes, bacterial toxins. Electron leakage occurs during mitochondrial electron transport of H$_2$O$_2$ to water; about 3-5% of total oxygen consumed is reduced univalently to superoxide ions (O$_2^-$) by the process called autoxidation $^{37-39}$.

In FFA metabolism the PPAR$\alpha$ gets activated first oxidizing FFAs to small chain fatty acids, with the release of H$_2$O$_2$ as byproduct. In this step no ATP is released. These smaller chains then taken by mitochondria for $\beta$-oxidation and release of adenosine triphosphate (ATPs). The H$_2$O$_2$ produced in first step is efficiently reduced to water and oxygen by catalase enzyme present abundantly in peroxisomes $^{40}$. Enzymes that are responsible for the removal of oxygen radicals like hydrogen peroxide are superoxide dismutase (SOD) and glutathione peroxidase (GPX). SOD removes H$_2$O$_2$ by converting it into water and oxygen while GPX utilize glutathione (GSH) to convert it into water. The glutathione (GSH) get oxidized in this process to glutathione disulphide (GSSG), which again recovered by reaction of oxidized form with NADPH in the presence of glutathione reductase enzyme $^{41}$.

Peroxisomes possess two potential enzymatic sources of superoxide (O$_2^-$) and NO$^\cdot$, the xanthine oxidase that produces H$_2$O$_2$ and O$_2^-$ as byproduct and inducible form
of nitric oxide synthase. The damage or excessive pathological up regulation of peroxisomes results in excessive release of H$_2$O$_2$ and O$_2$$^-$ into cytosol. The inducible nitric oxide synthase releases O$_2$$^-$ along with NO$^*$ as reactive nitrogen species leading to nitrosative stress. Both can react to form ONOO$^-$ a strong oxidizing and nitrating agent, that can activate series of lethal events. Moreover peroxisomes may act as source, sink or as target for the ROS and reactive nitrogen species (RNS) 42.

These ROS/ RNS not only produced aerobically but also produced in hypoxic and reoxygenated conditions. High quantities of superoxide (O$_2$$^-$) and H$_2$O$_2$ are released interacting with proteins and lipids. Factors releasing ROS in hypoxic conditions includes xanthine dehydrogenase oxidase that acts mainly in liver and intestine. Ferrylhemoglobin and myoglobin released in blood after trauma mediates endothelial cell oxidative stress and lipid peroxidation after exposure even increases in reoxygenation. Other includes NADPH oxidase which is membrane enzyme having main role in ROS generation during lung ischemia 43.

1.4.2. Potential roles of reactive oxygen species

ROS possess the potential of double edge sword and can both prevent and promote disease. They are involved in various physiological and pathological conditions. Various studies have been conducted upon their roles in both disease and healthy conditions that includes the cancer, cardio vascular diseases (CVD), their effects on skeletal muscles, Alzheimer and other neurodegenerative disorders, immune responses and apoptotic cell degradation and elimination 44.
1.4.3. Potential role in physiological conditions

ROS are very important in maintaining the physiological functions when produced at normal levels. They act as second messengers in the signals transduction pathways, regulating protein modification by controlling the gene expression. Promoting immunity in macrophages and neutrophils against bacterial and other infections \(^{45}\). The induced nitric oxide (NO\(^\cdot\)) is the RNS produced from arginine metabolism by nitric oxide synthase plays an important role in neurotransmission, blood pressure, smooth muscles relaxation and immune regulation \(^{46-47}\).

1.4.4. Potential role in pathological conditions

The ROS produced during metabolic processes are in balance with the body antioxidant system which scavenges these ROS from within the cell and general circulation maintaining redox homeostasis. In cell it is mostly controlled by GSH in balance with its oxidized form GSSG. In pathological stress the over production of ROS disrupts this balance and results in depletion of enzymatic or non enzymatic antioxidant systems for the recovery of redox environment. This ultimately increases the damage susceptibility of DNA, lipids cell membranes and endothelial cell lining \(^{48}\). Besides this severe oxidative stress was observed when GSH was depleted in rats resulting in hypertension. Evidence showed increased NADPH oxidase activation and leakage of superoxide radicals due to hyperglycemia and high FFA blood levels. This raised levels of ROS resulted in impaired endothelial dependent vasorelaxation in both type-1 and type-2 diabetes mellitus (DM) models \(^{49}\).
1.4.5. Potential role of reactive oxygen species in hyperlipidemia

ROS are the aerobic byproducts released during physiological conditions and interacts with lipids, proteins, DNA and carbohydrates. In hyperlipidemia oxidative modification of LDL-C particles, protein glycation and glucose autooxidation leads to excessive production of lipid peroxidative products elevating oxidative stress 50.

LDL-C contains about more than 60% of total serum cholesterol and less amount of triglycerides, and derived from TG rich VLDL-C particles. They transport lipids to organs either by attachment with LDL-C receptors or by non-specific endocytosis. In liver they are utilized to form bile acids which are released in duodenum, while other organs use LDL-C for synthesis of steroids, cell membrane and energy. LDL-C carries mostly polyunsaturated fatty acids (PUFA) mostly lenoliec acid of cholesteryl esters as major component and because of having the double bond they can be easily attacked by radicals. They are protected from oxidation by lipophilic endogenous antioxidants in which α-tocopherol is present in highest quantity 11. When LDL-C is attacked by free radicals it abstract hydrogen atom from PUFA to form an unstable lipid radical (L'), which than react with molecular oxygen to form lipid peroxyl radical (LOO'). This radical can abstract hydrogen ion from adjacent fatty acid to form lipid hydroperoxides (LOOH) and another lipid radical. The LOOH can be further cleaved by reduced metal ion like iron (Fe^{++}) forming lipid alkoxyl radical (LO') as shown in figure 1.3. Both peroxyl and alkoxyl radical initiates a chain reaction of lipid peroxidation by abstracting H-ion from other fatty acid molecules 51-52. The degradation of LOOH can give products in the form of alcohols, ketones, ethers, aldehydes and epoxides through several pathways. Among them aldehydic product called malondialdehyde (MDA) is reactive.
having long life and produced in large amount. It can form adducts (complexes) with other biological compounds especially with the macrophages, endothelial cells and glutathione. Therefore it is used as a biomarker for the quantification of lipid peroxidation\textsuperscript{53-54}.

The oxidized LDL (LDL-oxi) attracts other lipid molecules forming aggregates and are detected and engulfed by tissue macrophages especially of blood vessels as foreign bodies. The resulting macrophage loaded LDL-oxi leads to the formation of foam cells within vessel walls, a sign of earliest atherosclerotic lesion that propagates towards atherogenesis\textsuperscript{55}.
1.5. **Defensive mechanisms against hyperlipidemia associated lipid peroxidation**

Human body has been exposed to oxidative stress through numerous ways against which the immune system has been developed to control these stress situations. These mechanisms can be broadly classified in direct and indirect mechanisms. The indirect mechanisms involve the control of endogenous production sources of ROS such as NADPH oxidase. Human body also possesses a defensive system of antioxidants that protects from oxidants generating within the body by direct mechanism. They are comprised of antioxidant enzymes and low molecular weight antioxidants (LMWA).

1.5.1. **Enzymatic components of biological redox homeostasis**

Enzymes that are responsible for the removal of oxygen radicals like hydrogen peroxide are SOD and GPX. SOD has two sub classes the copper and zinc (Cu-Zn-SOD) and manganese (Mn-SOD). It contains two subunits of proteins and are present in cytoplasm and removes $\text{H}_2\text{O}_2$ by converting it into water and oxygen. The GPX utilize glutathione (GSH) to convert it into water. The GSH get oxidized in this process to glutathione disulphide (GSSG), which again recovered by reaction of oxidized form with NADPH in the presence of glutathione reductase enzyme. Catalase enzymes are tetrameric heme containing molecule catalyzes dismutation of two $\text{H}_2\text{O}_2$ molecules into water and oxygen. Ascorbate peroxidase (APX) is an important component of ascorbate-glutathione cycle and act intracellularly. It utilizes two molecules of ascorbic acid to convert $\text{H}_2\text{O}_2$ into water along with generation of two mono-dehydroascorbate (MDHA) molecules. The extensive distribution in cell organelles and cytoplasm and strong affinity for $\text{H}_2\text{O}_2$ makes APX as most efficient scavenger of $\text{H}_2\text{O}_2$. The MDHA radicals produced
by APX activity is reduced by MDHA-reductase for regeneration of ascorbic acid using NADPH as electron donor. Similarly dehydroascorbate reductase utilizes glutathione as substrate for reduction of dehydroascorbate radical to ascorbic acid maintaining its level in reduced form. The glutathione reductase belongs to the flavoenzymes and contains a disulfide group catalyzing the reduction of glutathione disulfide (GSSG) to glutathione in the presence of NADPH, maintaining higher ratio of cellular GSH/GSSG.57

1.5.2. Nonenzymatic components of biological redox homeostasis

Non-enzymatic antioxidants that are involved against cellular oxidative stress can be broadly classified into water soluble which remains in cytosol and lipid soluble which resides in and prevents cell membrane from peroxidation. They includes vitamin-E (both tocopherol and tocotrienol), ascorbic acid (AA), sulfhydryl group containing compounds; glutathione (GSH), cystine (CySS), homocysteine (Hcy), cysteine (Cys), methionine (Meth) and N-acetylcysteine (NAC). Others are lipoic acid, uric acid, carotenes, ubiquinol and phenols.57-58

Glutathione (glutamyl-cysteinyl-glycine) is a tripeptide and most abundant antioxidant synthesized in mitochondria. It is present in cell cytosol in concentrations of 1-11mM. It is one of the low molecular weight non protein thiols perform major role against oxidative stress intracellularly. Mostly available in reduced forms and abundantly in dietary items. It act as cofactor for glutathione peroxidase and dehydroascorbate reductase, act against peroxidases mediated cell injury and maintains the level of reduced forms of vitamin E and C in biological fluids. Glutathione disulphide (GSSG) the oxidized form produced in redox reactions converts back to GSH by glutathione
reductase in the presence of NADPH. GSH is also produced by cystine a disulphide oxidized form of cysteine by cystine/glutamate exchange system. \( \text{H}_2\text{O}_2 \) produced in mitochondria stimulates monocytes for adhesion to endothelial cell lining but quickly reduced by over production of mitochondrial thioredoxin (Trx2), a protein antioxidant presents in high quantity along with GSH. Trx2 is oxidized by extracellular cystine while the redox state of thioredoxin in cytoplasm and nucleus (Trx1) remains unaffected. Homocysteine is a non protein amino acid that is synthesized in the body from methionine and has two main roles either converts back to methionine by tetrahydrofolate or initiate synthesis of cysteine. In majority of cases it is reported that high level of Hcy is directly correlated with the production of free radicals and increases the level of lipid peroxidation end product malondialdehyde. Studies reported hyperhomocysteinemia as an independent risk factor for the development of CVDs, while folate is being reported to reduce this condition by converting the homocysteine back to methionine.

Cysteine a sulfhydryl group containing amino acid possesses the same chemical features like homocysteine. Its autoxidation results in both atherogenic and thrombogenic processes and formation of foam cells by promoting superoxide mediated LDL-C peroxidation within the vessel walls. This makes the plasma cysteine a risk factor along with homocysteine. It also reacts with nitric oxide and impairs endothelial function. N-acetylcysteine is a cysteine analogue amino acid and performs both directly by detoxifying the free radicals and indirectly by providing Cys for the synthesis of GSH during oxidative stress. It promotes the glutathione redox cycle and also work as cell permeable precursor in the synthesis of glutathione. Methionine is a proteinogenic essential two sulfur containing amino acid not synthesized in human cells and taken from
diet to run a cycle necessary for the synthesis of reduced glutathione. Methionine cycle is involved in basic cellular metabolic mechanisms and its defect may result in many complications of CVD, DNA methylation, cancer progression. It is first converted to homocysteine for glutathione synthesis, thus act as a precursor. Methionine is also reproduced from homocysteine by methionine synthase using methyltetrahydrofolate in the presence of Vit-B<sub>12</sub> as cofactor. Glutathione synthesis from methionine is shown in figure 1.4.

**Figure 1.4:** Synthesis pathway of glutathione from methionine cycle.
Plasma cysteine, homocysteine and all other amino acids coexist in oxidized, reduced and protein bound forms and interact with one another through redox and disulfide exchange system. Ascorbic acid is considered a powerful antioxidant because of its electron donor capacity to both enzymatic and non enzymatic reactions. In physiological level of 40-80 μM exert antioxidant effect and as co-vitamin with α-tocopherol (vit-E), preserving LDL-C from detectable oxidative damage. It protects membranes by reacting directly with superoxide and peroxide radicals and regenerating α-tocopherol from tocopheroxyl radical. It removes peroxide radicals by ascorbic acid-glutathione cycle. As ascorbate it can be regenerated from ascorbate radical and dihydro-ascorbate both enzymatically and non-enzymatically. Auto-oxidation makes ascorbate as pro-oxidant in combination with metal catalyst producing hydrogen peroxide. The α-tocopherol is a major lipophilic antioxidant and is capable in breaking lipid peroxidation propagation. Studies reported that it scavenges on peroxyl radical and has no potent scavenging effect on hydroxyl, alkoxyl and nitrogen radicals. While synergistic interaction was observed with ascorbic acid. Its antioxidant property is because of phenolic hydroxyl group, by donating hydrogen to peroxyl radicals. This results in formation of stable lipid molecule and oxidized α-tocopherol, which is regenerated back to reduced form by AA, GSH or coenzyme Q.

Polyphenols are the secondary metabolites possessing potent antioxidant activity because of aromatic rings with –OH and –OCH₃ groups. They can chelate metal ions, scavenge oxygen radicals directly and inhibit lipid peroxidation by scavenging lipid alkoxyl radicals. They also decrease membrane fluidity and hence hinder free radicals diffusion and peroxidative reactions. They indirectly up regulate antioxidant capacity.
by boosting antioxidant enzymes. Suppressed LDL oxidation, lipid peroxidation rate was observed in diabetes mellitus upon interventions with polyphenols along with increase in plasma HDL levels but negligible effects on health volunteers were reported\textsuperscript{77-78}.

Retinoic acid available as \textit{all}-trans retinol in physiologic form involved in cell differentiation and has antioxidant properties. It can easily penetrate into the cell aiding other antioxidants in maintaining redox state. In maintaining lipid homeostasis it binds to RXR and RAR receptors and down regulates the expression of CYP7A1 and CYP8B1 which are responsible for bile acid synthesis. This consequently reduces emulsification and absorption of cholesterol in gut and ultimately effects plasma lipid levels. In hyperlipidemia the elevated blood level of retinoic acid can be considered as biomarker for hyperlipidemic associated oxidative stress\textsuperscript{79}.

The role of antioxidants in human body against free radicals is explained in figure 1.5 \textsuperscript{48}. 
Figure 1.5: Mechanistic pathway for free radical detoxification.

The glutathione (GSH) is oxidized to glutathione oxidized form (GSSG) by glutathione peroxidase and reproduced by glutathione reductase enzyme. The α-tocopherol is oxidized by lipid radical and again reduced by expense of ascorbate and...
GSH molecules. Further the conversion of GSSG to GSH occurs by expense of dihydrolipoic acid (DHLA) to oxidized form α-lipoic acid (ALA). The malondialdehyde (MDA) produced can form adduct with DNA and attach itself to the inner side of blood vessels enhancing the formation of atherogenic plaque. The 4-hydroxynonenal produced in the second reaction is less reactive than MDA.  

1.6. Association of hyperlipidemia with other clinical conditions

1.6.1. Diabetes and hyperlipidemia

The most prevailing type of hyperlipidemia in diabetes is the hypertriglyceridemia associated with hypercholesterolemia both in type 1 and type 2 DM. In type 2 diabetes insulin resistance is high, with reduced particle size and high density LDL-C making them more atherogenic, and VLDL level is high while HDL level is low. Diabetes associated hyperlipidemia can make the disease more severe by accelerating the development of diabetic neuropathy. Study reported increased susceptibility for the development of peripheral diabetic neuropathy independent of blood glucose level. High lipid level is responsible for development of premature CVD in type 2 DM that includes artherosclerosis, stroke and infarction and also involved in progression of diabetic kidney disease i.e. nephropathy.

1.6.2. Obesity and hyperlipidemia

Obesity currently recognized as marker of “dysfunctional adipose tissues”, is another most commonly associated condition with hyperlipidemia and mostly affects adolescent and young children. In United States about 15% of children and adolescents are obese and are in great risk of having cardiovascular and metabolic diseases. It is
also now a common growing condition in adults, specially the abdominal obesity due to abnormality in lipid metabolism and over accumulation in adipose tissues. Obesity induced hyperlipidemia is due to high fat diet which makes liver to release high quantity of triglycerides rich VLDL-C particles. High levels of TG not only diminish the LPL activity but also reduce the liberation of HDL-C from their surfaces. It can occur with liver transplantation both in donor and receptor because of graft rejection, cardiovascular condition, hypertension, dyslipidemia or renal failure.

1.6.3. Cardiovascular diseases and hyperlipidemia

The ROS produced during oxidative hyperlipidemia and other associated pathologies modify phospholipids and protein leading to peroxidation of lipids. In addition these free radicals also promote the release of calcium ions from sarcoplasmic reticulum in cardiac and skeletal muscles. Accumulation of lipids cause excessive sodium (Na⁺) ions in cardiac cells and along with lack of ATPs, it results in imbalancement in calcium (Ca++) handling in sarcoplasmic reticulum. This condition leads to Ca++ overload in cardiac cells, moreover the reperfusion also enhances Ca++ uptake thus may worsen the condition. All these mechanisms in addition to the high cholesterol level and increased uptake of LDL-oxi in endothelial cells results in increased chances of developing atherosclerosis, hypertension, myocardial cell damage in reperfusion and hypertrophy. The direct reaction of O₂•− with NO• results in peroxynitrite which also increases the rate of lipid peroxidation. Presence of huge amount of iron in atherosclerotic lesions confirmed the involvement of iron in catalyzing free radical development. Nitric oxide (NO) is produced from L-arginine by nitric oxide synthase (NOS) and this enzyme is inhibited by asymmetric dimethylarginine (ADMA).
It was found that in hyperlipidemia there is decreased bioavailability of NO either due to increased effect of ADMA or excessive conversion of NO to peroxynitrite radical \(^91\).

### 1.6.4. Cerebral disorders and hyperlipidemia

High lipid levels and brain disorders are interconnected with each other, in some cases it may worse the condition such as Alzheimer’s in which excessive β-amyloid proteins are accumulated in brain. Its synthesis and elimination is controlled by cholesterol and untreated hyperlipidemia can aggravate the condition by excessive free radical production and lipid peroxidation \(^92\)–\(^93\). Another example is the schizophrenia in which use of antipsychotic drugs causes elevated blood lipid and glucose levels that may trigger CVDs or cerebrovascular diseases and associated oxidative stress \(^94\).

### 1.7. Current medication therapies

Research studies on diet control intervention and medication therapies in hyperlipidemic population confirmed the decrease in TC, LDL-C and cardiovascular diseases. Decrease in 1% of TC or LDL-C reduced cardiovascular events by 1% or more \(^95\). Antihyperlipidemic therapies are broadly classified into non-pharmacological and pharmacological classes.

#### 1.7.1. Non-Pharmacological therapies

It includes those parameters that if adopted, can produce effects in normalizing lipid profile, prevent developing of artherosclerosis and cardiovascular events.
1.7.1.1. Low intake of high fat and caloric diet

Dietary fats and hyperlipidemia are very closely related. Studies reported that the use of saturated fats and trans-fatty acids increases plasma TC and LDL-C. While the use of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of n-6 chain length series decreases TC and LDL-C and elevates HDL-C levels. PUFA are less effective than MUFA in raising plasma HDL-C level. Reported data showed high level of unsaturated fatty acids in olive oil, sunflower oil and rapeseed oil and effects in lowering TC level significantly. PUFA of n-3 series of FA has pronounced effects on TG rich VLDL-C. Yet they may raise LDL-C to some extent but the overall effect is reduction in progression in cardiovascular events. American heart association (AHA) strongly recommends the dietary cholesterol intake not more than 300 mg/day in healthier and less than 200 mg/day in diseased patients. Canadian health authority restricts the intake of saturated fats not more than 10% per day.

1.7.1.2. Diet containing carbohydrate and fiber

Several studies showed that replacing of saturated fats with carbohydrates decreases the plasma TC and LDL-C levels. However sole use of carbohydrates may raise plasma TG level to a higher extent with decrease in plasma HDL-C, and may act as independent CV risk factor. Sugars are found to increase plasma TG level with decrease in HDL-C level due to the over enrichment lipoproteins with TG enhancing their catabolism while has no significant effect on LDL-C level. Diet rich of fibers also helps in reducing plasma TC and LDL-C, they block the absorption of cholesterol from intestines. This effect is more significant when soluble or gel forming fibers are
used as part of diet. Addition of cereals, legumes in diet is essential for lipid control, use of 5-10 gm/day of psyllium, β-glucans and guar can reduce LDL-C by 5% $^{103}$.

1.7.1.3. **Obesity, weight control and physical activity**

Obesity is associated with uncontrolled lipid profile and risk of developing cardiovascular conditions. Studies have mentioned that weight loss and obesity control has little effect on plasma TC and LDL-C but more pronounced effect on TG and HDL-C levels $^{104}$. It was observed that 10kg loss in weight decrease the LDL-C level by 8.8 mg/dl in overweight patients and has also positive effect on glycemic control and blood pressure $^{105}$. Lack of physical activity has major contribution in aiding weight gain, lipid profile and other clinical parameters $^{106}$. Clinical studies has reported that regular exercise has positive effects on HDL-C level and can bring rise between 9%-50%, along with 19%-50% decrease in TG levels $^{107}$.

1.7.2. **Pharmacological treatment**

Several classes of drugs are being introduced for the treatment and management of hyperlipidemia. Some countries have published guidelines about the use of such drugs for the treatment that depends on the lipid profile $^{108-110}$. Various classes of medications that are currently are briefly discussed here.

1.7.2.1. **Statins first line drugs**

This class of drugs inhibits 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (HMG-Co-A reductase) and blocks the synthesis of cholesterol, which increases the clearance of LDL-C. As endogenous synthesis is blocked so for energy LDL-C utilization
results reduction in its plasma level \(^{111}\). Drugs include simvastatin, pravastatin and lovastatin are fungal derived while atorvastatin, fluvastatin, rosuvastatin, and pitavastatin are synthetic \(^{112}\). Several clinical data showed LDL-C lowering down to 35\% and decreasing total mortality rate by 30\% after more than five years of statin therapy \(^{113}\). But some experimental data reported inadequacy of statin monotherapy in reducing plasma LDL-C at optimal doses, thus pointing out the need of dose titration and lack of achievement \(^{114-115}\). Similarly significant reduction in cerebrovascular events was observed in two meta analysis in secondary prevention while no satisfactory results were observed in primary prevention of stroke \(^{116}\).

Combination of statin therapies with other lipid lowering classes resulted in synergistic effect in lowering the plasma lipid level LDL-C fractions, with ameliorating effect on other lipoprotein fractions due to the different mechanisms of actions \(^{117-118}\). Besides beneficial outcome, adaptation of polypharmacy has also increased side effects on liver and skeletal muscle \(^{119}\). A condition called myopathy is the skeletal muscle pain due to increased level of creatinine kinase almost 10 times. It may lead to rhabdomyolysis and acute renal failure if untreated \(^{120}\). Cases of statin induced hepatotoxicity were found in very few patients (<1\%) but still contraindicated in liver diseases \(^{121}\). If metabolic enzyme inhibitors are administered along with statins (lovastatin, simvastatin) it may lead to increased plasma level and chances of adverse effects. Combination of statin with fibrates and niacin can cause myopathy without effecting plasma level, while their low dose combinations were found safe \(^{116}\). Simvastatin is found to be neuroprotective by mediating release of neurotrophic factors thus enhancing nerogenesis especially after cerebral injury and in Alzheimer’s disease \(^{122}\).
Studies have reported in vivo antioxidant activity of statins against oxidative damage and lipid peroxidation by inhibiting the activation of NADPH oxidase mediated ROS generation and enhancing endothelial derived relaxing factor by decreasing plasma cholesterol level\textsuperscript{123-125}. Nitric oxide (NO) is produced either by endothelial nitric oxide synthase (eNOS), neuronal (nNOS) or inducible (iNOS), a radical form released by inflammatory response of macrophages. The eNOS released NO produce dilation effect in ischemia while nNOS activation results in activation and release of iNOS and over production of NO radicals’ leads to peroxidation and endothelial damage. Statin enhances the eNOS for production of NO while down regulating the nNOS and iNOS\textsuperscript{126-128}. An animal study reported that treatment with statin (atorvastation) after inducing ROS production, induced stimulation of mitochondrial biogenesis and hence antioxidant capacity in cardiac cells. While in skeletal muscles cells it rather worsen the condition which is the main reason behind the muscle pain and this effect was contracted by administration of quercetin\textsuperscript{129}.

### 1.7.2.2. Fibrates as PPARα activators

These are the fibric acid derivatives and include clofibrate, ciprofibrate, benzafibrate, gemfibrozil and fenofibrate (prodrug). They are the potent PPARα activators selectively and mediate the metabolism of TG and HDL-C by triggering LPL. They act against LDL-C if level is higher than baseline however less effective in reducing total mortality rate, therefore used in combinations with statins and other lipid modifying drugs\textsuperscript{130}. Fibrates increases the hepatic uptake of fatty acids (FA), their conversion to acyl-Co-A, initiation of β-oxidation and metabolism of FA thus reducing its level for TG synthesis. They increases LDL catabolism by enhancing the affinity for LDL receptors.
and enhances HDL synthesis by reverse cholesterol transport, consequently reduces TG rich lipoproteins levels in plasma\textsuperscript{131}.

Side effects are similar to that of statins, others includes myalgia and pruritus. Rhabdomyolysis induced death ratio increases when used in combination with statins \textsuperscript{132}. Most of fibrates are also reported to increase blood homocysteine and creatinine levels with different magnitudes (18\%-55\%) \textsuperscript{133-134}.

1.7.2.3. **Cholesterol absorption inhibitors**

Member of this class (e.g. ezetimibe) blocks the intestinal and biliary reabsorption of cholesterol by attachment with Niemann-Pick C-1 like-1 (NPC1L1) receptors. This leads in low dietary availability and expression of LDL receptors for uptake and catabolism of LDL particles \textsuperscript{135}. NPC1L1 is a transmembrane protein expressed locally in small intestine brush border to large extents and in liver of humans while mice lack this protein in liver \textsuperscript{136}. This protein promotes the transport of cholesterol across the small intestine by forming micelles, for absorptive enterocytes to deliver sterol to chylomicrons \textsuperscript{137}. Combination therapies with statins produced incremental effects in lowering plasma LDL-C and rising HDL-C levels \textsuperscript{138}. Another study reported the synergistic effects of ezetimibe with simvastatin in combination on TC level \textsuperscript{139} while no statistically satisfactory effects were observed on TG and HDL levels \textsuperscript{140}. Other combination therapy trials of ezetimibe with orlistat in hypercholesterolemic patients resulted in significant reduction of plasma LDL-C compared to monotherapies as both drugs inhibits lipid absorption by different mechanisms without having any serious side effects \textsuperscript{141-142}. 
This class is well tolerated having no any serious adverse effects. However, some rare side effects of darkening of stool and urine, other gastrointestinal tract (GIT) problems gum bleeding, rashes and fast heart beat were found in few patients.

1.7.2.4. Niacin

In spite of not reducing total mortality rate, niacin is still a proven drug in rising plasma HDL-C levels. It acts by blocking the synthesis of TG in liver by inhibiting the hepatic diacylglycerol acyltransferase (DGAT2) enzyme that promotes the final step in TG synthesis. It also selectively inhibits the uptake and removal of HDL-Apo A-I thus increases the concentration of Apo-A containing HDL-C. Study has shown the antioxidative activity of niacin by increasing the level of NADPH$_2$ and glutathione levels with inhibition of angiotensin II mediated ROS release and lipid peroxidation. Main adverse effect of niacin is facial flushing due to vasodilatation caused by the release of PGD2 and PGE2 from macrophages and immune cells. This effect can be blocked by pretreatment with aspirin or formulating it in extended release dosage form, that will release the drug in an extended time thus minimizing the immune cells reaction against niacin high levels. Studies have reported the synergistic effects of niacin in rising HDL-C and lowering cholesterol and TG levels when administered in combinations with statins, other drugs and plant sterols (such as polyphenols) for the treatment of dyslipidemia.

1.7.2.5. Herbal therapies

Herbal therapies are currently most focused area for the treatment of various chronic ailments. Various plants were evaluated in extracts and pure isolated form for
efficacies. Numerous clinical trials were conducted and most of them were fond effective in reducing total plasma lipid profiles. These activities were present because of various antioxidants in plants. They act at various sites such as GIT, liver, heart, blood vessels, skeletal muscle cells and adipose tissues. Among antioxidants; polyphenols are the most abundantly available in nature. These are the secondary metabolites and include oligomeric proanthocyanidins, quercetin, curcumin and resvaretrol. They are not effective against high lipid levels but also in other clinical conditions such as diabetes, hypertension, neurodegenerative disorders and in cancer. Various studies were conducted to evaluate lipid lowering effects of these herbal extracts in purified forms such as grape seed antioxidant extract in hypercholesterolemic adults and were found effective in reducing TC and LDL-C by 9% along with reduction in blood pressure. Similarly an in-vitro study on human erythrocytes concluded synergism when antioxidants were administered in combinations. Besides beneficial effects of natural antioxidants using them in pure form has some shortcomings such as becoming prooxidant. This phenomenon is because of their usage in high doses individually and their oxidation may cancel their own effects. Therefore combination therapies would be more suitable and were found superior over individual therapies in the previous reported data.

1.8. Antioxidants

Antioxidants are the chemical agents capable of inhibiting peroxidation caused by free oxygen radicals. Oxidation is a chemical reaction that involves the transfer of electron or hydrogen from a molecule to the oxidizing agent, resulting in the generation of free radicals which initiates chain reactions that can damage the cells membrane, DNA
and other macro molecular particles. Antioxidants scavenge these free radicals and prevent oxidation while gets oxidized itself. Antioxidants are widely explored and still extensively under research, and are used in the treatment of various clinical conditions such as coronary artery diseases, diabetes and cancer. However the data is still insufficient in concluding the beneficial aspects of majority antioxidants. Some studies have also reported no effects on retarding cancer progression and mortality rate hence exploration is still required in this area to uncover all aspects of antioxidants.

1.8.1. Dietary antioxidants

A range of antioxidants are present in daily dietary items, maximum in fruit bearing plants other includes nuts, grains, fish, poultry, orange colored and green colored vegetables are also rich in antioxidants. A general classification of antioxidants is provided in the figure 1.6, followed by details of antioxidants that are used in current study.
Figure 1.6: General classification of antioxidants \textsuperscript{171-173}. 
1.8.2. Proanthocyanidins

They are the most ubiquitously distributed secondary metabolite in plant kingdom, and due to their biological activity, they have been evaluated in various cellular and physiological processes. Proanthocyanidins also called condensed tannins are composed of monomers dimmers, trimers and tetramers belongs to subclass of flavonols of flavonoids. They are present in aglycon form as monomers and oligomers while other subclasses of flavonoids exist as glucosides or esterified with gallic acid. Proanthocyanidins or oligomeric proanthocyanidins (OPC) is composed of 2 aromatic rings connected by a pyron ring, mostly comprise of favan-3-ol (+) catechin (-) epicatechin, (+) gallocatechin and (-) epigallocatechin and (-) gallate (Figure 1.7). OPC has different chain lengths, degree of polymerization and different hydroxylation patterns and classified into several groups based on hydroxylation pattern, among them procyanidins is the most commonly found in nature.

![Figure 1.7: 1) Epicatechin one of the building block, 2) Oligomeric proanthocyanidins.](image-url)
1.8.2.1. Absorption

Enteric absorption of OPC is through passive diffusion and almost more than 60%. Still the data is controversial as long chains are unable get access to general circulation because of their massive structures and during passage they are conjugated within the intestinal cells and later in liver. The rate of absorption, and conjugated metabolite type mainly depends on the nature of PCs, not on the concentration. The polymeric-PC also influences the absorption of oligomeric-PC. PCs with less than 3 degree of polymerization (DP) are depolymerized in stomach acidic environment before absorption. With food this reaction may slowdown due to less acidic environment. While PCs with DP more than 10 doesn’t depolymerize and get degraded by colonic microflora, before transportation across intestinal walls.

1.8.2.2. Metabolism and excretion

PCs are metabolized through methylation, glucuronidation, sulfated conjugation and are present in blood along with parent compounds i.e. monomers, dimers and trimers, found maximum in glucuronide form. Some detected in maximum amount as methylated form. Among all the B1 and B2 dimer were detected in plasma, tissue and in urine samples human volunteers. PCs and their conjugated forms circulate in protein bounded form and their affinity varies according to their molecular shape and level of substitution. They are hydrophilic and therefore interact at physiologic pH with phospholipids through hydrogen bonding using hydroxyl groups. Mostly excreted in urine and plasma concentration of parent compound rarely exceeds 1µmol/l and in urine the recovery was 1% to 25% of ingested dose.
1.8.2.3. Pharmacological properties

Proanthocyanidins exert their pharmacological effects in various ways; locally in GIT by forming complexes, unabsorbed, and as absorbed form, only low molecular weight are absorbed form GIT e.g. monomers, dimmers and trimers and may produce effects in different organs. They exert various beneficial effects, as antibacterial, form complex with protein, lipids and carbohydrates reducing their absorption, blocks lipase activity and also possess immunomodulatory, anti-inflammatory and antioxidant capabilities. They also suppress atherosclerotic plaques formation and platelet aggregation and enhance vascular functions by potentiating nitric oxide (NO) release.

1.8.2.3.1. Role in lipid homeostasis

Lipid homeostasis is governed by membrane bounded transcriptional factor called SREBP. The SREBP 1 regulates lipogenesis enzymes expression and TG storage in liver, while SREPB 2 regulates genes involved in cholesterol synthesis. PCs significantly reduce these factors resulting in inhibition of TC and TG accumulation in liver. It acts both on apolipoprotein-B rich TG and intestinal chylomicrons formation resulting in reduced synthesis of hepatic VLDL-C and LDL-C particles. They also elevate plasma HDL-C level, but the extent varies due to different doses applied and percent of proanthocyanidins composition. PCs decrease the pancreatic lipase activity to digest lipids in gut, thus reducing the synthesis of TG and TC rich chylomicrons particles. This up-regulates the LPL activity for the production of energy from stored fats in body. Low TG levels in blood consequences in reduced lipogenesis and enhanced FFAs oxidation for energy.
In GIT fats hydrolysis by pancreatic lipase is blocked by PCs limiting TG absorption and this activity enhances with the degree of polymerization from dimers to pentamers, monomers have no such effect. Others reported that lowering cholesterol and bile acids absorption is due to decrease in micellar cholesterol solubility \(^{193-196}\) and synthesis of cholesteryl esters in CaCO\(_2\) cells \(^{197}\). Study had reported the upregulation of cholesteryl 7-\(\alpha\) hydroxylase (CYP7A1) enzyme by PCs leading the cholesterol towards bile acids conversion and excretion, resulting in removal of circulating cholesterol \(^{198}\). Studies reported that consumption of red wine and apple polyphenol extract has significantly reduced Apob48 (marker for chylomicrons) secretion in intestine because of the high concentrations of PCs in red wine and apple extract \(^{197,199}\). PCs has also mild enhancing effects on peroxisomes especially PPAR\(\gamma\) assisted \(\beta\)-oxidation of fattyacids \(^{181,200}\) resulting in the removal of circulating lipids. In GIT intestinal cells perform triglyceride synthesis by delivering FAs either to monoacylglycerol or glycerol 3-phosphate pathways. PCs suppress FA supply towards monoacylglycerol pathway in fed state while towards both pathways in fasting state \(^{201}\).

### 1.8.2.3.2. Role as antiperoxidative

PCs possess broad spectrum scavenging capability against ROS and oxidative stress induced by various pathological conditions \(^{202}\) depending upon chain length and the type of free radical they attacking \(^{203}\). Antioxidant effects of PCs and other flavonoids may be due to the activation of other endogenous phase-II antioxidant enzymes such as NADPH-quinone oxidoreductase, glutathione S-transferase etc, having antioxidant activity against electrophile toxicants and oxidative stress. These enzymes are mediated
by activation of electrophile responsive element (ERE) which activates a series of genes in phase II reactions triggering the redox cycle. Another mechanism is through the reduction of antioxidants present in blood such as \( \alpha \)-tocopherol, which is the major antioxidant found in LDL lipoproteins. In a clinical trial Natella et.al reported the reduction in lipid peroxidation and LDL oxidation level with PCs therapy in a group with high fat diet. Hirano et al proposed that flavonoids exert antioxidant effect via donating hydrogen ion to \( \alpha \)-tocopheryl radical a potential prooxidant resulting in regeneration of reduced tocopherol form. The ionic interaction of PCs with lipoproteins is between hydrophilic and lipophilic surfaces rather than hydrophilic only. Catechins the monomers of PCs were observed of having greater antioxidant effect than ascorbate in regenerating \( \alpha \)-tocopherol in human LDL-C.

Another mechanism proposed by Yokozawa et al is that antioxidant effects of PCs are due to upregulation of GSH which is the major non-enzymatic antioxidant. Thus preventing LDL from ROS and reestablishing the redox state in pathological condition. Similarly another study concluded the abrogation of glutathione oxidized form as possible mechanism of PCs as antiperoxidative. Others reported inhibitory effect of PCs on oxidative cytochrome P-450 enzymes thus GSH may be reproduced by this mechanism. In long term treatments reduced activity of antioxidant enzymes (GPx, SOD) along with reduced levels of MDA will be considered as indicator of improved antioxidant homeostasis. Thus PCs present itself as a signaling moiety in regulating antioxidant enzyme systems.
1.8.2.3.3. Effects on cardiovascular and vascular endothelial cells

Antiatherogenic property of PCs, is by inhibiting platelet aggregation and reducing their hyper activity, minimizing the risk of infarction in cardiac and blood vessels. PCs also promote the synthesis of endogenous nitric oxide synthase (eNOS) to synthesize nitric oxide while suppressing the transcription of inducible nitric oxide synthase (iNOS) mRNA thus helps in maintaining the normal vascular tone and blood pressure $^{181,213}$. Proper functioning of mitochondria is necessary to control free radical release during ATP production. Loss of organelle structure or function is considered an important aspect of coronary and other pathophysiologies. Calcium overload caused by myocardial ischemia results in excessive release of free radicals and swelling of mitochondria, triggering the cell death. These effects can be controlled by intake of dietary PCs especially the monomers and dimers which can reduce and prevent amplification in coronary infarct size $^{214-215}$. Activation of NADPH oxidase by superoxide ions is the major event in initiating the platelet activation and promoting thrombogenic phenomena. PCs modulate various pathways to inhibit such events $^{216}$. They have also suppressing effect on endothelial formation of cyclo-oxygenase (COX) derived vasoconstrictor factor (PGI$_2$), arachidonic acid metabolism and ADP activation thus preventing platelet aggregation $^{217}$.

1.8.2.3.4. Effects on blood glucose level

Proanthocyanidins specifically oligomeric form has greater antidiabetic activity by reducing circulating glucose and rate of protein glycosylation and also inhibits renal advanced glycation end products (AGEs) a major causative factor of diabetic
complications\textsuperscript{191}. AGEs are produced due to excessive intracellular glucose levels, leading to increased NADH and FADH levels and hence mitochondrial ROS production\textsuperscript{218}-\textsuperscript{219}. Other mechanisms of glucose homeostasis is through limiting the absorption of glucose from intestinal walls, reducing hepatic lipid accumulation as they can be easily converted to glucose, inhibition of \(\alpha\)-glucosidase and \(\alpha\)-amylase enzymes, and increasing insulin sensitivity\textsuperscript{220}-\textsuperscript{222}.

1.8.2.3.5. Effects as anti-inflammatory, anticancer, anti-obesity and antimicrobial

PCs in several studies had proved to possess anti inflammatory and anti cancer effects by decreasing the levels of proinflammatory proteins; the interleukin-6 (IL-6), tumor necrosis factor (TNF-\(\alpha\)), C-reactive proteins (CRP) and macrophage activating factor. It also enhances anti inflammatory cytokine adiponectin level\textsuperscript{223}-\textsuperscript{226}. Anti cancer effects of PCs in lungs cells is by the inhibition of COX-2 and prostaglandin E2 receptors\textsuperscript{227}. Both \textit{in-vivo} and \textit{in-vitro} data support the anticancer activity of PCs by antiproliferative and pro-apoptotic effects while toxicity data suggest that PCs has no genotoxic effect to normal cells\textsuperscript{228}-\textsuperscript{229}. They inhibits obesity associated chronic inflammation caused by infiltration of macrophages and abnormal proinflammatory proteins production, thus maintains normal adipose tissues tone to a greater extent\textsuperscript{230}. Anti-obesity effect of PCs was also observed by potentiation of adiponectin a protein hormone released from adipose tissues and regulates glucose and fatty acid \(\beta\)-oxidation thus prevents adiponectin imbalance associated obesity\textsuperscript{231}-\textsuperscript{232}. PCs and their conjugates are found to possess broad spectrum antimicrobial activities including antibacterial, antifungal and antiviral effects. Strong effects against \textit{helicobacter pylori}, gastric cancer causing microbes and microbials resistant to drugs like metronidazole and clarithromycin.
were reported \(^{233}\). PCs are also found effective in urinary tract infections, lower GIT infections and dental conditions against gram negative bacteria escherichia coli, oxacillin resistant staphylococcus aurous and clostridium. While no effects against beneficial gram positive probiotics lactic acid bacteria were observed \(^{234}\). They act on microbial cell growth by disrupting cytoplasmic membrane, increasing microbial cell permeability, blocking microbial enzymes, and chelating with iron and zinc necessary for their cell growth \(^{235}-^{236}\). Antiviral effects were observed against herpes simplex virus type-1 (HSV-1) such as influenza virus, by preventing virus entry into host cells the first step of HSV-1 infection initiation \(^{237}\).

Other activities includes prevention of diabetic peripheral neuropathy \(^{238}\), antidepressant effect by blocking monoamine oxidase (A & B) in dose dependant manner thus rising 5HT, noradrenalin and dopamine levels \(^{239}\). Nephroprotective effects were observed in cisplatin induced nephrotoxicity \(^{240}\). Amelioration of renal ischemia/reperfusion injury induced increased levels of serum creatinine, blood urea nitrogen (BUN), alanine aminotransferase (ALT) \(^{241}\) and inhibition of Alzheimer’s associated neuropathologies \(^{242}-^{243}\) are also reported.

**1.8.2.4. Adverse effects**

No such adverse effects were found after chronic administration of PCs, except some monomers may produce few mild side effects but they are very rare. Still dose should be observed before administration, and pure OPCs should be used instead of crude extracts of plants. It also possess enzyme inhibitory effect especially on cytochrome (CYP) 3A4 and may increase bioavailability of some drugs like benzodiazepines and terfenadine, therefore should be used with caution \(^{173,244-245}\). Prooxidant effects were
observed in cardiomyocytes and induced cardiac oxidative stress due to generation of ROS as the dose increases \textsuperscript{246-247} therefore dose should be selected cautiously.

\textbf{1.8.3. Pterostilbene}

Pterostilbene (PT) is chemically trans-3, 5-dimethoxy—hydroxystilbene and found abundantly in blue berries and heartwood of \textit{Pterocarpus marsupium}, and classified biologically as phytoalexin (plant antimicrobial substances). It is natural analog of resvaretrol and possess the antioxidant, anticancer and anti inflammatory properties \textsuperscript{248}.

It differs from resvaretrol at hydroxyl (OH) position, resvaretrol has three OH groups while PT has one OH group and the other two OH groups are replaced by methoxy groups (Figure 1.8) \textsuperscript{249}.

![Figure 1.8: (A) Resvaretrol, (B) Pterostilbene a natural occurring analog with two methoxy groups.](image)

\textbf{1.8.3.1. Pharmacokinetics}

Substitution with methoxy makes it more lipophilic and insoluble in water with enhanced bioavailability (1.5 hours) compared to resvaretrol (30min), thus can produce more prolonged effects \textsuperscript{250-251}. Studies have reported metabolic end products of PT
through glucoronidation and sulfation but in slower rate than resvaretrol. Excretion occurs through urine in the form of glucuronide conjugates \(^{249,252}\).

### 1.8.3.2. Pharmacological properties

PT has a potential of diverse pharmacological properties similar to OPCs but with different intensities. These includes antihyperlipidemic, antioxidant, antimicrobial, anticancer, anti-inflammatory and enzyme modulatory effects.

#### 1.8.3.2.1. Antihyperlipidemic and antioxidant properties

PT is found to be more potent activator of PPAR\(\alpha\) than resvaretrol. It acts by activating \(\beta\)-oxidation of fatty acids resulting in reduction of TG and VLDL-C levels via restraining hepatic Apo-C-III and enhancing hepatic LPL gene expression. In humans it promotes HDL-C level by expressing Apo-A in hepatic cells \(^{253-254}\) and suppress proatherosclerotic events by activating PPAR\(\alpha\) \(^{255}\). Effect as antioxidant is because of scavenging activity of ROS and GSH depletion prevention both individually and in combinations with other antioxidants where effects were synergistic \(^{159}\).

#### 1.8.3.2.2. Cardio protective and antiatherogenic effects

Studies have reported the protective effect of PT on cardiovascular health because of potentiating effect on antioxidant enzymes. They concluded that reduction in myocardial infarct size in a study is due to protective effect on ischemic cardiomyocytes against ROS resulting in reduced cardiac cells apoptosis \(^{256}\). Similarly upregulation of antioxidant enzymes such as SOD and GPx along with thioredoxin in Apo-E deficient atherosclerotic animal model were also reported. It also blocks vascular endothelial
derived H$_2$O$_2$ radicals preventing endothelial injury, platelet aggregation and plaque formation $^{257-258}$. A study reported anti-proatherosclerotic and anti-apoptotic processes by induction of engulfment of proteins with LDL-oxi via cyclic adenosine monophosphate (cAMP) mediated influx of Ca$^+$ ions in endothelial cells $^{259}$. Extended administration to chronic smokers reduced angiotensin converting enzyme activity and blood lipid hydroperoxides level $^{260}$. Another study reported inhibitory effects on platelet derived growth factor (PDGF)-BB which mediates the vascular smooth muscle cell (VSMC) proliferation; a causative factor of atherosclerosis and angioplasty restinosis $^{261}$.

1.8.3.2.3. Antidiabetic effect

PT releases insulin from pancreas $^{254}$. In streptozocin induced diabetic rat model PT therapy reduced blood glucose level by 42% in 20mg/kg dose $^{256}$. Similarly another study reported reduction in blood glucose by 50% and glycosylated hemoglobin (HbA1c) levels with increase in insulin level $^{262}$. The positive effects of PT on GSH, GPx, GST and SOD with the decrease of ROS in liver and kidney are the proposed mechanisms for antidiabetic effect $^{263}$.

1.8.3.2.4. Anticancer

Anticancer mechanism observed in human prostate cancer cells is the antiproliferative effects on cancerous cells and ROS release by boosting AMP activated protein kinase (AMPK), blocking key enzymes for lipogenesis like fatty acid synthase $^{264}$. Another Study on breast cancer cells concluded that PT initiates apoptosis in cancer cells by increasing GPx activity and release of H$_2$O$_2$ and O$_2$•$^-$ radicals altering the oxidation state $^{265}$. An in-vivo study confirmed PT anticancer effect on pancreatic cancerous cells
by enhancing the expression of antiproliferative bio-markers such as cytochrome-C and SOD.\textsuperscript{266}

**1.8.3.2.5. Other effects**

Pterostilbene like OPCs showed anti inflammatory effect by scavenging ROS and down regulation of lipopolysaccharide induced inflammatory iNOS and COX-2 expression in macrophages\textsuperscript{267-268}. Suppression of inflammatory chemical mediators; PGE\textsubscript{2} and TNF\textalpha in-vitro provided indication of effectiveness in inflammatory bowel syndromes, arthritis and other conditions\textsuperscript{269-270}. Anti obesity effect of PT is because of up regulation of adiponectin and suppression of leptin thus inhibiting lipogenesis in adipose tissues along with attenuation of PPAR\gamma expression in adipocytes\textsuperscript{263,271}. While it increased PPAR\gamma activity in hepatocytes, skeletal muscle cells and pancrease \beta-cells increasing the glucose uptake, metabolism and release of insulin\textsuperscript{253} thus acts as partial agonist. PT raised SOD levels in Alzheimer’s disease model via PPAR\alpha receptors expression and has more effect against cognition and cellular stress than resvaretrol\textsuperscript{272}. Hepatoprotective effect was observed against acetaminophen induced toxicity by reversing the depletion of GSH, SOD and catalase and retarding MDA and liver enzymes levels\textsuperscript{273}.

**1.8.3.3. Adverse effects**

Pterostilbene was observed for adverse reactions at different doses up to 250mg/day on humans. Results after biochemical analysis concluded no any adverse effect on glucose, hepatic and renal markers\textsuperscript{274}. However the phenoxyl radical produced due to oxidation of polyphenols are reactive and may act as prooxidant if no other antioxidant is available for scavenging. As they show prooxidant effect in cancerous cells
so there may its chances at higher doses in normal organs, therefore like OPC, PT dose should be monitored and antioxidant blends should be preferred to avoid such phenomenon \(^\text{159,275-276}\).

### 1.8.4. Niacin (nicotinic acid)

Niacin (3-picolonic acid, B\(_3\)) is an essential vitamin, a double faced organic compound, it act as vitamin (nicotinamide) in low doses while in high doses it act as lipid modifying agent (nicotinic acid) (Figure 1.9) \(^\text{277}\). Nicotinamide lack the property of lipid modification. It is synthesized from L-aspartate \(^\text{278}\) and widely distributed in nature specially legumes, peanuts, sunflower, fish etc \(^\text{279}\). It is also synthesized by human liver expending 60mg tryptophan for 1 mg of niacin \(^\text{280}\).

![Figure 1.9: A: Nicotinic acid, B: Nicotinamide](image)

### 1.8.4.1. Pharmacokinetics

It is rapidly absorbed from GIT and peak plasma concentration reaches in 30min to 1hr. Plasma half life is about 1hr. It is of high affinity and low capacity and gets activated on long acting slow release dosage forms. There are more chances of
hepatotoxicity with nonconjugative pathway. If immediate release dosage form is administered, it will quickly saturate the nonconjugative pathway and will activate the conjugation pathway which can cause facial flushing. It is partially metabolized by liver and excretion of both metabolites and parent drug occurs through urine.

1.8.4.2. Pharmacological effects

1.8.4.2.1. Effect on lipid regulation

Niacin acts at various steps in lipid regulation. It inhibits TG mobilization by blocking c-AMP activation in adipocytes, preventing release in general circulation. It suppresses the activity of hormone sensitive lipase activity and inhibits the release of fatty acids for the synthesis of TG in liver. It inhibits the fatty acid synthesis and esterification by blocking diacylglycerol (DAG) to form TG by suppressing directly diacylglycerol acyltransferase enzyme. This leads to decreased Apo-B lipidation in hepatocytes and translocation across endoplasmic reticulum with increase in its intracellular degradation. It suppresses large TG-rich VLDL-C particles synthesis and their conversion to LDL-C. Niacin is found to be effective in rising plasma HDL-C levels by reducing HDL-Apo-AI reuptake and degradation by hepatocytes by blocking HDL catabolic receptors. In general niacin enhances HDL half life, cholesterol efflux and reverse cholesterol transport (RCT) thus minimizing atherosclerotic plaque formation rate and coronary artery disease.

1.8.4.2.2. Effects on cardio vascular health

Atherogeneity associated endothelial dysfunction leads to narrowness of blood vessels and increased blood pressure along with suppressed NOS activity and NO
synthesis and enhanced vasoconstrictor endothelin release \(^{283}\). These effects are potently reduced by niacin as it rises HDL-C and decreases TG by 30\% and 50\% respectively as reported in studies \(^{285-287}\). Significant reduction in carotid wall area was observed in niacin with stain therapy \(^{288}\). Its antiatherogenic effect is also due to inhibition of pro-inflammatory chemokines release with expression of mRNA for the release of atheroprotective adipokine, adiponectin \(^{289}\). Niacin activates PPAR\(\gamma\) and intracellular cAMP in macrophages and monocytes thus mediates RCT and prevent macrophage foam cells formation and atherosclerosis \(^{290}\). It also upregulates liver X receptor (LRX)\(\alpha\) inducing ATP-binding cassette (ABC)-A1 mediated cholesterol hepatic metabolism via efflux towards HDL-C \(^{291}\).

### 1.8.4.2.3. Antioxidant effects

Niacin acts as precursor for the synthesis of cellular nicotinamide adenine dinucleotide (NAD\(^+\)) and nicotinamide adenine dinucleotide phosphate (NADPH). It upregulates the glucose-6-phosphate dehydrogenase (G6PD), a key source and rate limiting enzyme in the synthesis of cellular NADPH. Upregulation of NADPH enhances cellular ROS scavenging capacity either by ROS-generating oxidases regulation or reproducing antioxidants like GSH as reported in one study up to 98\% of normal level \(^{145}\). Niacin was also found to increase endothelial homocysteine levels, a negative effect \(^{292}\) but this effect can be compensated by production of GSH and enhancing redox state in endothelial cells. Besides that niacin also block NADPH oxidase enzyme in human aortic endothelial cells thus prevent angiotensin II stimulated endothelial NADPH oxidase ROS production \(^{145,293}\).
1.8.4.3. Adverse effects

The most common side effect is facial flushing, due to cutaneous vasodilation. But it subsides as the plasma peak concentration achieved and also along with the therapy. Facial flushing is due to formation of arachidonic acid which metabolizes into prostanoids like PGD<sub>2</sub> and PGE<sub>2</sub> which promotes vasodilation by activating Gs-coupled receptors. Several reported increased resistance to insulin by niacin therapy but this mechanism is still unclear. Other side effects include hepatotoxicity which depends on formulation, slow release may produce less flushing but increases the risk of hepatotoxicity.

1.9. Background for therapy based formulation development

Different formulations have been developed for antioxidants to treat various ailments. These include both herbal formulations such as polyherbal ayurvedic formulations and extracts for DM and hyperlipidemia. Pure drug based formulations such as in skin care, antidiarrheal formulations, for lipid lowering and rheumatoid arthritis are also included.

OPC effectively prevents diarrhea when coated with enteric polymer that releases the drug in intestinal pH. Similarly administration of OPC for the suppression of in-vitro gut epithelial cell invasiveness caused by E.coli along with probiotic in combined formulation resulted in significant reduction in invasion by different mechanisms. OPC combined in a formulation with policosanols and tocotrienol for treatment of hyperlipidemia (twice daily) in human population produced significant effects in normalizing lipid levels. Similarly also incorporated in liquid formulations for the
Chapter 1

INTRODUCTION

Treatment of CVDs, DM, hyperlipidemia, dementia and Alzheimer disease in combination with resvaretrol, co-enzyme Q10, quercetin, glutathione, curcumin and various vitamins.  

Pterostilbene formulations were used in combinations for the treatment of chronic illnesses. Combination with curcumin normalized lipid levels, suppresses oxidative stress and inflammation. They were incorporated up to 5000mg which is very high dose for every day administration in long term. Similarly in combination with statin, PT up to 250 mg was incorporated using numerous semi synthetic cellulose polymers. Results confirmed the superior effects of statin in combination with stilbenes as new class of PPARs agonists rather than with fibrates on CVDs. Another formulation was based on combination with grape juice extract up to 500 mg dose. The combined effects were found superior on individual low and high dose therapies. For formulation development numerous dosage forms were suggested that includes tablet, capsules, lozenges, suspensions, effervescent, oral powder and topical forms. Numerous excipients were suggested including cellulose derivates like hydroxy propyl methyl cellulose (HPMC), microcrystalline cellulose (MCC), talc, polyvinyl pyrololidone (PVP), natural gums, wetting agents, disintegrants and silicon dioxide. Natural antioxidants such as OPC and PT are compatible and can be formulated with a number of excipients.  

Niacin was formulated in various dosage forms that includes the delayed release niacin coated with polyvinylpyrrolidone and in combination with statins using ethyl cellulose (hydrophobic polymer) with microcrystalline cellulose for modified release. Niacin was incorporated up to 1000 mg for lipid lowering effect. Similarly floating tablets prepared for sustained release action and in combination with oxicam such as
piroxicam to control facial flushing $^{308-309}$. While the burst release is accompanied by facial flushing, this subsides with the therapy gradually. The modified release niacin is mostly favored because of dosing and therapeutic effect $^{309}$. Combined therapies of niacin with statins were proved more effective than individual therapies $^{310}$.

1.10. Aim and objectives

The present study is designed to:

- Develop a dose rationale and optimized formulation of included dietary antioxidants in combination against hyperlipidemia.
- Explain the roles of body antioxidants in preventing the development of hyperlipidemia associated oxidative stress.
- Determine the effects of natural antioxidant therapies in various ratios on lipid profile and antioxidant levels in serum samples.
- Formulation development and stability studies of blend of antioxidants in human equivalent dosage form to give a new strategy of using natural antioxidants in chronic condition.
1.11. Hypothesis

Human body has its own antioxidant system that provides defensive role against free radicals produced during pathological conditions such as metabolic syndrome (hyperlipidemia, diabetes, hypertension and obesity) and associated oxidative stress. These antioxidants are recycled to their reduced forms via various mechanisms and their depletion can lead to harmful effects on organs, tissues and cells. Such as α-tocopherol, a major antioxidant inside LDL-C, if not recycled to its reduced form may become prooxidant and promote atherosclerotic events. Natural/dietary antioxidants have proven to support endogenous homeostasis by boosting blood antioxidants regeneration and scavenging the free radicals. However, they were found to have ceiling effect and act dose dependently up to certain levels, above which their effects decrease/remain constant or may convert to prooxidant. They were found effective in various disease conditions in varied doses and in combinations, providing synergistic effects.

Our hypothesis is:

- Synergistic effects of natural antioxidants can be achieved in low dose combinations in hyperlipidemia.
- Low dose blends are more effective in normalizing disease conditions compared to individual high dose therapies.
- Stable and optimized formulations in conventional tablets for antioxidant blends can be prepared with granulation techniques.
Chapter 2

EXPERIMENTAL
2. EXPERIMENTAL

2.1. Drugs, reagents and compounds

Cholesterol (Avonchem, UK, % purity ≥99), cholesterol kit including TC, TG and HDL (Human diagnostics, GmbH, Germany), liver function tests (ALT, AST and ALP) (Erba Diagnostics, Mannheim, Germany), Oligomeric proanthocyanidins (OPC) (Shaanxi Run Time Bio-Technology Development Co., Ltd, China, % purity ≥ 98), pterostilbene (PT) (Shanghai Korey Pharm Co., Ltd, China, % purity ≥ 99), niacin (NA) (Scharlau Chemie, SA, Spain, % purity ≥ 98), cystine (CySS) (CAS: 56-89-3, % purity ≥ 99), cysteine (Cys) (CAS: 52-90-4, % purity ≥ 98.5), homocysteine (Hcy) (CAS: 454-29-5, % purity ≥ 99) ascorbic acid (AA) (CAS: 50-81-7, % purity ≥ 99), methionine (Meth) (CAS: 63-68-3, % purity ≥ 98), reduced glutathione (GSH) (CAS: 70-18-8, % purity ≥ 99), oxidized glutathione (GSSG) (CAS: 103239-24-3, % purity ≥ 98), N-acetyl cysteine (NAC) (CAS: 616-91-1, % purity ≥ 99), potassium bromide (KBr) (CAS: 7758-02-3, % purity ≥ 99) (Sigma-Aldrich, Oslo, Norway). 1,1,3,3-tetraethoxypropane (malondialdehyde bis-diethyl acetal) (CAS: 122-31-6, % purity ≥ 98) (Merck, NJ, USA) and dopamine (CAS: 62-31-7, % purity ≥ 98) rosuvastatin (CAS: 147098-20-2, % purity ≥ 98), simvastatin (CAS: 79902-63-9, % purity ≥ 98), atorvastatin (CAS: 344423-98-9, % purity ≥ 98) (Fluka-Chemika, Switzerland).

2.2. Chemicals

Trifluoroacetic acid (TFA) (CAS: 76-05-1, % purity ≥ 99), HPLC grade acetonitrile (ACN) (CAS: 75-05-8, % purity 99.8), HPLC grade methanol (MeOH) (CAS: 67-56-1, % purity ≥ 99.9), hydrochloric acid (HCl) (CAS: 7647-01-0, 36.5-
38.0%), metaphosphoric acid (CAS: 37267-86-0, 33.5-36.5%), triethylamine (TEA) (CAS: 121-44-8, % purity ≥ 99), amphiphilic ion pairing agent hexadecyltrimethylammonium bromide (CTAB) (CAS: 57-09-0, % purity > 95), formic acid (FA) (CAS: 64-18-6, % purity ≥ 96), butylated hydroxy toluene (BHT) (CAS: 128-37-0, % purity 98), ferric ammonium sulfate (CAS: 7783-83-7, % purity 99) (Sigma-Aldrich Oslo, Norway).

2.3. Excipients

Polyvinyl pyrollidone (PVP-K30) (I.S.P technology, Texas), Cross linked carboxymethyl cellulose sodium (cross carmellose sodium), Tablettose-80, micro crystalline cellulose, sodium starch glycolate (F.M.C International, Ireland) and talc (Dow Chemical company, Midland, USA), magnesium stearate (Coin Powder International Company Ltd, Taiwan).

2.4. Instrumentation

2.4.1. UV/Visible spectrophotometer

Perkin Elmer (Norwalk, USA) Lambda 25 double beam UV/Visible spectrophotometer controlled by UV Winlab 2.85 software.

2.4.2. Incubator

Incubator (Incucell Med Center GmbH, Germany)

2.4.3. Centrifuge machine

Centrifuge machine (Centurion scientific Ltd, UK).
2.4.4. High performance liquid chromatography (HPLC)

Perkin Elmer High performance liquid chromatography system (Norwalk, USA) having a pump (series 200), online vacuum degasser (200 series), auto-sampler (series 200), column oven (series 200). System was linked with detectors by Pe Nelson network chromatography interface (NCI) 900 and controlled by totalchrom workstation software.

2.4.5. Detectors

UV/VIS detector (series 200, Norwalk USA) and DECADE II Electrochemical Detector (ECD) (Antec Leyden, Netherland) with a flow cell having effective volume of 0.5µl, consists of three electrode configuration with a working glassy carbon electrode, Hy REF reference electrode (REF) and an auxiliary electrode KCl Ag/AgCl.

2.4.6. HPLC columns

Discovery Supelco HS C18 RP-HPLC chromatographic column (250 mm × 4.6 mm, 5 µm; Bellefonte, USA) and Athena C18-WP (100A, 4.6mm × 250mm, 5 µm; CNW technologies GmbH, Germany).

2.4.7. Distillation apparatus

Millipore (Milford, USA) distillation apparatus.

2.4.8. pH meter

Jenway 3505 pH meter (Bibby Scientific Ltd, Dunmow, UK)
2.4.9. **Filtration assembly**

Consist of vacuum pump connected with filtration assembly using 0.45 μm filtration membrane.

2.4.10. **Equipments incorporated in formulation development**

Digital balance (OHAUS Pioneer balances, USA), Fourier transform infra red (FTIR) spectrophotometer (Shimadzu, Kyoto, Japan) for drug excipients interaction, Granulation machine (S.T.C China), hot air oven (Memmert GmbH, Germany), for granule sieving and sizing electromagnetic sieve shaker (Cisa, Spain). Tablet compression machine ZP-17 (S.T.C China), tablet hardness tester (Pharma Test, Germany), friabiliator (Pharma Test Germany), Tablet disintegration apparatus (Pharma Test, Germany), Tablet dissolution apparatus (Instruemends, Lahore, Pakistan), stability chamber (JEIO Tech, Korea) and halogen moisture analyzer (Mettlor Toledo, Switzerland).

2.4.11. **Ethical Approval**

This research work was allowed to be carried out with the approval of ethical committee of Department of Pharmacy, University of Peshawar, Peshawar, Pakistan, under the certificate number 02/EC-15/Pharm. Animals used in experiments, were handled according to “Animals Scientific Procedure Act (1986) of United Kingdom (UK)”.
2.5. Study design

Study was carried out in the following phases:

Phase 1: Antihyperlipidemic animal studies
   a) Development of hyperlipidemic model
   b) Blood sampling procedure
   c) Lipid profiling using kit method
   d) Assessment of liver enzymes
   e) Treatment protocol

Phase 2: Analysis of antioxidants in blood, lipid peroxidation and natural antioxidants
   a) Analysis of fat soluble antioxidants in serum samples; \( \alpha \)-tocopherol and all-trans retinol.
   b) Simultaneous determination of water soluble antioxidants and malondialdehyde by RP-HPLC coupled with electrochemical detector in serum samples.
   c) Development of HPLC-UV method for simultaneous determination of niacin (hydrophilic) and pterostilbene (lipophilic) antioxidants.
   d) Validation of developed HPLC methods.
   e) Analysis of oligomeric proanthocyanidins.

Phase 3: Formulation development
   a) Pre-formulation studies.
   b) Conversion of animal dose into human equivalent dose
c) Development of formulation for the optimized active pharmaceutical ingredient (APIs) blend.

d) In-vitro evaluation studies.

e) Selection of optimum formulation(s)

f) Stability studies of optimized formulations.

2.5.1. Antihyperlipidemic animal studies

2.5.1.1. Development of hyperlipidemic model

Various models were reported for antihyperlipidemic study among them rats, guinea pigs and rabbits are the most suitable model. For the sake of multiple blood sampling and availability of space and animals, male albino rabbits of 1.2-1.6 kg were selected. Male gender is most suitable because it has less adipose tissue deposition than female. Hyperlipidemic model was developed according to the previously reported protocol. Animals were kept for 1 week acclimatization period at animal house facility at Department of Pharmacy University of Peshawar, Pakistan. Cholesterol (400 mg/kg) doses were administered per oral route with the help of flexible plastic tube attached with syringe. Cholesterol suspension was prepared in 3ml sunflower oil and initial administration was performed for two weeks to develop hyperlipidemia. Day and night cycle was maintained and animals were allowed to freely access food and water ad-libitum. Animals were kept at animal house facility and were fed on previously used normal chow diet (Purina, high fiber rabbit chow, No. 5326) composed of following ingredients (Tab. 2.1).
Table 2.1: Composition of experimental diet

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Nutrient composition (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat middling</td>
<td>Proteins (145)</td>
</tr>
<tr>
<td>Soybean Hull</td>
<td>Fats (66)</td>
</tr>
<tr>
<td>Alfalfa meal</td>
<td>Fiber (crude) (225)</td>
</tr>
<tr>
<td>Cane molasses</td>
<td>Starch (88)</td>
</tr>
<tr>
<td>Ground corn</td>
<td>Sucrose (30)</td>
</tr>
<tr>
<td></td>
<td>Mineral and vitamins (0.5-2)</td>
</tr>
</tbody>
</table>

2.5.1.2. Blood sampling procedure

Blood sampling was performed using sterilized blade for shaving ear and 1ml syringe (22G). Marginal veins were exposed and local anesthetic lignocaine was applied for 5-10 minutes before sampling. Blood samples were transferred to hard plastic tubes to clot centrifuged at 3000×g at 25 °C for 10 minutes and supernatant serums were separated for analysis.

2.5.1.3. Lipid profiling using kit method

Lipid profiling were performed using commercially available kits that includes total cholesterol (TC), triglycerides (TG) and high density lipoproteins (HDL-C) on Lambda 25 UV/Visible spectrophotometer. The LDL-C and VLDL-C were calculated using Friedewald formula\(^{313-314}\) equation 1 and 2 and atherogenic index was calculated using equation 3\(^{315}\).
2.5.1.3.1. Total cholesterol and triglyceride analysis

To the blank reagent (2 ml) (separate for cholesterol and triglyceride) serum/standard (20 μl) was added in UV/visible spectrophotometer cuvette, mixed and incubated at 37 °C for five minutes. At the end pinkish red or slight yellow color appears. The absorbance was than measured at 520 nm λ-max and reading was place in the following formula.

\[ \text{TC or TG in sample (mg/dL)} = \frac{\text{absorbance of sample}}{\text{absorbance of standard}} \times 200 \text{mg/dL} \ldots \text{eq: 4} \]

The 200 mg/dl is the concentration of cholesterol and triglyceride in standard solutions per 100 ml.

2.5.1.3.2. High density lipoproteins analysis

To reagent-A (750 μl, enzyme) distilled water (10 μl) was added and incubated for 5 minutes at 37 °C after which reagent-B (250 μl, substrate) was added to above solution and again incubated to be used as blank. In serum and standard cholesterol sample preparation, water was replaced by serum and standard cholesterol. Absorbance was measured at 593 nm λ-max and concentration of HDL-C was calculated using the following equation \(^{316}\).
2.5.1.4. Assessment of liver enzymes

Alanine transaminase (ALT or SGPT), aspartate transaminase (AST or SGOT) and alkaline phosphatase (ALK or ALP) were determined at the end of therapy duration in serum samples. The analysis for all is same only differ in reagents and calibrator. Overall the test method is as follows.

Reagent-1 (R1) was mixed with reagent-2 (R2) in 4:1 to make working reagent, and stored at 20-25 °C in dark. Working reagent (1000 μl) and serum sample or calibrator (for ALT and AST; 100 μl and for ALP; 20 μl) were mixed and incubated for 1 minute at 37 °C. Absorbance was measured at 340 nm for ALT, AST and 405nm for ALP at time 0 and after 1, 2 and 3 minutes against blank reagent. The 1 minute absorbance change was measured and results were calculated in U/L according to following formulae 317.

\[
\text{ALT or AST (U/L)} = \frac{\Delta A_{\text{sample}}/\text{min}}{\Delta A_{\text{calibrator}}/\text{min}} \times C_{\text{cal}} \quad \text{..... eq: 6}
\]

Where \(C_{\text{cal}}\) is the concentration of calibrator.

Or by applying factor as:

\[
\text{ALT/ AST (U/L)} = \Delta A_{\text{sample}}/\text{min} \times f \quad \text{..... eq: 7} \quad \text{[f (factor)= 1745 at 37\degree C]}
\]

\[
\text{ALP (U/L)} = \frac{\Delta A_{\text{sample}}/\text{min}}{\Delta A_{\text{calibrator}}/\text{min}} \times C_{\text{cal}} \quad \text{..... eq: 8}
\]

Where \(C_{\text{cal}}\) is the concentration of calibrator.
Or by applying factor as:

\[ \text{ALP (U/L)} = \Delta \text{A}_{\text{sample} / \text{min}} \times f \ldots \text{eq: 9} \quad [f \text{ (factor)} = 2764 \text{ at 405nm}] \]

2.5.1.5. Treatment protocol

Therapies were divided into the following stages: 1) Individual therapies, 2) Combinational therapies, 2.1) Two drug combinational therapies, 2.2) Three drug combinational therapies.

2.5.1.5.1. Selection of dose rationale

Dose rationale for current study was based on reported data in literature of natural antioxidants, the quantity administered in human equivalent doses previously (as in case of niacin)\textsuperscript{190,284,318-321}. In case of pterostilbene its safety profile is well documented up to large dose quantities but the dose dependent efficacies in individual and combinations therapies is lacking specially in hyperlipidemia\textsuperscript{322}. Observing overall literature 100 mg/kg was found to be most suitable for selected natural antioxidants. Therefore to develop dose rationale in combinations 100 mg/kg total dose quantity was chosen.

2.5.1.5.1.1. Individual therapies

Each selected compound was administered in 100 mg/kg individual doses along with cholesterol in each group (n= 6) per oral route for three months. Lipid profiles were measured and blood samples were collected for further HPLC analysis. Atorvastatin was administered to separate group for comparative evaluation.
2.5.1.5.1.2. Combinational therapies

These therapies were designed by administering the selected drugs in ratios such that total dose does not exceed 100 mg/kg. For this purpose each drug was combined with other two drugs in various ratios for two and three drug combinations.

- **Two drug combinational therapies:** Three combinations were made among all the three drugs i.e. OPC + NA, OPC + PT, NA + PT. Each combination was administered in three ratios i.e. 70:30, 50:50, 30:70. Plasma lipid profile was closely monitored for extent of synergistic effects among the groups. In each two drug combinations the combinatorial ratio possessing high lipid lowering effect was selected for three drug combinations.

- **Three drug combinational therapies:** The three drug combinations were made by reducing the quantity of drug with high ratio and incorporating the remaining candidate to the mixture. Overall schematic diagram is presented in figure 2.1.

![Figure 2.1: Scheme for antioxidant treatment therapies.](image-url)
In all drug combinations, final dose remained constant (100 mg/kg), in order to evaluate the synergistic effects since adding in equal doses i.e. 100 mg + 100 mg will provide additive effect and cannot be compared to single drug effect.

2.5.2. **Analysis of antioxidants present in blood and selected natural antioxidants**

2.5.2.1. **Analysis of fat soluble antioxidants in serum samples; \(\alpha\)-tocopherol and all-trans retinol**

\(\alpha\)-Tocopherol and all-trans retinol were observed following the UV based HPLC method \(^{323-324}\). Briefly in serum (100 μl), retinyl acetate (IS) was added to make final concentration of 1 μg/ml. After protein precipitation with ethanol, extraction was carried out with \(n\)-hexane followed by dichloromethane. After centrifugation at 5000×g at 4 °C the extraction process was repeated. The combined recovered extraction solvents were dried under nitrogen stream and the residue was reconstituted with mobile phase consisting of methanol and water in 95:05 ratios v/v. Analysis was performed at 292 nm \(\lambda\)-max using Athena C18-WP (100A, 4.6 mm×250 mm, 5 μm; CNW technologies GmbH Germany) HPLC column.
2.5.2.2. Simultaneous determination of water soluble antioxidants and malondialdehyde by RP-HPLC coupled with electrochemical detector in serum samples

A new method was developed and validated for simultaneous analysis of aminothiols along with ascorbic acid and lipid peroxidation determinant; malondialdehyde in serum samples.

2.5.2.2.1. Preparation of standard solutions

Aminothiols and ascorbic acid stock solutions (in mmol) were prepared in TFA aqueous (0.05%) separately in dark and stored at -20 °C. Fresh dilutions were prepared on daily basis with the same solvent. MDA solution was prepared in 0.1 M HCl in volumetric flask, holding it in boiling water for 5 minutes to complete the hydrolysis and release all the acetals. Further dilutions were prepared using TFA aqueous.

2.5.2.2.2. Blood collection, storage and sample preparation

Blood samples were collected from normal albino rabbits in gel clot tubes, serums were removed after centrifugation at 3000xg at 4 °C and stored at -20 °C until analyzed. Serum samples were thawed to melt at room temperature and subsequently spiked with appropriate concentrations of aminothiols, AA and MDA along with dopamine (10 µl) as internal standard (IS) making its final concentration of 1 µg/ml (equiv: 6.53 mmol/l) and extracted. The calibration curves were constructed in 6 concentrations range in spiked serum, standard solutions in mobile phase and spiked serum corrected for blank serum.
2.5.2.2.3. Sample extraction procedure

Freshly prepared meta-phosphoric acid (10%) was used for protein precipitation and further extracting with mobile phase for maximum recoveries. Serum samples were subsequently spiked appropriately with different concentrations and checked for recoveries.

To serum samples (100 µl), IS (10 µl) was added in 2 ml Eppendorf tubes and vortexed vigorously for 1 minute. The freshly prepared meta-phosphoric acid (100 µl) was added to each tube for protein precipitation and after vortexing diluted with mobile phase to volume (300 µl) for extraction. After vortexing, centrifuged at 14000×g at 4 °C for 10 minutes. Clear supernatants obtained were subsequently transferred to ambered colored autosampler vials for analysis using 5 µL injection volumes within 24 hours (Fig 2.2).

Figure 2.2: Extraction procedure of antioxidants from blood.
2.5.2.2.4. Chromatographic conditions and experimental procedures optimization

The effects of chromatographic conditions for simultaneous analysis were optimized for accuracy, sensitivity and robustness of current adopted method.

2.5.2.2.4.1. Selection of mobile phase

Mobile phase composition was optimized based on previous analytical methods. In organic phase methanol and acetonitrile was used in various ratios with aqueous phase for better resolution of compounds. TFA in 0.025-0.1% was evaluated at three pH levels 2.0, 2.25 and 2.45 for complete separation and sensitivities. Aqueous to organic phase ratios used were from 95:5 to 100:0.

2.5.2.2.4.2. Selection of flow rate

Flow rate ranging from 0.5 to 1.2 ml/min was evaluated for good separation and sensitivity.

2.5.2.2.4.3. Selection of column oven and electrochemical detector temperature

Various temperature levels (25-40 °C) of column oven and electrochemical detector were applied to observe effects on analyte resolution and sensitivity.

2.5.2.2.4.4. Selection of electrochemical detector electrode potential

Effect of electrode potential on the response of analytes was observed while keeping other parameters constant. Potential of 600-1000 mV was applied for optimum results.
2.5.2.2.5. Application of method

The method was optimized to determine level of oxidative stress in hyperlipidemic state by quantifying antioxidants levels in blood. They maintain balance between production of free radicals produced during metabolic processes and their scavenging and linked with one another for regeneration. In pathologic conditions such as diabetes, hyperlipidemia, hypertension and other neurodegenerative illnesses; excessive, abnormal and uncontrolled release of free radicals causes the disruption of such balance. Depletion of endogenous antioxidants propagates towards the development of oxidative stress. Interventions followed in such conditions helps in achievement of physiological redox state. For outcome assessment quantification of endogenous antioxidants is necessary along with lipid profile to correlate with the interventions.

2.5.2.3. Development and validation of HPLC-UV method for simultaneous determination of niacin (hydrophilic) and pterostilbene (lipophilic) natural antioxidants

Analysis of niacin and pterostilbene on UV/Visible spectrophotometer was difficult because of near $\lambda$-max causing interference in each other’s peak, while OPC can be easily quantified on UV/Visible spectrophotometer. Therefore a novel approach was followed for simultaneous analysis of NA and PT using quaternary ammonium amphiphilic ion pairing agent for retaining niacin on RP-columns. The method was found sensitive for simultaneous determination of both hydrophilic (NA) and lipophilic (PT) antioxidants on reverse phase C-18 HPLC chromatographic column.
2.5.2.3.1. Preparation of stock solutions

Stock solutions (1 mg/10 ml) of PT, NA and internal standard (IS) atorvastatin, rosvastatin were prepared in volumetric flask, and stored at -20 °C. Desired working concentrations were made by diluting the stock solutions with mobile phase composed of organic phase (80:20 ACN: MeOH) and aqueous phase (5 mM CTAB) adjusted at pH 2.5 with formic acid. The mobile phase was pumped in ratios in 60-70 organic: 40-30 aqueous phase at 1 ml/min flow rate. Calibration curves of both drugs were obtained at various concentration levels keeping the concentration of IS constant (1 μg/ml).

2.5.2.3.2. Preparation of blood samples and in-vitro dissolution samples

Blood samples were collected in EDTA tubes and centrifuged at 3000×g at 4 °C. Supernatant plasma was separated and stored in eppendorf tubes (2 ml) at -20 °C until used. For sample preparation plasma (200 μl) was spiked with appropriate concentrations of both drugs. IS (10 μl) was added to each tube making final concentration of 1 μg/ml. Sample deproteinization and extraction was carried out by adding mobile phase (800 μl) followed by vortexing and centrifugation at 14000×g at 4 °C for 10 minutes. Clear supernatants obtained were subsequently transferred to ambered colored autosampler vials and 50 μl volumes was injected onto HPLC column for analysis.

Same procedure was followed in analysis of in-vitro drug release profile. Samples from dissolution media obtained at various time intervals were filtered and after adding IS, diluted with mobile phase and vortexed, centrifuged at 3000×g for 5 minutes to sediment any visible impurity, and injected directly onto HPLC column for analysis.
2.5.2.3.3. **Calibration curves**

Calibration curves for both analytes in mobile phase and spiked plasma samples were obtained on Athena C18-WP (100A, 4.6 mm×250 mm, 5 µm; CNW technologies GmbH Germany) RP-HPLC column using rosuvastatin (1 µg/ml) IS.

2.5.2.3.4. **Chromatographic conditions optimization**

2.5.2.3.4.1. **Mobile phase composition**

For the enhancement of retention time (RT) of hydrophilic analytes CTAB was added to the aqueous phase at various concentrations (1-5 mmol/l CTAB) without effecting lipophilic analyte RT to large extent. The pH was adjusted with various acids such as formic acid in 2-4 pH range for peak separation, sensitivity, sharpness. The organic phase was composed of methanol, acetonitrile and their combined ratios and evaluated for separation and sharpness. The mobile phase was pumped at 1 ml/min in various ratios and the combination giving optimum results was selected for analysis.

2.5.2.3.4.2. **Flow rate**

Flow rate was checked between 0.7 to 1.2 ml/min for peak shape, selectivity and sensitivity for the analysis of both antioxidants.

2.5.2.3.4.3. **Column oven temperature**

Column oven temperature was evaluated between 25-50 °C, for optimum peak separation and sensitivity.
2.5.2.3.4.4. **Injection volume**

Injection volume ranging between 10 to 100 μl was evaluated to optimize detector response at specified wavelength.

2.5.2.3.4.5. **Selection of internal standard**

Rosuvastatin, simvastatin and atorvastatin were evaluated as internal standard (IS) for simultaneous method development. Compound with good separation and minimum effects on other peaks was selected as internal standard.

2.5.2.3.4.6. **Selection of detector wavelength**

Detector wavelength was checked at range of 220-280 nm for optimum response.

2.5.2.3.5. **Application of method**

The validated method was applied for simultaneous determination of both antioxidants in plasma and in *in-vitro* dissolution samples. This method can be applied to other hydrophilic and lipophilic compounds, vitamins and antioxidants for separation and determination simultaneously in single elution. Since water soluble vitamins have poor RT on reverse phase C-18 HPLC columns thus this method provides a mean of separating hydrophilic compounds efficiently along with the lipophilic compounds.

2.5.2.4. **Validation of HPLC methods of analysis**

For the two methods developed under this study for simultaneous determination of: 1) water soluble endogenous antioxidants, peroxidative biomarker and 2) natural
water/fat soluble antioxidants, different parameters were evaluated for validation on the basis of allowed variability. Methods were validated for their precision, sensitivity, selectivity, robustness and linearity along with lower limit of detection (LLOD) and lower limit of quantification (LLOQ). Suitability and repeatability were also checked according to standard guide lines for analytical methods. 

2.5.2.4.1. Specificity/selectivity

Specificity of method was determined for each of analyte both in standard solutions prepared in mobile phase and spiked plasma samples in order to detect any extraneous interfering peaks. Binary mixture (1:1) of each analyte in both analytical methods was prepared to determine peak separation.

2.5.2.4.2. Accuracy

Accuracy was measured in terms of percent recovery of analytes, by spiking serum samples with suitable concentrations of analytes in triplicate using equation:

\[ \% \text{ Recovery} = \frac{A_{ss}}{A_{mp} + A_{cs}} \times 100 \]...... eq: 10

A\text{ss}: response ratio of analyte to IS in spiked serum, A\text{mp}: response ratio of analyte to IS in mobile phase, A\text{cs}: response ratio of analyte to IS in control serum. Response ratio of each analyte is equal to the area of analyte divided by area of IS.

2.5.2.4.3. Linearity

Linearity was determined by constructing calibration curve for each analyte in spiked serum and mobile phase at six different concentrations in terms of analyte
response ratios to IS versus spiked concentration of each analyte \(^{329}\). Linear least square regression equation was used and the resulting correlation coefficient \((r)\), intercepts and slope \((b)\) were calculated using graph pad prism 5.01-software.

2.5.2.4.4. Precision

Precision was measured by injection repeatability and analysis repeatability of standard and spiked samples.

2.5.2.4.4.1. Injection repeatability

Precision in terms of injection repeatability was assessed by repeated injections of spiked serum samples with appropriate concentration of each analyte on to HPLC system. The retention time and peak areas obtained were presented as mean \(\pm\) SD and covariance \((%\text{RSD})\).

2.5.2.4.4.2. Intermediate precision

This includes intra-day and inter-day repeatability of spiked samples \(n=6\) with each analyte and IS into HPLC and measured by retention time and peak area as mean, standard deviation \((\pm\text{SD})\) and covariance \((%\text{RSD})\). Repeatability studies were evaluated at three times intervals 08:00, 16:00 and 24:00 hours for three consecutive days \(^{330}\) and following equation was used to determine the recovered quantity.

\[
c = \frac{x}{y} \times \frac{a}{b} \times C_s \times F_D \quad \text{eq: 11}
\]

Where \(x\) and \(y\) are the peaks of analyte in plasma and 1:1 mixture. \(a\) and \(b\) are the peaks of IS in 1:1 mixture and plasma sample. \(C_s\) is the analyte concentration in 1:1 mixture and \(F_D\) is the dilution factor.
2.5.2.4.5. Sensitivity

Sensitivity of each method was assessed by LOD and LLOQ for each analyte at signal to noise ratio (S/N) of 3 and 10, indicating the minimum concentration of analyte that can be measured with optimal accuracy, producing signal 10 times to noise level 331.

2.5.2.4.6. Robustness

Methods robustness was determined by performing deliberate changes in chromatographic conditions including mobile phase composition, temperature, flow rate, electrode potential and wave length.

2.5.2.4.7. Stability of samples

Stability studies were performed by comparing the peak area of each analyte in different timings with the initial reading 328. Both standard solutions and spiked samples were checked at room (25 °C), refrigerator (4 °C) and at freezing (-20 °C) temperatures. Percent stability and percent loss were calculated using the following equations 326.

\[
\% \text{ stability} = \frac{S_t}{S_0} \times 100 \quad \text{eq: 11}
\]

\[
\% \text{ loss} = \frac{(S_0 - S_t)}{S_0} \times 100 \quad \text{eq: 12}
\]

In which \( S_0 \) and \( S_t \) are the concentrations of each analyte initially and after time \( t \).
2.5.2.5. UV/Visible spectrophotometric analysis of oligomeric proanthocyanidins in combinatorial dosage forms.

For in-vitro assay of OPC in dosage form, previously reported method of Porter et al. was followed. Briefly two reagents R1 (2 gm of ferric ammonium sulfate in 2 M HCl) and R2 (n-butanol with HCl in 95:5 ratios) were prepared. Standard solution of OPC (30 mg/10 ml methanol) was prepared in volumetric flask. Taking volume containing 100 μg OPC from the above solution into 10 ml plug tube and adding 0.2 ml of R1 and 6 ml of R2, the tube was caped and vortexed. After which cap was loosened and tube was placed in hot water bath at 95 °C for 1 hour to complete reaction. The tube was than cooled immediately with icy water adjusting volume (10 ml) (10 μg/ml concentration) with R2 to get standard solution. Drug release profile from formulated dosage forms was observed by preparing samples in the same manner by taking 5 ml from dissolution media. After filtration through Wattman filter paper, pipetting 3 μl into 10 ml capped tube and diluting with R1 and R2 followed by the steps mentioned above. Measurement was performed at 546 nm λ-max on lambda-25 UV/Vis spectrophotometer.

2.5.3. Formulation development

2.5.3.1. Pre-formulation studies

2.5.3.1.1. Drug excipient compatibility studies

Drug excipient compatibilities were checked by drug contents and FTIR spectras. Samples were prepared in binary ratios among APIs and excipients. Stability was performed by employing the thermal-isothermal stress testing of binary admixtures of
drug-excipient kept under stress conditions at 40 °C and 75% relative humidity for a month.

2.5.3.1.2. Sample preparation

Drug samples were analyzed individually and in binary mixtures (1:1). This ratio was followed in each drug excipient admixtures as mentioned in table 2.2.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Sample composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Oligomeric proanthocyanidins (OPC) (50 mg)</td>
</tr>
<tr>
<td>02</td>
<td>Niacin (NA) (50 mg)</td>
</tr>
<tr>
<td>03</td>
<td>Pterostilbene (PT) (50 mg)</td>
</tr>
<tr>
<td>04</td>
<td>Tablettose-80 + Ac-Di-Sol + Talc + PVP-K30 + cross carmellose sodium + micro crystalline sodium (50 mg each).</td>
</tr>
<tr>
<td>05</td>
<td>OPC (50 mg) + all excipients (50 mg)</td>
</tr>
<tr>
<td>06</td>
<td>NA (50 mg) + all excipients (50 mg)</td>
</tr>
<tr>
<td>07</td>
<td>PT (50 mg) + all excipients (50 mg)</td>
</tr>
</tbody>
</table>

2.5.3.1.3. FTIR spectral analysis

FTIR spectral data was obtained using FTIR spectrometer (Prestige, Shimadzu, Japan) having IR solutions version 1.10 software. KBr pellet method was followed in sample preparation. Sample (2%) prepared in KBr, pulverized and compacted in a disc. Spectra were recorded in range of 400 – 4000/ cm at 8/ cm resolution in absorbance mode.
2.5.3.1.4. Drug content determination

Drug content in each mixture was determined using HPLC-UV and VU/Visible spectrophotometric method of analysis. Both standard and samples were analyzed under same conditions. Drug content was calculated in percentage at time zero and after 30 days of storage under accelerated conditions using the equation 13.

\[
\text{Drug Content (\%)} = \frac{A_{\text{spl}}}{A_{\text{std}}} \times 100\% \quad \text{eq: 13}
\]

Where \( A_{\text{spl}} \) and \( A_{\text{std}} \) is the peak area of sample and standard. All the analysis was performed in triplicate and data was arranged in mean ± standard deviation.

2.5.3.1.5. Physical consistency

Binary admixtures were also checked for any change in physical inconsistency including change in color, any unknown agglomerations.

2.5.3.2. Conversion of animal dose into human equivalent dose

Animal dose is first converted into human equivalent dose based on body surface area (BSA), because BSA as a translating tool correlates best among several mammalian species with various biological characteristics including oxygen consumption, basal metabolic rate, calorie expenditure, blood volume and renal function. A formula developed by food and drug administration (FDA) based on BSA for dose translation is as follows \(^{335}\).

\[
\text{Human Equivalent Dose (HED)} = \frac{\text{Animal Dose (mg/Kg)} \times \frac{\text{Animal } K_m}{\text{Human } K_m}} \quad \text{eq: 14}
\]
Where $K_m$ is the factor calculated by dividing the body weight (kg) by BSA (m$^2$) to convert mg/kg dose to mg/m$^2$. The $K_m$ values are mentioned in table 2.3.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Specie</th>
<th>Weight (kg)</th>
<th>BSA (m$^2$)</th>
<th>$K_m$ factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Human</td>
<td>Adult 60</td>
<td>1.6</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Child 20</td>
<td>0.8</td>
<td>25</td>
</tr>
<tr>
<td>02</td>
<td>Rabbit</td>
<td>1.8</td>
<td>0.15</td>
<td>12</td>
</tr>
</tbody>
</table>

### 2.5.3.3. Development of formulation for the optimized APIs blends

The aims of formulation are:

- To reduce the tablet bulk and obtain best powder flowability by performing granulation techniques.
- Acquire disintegration within limits using minimum quantity of excipients.
- To give a research based finished against marketed products as they offer benefits of these drugs in bulk doses which is a wastage of capital, drugs and may result harmful effects rather than beneficial.

Preliminary tests showed poor flow properties of these drugs, therefore formulations were prepared using granulation technique by both wet and dry methods. Drug content per tablet is high enough and especially OPC has the property of retaining water for some time preventing disintegrating agents from performing their function. To optimize the disintegration time (DT) and dissolution profiles, commonly used excipients were evaluated for desired results. Tablettose-80, micro crystalline cellulose, cross
carmellose sodium and sodium starch glycolate were used to achieve DT time within limits.

The APIs dose was divided into two halves (i.e. 750 mg/tab) and their formulations were developed in the pattern summarized in table 2.4.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Ingredients (mg/tab)</th>
<th>Formulations</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Blend of APIs (OPC + NA + PT)</td>
<td>F1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>85</td>
</tr>
<tr>
<td>02</td>
<td>Tabletose-80</td>
<td>10</td>
</tr>
<tr>
<td>03</td>
<td>Micro crystalline cellulose</td>
<td>-</td>
</tr>
<tr>
<td>04</td>
<td>Cross carmelloose sodium</td>
<td>-</td>
</tr>
<tr>
<td>05</td>
<td>Sodium starch glycolate</td>
<td>-</td>
</tr>
<tr>
<td>06</td>
<td>Talc</td>
<td>2</td>
</tr>
<tr>
<td>07</td>
<td>Magnesium stearate</td>
<td>3</td>
</tr>
</tbody>
</table>

*Quantities added are in % w/w per tablet.

All these formulations were prepared in the following three ways.

- Dry granulation (slugging method).
- Wet granulation with de-ionized water.
- Wet granulation using 2% aqueous PVP-K30 solution.

In dry granulation method slugging was performed which were subsequently crushed and passed through mesh #12. In dry granulation APIs blend was mixed with half quantity of disintegrating agent and slugged. Then these slugs were crushed, sifted and mixed with rest of disintegrant. After passing through mesh #60 lubricants were
added, mixed and compressed into oblong double convex tablet of 19 mm×8 mm dimensions using rotary compression machine. A batch of 100 tablets was prepared for each formulation.

Wet granulation was performed by de-ionized water and aqueous 2% PVP-K30 to observe effects on powder bulk and flowability. APIs were mixed with half of disintegrant before granulation to enhance granules rupturing and drug release. De-ionized water or Aqueous 2% PVP K-30 solution was added slowly to the mixture with continuous mixing to make lumps. These were when half dried at 40-50 °C in hot air oven, passed from mesh # 8 and left for complete drying until moisture content decreases to less than 2%. The granules were further passed through mesh #12 and after adding remaining disintegrant and lubricant, compressed into tablet. Active pharmaceutical ingredients (APIs) blend quantity in each tablet was reduced to half of actual dose to control tablet thickness, disintegration, and elegance.

2.5.3.4. In-vitro evaluation studies

In-vitro evaluation was conducted in two steps; precompresion and post compression evaluation studies.

2.5.3.4.1. Pre-compression studies

Precompresion studies were preformed for all the three active drugs and excipient blends for flowability, compressibility, bulk density, tape density, angle of repose, Carr’s index and Hausner ratio \(^{336}\). All these parameters were determined in triplicate and data was tabulated in mean ± SD.
2.5.3.4.1.1. **Bulk densities**

Bulk densities of both powder APIs and formulated granules were determined using the procedure mentioned in United States Pharmacopoeia (USP 32)\(^{336}\). Bulk density was calculated by taking weighed quantities in graduated cylinder and measuring volumes occupied using following formula.

\[
\text{Bulk Density (Db)} = \frac{W}{V} \quad \text{eq: 15}
\]

Here, Db = Bulk density (gm/ml), W = Weight of powder/ granule bulk (gm), V = Volume occupied by weighed bulk (ml).

2.5.3.4.1.2. **Tape densities**

Tape densities of APIs powder / granules were measured by taping 100 times the weighed quantity in graduated cylinder against table top. After taping volume occupied was observed and tape density was calculated using equation 16.

\[
\text{Tapped Density (Dt)} = \frac{W_t}{V_t} \quad \text{eq: 16}
\]

Where; Dt = Tape density, Wt = Weight of taped bulk, Vt = Volume of tapped bulk

2.5.3.4.1.3. **Carr’s index (IC)**

Carr’s index was calculated from the values of bulk and tape densities applying equation 17.

\[
\text{Carr's Index (IC)} = \frac{Dt - Db}{Dt} \times 100 \quad \text{eq: 17}
\]

Where; IC = Carr’s Index (%), Dt = Tape density (gm/ml), Db = Bulk density (gm/ml)
2.5.3.4.1.4. **Hausner ratio (Hr)**

Hausner ratio was calculated by bulk and tape densities values using equation 18.

\[
\text{Hausner Ratio (Hr)} = \frac{D_t}{D_b} \quad \text{eq: 18}
\]

Where; \( D_t \) = Tape density (gm/ml), \( D_b \) = Bulk density (gm/ml)

2.5.3.4.1.5. **Angle of repose (\( \alpha \))**

Angle of repose was determined using funnel method in which powder and granules were allowed to flow through funnel held 3-4 cm above plan surface. Height and radius was placed in the equation 19.

\[
\text{Angle of Repose (} \alpha \text{)} = \tan^{-1}\left(\frac{H}{r}\right) \quad \text{eq: 19}
\]

Where; \( H \) and \( r \) is the height (cm) and radius of powder/granules formed on surface.

2.5.3.4.2. **Post-compression studies**

2.5.3.4.2.1. **Tablet hardness and thickness test (USP)**

Prepared tablets were subjected to hardness test to ensure their strength within limits. From each batch \( n=10 \) tablets were taken and tested on digital hardness tester. Cushing strength of tablets was determined in mean ± SD along with tablet thickness with the same instrument.

2.5.3.4.2.2. **Tablet friability test (USP)**

Friability test was performed on \( n=10 \) tablets using digital friabiliator to determine their strength against physical stress. Tablets were weighed and subjected to
100 revolutions and after completion of session re-weighed and friability was calculated in terms of percent loss by equation 20.

\[
\text{Friability (\%) = } \frac{W_1 - W_2}{W_1} \times 100 \quad \text{eq: 20}
\]

Where; W1 and W2 are the initial and final weights of tablets before and after 100 revolutions.

2.5.3.4.2.3. Tablet disintegration test (USP)

Granules prepared were compressed into tablet preventing capping and lamination and their DT were determined on n= 6 tablets from each batch. USP disintegration apparatus was used with and without disc having deionized water at 37 °C. Readings were taken in triplicate and data was presented in mean ± SD.

2.5.3.4.2.4. In-vitro drug release profiling (Dissolution test, USP)

In-vitro drug release profiles of formulated dosage form were determined using dissolution apparatus-II (paddle method) using 0.1 N HCl (900 ml) solution as dissolution media at 37 °C. The paddle revolution per minute (RPM) was maintained at 60 and samples (5 ml) were withdrawn at time 0, 5, 15, 30, 45 and 60 minutes, diluted appropriately and injected onto HPLC column for analysis of niacin and pterostilbene. While for OPC UV/Visible spectrophotometric analysis was followed. Each sample volume was replaced with fresh 0.1 N HCl solution.

According to USP35 all the nutritional supplements that comes under class II to VI and formulated in to tablet or capsule are subjected to dissolution test as shown in table 2.5.
Table 2.5: Dissolution testing criteria of nutraceuticals according to USP

<table>
<thead>
<tr>
<th>USP Class</th>
<th>Combination of nutraceuticals</th>
<th>Dissolution requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Oil-soluble</td>
<td>Not applicable</td>
</tr>
<tr>
<td>II</td>
<td>Water soluble</td>
<td>One index compound; folic acid (if present)</td>
</tr>
<tr>
<td>III</td>
<td>Water soluble nutraceuticals with minerals</td>
<td>One index compound and one index elemental; folic acid (if present)</td>
</tr>
<tr>
<td>IV</td>
<td>Oil soluble and water soluble</td>
<td>One index compound; folic acid (if present)</td>
</tr>
<tr>
<td>V</td>
<td>Oil soluble and water soluble with minerals</td>
<td>One index compound and one index elemental; folic acid (if present)</td>
</tr>
<tr>
<td>VI</td>
<td>Minerals</td>
<td>One index elemental</td>
</tr>
</tbody>
</table>

Therefore on the basis of these criteria dissolution test for pterostilbene is not necessary because we have two hydrophilic compounds as dissolution markers in our formulations; besides that pterostilbene dissolution was also observed. For ease of analysis choice of adding surfactants in appropriate concentrations to the dissolution media to enhance lipophilic drug dissolution was also applicable.

2.5.3.5. Selection of optimized formulation(s)

The optimum formulations were selected on the basis of tablet crushing strength-friability/disintegration time ratio (CSFR/DT). There are no standards for calculating this ratio and it totally depends on the individual tablet formulation technique. It provides a suitable index for determining tablet strength or weakness and their effects on DT. Higher value represents high balance between tablet binding and disintegration and indicates better DT.
2.5.3.6. Stability studies of optimized formulation(s)

Optimized formulations of antioxidants therapeutic blends were subjected to accelerated stability tests at 40 °C and 75% RH for 3-months in terms of friability, disintegration and degradation half life ($t_{1/2} = 0.693/k$).
Chapter 3

RESULTS

AND DISCUSSIONS
3. RESULTS AND DISCUSSIONS

3.1. Assessment of lipid profile

After two weeks oral cholesterol (400 mg/kg) feeding, almost 2-10 folds elevation in lipid profile was observed indicating that taking high dietary cholesterol only, produces affects on all the lipid parameters consequently, even if triglyceride intake is normal and the same mechanism is for high intake of triglycerides. This phenomenon is due to the excess intake of either of lipid ingredient activate synthesis of other through acetyl-CoA pathway. Thus both parameters perform their part in development of atherogenic events. Lipid endogenous regulation is explained in figure 3.1.

![Diagram of lipid synthesis](image)

**Figure 3.1:** Scheme for *de novo* synthesis of cholesterol and triglycerides.
Lipid profile including TC, TG, LDL-C, HDL-C, VLDL-C of normal healthy group and high cholesterol diet fed group are calculated in mg/dl along with LDL-C/HDL-C ratio and atherogenic index (AI). Data is presented as mean ± SEM along with p-values in table 3.1. After two weeks of cholesterol dosing significant elevation in all lipid parameters \( p< 0.0001 \) was observed. The LDL-C/HDL-C rose from 0.524 ± 0.035 to 3.536 ± 0.040 and rising of AI from 0.331 ± 0.014 to 0.458 ± 0.040 confirmed ascend in triglyceride level \( (140 ± 2.465 \text{ mg/dl}) \) compared to healthy rabbits \( (67 ± 0.635 \text{ mg/dl}) \).

### Table 3.1: Lipid profile of normal healthy and hyperlipidemic groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lipid profile of healthy rabbits group (mg/dl)</th>
<th>Lipid profile control group after 2 weeks cholesterol dosing (mg/dl)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>61 ±2.468</td>
<td>249 ±7.425</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>67 ±0.635</td>
<td>140 ±2.465</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>LDL-Cholesterol</td>
<td>16.36 ±0.618</td>
<td>172.3 ±5.557</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HDL-Cholesterol</td>
<td>31.24 ±1.241</td>
<td>48.72 ±2.211</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>VLDL-Cholesterol</td>
<td>30.45 ±1.002</td>
<td>63.64 ±2.113</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>LDL-C/HDL-C ratio</td>
<td>0.524 ±0.035</td>
<td>3.536 ±0.040</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Atherogenic Index (AI)</td>
<td>0.331 ±0.014</td>
<td>0.458 ±0.040</td>
<td>0.0071</td>
</tr>
</tbody>
</table>

#### 3.1.1. Treatment therapies

Lipid profile of each group including disease control and treatment groups are calculated in mean ± SEM. Evaluating baseline, all the parameters raised in 12 weeks of cholesterol dosing. The LDL-C/HDL-C ratio and AI incremented parallel from 3.536 ± 0.057, \( 0.458 ± 0.057 \) to 30.50 ± 0.686 and 1.096 ± 0.041 respectively. However HDL-C decreased from 48.72 ± 3.13 to 27.83 ± 0.82 mg/dl as shown in table 3.2.
Table 3.2: Lipid profile of disease control after twelve weeks of cholesterol dosing

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>249 ± 10.50</td>
<td>462 ± 11.52</td>
<td>708 ± 15.71</td>
<td>946 ± 38.53</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>140 ± 3.49</td>
<td>210 ± 7.57</td>
<td>283 ± 9.27</td>
<td>347 ± 10.04</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>172.3 ± 7.86</td>
<td>379.66 ± 11.78</td>
<td>617.02 ± 17.37</td>
<td>848.8 ± 34.19</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>48.72 ± 3.13</td>
<td>40.34 ± 2.24</td>
<td>34.38 ± 1.44</td>
<td>27.83 ± 0.82</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>63.64 ± 2.99</td>
<td>95.45 ± 5.74</td>
<td>128.64 ± 3.95</td>
<td>157.7 ± 2.69</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.536 ± 0.057</td>
<td>9.41 ± 0.421</td>
<td>17.95 ± 0.543</td>
<td>30.50 ± 0.686</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.458 ± 0.057</td>
<td>0.716 ± 0.065</td>
<td>0.915 ± 0.037</td>
<td>1.096 ± 0.041</td>
</tr>
</tbody>
</table>

3.1.1.1. Individual therapies of selected antioxidants

Comparing the therapies with disease control the atorvastatin therapy significantly suppressed the elevation in blood lipid levels. Overall the LDL-C/HDL-C and AI were 14.45 ± 0.97 and 0.808 ± 0.06 respectively as shown in table 3.3.

Table 3.3: Lipid profile of atorvastatin group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>240 ± 9.21</td>
<td>366 ± 14.21</td>
<td>510 ± 12.88</td>
<td>625 ± 17.42</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>147 ± 4.67</td>
<td>175 ± 8.71</td>
<td>209 ± 9.34</td>
<td>240 ± 5.491</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>164.17 ± 7.57</td>
<td>288.17 ± 10.71</td>
<td>428.36 ± 8.91</td>
<td>539.65 ± 15.01</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>46.43 ± 2.32</td>
<td>42.83 ± 1.49</td>
<td>39.84 ± 5.17</td>
<td>37.35 ± 1.72</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>66.82 ± 3.44</td>
<td>79.55 ± 1.76</td>
<td>95.0 ± 2.96</td>
<td>109.1 ± 3.35</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.54 ± 0.245</td>
<td>6.73 ± 0.592</td>
<td>10.75 ± 1.35</td>
<td>14.45 ± 0.97</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.50 ± 0.037</td>
<td>0.611 ± 0.029</td>
<td>0.72 ± 0.049</td>
<td>0.808 ± 0.06</td>
</tr>
</tbody>
</table>
Progressive changes in lipid profiles during individual antioxidant treatments were observed by diminished increments in lipid parameters compared to disease control. In individual antioxidant therapies the maximum effects in decreasing TC was observed in PT group 698 ± 14.19 mg/dl, while in OPC and NA groups the TC was 733 ± 21.42 mg/dl and 806 ± 16.51 mg/dl respectively (Tab. 3.4-3.6). This elevated effect in PT group is due to its potent PPAR-α activating mechanism enhancing metabolism. The reduced value of AI in OPC 0.893 ± 0.033 compared NA and PT group 0.896 ± 0.053 and 0.971 ± 0.057 respectively, signify the concept; that total cholesterol itself is not responsible solely in atherosclerosis. Other lipid parameters like increased TG and declined HDL-C may also play important role in its initiation and propagation.$^{342-343}$

### Table 3.4: Lipid profile of oligomeric proanthocyanidins (OPC) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>258 ± 9.69</td>
<td>404 ± 10.39</td>
<td>590 ± 17.56</td>
<td>733 ± 21.42</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>145 ± 3.61</td>
<td>180 ± 3.041</td>
<td>225 ± 3.49</td>
<td>260 ± 9.104</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>179.48 ± 6.40</td>
<td>324.6 ± 7.48</td>
<td>506.77 ± 7.06</td>
<td>647.8 ± 19.34</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>49.52 ± 2.69</td>
<td>43.40 ± 2.29</td>
<td>38.23 ± 1.65</td>
<td>33.24 ± 2.09</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>65.9 ± 1.36</td>
<td>81.81 ± 1.87</td>
<td>102.3 ± 1.76</td>
<td>118.2 ± 2.09</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.62 ± 0.229</td>
<td>7.48 ± 0.425</td>
<td>13.25 ± 0.950</td>
<td>19.49 ± 0.866</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.467 ± 0.033</td>
<td>0.618 ± 0.029</td>
<td>0.77 ± 0.033</td>
<td>0.893 ± 0.033</td>
</tr>
</tbody>
</table>
Table 3.5: Lipid profile of niacin (NA) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>246 ± 10.49</td>
<td>422 ± 9.17</td>
<td>620 ± 14.52</td>
<td>806 ± 16.51</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>144 ± 3.71</td>
<td>185 ± 3.72</td>
<td>237 ± 6.50</td>
<td>278 ± 8.66</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>169.7 ± 7.47</td>
<td>342.36 ± 11.97</td>
<td>532.28 ± 17.64</td>
<td>715.1 ± 19.87</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>47.50 ± 3.14</td>
<td>42.64 ± 3.10</td>
<td>38.32 ± 2.09</td>
<td>35.29 ± 1.85</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>65.45 ± 2.35</td>
<td>84.09 ± 1.91</td>
<td>107.72 ± 3.43</td>
<td>126.4 ± 4.79</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.57 ± 0.159</td>
<td>8.02 ± 0.723</td>
<td>13.89 ± 1.192</td>
<td>20.26 ± 0.94</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.482 ± 0.024</td>
<td>0.637 ± 0.041</td>
<td>0.809 ± 0.061</td>
<td>0.896 ± 0.053</td>
</tr>
</tbody>
</table>

Table 3.6: Lipid profile of pterostilbene (PT) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>239 ± 10.50</td>
<td>419 ± 9.70</td>
<td>579 ± 15.39</td>
<td>698 ± 14.19</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>147 ± 3.49</td>
<td>194 ± 3.85</td>
<td>252 ± 4.19</td>
<td>296 ± 3.64</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>163.68 ± 7.86</td>
<td>338.58 ± 7.89</td>
<td>491.74 ± 14.91</td>
<td>607.16 ± 14.45</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>45.92 ± 3.13</td>
<td>41.62 ± 3.72</td>
<td>36.86 ± 1.84</td>
<td>31.64 ± 3.03</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>66.82 ± 2.14</td>
<td>88.18 ± 2.89</td>
<td>114.54 ± 2.63</td>
<td>134.5 ± 6.30</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.564 ± 0.20</td>
<td>8.135 ± 0.763</td>
<td>13.34 ± 0.923</td>
<td>19.19 ± 0.906</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.505 ± 0.024</td>
<td>0.668 ± 0.041</td>
<td>0.835 ± 0.041</td>
<td>0.971 ± 0.057</td>
</tr>
</tbody>
</table>

3.1.1.2. Combination therapies of selected antioxidants

The combinational therapies were performed for the following reasons:

- Natural antioxidants specially the flavonoids are found most often ineffective or with reduced effects at large doses producing a nonlinear response \(^{344-345}\). In current study we had correlated therapy outcome with different doses in blends to the blood lipid profile and blood antioxidants in hypercholesterolemic animal model.
Drugs selected for the current study were mostly used individually or in two drug combinations in additive ratios, especially niacin with OPC\textsuperscript{346-347}. Pterostilbene was not evaluated separately in low doses with OPC and niacin for lipid lowering potential in combination. In our study they were tested in individual as well as in reduced dose combinations.

Natural antioxidants have been reported of having ceiling effects above which they respond as prooxidant\textsuperscript{345,348}. In current study we developed our own dose rationale by optimizing combination dose ratios for maximum lipid lowering effects and to prevent ceiling effects.

### 3.1.1.2.1. Oligomeric proanthocyanidins and niacin combination therapies

Evaluating OPC: NA combination ratios most optimum lipid lowering effect was observed in 70:30 ratio, where LDL-C/HDL-C ratio and AI values were 15.36 ± 0.984 and 0.843 ± 0.041 respectively. While in case of 50:50 (Table 3.8) and 30:70 (Table 3.7); the LDL-C/HDL-C was 17.33 ± 0.751 and 16.77 ± 0.694 and AI were 0.887 ± 0.037 and 0.837 ± 0.045 respectively. Moreover the TC and TG levels were also lower; 642 ± 13.31 mg/dl and 252 ± 8.54 mg/dl in 70:30 (Table 3.9) compared to other two groups making it most favorable to be incorporated further among the three combinations.
Table 3.7: Lipid profile of OPC:NA (30:70) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>247 ± 11.61</td>
<td>422 ± 9.85</td>
<td>598 ± 12.48</td>
<td>725 ± 15.92</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>145 ± 3.88</td>
<td>188 ± 4.53</td>
<td>231 ± 3.83</td>
<td>260 ± 6.26</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>180.69 ± 8.68</td>
<td>340.42 ± 8.38</td>
<td>511.71 ± 10.84</td>
<td>635.1 ± 14.62</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>48.19 ± 3.51</td>
<td>43.88 ± 3.54</td>
<td>40.09 ± 2.53</td>
<td>37.86 ± 1.88</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>65.9 ± 2.99</td>
<td>85.45 ± 2.63</td>
<td>105 ± 3.65</td>
<td>118.2 ± 3.93</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.75 ± 0.057</td>
<td>7.76 ± 0.445</td>
<td>12.76 ± 0.678</td>
<td>16.77 ± 0.694</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.478 ± 0.049</td>
<td>0.632 ± 0.037</td>
<td>0.760 ± 0.037</td>
<td>0.837 ± 0.045</td>
</tr>
</tbody>
</table>

Table 3.8: Lipid profile of OPC:NA (50:50) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>255 ± 11.85</td>
<td>410 ± 11.20</td>
<td>572 ± 13.72</td>
<td>704 ± 13.15</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>148 ± 3.96</td>
<td>185 ± 5.65</td>
<td>239 ± 9.20</td>
<td>273 ± 8.68</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>177.52 ± 8.75</td>
<td>330.7 ± 7.12</td>
<td>485.48 ± 11.80</td>
<td>614.0 ± 12.58</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>47.88 ± 3.12</td>
<td>42.30 ± 2.38</td>
<td>38.72 ± 2.07</td>
<td>35.42 ± 1.36</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>67.27 ± 2.99</td>
<td>84.09 ± 3.63</td>
<td>108.64 ± 4.53</td>
<td>124.1 ± 3.76</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.71 ± 0.282</td>
<td>7.82 ± 0.416</td>
<td>12.54 ± 0.825</td>
<td>17.33 ± 0.751</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.490 ± 0.037</td>
<td>0.641 ± 0.037</td>
<td>0.790 ± 0.024</td>
<td>0.887 ± 0.037</td>
</tr>
</tbody>
</table>
Table 3.9: Lipid profile of OPC:NA (70:30) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>237 ± 10.50</td>
<td>402 ± 11.97</td>
<td>546 ± 11.75</td>
<td>642 ± 13.31</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>143 ± 4.71</td>
<td>177 ± 5.93</td>
<td>232 ± 7.83</td>
<td>252 ± 8.54</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>161.46 ± 6.23</td>
<td>323.75 ± 6.08</td>
<td>460.73 ± 10.06</td>
<td>555.4 ± 11.65</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>46.94 ± 2.78</td>
<td>42.85 ± 2.01</td>
<td>38.87 ± 2.13</td>
<td>36.16 ± 2.13</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>65.0 ± 3.06</td>
<td>80.45 ± 4.09</td>
<td>105.45 ± 3.44</td>
<td>114.5 ± 3.38</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.44 ± 0.159</td>
<td>7.55 ± 0.494</td>
<td>11.85 ± 0.678</td>
<td>15.36 ± 0.984</td>
</tr>
<tr>
<td>AI [\log(TG/HDL-C)]</td>
<td>0.484 ± 0.045</td>
<td>0.616 ± 0.020</td>
<td>0.776 ± 0.033</td>
<td>0.843 ± 0.041</td>
</tr>
</tbody>
</table>

3.1.1.2.2. Oligomeric proanthocyanidins and pterostilbene combination therapies

Among combinations 30:70, 50:50 and 70:30 the most prominent results were obtained in 70:30 ratio of OPC and PT. The LDL-C/HDL-C ratio and AI were 15.36 ± 0.984 and 0.851 ± 0.041 (Table 3.12) respectively. In 50:50 and 30:70 blend groups the LDL-C/HDL-C ratios were 16.33 ± 0.751 and 15.19 ± 0.416 and AI were 0.906 ± 0.037 and 0.929 ± 0.057 respectively (Table 3.10, 3.11). Similarly significant retarding effects in TG levels were observed in 70:30 blend therapy (252 ± 8.95 mg/dl) compared to other groups, and thus selected for three drug combination studies.
### Table 3.10: Lipid profile of OPC:PT (30:70) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>248 ± 9.60</td>
<td>390 ± 9.29</td>
<td>470 ± 11.66</td>
<td>598 ± 14.01</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>139 ± 3.48</td>
<td>196 ± 4.04</td>
<td>257 ± 4.98</td>
<td>284 ± 3.81</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>172.06 ± 7.43</td>
<td>308.37 ± 7.89</td>
<td>381.48 ± 9.83</td>
<td>507.8 ± 10.38</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>48.14 ± 2.68</td>
<td>42.43 ± 1.90</td>
<td>37.12 ± 2.25</td>
<td>33.42 ± 1.68</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>63.18 ± 3.07</td>
<td>89.09 ± 3.44</td>
<td>116.8 ± 3.40</td>
<td>129.1 ± 4.17</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.574 ± 0.179</td>
<td>7.27 ± 0.416</td>
<td>10.28 ± 0.457</td>
<td>15.19 ± 0.416</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.460 ± 0.045</td>
<td>0.664 ± 0.033</td>
<td>0.840 ± 0.037</td>
<td>0.929 ± 0.057</td>
</tr>
</tbody>
</table>

### Table 3.11: Lipid profile of OPC:PT (50:50) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>257 ± 10.50</td>
<td>400 ± 10.86</td>
<td>535 ± 13.66</td>
<td>642 ± 15.60</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>142 ± 3.49</td>
<td>181 ± 3.36</td>
<td>237 ± 6.25</td>
<td>273 ± 8.68</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>179.66 ± 8.68</td>
<td>320.58 ± 11.65</td>
<td>499.43 ± 13.55</td>
<td>553.5 ± 14.66</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>48.94 ± 3.25</td>
<td>43.22 ± 2.28</td>
<td>38.17 ± 2.99</td>
<td>33.89 ± 2.17</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>64.54 ± 3.03</td>
<td>82.27 ± 3.72</td>
<td>107.73 ± 4.27</td>
<td>124.1 ± 3.76</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.67 ± 0.179</td>
<td>7.42 ± 0.498</td>
<td>11.77 ± 0.674</td>
<td>16.33 ± 0.751</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.458 ± 0.033</td>
<td>0.613 ± 0.250</td>
<td>0.793 ± 0.037</td>
<td>0.906 ± 0.037</td>
</tr>
</tbody>
</table>
### Table 3.12: Lipid profile of OPC:PT (70:30) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>244 ± 11.64</td>
<td>377 ± 10.35</td>
<td>502 ± 10.35</td>
<td>608 ± 15.06</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>142 ± 4.23</td>
<td>183 ± 4.17</td>
<td>220 ± 6.33</td>
<td>252 ± 8.95</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>168.2 ± 7.31</td>
<td>297.17 ± 5.66</td>
<td>418.79 ± 10.35</td>
<td>518.0 ± 12.37</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>47.40 ± 2.84</td>
<td>43.23 ± 3.38</td>
<td>39.21 ± 2.46</td>
<td>35.47 ± 2.16</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>64.54 ± 3.13</td>
<td>83.18 ± 4.19</td>
<td>100 ± 3.31</td>
<td>114.5 ± 3.38</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.549 ± 0.155</td>
<td>6.87 ± 0.551</td>
<td>10.68 ± 0.768</td>
<td>15.36 ± 0.984</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.477 ± 0.041</td>
<td>0.627 ± 0.033</td>
<td>0.749 ± 0.037</td>
<td>0.851 ± 0.041</td>
</tr>
</tbody>
</table>

#### 3.1.1.2.3. Niacin and pterostilbene combination therapies

Comparing all three ratios of NA-PT, the 70:30 was found most effective ratio. The LDL-C/HDL-C ratio (15.16 ± 0.890) was similar to that observed in 50:50 (15.16 ± 0.841) while significantly lower than 30:70 (15.75 ± 0.531). The AI values of all three ratios (70:30, 50:50, 30:70) were 0.858 ± 0.053, 0.868 ± 0.037 and 0.919 ± 0.037 respectively. Other lipid parameters differ in less magnitude except TC and HDL-C values. Overall results were found parallel to reported data by providing synergism in five antioxidant combinations as shown in table 3.13, 3.14, 3.15.
Table 3.13: Lipid profile of NA:PT (30:70) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>249 ± 12.26</td>
<td>399 ± 10.52</td>
<td>535 ± 11.56</td>
<td>615 ± 15.02</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>142 ± 3.21</td>
<td>197 ± 3.36</td>
<td>241 ± 4.22</td>
<td>277 ± 11.07</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>172.51 ± 7.86</td>
<td>317.3 ± 7.54</td>
<td>449.22 ± 12.03</td>
<td>526.2 ± 9.57</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>48.09 ± 3.01</td>
<td>42.30 ± 2.25</td>
<td>37.58 ± 2.14</td>
<td>33.41 ± 1.61</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>64.54 ± 3.55</td>
<td>89.54 ± 3.45</td>
<td>109.5 ± 2.61</td>
<td>125.9 ± 3.04</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.587 ± 0.118</td>
<td>7.5 ± 0.60</td>
<td>11.95 ± 0.457</td>
<td>15.75 ± 0.531</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.470 ± 0.041</td>
<td>0.668 ± 0.028</td>
<td>0.807 ± 0.037</td>
<td>0.919 ± 0.037</td>
</tr>
</tbody>
</table>

Table 3.14: Lipid profile of NA:PT (50:50) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>234 ± 9.24</td>
<td>408 ± 10.37</td>
<td>549 ± 12.95</td>
<td>646 ± 13.97</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>138 ± 3.51</td>
<td>179 ± 5.17</td>
<td>232 ± 5.62</td>
<td>270 ± 7.07</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>159.48 ± 7.66</td>
<td>329.41 ± 7.92</td>
<td>463.45 ± 10.83</td>
<td>555.4 ± 12.30</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>46.92 ± 3.12</td>
<td>42.79 ± 2.42</td>
<td>39.15 ± 2.25</td>
<td>36.63 ± 1.54</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>62.73 ± 3.09</td>
<td>81.36 ± 4.04</td>
<td>105.45 ± 3.86</td>
<td>122.7 ± 4.34</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.399 ± 0.098</td>
<td>7.698 ± 0.649</td>
<td>11.84 ± 0.747</td>
<td>15.16 ± 0.841</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.469 ± 0.037</td>
<td>0.621 ± 0.033</td>
<td>0.773 ± 0.037</td>
<td>0.868 ± 0.037</td>
</tr>
</tbody>
</table>
### Table 3.15: Lipid profile of NA:PT (70:30) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>243 ± 10.40</td>
<td>403 ± 9.08</td>
<td>565 ± 11.22</td>
<td>659 ± 13.11</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>147 ± 3.24</td>
<td>202 ± 5.09</td>
<td>247 ± 4.19</td>
<td>270 ± 5.91</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>167.58 ± 7.77</td>
<td>320.27 ± 7.10</td>
<td>475.74 ± 9.04</td>
<td>567.6 ± 10.70</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>46.02 ± 3.13</td>
<td>42.33 ± 2.61</td>
<td>39.86 ± 2.16</td>
<td>37.44 ± 2.26</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>66.82 ± 3.01</td>
<td>91.81 ± 3.18</td>
<td>112.27 ± 3.40</td>
<td>122.7 ± 3.86</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.641 ± 0.057</td>
<td>7.57 ± 0.539</td>
<td>11.94 ± 0.694</td>
<td>15.16 ± 0.890</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.504 ± 0.029</td>
<td>0.679 ± 0.033</td>
<td>0.792 ± 0.041</td>
<td>0.858 ± 0.053</td>
</tr>
</tbody>
</table>

3.1.1.2.4. **Oligomeric proanthocyanidins, niacin and pterostilbene blend in three drug combination therapies**

The combination ratio among two drugs (OPC:NA, OPC:PT and NA:PT) delivering most pronounced effects were selected and incorporated in three drug combinations (Table 3.16, 3.17, 3.18). Three drug combination therapies were designed to evaluate the extent of synergism among antioxidants acting through various mechanisms and to develop our own therapeutic dose rationale among the selected drugs. The drug with highest ratio (i.e. 70%) was reduced by 20 mg, and this amount was replaced by incorporating the remaining third drug and executed for 90 days chronic study under same experimental conditions.

The most effective combination found among them was OPC:NA:PT in 50:30:20 ratio, that suppressed LDL-C/HDL-C and AI to 12.41 ± 0.72 and 0.821 ± 0.041 compared to which other two combinations 50:20:30 and 20:50:30 were able to suppress LDL-C/HDL-C and AI to 12.85 ± 0.776 and 0.839 ± 0.037 and 12.86 ± 0.923 and 0.827
± 0.029 respectively. It was evident from the outcome that using natural multi antioxidant blends alone or in conjunction with conventional therapies to achieve more beneficial results that will eventually reduce the dose of conventional therapies.

### Table 3.16: Lipid profile of OPC:NA:PT (50:30:20) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>242 ± 9.64</td>
<td>376 ± 8.79</td>
<td>486 ± 11.61</td>
<td>563 ± 13.31</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>131 ± 3.77</td>
<td>174 ± 5.61</td>
<td>219 ± 3.85</td>
<td>253 ± 6.65</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>169.78 ± 7.89</td>
<td>298.81 ± 6.41</td>
<td>402.38 ± 9.19</td>
<td>474.2 ± 11.55</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>46.02 ± 3.05</td>
<td>42.39 ± 1.91</td>
<td>39.82 ± 2.09</td>
<td>38.20 ± 1.89</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>59.54 ± 2.13</td>
<td>79.1 ± 1.93</td>
<td>99.54 ± 2.59</td>
<td>115.0 ± 3.04</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.69 ± 0.351</td>
<td>7.05 ± 0.449</td>
<td>10.10 ± 0.739</td>
<td>12.41 ± 0.72</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.454 ± 0.037</td>
<td>0.613 ± 0.020</td>
<td>0.740 ± 0.033</td>
<td>0.821 ± 0.041</td>
</tr>
</tbody>
</table>

### Table 3.17: Lipid profile of OPC:NA:PT (50:20:30) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>250 ± 6.41</td>
<td>382 ± 7.51</td>
<td>489 ± 11.73</td>
<td>572 ± 13.15</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>136 ± 3.47</td>
<td>179 ± 4.91</td>
<td>229 ± 8.03</td>
<td>265 ± 9.46</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>178.83 ± 7.61</td>
<td>304.98 ± 12.06</td>
<td>404.43 ± 9.88</td>
<td>481.47 ± 8.29</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>43.97 ± 2.07</td>
<td>41.22 ± 2.13</td>
<td>38.77 ± 2.74</td>
<td>37.53 ± 1.34</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>61.82 ± 3.22</td>
<td>81.36 ± 4.04</td>
<td>104.09 ± 4.12</td>
<td>120.5 ± 5.08</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>4.07 ± 0.351</td>
<td>7.4 ± 0.439</td>
<td>10.43 ± 0.416</td>
<td>12.85 ± 0.776</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.490 ± 0.033</td>
<td>0.638 ± 0.025</td>
<td>0.771 ± 0.033</td>
<td>0.839 ± 0.037</td>
</tr>
</tbody>
</table>
Table 3.18: Lipid profile of OPC:NA:PT (20:50:30) group after twelve weeks of therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline (After 2 weeks of CHO dosing) Mean ± SEM</th>
<th>After 4 weeks Mean ± SEM</th>
<th>After 8 weeks Mean ± SEM</th>
<th>After 12 weeks Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>258 ± 8.16</td>
<td>390 ± 9.06</td>
<td>497 ± 10.66</td>
<td>588 ± 11.73</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>153 ± 3.81</td>
<td>182 ± 3.71</td>
<td>228 ± 3.08</td>
<td>260 ± 2.81</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>180.91 ± 8.66</td>
<td>311.17 ± 9.55</td>
<td>411.61 ± 11.25</td>
<td>497.32 ± 13.07</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>46.49 ± 2.31</td>
<td>42.43 ± 1.78</td>
<td>39.79 ± 1.68</td>
<td>38.68 ± 2.37</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>69.54 ± 1.96</td>
<td>82.73 ± 2.71</td>
<td>103.64 ± 3.33</td>
<td>118.2 ± 2.83</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.98 ± 0.192</td>
<td>7.33 ± 0.465</td>
<td>10.34 ± 0.441</td>
<td>12.86 ± 0.923</td>
</tr>
<tr>
<td>AI [log(TG/HDL-C)]</td>
<td>0.517 ± 0.037</td>
<td>0.632 ± 0.037</td>
<td>0.758 ± 0.033</td>
<td>0.827 ± 0.029</td>
</tr>
</tbody>
</table>

3.1.2. Conclusion of lipid lowering therapies outcome

The multi-drugs therapies having drug candidates with different mechanisms improved lipid profile synergistically compared to individual therapies, even three drug combinations showed more impressive results than the two drug blends. Improvements were observed by comparing LDL-C/HDL-C and AI values among various treatment groups. Incorporating antioxidants blends along with conventional antihyperlipidemic drugs can provide synergism \(^{350-351}\), by reducing the dose of conventional drugs and ultimately their side effects.

3.1.2.1. Overall results of lipid lowering therapies

The final values of lipid profiles are summarized in table 3.19 as mean ± SEM at significance level \(p < 0.05\%) against the disease control. One way analysis of variance (ANOVA) test was applied followed by suitable comparison tests.
### Table 3.19: Blood lipid profile of various groups after twelve weeks treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>GROUP</th>
<th>TC value (mg/dl) ± SEM</th>
<th>LDL-C value (mg/dl) ± SEM</th>
<th>HDL-C value (mg/dl) ± SEM</th>
<th>TG value (mg/dl) ± SEM</th>
<th>VLDL-C value ± SEM</th>
<th>LDLC score</th>
<th>Atherogenic index ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blank Control</td>
<td>105 ± 5.013</td>
<td>58.75 ± 18.25</td>
<td>30.65 ± 1.74</td>
<td>78 ± 3.527</td>
<td>35.45 ± 3.074</td>
<td>1.917 ± 0.56</td>
<td>0.406 ± 0.037</td>
</tr>
<tr>
<td></td>
<td>Disease Control</td>
<td>946 ± 38.53</td>
<td>848.8 ± 34.19</td>
<td>27.83 ± 0.82</td>
<td>347 ± 10.04</td>
<td>157.7 ± 2.29</td>
<td>30.50 ± 0.69</td>
<td>1.096 ± 0.040</td>
</tr>
<tr>
<td></td>
<td>Atorvastatin</td>
<td>625 ± 17.42</td>
<td>539.65 ± 15.01</td>
<td>37.35 ± 1.72</td>
<td>240 ± 5.49</td>
<td>109.1 ± 3.35</td>
<td>14.45 ± 0.99</td>
<td>0.808 ± 0.061</td>
</tr>
<tr>
<td></td>
<td>Proanthocyanidins (OPC)</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Niacin (NA)</td>
<td>733 ± 21.42</td>
<td>647.8 ± 19.34</td>
<td>33.24 ± 2.09</td>
<td>260 ± 9.10</td>
<td>118.2 ± 2.09</td>
<td>19.49 ± 0.87</td>
<td>0.893 ± 0.033</td>
</tr>
<tr>
<td></td>
<td>Pterostilbene (PT)</td>
<td>698 ± 14.19</td>
<td>607.16 ± 14.45</td>
<td>31.64 ± 3.03</td>
<td>296 ± 3.64</td>
<td>134.5 ± 6.30</td>
<td>19.19 ± 0.91</td>
<td>0.971 ± 0.073</td>
</tr>
<tr>
<td></td>
<td>OPC : NA (30:70)</td>
<td>725 ± 15.92</td>
<td>635.1 ± 14.62</td>
<td>37.86 ± 1.88</td>
<td>260 ± 6.26</td>
<td>118.2 ± 3.93</td>
<td>16.77 ± 0.69</td>
<td>0.837 ± 0.045</td>
</tr>
<tr>
<td></td>
<td>OPC : NA (50:50)</td>
<td>704 ± 13.15</td>
<td>614.0 ± 12.58</td>
<td>35.42 ± 1.36</td>
<td>273 ± 8.68</td>
<td>124.1 ± 3.76</td>
<td>17.33 ± 0.75</td>
<td>0.887 ± 0.037</td>
</tr>
<tr>
<td></td>
<td>OPC : NA (70:30)</td>
<td>642 ± 13.31</td>
<td>555.4 ± 11.65</td>
<td>36.16 ± 2.13</td>
<td>252 ± 8.54</td>
<td>114.5 ± 3.38</td>
<td>15.36 ± 0.98</td>
<td>0.843 ± 0.041</td>
</tr>
<tr>
<td></td>
<td>OPC : PT (30:70)</td>
<td>598 ± 14.01</td>
<td>507.8 ± 10.38</td>
<td>33.42 ± 1.68</td>
<td>284 ± 3.81</td>
<td>129.1 ± 4.17</td>
<td>15.19 ± 0.42</td>
<td>0.929 ± 0.057</td>
</tr>
<tr>
<td></td>
<td>OPC : PT (50:50)</td>
<td>642 ± 15.60</td>
<td>553.5 ± 14.66</td>
<td>33.89 ± 2.12</td>
<td>273 ± 8.68</td>
<td>124.1 ± 3.76</td>
<td>16.33 ± 0.75</td>
<td>0.906 ± 0.037</td>
</tr>
<tr>
<td></td>
<td>OPC : PT (70:30)</td>
<td>608 ± 15.06</td>
<td>518.0 ± 12.37</td>
<td>35.47 ± 2.16</td>
<td>252 ± 8.95</td>
<td>114.5 ± 3.38</td>
<td>14.60 ± 0.98</td>
<td>0.851 ± 0.041</td>
</tr>
<tr>
<td></td>
<td>NA : PT (30:70)</td>
<td>615 ± 15.02</td>
<td>526.2 ± 9.57</td>
<td>33.41 ± 1.61</td>
<td>277 ± 11.1</td>
<td>125.9 ± 3.04</td>
<td>15.75 ± 0.53</td>
<td>0.919 ± 0.037</td>
</tr>
<tr>
<td></td>
<td>NA : PT (50:50)</td>
<td>646 ± 13.97</td>
<td>555.4 ± 12.30</td>
<td>36.63 ± 1.54</td>
<td>270 ± 7.07</td>
<td>122.7 ± 4.34</td>
<td>15.16 ± 0.84</td>
<td>0.868 ± 0.037</td>
</tr>
<tr>
<td></td>
<td>NA : PT (70:30)</td>
<td>659 ± 13.11</td>
<td>567.6 ± 10.70</td>
<td>37.44 ± 2.26</td>
<td>270 ± 5.91</td>
<td>122.7 ± 3.86</td>
<td>15.16 ± 0.89</td>
<td>0.858 ± 0.053</td>
</tr>
<tr>
<td></td>
<td>OPC : NA : PT (50:30:20)</td>
<td>563 ± 13.31</td>
<td>474.2 ± 11.55</td>
<td>38.20 ± 1.89</td>
<td>253 ± 6.65</td>
<td>115.0 ± 3.04</td>
<td>12.41 ± 0.72</td>
<td>0.821 ± 0.041</td>
</tr>
<tr>
<td></td>
<td>OPC : NA : PT (50:20:30)</td>
<td>572 ± 13.15</td>
<td>482.67 ± 8.29</td>
<td>37.53 ± 1.34</td>
<td>259 ± 9.46</td>
<td>120.5 ± 5.08</td>
<td>12.86 ± 0.78</td>
<td>0.839 ± 0.037</td>
</tr>
<tr>
<td></td>
<td>OPC : NA : PT (20:50:30)</td>
<td>388 ± 11.73</td>
<td>497.32 ± 13.06</td>
<td>38.68 ± 2.37</td>
<td>260 ± 2.81</td>
<td>118.2 ± 2.83</td>
<td>12.86 ± 1.17</td>
<td>0.827 ± 0.029</td>
</tr>
</tbody>
</table>

For each group (n=6), the lipid profile was calculated as mean ± SEM, using ANOVA followed by Dunnett’s test (*p < 0.05, ** p < 0.01, ***p < 0.001).
3.1.2.2. Percent change in lipid profile

Percent changes in lipid profile were calculated as percent difference at specific sampling time against disease control at 95% CI (confidence interval). Observing the percent decrease in all lipid parameters especially LDL-C/HDL-C ratios and AI, which were believed to be conclusive in determining atherosclerotic events by two ideological, research groups. The three drug combinations (OPC: NA: PT in 50:30:20, 50: 20: 30 and 20: 50: 30) produced more significant effects and retarded both LDL-C/HDL-C ratios by -59.30, -57.84 and -57.80% and AI by -25.09, -23.45, -24.50% respectively compared to individual and two drug combinations as shown in table 3.20 and figures 3.2, 3.3, 3.4, 3.5, 3.6, 3.7 and 3.8. The retarded elevation in LDL-C/HDL-C was controlled by decreasing blood TC and LDL-C levels with enhanced HDL-C levels, while in case of AI the retarding effect in TG levels governs the reduction of atherogenic events. Antioxidants effects were found higher compared to statin therapy which retarded LDL/HDL by -52.66% while similar effects were observed on AI value (-26.28%).
### Table 3.20: Percent change in lipid profile after twelve weeks in each treatment group

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TC value (95 % CI)</th>
<th>LDL-C value (95 % CI)</th>
<th>HDL-C value (95 % CI)</th>
<th>TG value (95 % CI)</th>
<th>VLDL-C (95 % CI)</th>
<th>LDL-C/ HDL-C (95 % CI)</th>
<th>Atherogenic index (95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Atorvastatin</td>
<td>-34.0 (-36.43, -31.57)</td>
<td>-36.42 (-39.00, -33.80)</td>
<td>34.20 (29.78, 38.62)</td>
<td>-30.80 (-32.74, -28.86)</td>
<td>-30.80 (-33.58, -28.02)</td>
<td>-52.66 (-62.56, -42.76)</td>
<td>-26.28 (-31.97, -20.59)</td>
</tr>
<tr>
<td>2 Proanthocyanidins (OPC)</td>
<td>-22.50 (-24.19, -20.81)</td>
<td>-23.68 (-25.50, -21.86)</td>
<td>19.44 (16.30, 22.58)</td>
<td>-25.07 (-27.33, -22.81)</td>
<td>-25.05 (-26.23, -23.92)</td>
<td>-36.09 (-40.21, -31.97)</td>
<td>-18.52 (-20.26, -16.78)</td>
</tr>
<tr>
<td>5 OPC : NA 30:70</td>
<td>-23.36 (-24.68, -22.04)</td>
<td>-25.18 (-26.67, -23.69)</td>
<td>36.0 (31.41, 40.59)</td>
<td>-25.07 (-26.62, -23.52)</td>
<td>-25.05 (-27.19, -22.91)</td>
<td>-45.02 (-49.82, -40.22)</td>
<td>-23.63 (-26.88, -20.38)</td>
</tr>
<tr>
<td>6 OPC : NA 50:50</td>
<td>-25.58 (-26.81, -24.35)</td>
<td>-27.66 (-29.11, -26.21)</td>
<td>27.27 (24.54, 30.00)</td>
<td>-21.32 (-23.07, -19.57)</td>
<td>-21.31 (-22.97, -19.65)</td>
<td>-43.2 (-48.02, -38.38)</td>
<td>-19.07 (-21.10, -17.04)</td>
</tr>
<tr>
<td>7 OPC : NA 70:30</td>
<td>-32.13 (-33.84, -30.42)</td>
<td>-34.57 (-37.89, -31.25)</td>
<td>29.9 (25.39, 34.41)</td>
<td>-27.4 (-29.81, -24.99)</td>
<td>-27.4 (-29.48, -25.32)</td>
<td>-49.64 (-57.83, -41.45)</td>
<td>-23.08 (-25.94, -20.22)</td>
</tr>
<tr>
<td>8 OPC : PT 30:70</td>
<td>-36.79 (-39.00, -34.58)</td>
<td>-40.17 (-42.27, -38.07)</td>
<td>20.09 (17.50, 22.68)</td>
<td>-18.16 (-18.79, -17.53)</td>
<td>-18.13 (-19.63, -16.63)</td>
<td>-50.2 (-53.74, -46.66)</td>
<td>-15.24 (-17.65, -12.83)</td>
</tr>
<tr>
<td>9 OPC : PT 50:50</td>
<td>-32.13 (-34.13, -30.13)</td>
<td>-34.97 (-37.36, -32.58)</td>
<td>27.27 (22.98, 31.56)</td>
<td>-21.33 (-23.07, -19.59)</td>
<td>-21.31 (-22.97, -19.65)</td>
<td>-48.9 (-54.96, -42.84)</td>
<td>-19.07 (-21.11, -17.03)</td>
</tr>
<tr>
<td>10 OPC : PT 70:30</td>
<td>-35.73 (-38.01, -33.45)</td>
<td>-38.97 (-41.34, -36.60)</td>
<td>27.45 (23.16, 31.74)</td>
<td>-27.38 (-29.88, -24.88)</td>
<td>-27.39 (-29.47, -25.31)</td>
<td>-49.64 (-57.82, -41.46)</td>
<td>-22.35 (-25.11, -19.59)</td>
</tr>
<tr>
<td>11 NA : PT 30:70</td>
<td>-34.99 (-37.18, -32.80)</td>
<td>-38.0 (-39.77, -32.23)</td>
<td>20.05 (17.56, 17.56)</td>
<td>-20.17 (-22.24, -18.10)</td>
<td>-20.16 (-21.41, -18.91)</td>
<td>-48.36 (-52.55, -44.17)</td>
<td>-16.15 (-17.81, -14.49)</td>
</tr>
<tr>
<td>12 NA : PT 50:50</td>
<td>-31.71 (-33.47, -29.95)</td>
<td>-34.57 (-36.54, -32.60)</td>
<td>31.62 (28.20, 35.04)</td>
<td>-22.19 (-23.68, -20.70)</td>
<td>-22.19 (-24.20, -20.18)</td>
<td>-50.30 (-57.47, -43.13)</td>
<td>-20.8 (-23.07, -18.53)</td>
</tr>
<tr>
<td>13 NA : PT 70:30</td>
<td>-30.34 (-29.62, -26.82)</td>
<td>-33.13 (-32.21, -29.33)</td>
<td>34.53 (32.17, 36.89)</td>
<td>-22.2 (-23.45, -20.95)</td>
<td>-22.2 (-23.99, -20.41)</td>
<td>-50.30 (-55.64, -41.48)</td>
<td>-21.72 (-25.18, -18.26)</td>
</tr>
<tr>
<td>14 OPC : NA : PT 50:30:20</td>
<td>-40.49 (-42.95, -38.03)</td>
<td>-44.1 (-46.86, -41.34)</td>
<td>37.26 (36.21, 41.91)</td>
<td>-27.09 (-28.82, -25.26)</td>
<td>-27.08 (-28.92, -25.24)</td>
<td>-59.30 (-68.18, -50.42)</td>
<td>-25.09 (-28.30, -21.88)</td>
</tr>
<tr>
<td>15 OPC : NA : PT 50:20:30</td>
<td>-39.53 (-41.86, -37.20)</td>
<td>-43.13 (-45.10, -41.28)</td>
<td>32.5 (29.48, 35.52)</td>
<td>-25.36 (-27.52, -23.20)</td>
<td>-23.59 (-26.15, -21.03)</td>
<td>-57.84 (-66.56, -49.12)</td>
<td>-23.45 (-25.85, -21.05)</td>
</tr>
<tr>
<td>16 OPC : NA : PT 20:50:30</td>
<td>-37.80 (-39.74, -35.86)</td>
<td>-41.4 (-44.20, -38.60)</td>
<td>38.99 (32.84, 45.14)</td>
<td>-25.07 (-25.76, -24.38)</td>
<td>-25.05 (-26.59, -23.51)</td>
<td>-57.80 (-68.45, -47.15)</td>
<td>-24.50 (-26.67, -22.33)</td>
</tr>
</tbody>
</table>

% reductions were calculated against the disease control.
3.1.2.3. Graphical presentation of percent change in each lipid parameter

Each parameter in all treatment groups is presented in individual graph as mean percent change ± SEM. Baseline value shows the initiation of therapy considering zero change at that time.

**Figure 3.2:** Mean percent change ± SEM in total cholesterol (TC) level at four week interval in all treatment groups against disease control.
Figure 3.3: Mean percent change ± SEM in triglycerides (TG) level at four week interval in all treatment groups against disease control.
Figure 3.4: Mean percent change ± SEM in low density lipoproteins (LDL-C) level at four week interval in all treatment groups against disease control.
Figure 3.5: Mean percent change ± SEM in high density lipoproteins (HDL-C) level at four week interval in all treatment groups against disease control.
Figure 3.6: Mean percent change ± SEM in very low density lipoproteins (VLDL-C) level at four week interval in all treatment groups against disease control.
Figure 3.7: Mean percent change ± SEM in low density lipoproteins / high density lipoproteins ratio (LDL-C/HDL-C Ratio) level at four week interval in all treatment groups against disease control.
**Figure 3.8:** Mean percent change ± SEM in atherogenic index (AI) level at four week interval in all treatment groups against disease control.
3.1.2.4. Difference between individual and maximum effective combinational therapies

The difference between individual therapy and respective combinational therapy outcome with maximum lowering effect in each two drug combination and all three drug combination groups were observed. Increased number of drugs in the treatment has more pronounced effects than individual drug therapies while keeping total dose constant. This provided an evidence of combinational therapy superiority that leads into decreased dose dumping and chances of adverse effects that might be associated with the individual bulk doses therapies. Data is presented as mean percent difference ± SEM of optimum combination vs. each individual therapy in table 3.21.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>PARAMETER</th>
<th>TC ± SEM</th>
<th>LDL-C ± SEM</th>
<th>HDL-C ± SEM</th>
<th>TG ± SEM</th>
<th>VLDL-C ± SEM</th>
<th>LDL-C/HDL-C ± SEM</th>
<th>Atherogenic index ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. a</td>
<td>OPC : NA (70:30) vs. Proanthocyanidins (OPC)</td>
<td>-9.63 ± 0.384</td>
<td>-10.89 ± 0.220</td>
<td>10.46 ± 0.617</td>
<td>-2.33 ± 0.078</td>
<td>-2.35 ± 0.069</td>
<td>-13.55 ± 0.869</td>
<td>-4.56 ± 0.221</td>
</tr>
<tr>
<td></td>
<td>OPC : NA (70:30) vs. Niacin (NA)</td>
<td>-17.23 ± 0.416</td>
<td>-18.82 ± 0.396</td>
<td>3.1 ± 0.184</td>
<td>-7.52 ± 0.253</td>
<td>-7.55 ± 0.224</td>
<td>-16.07 ± 1.029</td>
<td>-4.83 ± 0.233</td>
</tr>
<tr>
<td>2. a</td>
<td>OPC : PT (70:30) vs. Proanthocyanidins (OPC)</td>
<td>-13.23 ± 0.416</td>
<td>-15.29 ± 0.363</td>
<td>8.01 ± 0.486</td>
<td>-2.31 ± 0.082</td>
<td>-2.34 ± 0.069</td>
<td>-13.55 ± 0.869</td>
<td>-3.83 ± 0.184</td>
</tr>
<tr>
<td></td>
<td>OPC : PT (70:30) vs. Pterostilbene (PT)</td>
<td>-9.51 ± 0.298</td>
<td>-10.50 ± 0.251</td>
<td>13.76 ± 0.837</td>
<td>-12.68 ± 0.449</td>
<td>-12.68 ± 0.376</td>
<td>-12.56 ± 0.804</td>
<td>-10.85 ± 0.519</td>
</tr>
<tr>
<td>3. a</td>
<td>NA : PT (70:30) vs. Niacin (NA)</td>
<td>-13.32 ± 0.339</td>
<td>-15.02 ± 0.274</td>
<td>7.73 ± 0.465</td>
<td>-2.32 ± 0.049</td>
<td>-2.35 ± 0.073</td>
<td>-14.99 ± 0.849</td>
<td>-3.47 ± 0.216</td>
</tr>
<tr>
<td></td>
<td>NA : PT (70:30) vs. Pterostilbene (PT)</td>
<td>-2.9 ± 0.049</td>
<td>-2.30 ± 0.041</td>
<td>20.84 ± 1.256</td>
<td>-7.5 ± 0.163</td>
<td>-7.49 ± 0.237</td>
<td>-11.48 ± 0.649</td>
<td>-10.32 ± 0.637</td>
</tr>
<tr>
<td>4. a</td>
<td>OPC : NA : PT (50:30:20) vs. Proanthocyanidins (OPC)</td>
<td>-17.99 ± 0.649</td>
<td>-20.42 ± 0.498</td>
<td>17.16 ± 0.849</td>
<td>-2.02 ± 0.053</td>
<td>-2.03 ± 0.053</td>
<td>-23.21 ± 1.35</td>
<td>-6.57 ± 0.327</td>
</tr>
<tr>
<td></td>
<td>OPC : NA : PT (50:30:20) vs. Niacin (NA)</td>
<td>-25.59 ± 0.919</td>
<td>-28.35 ± 0.689</td>
<td>10.46 ± 0.519</td>
<td>-7.21 ± 0.188</td>
<td>-7.23 ± 0.192</td>
<td>-25.73 ± 1.498</td>
<td>-6.84 ± 0.339</td>
</tr>
<tr>
<td></td>
<td>OPC : NA : PT (50:30:20) vs. Pterostilbene (PT)</td>
<td>-14.27 ± 0.514</td>
<td>-15.63 ± 0.379</td>
<td>23.57 ± 1.164</td>
<td>-12.39 ± 0.327</td>
<td>-12.37 ± 0.327</td>
<td>-22.22 ± 1.294</td>
<td>-13.69 ± 0.682</td>
</tr>
</tbody>
</table>
3.1.3. Discussion

Enhanced lipid peroxidation and blood endogenous antioxidant system alteration are in close association with hyperlipidemia. That may initiate a number of atherogenic effects including plaque formation in the vessel lumen and accelerating foam cells formation within vessel walls. Preventive effects of oral antioxidants are helpful in suppressing the progression of oxidative stress severity induced by chronic pathological conditions such as hyperlipidemia. Natural antioxidants including those used in current study were applied in many chronic conditions to prevent hyperlipidemia and associated conditions.

Studies have shown the effectiveness of natural antioxidants specially polyphenols up to certain levels above which they were found as pro-oxidant and cancelling their own effects. In current study antihyperlipidemic effects were observed in combinations to demonstrate dose dependant effect. Results were found in close agreement with the previous data available on various disease conditions confirming the hypothesis of enhancing efficacy of natural antioxidants in concurrent administration. Lipid lowering effects were significant at low doses combination blends as compared to large dose individual therapies; amplifying the insight of reducing the chances of pro-oxidant effect. Studies have reported that treatment with multiple compounds has positive outcome on lipid profile in hyperlipidemia and regenerating endogenous defense system at various levels applying through separate mechanisms of actions. The decrease in LDL-C/HDL-C ratio and AI in combination drug therapies validate the beneficial aspects and effectiveness of natural dietary antioxidants in controlling chronic pathologies. Similar results were also reported in a diabetic rat model. The peroxidation of
unsaturated lipids in LDL-C is controlled by α-tocopherol a major antioxidant within LDL-C particles \(^{75}\) by scavenging free radicals and getting oxidized. It is recycled by other endogenous antioxidants like ascorbic acid and glutathione. In pathological situation when they get depleted and administering α-tocopherol alone will increase cardiovascular risk rather than decreasing it because of lack of recycling process. Exogenous antioxidants interventions were found facilitating in regenerating α-tocopherol and spare other antioxidants from depletion \(^{159,362}\). Plant phenolic compounds act synergistically with vitamin-C in enhancing LDL-C resistance towards oxidation \(^{363}\). Similarly synergistic effects were observed by combining phenolic compounds with niacin confirmed by decelerated production of lipid peroxidation end product (MDA). It is reported that use of single antioxidant cannot be able to take the place of combination antioxidant therapy. Administering single drug in mega doses may behave like pro-oxidant because of not recycling into reduced form after oxidation that might act as hapten or antigen for immune cells, as in case of ascorbic acid, curcumin and other natural antioxidants may become toxic rather that beneficial \(^{344,364}\).

Antioxidant effects of natural compounds like catechin, hesperidin, ferulic acid and quercetin were observed at low doses individually up to certain levels but became pro-oxidant as the dose increased above that threshold level, while combinations resulted in enhanced antiperoxidative effect at various doses \(^{345}\). This effect is due to the preventive behavior for one another from becoming pro-oxidant. The same initiative was focused in current research to reduce the chances of adverse effects, single drug dose bulking and system saturation due to higher doses. As reported previously in an
antihypertensive study of OPC that acts dose dependently up to some level when administered in low doses and acted poorly in high doses may be because of this effect reducing its own activity. Niacin efficacy in combination therapies with conventional drugs and with other antioxidants are frequently reported for improving blood lipid profile and cardiovascular health synergistically in clinical and preclinical studies. It act dose dependently up to large doses but along with this, chances of adverse effects like hepatotoxicity and hyperhomocysteinaemia are also reported. If combined in low doses with other drugs can act synergistically and minimize adverse effects. OPC acts as antihyperlipidemic by inhibiting lipid absorption from GIT, endogenous cholesterol synthesis and enhancing metabolism in liver and other organs. PT enhances the long chain fatty acids metabolism by activating PPARs. The synergism observed at lower concentrations in our study was found parallel to an antioxidant combinational therapy in-vitro. In our study we not only determined the level of synergism on lipid profiles but also their modulatory effects on blood antioxidants. An animal study conducted on safety profiles of pterostilbene and quercetin at various doses found them safe and effective in normalizing lipid profile at lower doses while no promising effects at higher doses were verified, indicating the possibility ceiling effects of natural antioxidants. The possible mechanisms by which all the three candidates applied their effects on lipid profile modulation are described schematically in figure 3.9.
3.2. Body weight changes during study

Changes in body weight during therapy were measured in blank control, negative control and treatment groups. Results showed increase in mean body weights after 90 days in all groups compared to initial weights. The percent changes (increment) in all treatment groups were in similar manner (4.40-6.02%) but slightly higher than normal group (3.13%), while varied significantly compared to disease control (10.20%). These effects were due to inhibition of absorption and storage of dietary lipids along with accelerated stored lipid mobilization and metabolism by natural antioxidants. Mean percent change ± SD in body weights was calculated from initial and final weights per group (n= 6) are summarized graphically in figure 3.10.
3.3. Effects of antioxidant therapies on liver enzymes

Changes in normal levels of liver enzymes in presence of circulating high blood lipids and the decelerating effects of natural antioxidants on these enzymes has already been reported \(^{373-376}\). Antioxidants especially polyphenolics were found effective in normalizing liver enzymes. In case of niacin both positive and negative conclusions were reported \(^{369,377-378}\). In current study the levels of liver enzymes; SGPT, SGOT and ALP in various groups were determined as mean ± SD (SEM) in U/L as shown in table 3.22 and figures 3.11, 3.12 and 3.13.

High cholesterol diet elevated their levels as in disease control the levels of ALT, AST and ALP were 125.27 ± 22.56 (9.21) U/L, 109 ± 14.53 (5.93) U/L and 204.57 ±
25.72 (10.50) U/L compared to blank control 51.68 ± 12.65 (5.16) U/L, 39.24 ± 5.67 (2.32) U/L and 67.22 ± 12.40 (5.06) U/L respectively. In treatment groups the elevations in liver enzymes were controlled significantly (p > 0.01-0.001). However less significant difference between the treatment groups were observed, presuming the fact that all currently applied drugs retarded elevation in liver enzymes. Using niacin in combinations showed less to no effects similar to other combination groups producing less stress on hepatic cells and prevented enzyme elevation.

Table 3.22: Effect of antioxidants therapies on liver enzymes in hyperlipidemic rabbits

<table>
<thead>
<tr>
<th>GROUP</th>
<th>ALT (SGPT) Final value ± SD (SEM)</th>
<th>AST (SGOT) Final value ± SD (SEM)</th>
<th>ALP Final value ± SD (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank Control 51.68 ± 12.65 (5.16)</td>
<td>39.24 ± 5.67 (2.32)</td>
<td>67.22 ± 12.40 (5.06)</td>
</tr>
<tr>
<td>2</td>
<td>Disease Control 125.27 ± 22.56 (9.21)</td>
<td>109 ± 14.53 (5.93)</td>
<td>204.57 ± 25.72 (10.50)</td>
</tr>
<tr>
<td>3</td>
<td>Atorvastatin *** 89.28 ± 14.67 (5.99)</td>
<td>78.53 ± 12.68 (5.18)</td>
<td>133.61 ± 25.06 (10.23)</td>
</tr>
<tr>
<td>4</td>
<td>Proanthocyanidins (OPC) *** 77.53 ± 12.39 (5.06)</td>
<td>65.50 ± 10.77 (4.39)</td>
<td>102.45 ± 15.78 (6.44)</td>
</tr>
<tr>
<td>5</td>
<td>Niacin (NA) ** 98.34 ± 10.43 (4.26)</td>
<td>77.59 ± 14.82 (6.05)</td>
<td>161.42 ± 18.55 (7.57)</td>
</tr>
<tr>
<td>6</td>
<td>Pterostilbene (PT) *** 65.59 ± 8.64 (3.53)</td>
<td>70.83 ± 17.92 (7.32)</td>
<td>111.56 ± 21.56 (8.80)</td>
</tr>
<tr>
<td>7</td>
<td>OPC: NA (70:30) *** 71.68 ± 11.71 (4.78)</td>
<td>58.42 ± 10.55 (4.31)</td>
<td>105.82 ± 19.46 (7.95)</td>
</tr>
<tr>
<td>8</td>
<td>OPC: PT (70:30) *** 60.34 ± 11.50 (4.69)</td>
<td>50.27 ± 10.08 (4.12)</td>
<td>95.55 ± 12.48 (5.09)</td>
</tr>
<tr>
<td>9</td>
<td>NA: PT (70:30) *** 82.49 ± 13.88 (5.67)</td>
<td>69.44 ± 13.66 (5.58)</td>
<td>118.21 ± 26.09 (10.65)</td>
</tr>
<tr>
<td>10</td>
<td>OPC : NA : PT (50:30:20) *** 57.34 ± 08.53 (3.48)</td>
<td>61.82 ± 09.72 (3.97)</td>
<td>104.35 ± 10.58 (4.32)</td>
</tr>
<tr>
<td>11</td>
<td>OPC : NA : PT (50:20:30) *** 57.78 ± 09.74 (3.98)</td>
<td>50.98 ± 10.45 (4.27)</td>
<td>96.86 ± 17.49 (7.14)</td>
</tr>
<tr>
<td>12</td>
<td>OPC : NA : PT (20:50:30) *** 63.79 ± 12.76 (5.21)</td>
<td>53.42 ± 09.34 (3.81)</td>
<td>132.34 ± 31.41 (12.82)</td>
</tr>
</tbody>
</table>

For each group (n=6), the liver enzymes were calculated as mean ± SD (SEM), using ANOVA followed by Dunnett’s test (*p < 0.05, **p < 0.01, ***p < 0.001).
Figure 3.11: Mean levels of alanine transaminase in normal, disease control and treatment groups with standard error bars.

Figure 3.12: Mean levels of aspartate transaminase in normal, disease control and treatment groups with standard error bars.
### Figure 3.13: Mean levels of alkaline phosphatase in normal, disease control and treatment groups with standard error bars.

#### 3.4. Effects of natural antioxidants therapies on endogenous antioxidants (fat soluble and water soluble) and lipid peroxidation biomarker

An electrochemical detection (ECD) based method was developed and validated according to standard guidelines for simultaneous determination water soluble endogenous antioxidants and lipid peroxidation analysis and for the analysis of fat soluble antioxidants previously reported method was followed \(^{323, 325, 379}\).
3.4.1. Method development for simultaneous determination of water soluble antioxidants and lipid peroxidative end product in serum samples

For optimization and validation of developed RP-HPLC-ECD method various chromatographic conditions were evaluated. All the analytes were determined in single elution using minimal sample volume (5 µl) and applied to the analysis of serum samples of test animals used. Both defensive antioxidant compounds and lipid peroxidative end product (MDA) were analyzed compared with the disease control.

3.4.1.1. Serum sample preparation procedure

For sample extraction methanol, acetonitrile and meta-phosphoric acid (10%) were evaluated. Organic solvents showed better protein precipitation but failed in complete extraction because of solubility of most analytes. Application of freshly prepared meta-phosphoric acid provided optimum extraction and protein precipitation. To serum samples (100 µl), IS (10 µl) was added, vortexed for 2 minutes and extracted with freshly prepared meta-phosphoric acid (100 µl). The mixture was diluted with mobile phase to 300 µl volume, vortexed and centrifuged at 14000×g at 4 °C for 10 minutes. Clear supernatants obtained were subsequently transferred to ambered colored auto sampler vials for analysis. The extracted samples showed complete protein precipitation, maximum recovery and analytes stabilities.

3.4.1.2. Optimization of chromatographic conditions

Assorted chromatographic conditions and investigational parameters were optimized and found suitable for estimation of analytes simultaneously.
3.4.1.2.1. Mobile phase composition and flow rate optimization

For optimum resolution and sensitivity TFA in 0.025, 0.05 and 0.1% concentrations was checked. Increasing TFA concentration above 0.05% showed no effect and produced overlapping peaks while low concentrations have lower sensitivities. Therefore TFA in 0.05% was selected.

Three pH levels 2.0, 2.25 and 2.45 of aqueous portion of mobile phase in ratio with methanol from 95:5 to 100:0 and elution rates in 0.5-1.2 ml/min range were investigated for better resolution and separation. Results showed that pumping mobile phase at pH 2.25 in 98.5:1.5 ratio at flow rate of 0.6 ml/min produced sharp peaks and optimum response at selected electrode potential.

3.4.1.2.2. Detector potential optimization

Analytics showed optimum sensitivity in the potential range of 900-1000 mV, above and below of which produced less response and some analytes were undetectable below 600 mV. Cystine, methionine and oxidized glutathione showed rapid reduction in sensitivity below 850 mV, others showed fewer differences down to 850 mV potential. Therefore 900 mV was selected as working electrode potential. A hydrodynamic voltamgram was constructed showing response of analytes against various applied electrode potentials as shown in figure 3.14.
3.4.1.2.3. Column oven and detector temperature optimization

Decreased temperature (25 °C) produced low resolution peaks while higher temperature (40 °C) resulted in lesser retention times and overlapping peaks. Thus for optimum sensitivity and resolution 35 °C was preferred for analysis.

3.4.1.3. Method validation

The established method was validated for linearity, precision, accuracy, recoveries, LLOD and LLOQ for the simultaneous determination of aminothiols, ascorbic acid and malondialdehyde in serum samples.
3.4.1.3.1. Linearity

Calibration curves from six concentration levels were developed for each analyte (ranging from 0.33-83.2 mmol/l) in standard solutions and spiked serum samples. The regression equation, correlation coefficient (r), slopes and intercepts revealed optimal linearity of the current method as shown in table 3.23 and in figure 3.15, 3.16 and 3.17.

Figure 3.15: Calibration curves for cystine, cysteine and homocysteine. The green line shows the spiked serum samples, red line shows the analytes in standard solutions and blue line shows the spiked serum samples corrected for blank samples.

Figure 3.16: Calibration curves for ascorbic acid, methionine and glutathione reduced. The green line shows the spiked serum samples, red line shows the analytes in standard solutions and blue line shows the spiked serum samples corrected for blank samples.
3.4.1.3.2. Accuracy

Accuracy was determined on the basis of percent recovery from spiked serum samples at appropriate concentrations of all analytes and summarized in table 3.23.

3.4.1.3.3. Precision

The precision studies were performed in terms of injection repeatability, inter-day and intra-day repeatability studies of spiked samples. Precision data is provided in table 3.24 and in figure 3.18.
Figure 3.18: Overlay chromatogram showing method precision in serum samples spiked with various concentrations of ascorbic acid, aminothiols and malondialdehyde. Peaks: 1: Cystine, 2: cysteine, 3: homocysteine, 4: ascorbic acid, 5: methionine, 6: glutathione reduced, 7: glutathione oxidized, 8: malondialdehyde, 9: dopamine and 10: N-acetylcysteine. Chromatogram was obtained using HPLC–ECD at potential 900 mV, mobile phase: TFA aqueous (0.05 %): Methanol at 98.5:1.5 ratio with flow rate 0.6 ml/min.

3.4.1.3.4. Sensitivity

Method sensitivity for the determination of biomarkers was calculated in terms of LLOD and LLOQ and provided in table 3.23.
Table 3.23: Linearity, recovery and sensitivity parameters of developed method

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameters</th>
<th>Cystine (mmol/l)</th>
<th>Cysteine (mmol/l)</th>
<th>Homo-cysteine (mmol/l)</th>
<th>Ascorbic acid (mmol/l)</th>
<th>Methionine (mmol/l)</th>
<th>Glutathione reduced (mmol/l)</th>
<th>Glutathione oxidized (mmol/l)</th>
<th>Malondialdehyde (mmol/l)</th>
<th>N-acetyl cysteine (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>A Standard Solutions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Regression equation</td>
<td>y = 0.02463 + x0.006708x</td>
<td>y = 0.3198 + x0.5835x</td>
<td>y = 0.08981 + x0.6344x</td>
<td>y = 0.07012 + x0.09770x</td>
<td>y = 0.03216 + x0.1149x</td>
<td>y = 0.02016 + x0.1210x</td>
<td>y = 0.005322 + x0.003776x</td>
<td>y = 0.01453 + x0.09322x</td>
<td>y = 0.5270 + x0.8678x</td>
</tr>
<tr>
<td></td>
<td>Correlation coefficient, r</td>
<td>0.9993</td>
<td>0.9995</td>
<td>0.9997</td>
<td>0.9997</td>
<td>0.9993</td>
<td>0.9998</td>
<td>0.9998</td>
<td>0.9996</td>
<td>0.9996</td>
</tr>
<tr>
<td>02</td>
<td>B Spiked serum Samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Regression equation</td>
<td>y = 0.03042 + x0.006777x</td>
<td>y = 0.3487 + x0.5878x</td>
<td>y = 0.1345 + x0.6726x</td>
<td>y = 0.0805 + x0.1052x</td>
<td>y = 0.03912 + x0.1211x</td>
<td>y = 0.03182 + x0.1219x</td>
<td>y = 0.00886 + x0.003818x</td>
<td>y = 0.02131 + x0.09375x</td>
<td>y = 0.5800 + x0.8738x</td>
</tr>
<tr>
<td></td>
<td>Correlation coefficient, r</td>
<td>0.9997</td>
<td>0.9995</td>
<td>0.9997</td>
<td>0.9994</td>
<td>0.9993</td>
<td>0.9998</td>
<td>0.9997</td>
<td>0.9997</td>
<td>0.9995</td>
</tr>
<tr>
<td>03</td>
<td>C Corrected serum samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Regression equation</td>
<td>y = 0.01787 + x0.00655x</td>
<td>y = 0.2933 + x0.5667x</td>
<td>y = 0.05740 + x0.6026x</td>
<td>y = 0.06146 + x0.09495x</td>
<td>y = 0.02454 + x0.1087x</td>
<td>y = 0.01143 + x0.1206x</td>
<td>y = 0.002546 + x0.003750x</td>
<td>y = 0.009443 + x0.09310x</td>
<td>y = 0.4763 + x0.8662x</td>
</tr>
<tr>
<td></td>
<td>Correlation coefficient, r</td>
<td>0.9997</td>
<td>0.9997</td>
<td>0.9998</td>
<td>0.9999</td>
<td>0.9997</td>
<td>0.9998</td>
<td>0.9997</td>
<td>0.9998</td>
<td>0.9998</td>
</tr>
<tr>
<td>02</td>
<td>Recovery from serum samples (% Recovery ±SD; % RSD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mmol/l</td>
<td>92.8 ± 2.04; 94.2 ± 1.6; 94.5 ± 1.31; 99.4 ± 1.08; 92.9 ± 1.26; 98.2 ± 0.89; 96.2 ± 0.91; 99.9 ± 0.61; 94.8 ± 1.54; 2.19; 1.7; 1.44; 1.09; 1.36; 0.91; 0.94; 0.61; 1.63</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 mmol/l</td>
<td>93.5 ± 2.41; 93.6 ± 1.4; 95.8 ± 1.37; 98.6 ± 1.43; 94.2 ± 1.16; 98.7 ± 0.55; 95.3 ± 0.77; 99.2 ± 0.61; 92.3 ± 1.79; 2.46; 1.93; 1.65; 1.18; 1.54; 1.12; 1.36; 0.97; 2.17</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>03</td>
<td>Sensitivity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LLOD (mmol/l)</td>
<td>0.1</td>
<td>0.01</td>
<td>0.012</td>
<td>0.018</td>
<td>0.026</td>
<td>0.012</td>
<td>0.40</td>
<td>0.12</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>LLOQ (mmol/l)</td>
<td>0.25</td>
<td>0.015</td>
<td>0.016</td>
<td>0.02</td>
<td>0.035</td>
<td>0.02</td>
<td>0.70</td>
<td>0.18</td>
<td>0.04</td>
</tr>
</tbody>
</table>

\( ^a \): It is for the recovered amount of added analytes to serum samples and calculated by = total amount of analyte in spiked sample - amount of analyte present endogenously in serum sample.
### Table 3.24: Repeatability studies performed for aminothiols, ascorbic acid and malondialdehyde

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cystine</th>
<th>Cysteine</th>
<th>Homocysteine</th>
<th>Ascorbic acid</th>
<th>Methionine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Repeatability</strong></td>
<td>Mean ± SD; %RSD</td>
<td>Mean ± SD; %RSD</td>
<td>Mean ± SD; %RSD</td>
<td>Mean ± SD; %RSD</td>
<td>Mean ± SD; %RSD</td>
</tr>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Injection repeatability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1 Retention time</strong></td>
<td>4.08 ± 0.02; 1.23</td>
<td>4.50 ± 0.01; 1.01</td>
<td>5.37 ± 0.02; 0.68</td>
<td>6.95 ± 0.03; 0.44</td>
<td>7.85 ± 0.05; 0.78</td>
</tr>
<tr>
<td><strong>2 Peak areas</strong></td>
<td>(4.16 mmol/l)</td>
<td>(8.25 mmol/l)</td>
<td>(7.4 mmol/l)</td>
<td>(5.7 mmol/l)</td>
<td>(6.7 mmol/l)</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Intra-day repeatability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1 mmol/l</strong></td>
<td>0.94 ± 0.024; 2.34</td>
<td>0.95 ± 0.017; 1.35</td>
<td>0.88 ± 0.012; 3.15</td>
<td>0.96 ± 0.018; 3.62</td>
<td>0.84 ± 0.035; 4.50</td>
</tr>
<tr>
<td><strong>2 mmol/l</strong></td>
<td>1.89 ± 0.035; 2.89</td>
<td>1.95 ± 0.022; 1.89</td>
<td>1.82 ± 0.026; 2.56</td>
<td>1.97 ± 0.014; 1.45</td>
<td>1.70 ± 0.041; 4.85</td>
</tr>
<tr>
<td><strong>4 mmol/l</strong></td>
<td>3.94 ± 0.049; 1.66</td>
<td>3.93 ± 0.029; 2.46</td>
<td>3.75 ± 0.043; 4.69</td>
<td>3.75 ± 0.029; 2.21</td>
<td>3.77 ± 0.029; 2.41</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Inter-day repeatability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1 mmol/l</strong></td>
<td>0.83 ± 0.121; 20.45</td>
<td>0.76 ± 0.156; 25.06</td>
<td>0.80 ± 0.110; 33.67</td>
<td>0.85 ± 0.162; 22.12</td>
<td>0.71 ± 0.118; 15.33</td>
</tr>
<tr>
<td><strong>2 mmol/l</strong></td>
<td>1.72 ± 0.198; 24.55</td>
<td>1.86 ± 0.236; 31.92</td>
<td>1.74 ± 0.234; 26.35</td>
<td>1.84 ± 0.221; 14.78</td>
<td>1.57 ± 0.246; 11.84</td>
</tr>
<tr>
<td><strong>4 mmol/l</strong></td>
<td>3.56 ± 0.348; 18.60</td>
<td>3.75 ± 0.403; 33.80</td>
<td>3.58 ± 0.357; 35.12</td>
<td>3.43 ± 0.355; 18.54</td>
<td>3.53 ± 0.418; 25.77</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Repeatability</strong></th>
<th>Glutathione reduced</th>
<th>Glutathione oxidized</th>
<th>Malondialdehyde</th>
<th>Dopamine</th>
<th>N-acetyl-cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Repeatability</strong></td>
<td>Mean ± SD; %RSD</td>
<td>Mean ± SD; %RSD</td>
<td>Mean ± SD; %RSD</td>
<td>Mean ± SD; %RSD</td>
<td>Mean ± SD; %RSD</td>
</tr>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Injection repeatability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1 Retention time</strong></td>
<td>8.95 ± 0.05; 0.82</td>
<td>10.01 ± 0.03; 0.66</td>
<td>11.12 ± 0.04; 0.51</td>
<td>12.15 ± 0.06; 0.36</td>
<td>17.77 ± 0.09; 0.92</td>
</tr>
<tr>
<td><strong>2 Peak areas</strong></td>
<td>(3.25 mmol/l)</td>
<td>(7.0 mmol/l)</td>
<td>(4.0 mmol/l)</td>
<td>(6.53 mmol/l)</td>
<td>(6.13 mmol/l)</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Intra-day repeatability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1 mmol/l</strong></td>
<td>0.90 ± 0.022; 4.61</td>
<td>0.88 ± 0.036; 3.17</td>
<td>0.97 ± 0.017; 1.77</td>
<td>0.97 ± 0.038; 4.23</td>
<td>0.95 ± 0.023; 5.22</td>
</tr>
<tr>
<td><strong>2 mmol/l</strong></td>
<td>1.49 ± 0.038; 2.92</td>
<td>1.73 ± 0.040; 4.87</td>
<td>1.94 ± 0.023; 2.12</td>
<td>---</td>
<td>1.92 ± 0.027; 4.06</td>
</tr>
<tr>
<td><strong>4 mmol/l</strong></td>
<td>3.71 ± 0.035; 3.77</td>
<td>3.56 ± 0.049; 9.56</td>
<td>3.88 ± 0.046; 2.85</td>
<td>---</td>
<td>3.84 ± 0.039; 4.74</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Inter-day repeatability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1 mmol/l</strong></td>
<td>0.82 ± 0.098; 38.40</td>
<td>0.70 ± 0.112; 34.78</td>
<td>0.89 ± 0.087; 08.59</td>
<td>0.85 ± 0.079; 18.30</td>
<td>0.81 ± 0.120; 21.55</td>
</tr>
<tr>
<td><strong>2 mmol/l</strong></td>
<td>1.35 ± 0.168; 35.22</td>
<td>1.45 ± 0.189; 42.64</td>
<td>1.85 ± 0.094; 11.85</td>
<td>---</td>
<td>1.84 ± 0.156; 35.86</td>
</tr>
<tr>
<td><strong>4 mmol/l</strong></td>
<td>3.54 ± 0.334; 28.49</td>
<td>3.33 ± 0.329; 31.51</td>
<td>3.73 ± 0.155; 09.64</td>
<td>---</td>
<td>3.69 ± 0.277; 35.20</td>
</tr>
</tbody>
</table>
3.4.1.3.5. Robustness

Method robustness was checked by making deliberate minor changes in chromatographic conditions like mobile phase composition, phase ratio and flow rate, temperature and detector electrode potential in ± 2%. All these changes showed negligible effects on linearity, sensitivity and retention times.

3.4.1.3.6. Stability studies

Stabilities of both standard and spiked serum samples were checked at ambient (25 °C), cold (4 °C) and -20 °C temperatures. Analytes were stable in initial hours after extraction at room temperature, while both standard and samples were remained stable for 1 week and 24 hours at 4 °C and -20 °C respectively. Cystine, ascorbic acid and oxidized glutathione were degraded more quickly at 25 °C than other analytes. Therefore samples were stored at -20 °C for maximum stability. Table 3.25 summarizes the percent loss after 1 week storage of standard solutions at three temperatures.
Table 3.25: One week stability studies of analytes standard solutions at 25 °C, 4 °C and -20 °C

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention Time (minutes)</th>
<th>Initial peak areas</th>
<th>Peak area after 7 days storage at 25 °C</th>
<th>Percent loss</th>
<th>Peak area after 7 days storage at 4 °C</th>
<th>Percent loss</th>
<th>Peak area after 7 days storage at -20 °C</th>
<th>Percent loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystine</td>
<td>4.08</td>
<td>411677</td>
<td>102795</td>
<td>75.03%</td>
<td>199128</td>
<td>51.63%</td>
<td>255733</td>
<td>37.88%</td>
</tr>
<tr>
<td>Cysteine</td>
<td>4.50</td>
<td>2715160</td>
<td>2218791</td>
<td>18.28%</td>
<td>2347255</td>
<td>13.55%</td>
<td>2436584</td>
<td>10.26%</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>5.37</td>
<td>2195550</td>
<td>1378328</td>
<td>37.22%</td>
<td>1548082</td>
<td>29.49%</td>
<td>1856996</td>
<td>15.42%</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>6.95</td>
<td>1212378</td>
<td>618432</td>
<td>48.99%</td>
<td>828781</td>
<td>31.64%</td>
<td>951837</td>
<td>21.49%</td>
</tr>
<tr>
<td>Methionine</td>
<td>7.85</td>
<td>633238</td>
<td>429490</td>
<td>32.18%</td>
<td>471572</td>
<td>25.53%</td>
<td>502094</td>
<td>20.71%</td>
</tr>
<tr>
<td>Glutathione reduced</td>
<td>8.95</td>
<td>539460</td>
<td>355236</td>
<td>34.15%</td>
<td>373090</td>
<td>30.84%</td>
<td>402868</td>
<td>25.32%</td>
</tr>
<tr>
<td>Glutathione oxidized</td>
<td>10.01</td>
<td>127367</td>
<td>41462</td>
<td>67.45%</td>
<td>70191</td>
<td>44.89%</td>
<td>78814</td>
<td>38.12%</td>
</tr>
<tr>
<td>Malondialdehyde</td>
<td>11.12</td>
<td>164085</td>
<td>116732</td>
<td>28.86%</td>
<td>120060</td>
<td>26.83%</td>
<td>135665</td>
<td>17.32%</td>
</tr>
<tr>
<td>N-acetylcysteine</td>
<td>17.77</td>
<td>1116570</td>
<td>834510</td>
<td>25.26%</td>
<td>923403</td>
<td>17.30%</td>
<td>971639</td>
<td>12.98%</td>
</tr>
</tbody>
</table>
3.4.1.4. Application of method

This method was applied to determine level of oxidative stress in diseased state, and was successfully applied to assess endogenous; aminothiols, AA and MDA in serum samples of various groups. Overlay of analytes responses present in healthy rabbits serum samples under the validated chromatographic conditions are given the in figure 3.19.

**Figure 3.19:** Hydrodynamic voltogram of endogenous compounds at 900mV electrode potential of normal healthy albino rabbits after adding IS and extraction. Peaks: 1: cystine, 2: cysteine, 3: homocysteine, 4: ascorbic acid, 5: methionine, 6: glutathione reduced, 7: glutathione oxidize, 8: malondialdehyde, 9: dopamine and 10: N-acetylcysteine. Chromatograms were obtained using mobile phase: TFA aqueous (0.05%): Methanol in 98.5:1.5 ratios pumped at 0.6 ml/min, injecting 5µl of sample for analysis.
3.4.2. Effect of exogenous antioxidant therapy on endogenous antioxidants

Effect of natural antioxidants therapies on endogenous antioxidants were evaluated systematically. Serum levels of both hydrophilic and lipophilic antioxidants such as glutathione reduced (GSH) and oxidized forms (GSSG), cystine, cysteine, homocysteine, ascorbic acid, methionine, N-acetylcysteine, α-tocopherol and all-trans retinol were quantified in normal, disease control, and various treatment groups. Lipid peroxidative extent was also observed by quantifying malondialdehyde level. Data was calculated in mean ±SEM in each group (n=6) at p<0.05%. Data showed positive correlation of natural antioxidant interventions with blood glutathione reduced form, methionine, ascorbic acid, N-acetylcysteine and α-tocopherol while negative correlation was observed with cystine, cysteine, homocysteine, all-trans retinol and malondialdehyde. All the combinations showed effects at varied extents depending upon the amount of drug incorporated in specific combination, confirming that oral therapies prevented the deleterious effects of hyperlipidemia on beneficial endogenous compounds by acting in gastrointestinal tract, liver and adipose tissues. Quantified values of these bio-compounds are summarized in table 3.26.
### Table 3.26: Quantification of endogenous antioxidants and malondialdehyde levels in various test groups

<table>
<thead>
<tr>
<th>Parameters (µmol/l) in mean ± SEM</th>
<th>Baseline</th>
<th>After 2 weeks of CHO-dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystine</td>
<td>52.95 ± 1.68</td>
<td>93.35 ± 3.81</td>
</tr>
<tr>
<td>Cysteine</td>
<td>13.66 ± 0.87</td>
<td>14.31 ± 1.09</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>8.72 ± 0.87</td>
<td>9.08 ± 0.96</td>
</tr>
<tr>
<td>Methionine</td>
<td>38.05 ± 1.55</td>
<td>34.97 ± 1.77</td>
</tr>
<tr>
<td>Glutathione reduced</td>
<td>3.75 ± 0.384</td>
<td>4.13 ± 0.245</td>
</tr>
<tr>
<td>Glutathione oxidized</td>
<td>0.87 ± 0.037</td>
<td>1.25 ± 0.041</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>4.42 ± 0.085</td>
<td>3.26 ± 0.067</td>
</tr>
<tr>
<td>N-acetyl cysteine</td>
<td>4.57 ± 0.457</td>
<td>4.38 ± 0.441</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>18.46 ± 1.74</td>
<td>17.29 ± 1.56</td>
</tr>
<tr>
<td>All-trans retinoic acid</td>
<td>1.83 ± 0.11</td>
<td>2.45 ± 0.15</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>5.59 ± 0.58</td>
<td>5.45 ± 0.67</td>
</tr>
<tr>
<td>Malondialdehyde</td>
<td>3.37 ± 0.233</td>
<td>4.08 ± 0.269</td>
</tr>
</tbody>
</table>

#### RESULTS AND DISCUSSIONS

In each group (n=6), the endogenous antioxidants were calculated as mean ± SEM, using ANOVA followed by Dunnett’s test (*p<0.05, **p<0.01, ***p<0.001)
3.4.2.1. **Analysis of serum cystine (CySS), cysteine (Cys) and methionine (Meth) levels in normal, disease control and treatment groups**

Serum levels of CySS and Cys after initial two week cholesterol dosing were elevated from baseline levels 42.95 ± 1.68 µmol/l and 13.66 ± 0.87 µmol/l to 75.35 ± 3.81 µmol/l and 14.31 ± 1.09 µmol/l respectively. The rise in both endogenous compounds disclosed their roles as risk factor in developing oxidative stress. However Meth levels were reduced from 38.05 ± 1.55 µmol/l to 34.97 ± 1.77 µmol/l because of its utilization in GSH generation.

Further at the end of study duration the CySS and Cys levels were significantly increased in disease control (110.8 ± 6.41 µmol/l and 42.36 ± 2.62 µmol/l) compared to normal group (50.37 ± 2.60 µmol/l and 14.70 ± 1.49 µmol/l). While comparing the treatment groups with the disease control, significant depressing effects in CySS levels (67.44 ± 3.87 - 77.23 ± 2.94 µmol/l) (p <0.001) were observed in most groups. While in case of Cys, the individual therapies including atorvastatin produced non-significant results as compared to which the blends produced 29.35 ± 1.93 - 33.51 ± 1.41 µmol/l results with most significant effects observed in 20:50:30 blend of OPC, NA and PT (29.35 ± 1.93 µmol/l, p <0.001).

Severe reduction in Meth level was observed in disease control (19.30 ± 1.18 µmol/l) almost half to normal group (39.23 ± 1.88 µmol/l). In treatment groups the OPC and combinational groups controlled its depletion significantly to varied extents (24.73 ± 1.04 - 35.08 ± 1.60 µmol/l). Most significant effects were observed in 50:30:20 blend of OPC, NA and PT (35.08 ± 1.60 µmol/l, p < 0.001).
Exogenous antioxidant treatment spared methionine by modulating lipid levels. Our results were similar to the previous work in terms of antioxidant activity and endogenous antioxidant capacity as shown in table 3.26 and in figure 3.20.

![Figure 3.20](image)

**Figure 3.20:** Mean serum levels of cystine, cysteine and methionine in normal, disease control and treatment groups with standard bars showing SEM of mean values.

### 3.4.2.2. Analysis of serum homocysteine (Hcy) and N-acetyl cysteine (NAC) levels in normal, disease control and treatment groups

From the baseline (8.72 ± 0.87 µmol/l) less increment was observed in Hcy level (9.08 ± 0.96 µmol/l) after two weeks of cholesterol dosing. However at the end of study the serum Hcy level in disease control was significantly elevated to 14.78 ± 1.49 µmol/l
compared to normal (blank) control group 8.56 ± 1.31 µmol/l. In treatments groups only few combinational groups showed significant effects (p <0.01) in suppressing Hcy levels 9.09 ± 1.39 µmol/l - 11.79 ± 1.63 µmol/l with most significant effect observed in 50:30:20 and 50:20:30 blends of OPC, NA and PT (9.09 ± 1.39 µmol/l and 9.64 ± 1.13 µmol/l p <0.01 respectively).

Serum levels of NAC after two week cholesterol dosing yield a decrease to 4.38 ± 0.441 µmol/l compared to blank control level 4.57 ± 0.457 µmol/l due to conversion into GSH \(^{383}\). At the end of study, the level decreased further in disease control by 3.70 ± 0.412 µmol/l compared to normal group 4.49 ± 0.429 µmol/l. In most of treatment groups NAC level varied from disease control insignificantly. Only three drug combinations showed some prominent effects and raised NAC levels above 4.0 µmol/l. Significant result was observed in 50:20:30 blend of OPC, NA and PT (4.21 ± 0.425 µmol/l, p<0.01) as shown in table 3.26 and in figure 3.21.
3.4.2.3. Analysis of serum glutathione reduced (GSH), glutathione oxidized (GSSG) levels and their ratio (GSH/GSSG) in normal, disease control and treatment groups

Serum levels of GSH, GSSG and GSH/GSSG ratio in normal healthy rabbits were 3.75 ± 0.384 µmol/l, 0.87 ± 0.037 µmol/l and 4.42 ± 0.098 respectively. After initial cholesterol feeding both GSH and GSSG were increased to 4.13 ± 0.245 µmol/l and 1.25 ± 0.041 µmol/l respectively. Hyperlipidemic condition induced accelerated production of free radicals in cells and circulation triggering the synthesis of GSH from NAC and
methionine (precursors). Besides the increased GSH level the GSH/GSSG ratio decreased to $3.26 \pm 0.127$ due to over production of GSSG leading to redox disruption.

Evaluating end results of study the GSH levels of the disease control decreased to $3.01 \pm 0.28 \mu M$ compared to normal group level $3.78 \pm 0.35 \mu M$. This fall after initial rise is because of burning of GSH precursors rapidly and unavailability for recovery leads to decrease in serum GSH levels. Besides that GSSG levels raised in disease control to $1.44 \pm 0.041 \mu M$ compared to normal group level $0.92 \pm 0.029 \mu M$, which further reduced the GSH/GSSG ratio significantly to $2.12 \pm 0.120$ in comparison with normal group; $4.18 \pm 0.102$. Treatment groups showed significant results ($p < 0.01, 0.001$) in elevating GSH levels ranging $3.32 \pm 0.31 \mu M$ to $3.82 \pm 0.47 \mu M$. Maximum result was observed in 50:30:20 and 50:20:30 blends of OPC, NA and PT ($3.82 \pm 0.47 \mu M$ and $3.77 \pm 0.28 \mu M$ ($p < 0.001$) respectively) testifying the superiority of combinational therapies.

The elevation in GSSG levels were controlled in most groups significantly from $1.24 \pm 0.045 \mu M$ ($p < 0.05$) down to $1.12 \pm 0.057 \mu M$ ($p < 0.001$), with most significant effects observed in three drug combinations ($p < 0.001$). As a result the GSH/GSSG ratios elevated significantly $2.51 \pm 0.092$ ($p < 0.05$) to $3.40 \pm 0.097$ ($p < 0.001$) maximum in 50:30:20 blend of OPC, NA and PT ($3.40 \pm 0.097$, $p < 0.001$) (Table 3.26, Figure 3.22).
Blank control
Disease control
Atorvastatin
Proanthocyanidins (OPC)
Niacin (NA)
Pterostilbene (PT)
OPC: NA  (70:30)
OPC: PT  (70:30)
NA: PT  (70:30)
OPC: NA: PT (50:30:20)
OPC: NA: PT (50:20:30)
OPC: NA: PT (20:50:30)

0
1
2
3
4
5 Glutathione Oxidized
Glutathione Reduced
GSH/GSSG
Conc. in µmol/L

Figure 3.22: Mean serum levels of GSH, GSSG and GSH/GSSG in normal, disease control and treatment groups with standard bars showing SEM of mean values.

3.4.2.4. Analysis of serum α-tocopherol (vit-E) and all-trans retinoic acid (RTA) levels in normal, disease control and treatment groups

Serum levels of vit-E after cholesterol dosing was reduced from 18.46 ± 1.74 µmol/l to 17.29 ± 1.56 µmol/l possibly due to over production of free radical induced vit-E oxidation. In the end of study duration, its level in disease control reduced significantly to 11.30 ±1.38µmol/l as compared to the normal group level; 18.87 ± 1.49 µmol/l. Oral antioxidants prevented vit-E depression significantly (12.57 µmol/l to 14.78 µmol/l), with most significant result observed in 70:30 blend of OPC and NA.
(14.78 µmol/l, p <0.001) and 20:50:30 blend of OPC, NA and PT (14.40 ± 1.75 µmol/l, p <0.001).

The RTA level in disease group rose to 2.45 ± 0.1511 µmol/l compared to the baseline level 1.83 ± 0.1143 µmol/l after two weeks of cholesterol dosing and further to 4.48 ± 0.24 µmol/l compared to normal group level 1.80 ± 0.10 µmol/l in 90 days. This is because of up-regulation of RTA genes by cholesterol and its metabolites. In treatment groups serum RTA levels declined significantly to 3.65 ± 0.22 µmol/l - 3.26 ± 0.12 µmol/l) with maximum effect observed in 70:30 blend of OPC and PT (3.26 ± 0.12 µmol/l, p <0.001) and 50:30:20 blend of OPC, NA and PT (3.29 ± 0.21 µmol/l, p <0.01) as shown in table 3.26 and figure 3.23.

**Figure 3.23:** Mean serum levels of α-tocopherol and all-trans retinoic acid in normal, disease control and treatment groups with standard bars showing SEM of mean values.
3.4.2.5. Analysis of serum ascorbic acid (AA) and malondialdehyde (MDA) levels in normal, disease control and treatment groups

Ascorbic acid (AA) levels in normal and after two week cholesterol dosing differ slightly, and decreased from $5.59 \pm 0.58 \text{ µmol/l}$ to $5.45 \pm 0.67 \text{ µmol/l}$ with significant increase in MDA from $3.37 \pm 0.233 \text{ µmol/l}$ to $4.08 \pm 0.269 \text{ µmol/l}$. At the end study the difference in AA levels was significant between normal and disease control; $5.53 \pm 0.72 \text{ µmol/l}$ and $4.90 \pm 0.59 \text{ µmol/l}$ respectively, similarly MDA level increased significantly in disease control by $5.90 \pm 0.404 \text{ µmol/l}$ as compared to blank group level $3.49 \pm 0.318 \text{ µmol/l}$.

Comparing the antioxidants therapies effects on AA, only combinations showed significant effects in preventing the decrease in serum levels ($5.06 \pm 0.76 \text{ µmol/l}$ (p <0.05) to $5.25 \pm 0.51 \text{ µmol/l}$ (p <0.001)) with maximum effect observed in 50:30:20 blends of OPC, NA and PT. While in case of MDA significant inhibitory effects were observed in all treatment groups including statin. MDA was retarded ranging $5.01 \pm 0.261 \text{ µmol/l}$ (NA group, p <0.05) to $3.96 \pm 0.335 \text{ µmol/l}$ (50:20:30 blend of OPC, NA and PT, p <0.001) as shown in table 3.26 and figure 3.24.
3.4.3. Discussion

In developed method of simultaneous analysis, lipid peroxidation was determined by measuring the level of malondialdehyde which is the end product of peroxidation and normally determined by colorimetric method making adducts with thiobarbituric acid (TBA). This is a non-specific method because other aldehydes in sample may also form complex thus producing false results than actual quantity, consequently in current method we have managed to detect MDA in its biological form without forming adduct, thus acquiring actual blood levels.
Association of ROS generation, levels of endogenous antioxidants (aminothiols, AA, vit-E) with pathological conditions like hyperlipidemia, cardiovascular diseases, obesity and diabetes had received much attention. The depletion of endogenous antioxidants in pathological condition results in imbalancement of ROS level and redox homeostasis which intern affect every vital organ systems thus worsening the disease. A review concluded that hyperhomocysteinemia alone is capable of initiating and worsening atherosclerotic events, lipid deregulation and cellular redox imbalancement. Similarly elevated cysteine level and its oxidized form cystine are also considered as risk factors especially in cardiovascular related pathologies. High levels of cysteine and homocysteine observed in our hypercholesterolemic animal model compared to blank control was found parallel to a study based on atherothrombotic events. The LDL-C carries mostly poly-unsaturated fatty acids (PUFA) and cholesteryl esters as major component and because of having the double bond they are being easily attacked and oxidized by free radicals. The α-tocopherol which is the major antioxidant in LDL-C scavenges these radicals and prevents lipid peroxidation. The GSH performs multi tasks such as regenerating α-tocopherol and forming adducts with lipid peroxidation end products which are then further removed from the body. In chronic condition these antioxidants gets depleted. Natural antioxidants were found effective in preventing peroxidative process and endogenous antioxidants depletion in many pathological conditions. From our study it was confirmed that exogenous antioxidants interventions in combinations delivers synergistic effects in lower doses compared to individual high doses. This was also confirmed by ascorbic acid intervention in preventing pro-oxidant effects of LDL-C-associated α-tocopherol.
In current study we correlated the endogenous antioxidants levels with the exogenous natural antioxidants therapies in hyperlipidemia and observed the disruption of balance between the oxidants and antioxidants levels in the body. From this study we also concluded that cystine, cysteine and homocysteine are the aminothiols that are associated with the worsening of pathologic consequences and can be used as biomarker along with MDA in estimation of oxidative stress.

Ameliorative effect of OPC was recorded in diabetic models by enhancing GSH/GSSG ratio similar to our results where more pronounced effects were observed in combinational therapies. The increase in RTA in the presence of high blood lipid levels is due to upregulation of retinal dehydrogenases which in turn suppress key enzymes of bile acid synthesis the CYP7A1 and CYP8B1. Thus reducing the bile acid synthesis and hence lipid metabolism and absorption in gut. In hyperlipidemia interventions with dietary exogenous antioxidants like alpha-tocopherol, ascorbic acid, niacin, proanthocyanidins, quercetin, and pterostilbene may help in intercepting the development of oxidative stress and maintaining the redox status in the body. The natural antioxidants have mostly been tested in diabetic models whereas in hyperlipidemic models data is much less available. In the current study, antioxidants were evaluated and compared in individual and 1:1 combination and other ratios to get an optimized dose rationale in a combination with optimum effects.
3.5. Formulation development and optimization studies

Formulations were pursued for the combination delivered maximum lipid lowering effects. The optimum effects were observed in 50:30:20 ratio of OPC: NA: PT and consequently selected for formulation studies. Various preformulation and in-vitro parameters were evaluated for the development of suitable and stable formulation. Compressed tablet dosage form was selected, as the total dose was 1500 mg therefore the formulations were performed on divided doses (i.e. APIs weight 750 mg/tab).

3.5.1. Grounds for developing dose rationale based formulation(s)

- Oligomeric proanthocyanidins (OPC) has problem of slow wetting because of polymeric nature making a hydrated outer layer preventing the tablet from disintegration. These were confirmed from preliminary experiments on raw and compressed tablet and the problem exaggerates with pterostilbene which is lipophilic.

- A number of formulations are available in local market with high doses of currently used drugs (>1000 mg/tab) especially OPC. These high doses can cause dumping, cost burden and loss of drug in GIT and may be harmful in long term use. Our formulation will provide a research base product to the current market with the aim of treating chronic ailments.

- Since OPC holds disintegration problem, therefore disintegrant selection and quantity optimization was also necessary.

- Blend formulation will also reduce the chances of adverse effects of niacin i.e. facial flushing and liver enzyme elevation, because using 450 mg/day instead of 1000-1500 mg will undoubtedly minimize these side effects. Though its beneficial outcome will
be reduced in monotherapy but using with other antioxidants will improve the outcome as confirmed in this study.

3.5.2. Pre-formulation studies

Pre-formulation studies were based on compatibility tests among drug-drug, drug-excipient and excipient-excipient mixtures at initial and in storage conditions under controlled humidity and temperature. They are tested in various ways to authenticate the stability of desired dosage form.

3.5.2.1. Drug excipient compatibility studies

Excipients are pharmacologically inert but play major roles in developing dosage form that includes binding effect, lubrication and disintegration. Various binary combinations were evaluated for compatibility and analyzed by;

1) FTIR spectral analysis
2) Drug content
3) Physical consistency

Drug content test was applied to those mixtures that contains drug.

3.5.2.1.1. FTIR spectral analysis

FTIR analysis was performed to check any chemical incompatibility. Change in IR spectra due to alteration in functional group is the sign of degradation process. Results showed no incompatibility in APIs and excipient mixtures. The IR spectra at initial and after 30 days under stress condition were in close agreement with each other. Data is presented in table 3.31 and the spectra are attached in the appendix section.
3.5.2.1.2. Drug content

Decrease in drug content is an indication of degradative process. Since heat and moisture are the main sources of catalyzing. Furthermore a hygroscopic excipient can degrade the API(s). Drug content was observed in triplicate and overall the percent drug content of all APIs was between 99.2% -100.1% at initial time. After 30 days under stress conditions (40 °C, 75% RH) were between 99.1% - 100.4% as shown in table 3.31, confirming that APIs are physically and chemically stable.

3.5.2.1.3. Physical consistency

Samples stored under accelerated conditions didn’t show any physical inconsistency. Samples colors, powder particle size (after sifting) remained the same. Moisture content slightly rose due to entrapment by OPC and some excipients up to 1.5%, yet has no deleterious effects as shown in table 3.27.
<table>
<thead>
<tr>
<th>Analysis time</th>
<th>Parameter</th>
<th>Spl 01</th>
<th>Spl 02</th>
<th>Spl 03</th>
<th>Spl 04</th>
<th>Spl 05</th>
<th>Spl 06</th>
<th>Spl 07</th>
<th>Spl 08</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day-01</td>
<td>FTIR Spectra</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
</tr>
<tr>
<td></td>
<td>*Drug Content (%)</td>
<td>99.15 ± 0.23</td>
<td>99.20 ± 0.15</td>
<td>99.67 ± 0.43</td>
<td>---</td>
<td>99.44 ± 0.21</td>
<td>99.66 ± 0.32</td>
<td>100.1 ± 0.12</td>
<td>99.66 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>Physical Consistency</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
</tr>
<tr>
<td>Day-30</td>
<td>FTIR Spectra</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
</tr>
<tr>
<td></td>
<td>*Drug Content (%)</td>
<td>99.66 ± 0.46</td>
<td>99.28 ± 0.20</td>
<td>99.12 ± 0.24</td>
<td>---</td>
<td>99.89 ± 0.60</td>
<td>99.17 ± 0.57</td>
<td>99.12 ± 0.34</td>
<td>99.80 ± 0.59</td>
</tr>
<tr>
<td></td>
<td>Physical Consistency</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
<td>Complies</td>
</tr>
</tbody>
</table>

* is the mean ± SD, and in sample-08 it shows the % mean content of all the drugs.
Complies indicate the similarity of IR spectra.
3.5.3. Conversion of animal dose into human equivalent dose

The combination OPC: NA: PT in 50:30:20 ratio was selected and dose was extrapolated into human equivalent dose (HED) as per FDA approved formula. Each drug dose ratio was calculated individually and then combined in single dosage form.

Average weight of animals used was 1.4kg and calculating the average body surface area (BSA), drug quantity in HED was calculated by putting the amount of drug used for animals in the formula as given below.

HED of OPC = 50 × 9.33/37 = 12.6 mg/kg or 12.6 × 60 = 750 mg/60 kg

HED of NA = 30 × 9.33/37 = 7.56 mg/kg or 7.56 × 60 = 450 mg/60 kg

HED of PT = 20 × 9.33/37 = 5.04 mg/kg or 5.04 × 60 = 300 mg/60 kg

Combining all the three drug ratios:

OPC + NA + PT = 750 + 450 + 300 = 1500 mg/day.

The total dose that should be administered to human population to get comparable results is 1500 mg/day of APIs blend in single or divided doses.
3.5.4. Development and validation of HPLC-UV method for simultaneous
determination of niacin (hydrophilic) and pterostilbene (lipophilic) natural
antioxidants

The purpose of developing this method was to omit the interferences caused by
both drugs on each other, observed during UV/Visible spectrophotometric analysis.
Method was developed and validated according to standard guidelines for simultaneous
determination. Hydrophilic compounds containing carboxylic acid groups possess poor
retention on reverse phase C-18 columns. In order to retain niacin on C-18 RP-HPLC
column, an amphiphilic ion pairing agent “hexa-decyltrimethylammonium bromide”
(cetrimide/CTAB) was used having both hydrophilic and lipophilic ends.

3.5.4.1. Extraction procedure optimization

Methanol, acetonitrile and mobile phase were evaluated for extraction procedures.
Experiments were performed on 200 µl of plasma samples and dissolution samples.
Optimum recovery was observed with mobile phase composed of aqueous phase (5 mmol
CTAB) and organic phase (80:20 v/v ACN: MeOH) in 30:70 v/v. as shown in table 3.28.

<table>
<thead>
<tr>
<th>Extraction solvent</th>
<th>Niacin (Percent recovery) Mean ± SD</th>
<th>Pterostilbene (Percent recovery) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>88.56 ± 1.56</td>
<td>95.38 ± 1.08</td>
</tr>
<tr>
<td>Methanol</td>
<td>83.42 ± 1.13</td>
<td>90.22 ± 1.19</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>80.27 ± 1.36</td>
<td>88.50 ± 1.04</td>
</tr>
</tbody>
</table>

Table 3.28: Percent recoveries of various solvents from spiked plasma samples

Mobile phase: Aq (5mM CTAB): Org (80:20 ACN: MeOH) in 30:70 v/v.
3.5.4.2. Chromatographic conditions optimization

3.5.4.2.1. Mobile phase composition

Both aqueous and organic phases were observed for optimum resolution and sensitivity. To retain niacin on RP-C18 column, amphiphilic ion pairing agent CTAB solution was prepared in 1-5mM concentrations. Results showed that with increase in concentration of CTAB, the retention time (RT) of niacin increases while has slight reducing effect on pterostilbene RT without effecting its sensitivity. Above 5mM concentration the sensitivity and sharpness of both analytes decreased. Organic solvents alone did not produce satisfactory results so their mixtures from 60:40 to 90:10 ratios of ACN: MeOH were evaluated. The ratio of 80:20 produced optimum separation from solvent front, plasma peak and between drugs and IS as shown in figure 3.25.

![Figure 3.25: Effect of organic phase composition (ACN: MeOH) on peak separation keeping aqueous phase constant at 5mM CTAB. Colors: black: 60:40, green: 70:30, blue: 80:20, pink 90:10.](image_url)
Aqueous and organic ratios were checked for optimum resolution. Preliminary findings revealed the suitability of 30:70 aqueous to organic ratio for optimum results.

3.5.4.2.2. Selection of mobile phase pH and flow rate

Effect of pH was observed in range of 2-5 adjusted with various acids including phosphoric acid, acetic acid glacial, trichloro acetic acid and formic acid. The reason for using these acids is to enhance sensitivity and separation without promoting the interaction of niacin peak with the solvent front. Adjusting pH with high molecular weight (MW) acids produced negative effects on niacin retention time. Higher the molecular weight of pH adjusting acid, more the decrease in RT. Thus formic acid (low MW) was found suitable for current method as it showed least impact on niacin RT as shown in table 3.29.

<table>
<thead>
<tr>
<th>Acids used for pH adjustment with CTAB (5 mM/l) (Target pH= 2.5)</th>
<th>Effects on retention time of analytes and internal standard (minutes) mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Niacin</td>
</tr>
<tr>
<td>Formic acid</td>
<td>4.28 ± 0.08</td>
</tr>
<tr>
<td>Acetic acid glacial</td>
<td>2.68 ± 0.11</td>
</tr>
<tr>
<td>Phosphoric acid</td>
<td>1.98 ± 0.09</td>
</tr>
<tr>
<td>Trichloro acetic acid</td>
<td>1.46 ± 0.16</td>
</tr>
</tbody>
</table>

Decreasing pH enhances sensitivity of niacin with decrease in its RT. Therefore for sample analysis pH 2.5 was selected (Fig. 3.26).
Flow rates from 0.7 to 1.2 ml/min were evaluated. Reducing flow rate resulted in mild peak broadening in pterostilbene and internal standard with slight increase in peak heights, whereas the flow rate of 1.2 ml/min reduced peak heights (sensitivity). Thus for optimum analysis 1 ml/min flow rate was selected.

3.5.4.2.3. Selection of column oven temperature

Effect of column oven temperature was observed in range of 25-40 °C for maximum resolution of analytes. Increasing temperature above ambient temperature enhanced sensitivity but lost the resolution between internal standard and pterostilbene, while niacin lost its retention. Thus beyond 25 °C no temperature level was found suitable for analysis procedures.
3.5.4.2.4. Injection volume selection

Drug sensitivities were checked at 1 μg/ml using various injections volumes (10-100 μl) under rated chromatographic conditions. Both drugs and internal standard responded exponentially with the sample volume as compared to the theoretically calculated response values. Peaks were sharp and narrow up to 60 μl, above which the peaks become broader, therefore 50 μl volume was selected.

3.5.4.2.5. Selection of internal standard

Rosuvastatin and atorvastatin produced sufficient peaks at 250 nm λ-max, while simvastatin peak was very weak. Analyzing sensitivity and resolution, rosuvastatin was found suitable as internal standard, as shown in figure 3.27.

![Figure 3.27: Chromatograms of standard and spiked plasma samples of 1) Niacin, 2) Internal standard (Rosuvastatin) and 3) Pterostilbene.](image)
3.5.4.2.6. Selection of detector wavelength

Detector wavelength was selected by scanning both drugs and IS in range of 220-280 nm. At lower wavelengths, analytes showed high sensitivity along with some unknown overlapping peaks causing interruption. At 260 nm and above all peak height decreased. At 250 nm all peaks were sufficiently tall with no interfering peaks, and selected for analysis (Figure 3.28).

Figure 3.28: Effect of detector wavelength on drugs sensitivities under rated chromatographic conditions. Chromatograms obtained with mobile phase: 30% aq (5mM CTAB): 70% org (80:20 ACN: MeOH) pH 2.5 pumped at 1 ml/min. Blue: 220 nm, red: 250 nm and green: 280 nm wavelengths.

3.5.4.3. Validation of HPLC-UV analysis method

The developed method was evaluated for its specificity/selectivity, linearity, accuracy, precision, sensitivity and stability for simultaneous determination of niacin and pterostilbene.
3.5.4.3.1. Specificity/selectivity

Method specificity was checked for any interfering peaks developed during analysis in blank, plasma sample spiked with IS only and with 1:1 mixtures. Results obtained were acceptable as no interfering peaks were observed. Dissolution media samples were also eluted and similar results were obtained signifying the specificity of developed method.

3.5.4.3.2. Linearity

Linearity of method was determined with calibration curves constructed on standard and spiked plasma samples. Six different concentrations were used from 0.01 to 10 µg/ml. Results showed linear response under rated conditions. The standard chromatogram and overlays of various concentrations of drugs in standard and spiked plasma samples are given in figures 3.29-3.31.

![Chromatogram of standard solution mixture](image)

**Figure 3.29:** Chromatogram of standard solution mixture of 1 µg/ml niacin, pterostilbene and rosuvastatin (IS), at 250 nm with mobile phase; 30% aq (5mM CTAB): 70% org (80:20 ACN: MeOH) pH 2.5 pumped at 1 ml/min.
Figure 3.30: Overlay of natural antioxidants at various concentrations in standard solutions.

Figure 3.31: Overlay of natural antioxidants at various concentrations in spiked plasma samples.
The calibration curves of niacin and pterostilbene in standard and spiked plasma samples are given in figure 3.32. The regression equation and correlation coefficients are provided in table 3.30.

![Calibration curves of niacin and pterostilbene in standard and spiked plasma samples.](image)

**Figure 3.32:** Calibration curves of niacin and pterostilbene in standard and spiked plasma samples. Red line represents response ratio in standard solutions while green line represents response ration in spiked plasma samples.

### 3.5.4.3.3. Accuracy

Accuracy was measured in terms of percent recovery ± SD and % RSD at 0.5, 1 and 2 µg/ml concentration levels showing the suitability of method for optimum recovery and quantification of both analytes on C-18 RP-HPLC column (Tab. 3.30).
Table 3.30: Linearity, accuracy, precision and sensitivity data of developed method

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameters</th>
<th>Niacin</th>
<th>Pterostilbene</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td><strong>Linearity</strong> (quantity used in μg/ml)</td>
<td>0.010-20</td>
<td>0.010-20</td>
</tr>
<tr>
<td></td>
<td><strong>Standard solutions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Regression equation</td>
<td>y = 0.8018x + 0.2868</td>
<td>y = 1.342x + 0.6412</td>
</tr>
<tr>
<td></td>
<td>Correlation coefficient, $r$</td>
<td>0.9994</td>
<td>0.9998</td>
</tr>
<tr>
<td>02</td>
<td><strong>Accuracy</strong> (% Recovery ± SD; %RSD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 μg/ml</td>
<td>90.42 ± 2.44; 2.61</td>
<td>96.80 ± 2.56; 2.64</td>
</tr>
<tr>
<td></td>
<td>1 μg/ml</td>
<td>91.65 ± 1.81; 1.91</td>
<td>97.31 ± 2.25; 2.31</td>
</tr>
<tr>
<td></td>
<td>2 μg/ml</td>
<td>90.57 ± 2.15; 2.29</td>
<td>95.85 ± 2.72; 2.84</td>
</tr>
<tr>
<td>03</td>
<td><strong>Precision</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Injection repeatability</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peak areas (1 μg/ml) ± SD; %RSD</td>
<td>84265 ± 523; 0.62</td>
<td>135088 ± 1165; 0.86</td>
</tr>
<tr>
<td></td>
<td>Retention time ± SD; %RSD</td>
<td>4.28 ± 0.08; 1.87</td>
<td>7.76 ± 0.04; 0.52</td>
</tr>
<tr>
<td>04</td>
<td><strong>Intermediate precision</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Conc. Recovered</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intra-day repeatability</td>
<td>Mean ± SD; % RSD</td>
<td>Mean ± SD; % RSD</td>
</tr>
<tr>
<td></td>
<td>Spiked conc. 0.25 μg/ml</td>
<td>0.22 ± 0.01; 4.55</td>
<td>0.23 ± 0.01; 4.35</td>
</tr>
<tr>
<td></td>
<td>0.50 μg/ml</td>
<td>0.43 ± 0.03; 6.98</td>
<td>0.46 ± 0.02; 4.35</td>
</tr>
<tr>
<td></td>
<td>1.0 μg/ml</td>
<td>0.91 ± 0.02; 2.20</td>
<td>0.95 ± 0.03; 3.13</td>
</tr>
<tr>
<td></td>
<td>Inter-day repeatability</td>
<td>Mean ± SD; % RSD</td>
<td>Mean ± SD; % RSD</td>
</tr>
<tr>
<td></td>
<td>Spiked conc. 0.25 μg/ml</td>
<td>0.21 ± 0.01; 4.76</td>
<td>0.23 ± 0.01; 4.35</td>
</tr>
<tr>
<td></td>
<td>0.50 μg/ml</td>
<td>0.43 ± 0.02; 4.65</td>
<td>0.44 ± 0.01; 2.27</td>
</tr>
<tr>
<td></td>
<td>1.0 μg/ml</td>
<td>0.92 ± 0.04; 4.35</td>
<td>0.94 ± 0.03; 3.19</td>
</tr>
<tr>
<td>04</td>
<td><strong>Sensitivity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LLOD (ng/ml)</td>
<td>08</td>
<td>04</td>
</tr>
<tr>
<td></td>
<td>LLOQ (ng/ml)</td>
<td>20</td>
<td>12</td>
</tr>
</tbody>
</table>
3.5.4.3.4. Precision

Precision was determined by injection repeatability, analysis repeatability, and intermediate precision (Intra and inter day repeatability) studies on spiked plasma samples are presented as mean ± SD along with % RSD in table 3.30.

3.5.4.3.5. Sensitivity

Method sensitivity in spiked plasma samples was determined as LLOD and LLOQ. The LLOD of niacin and pterostilbene were 08 ng/ml and 04 ng/ml, while LLOQ were 25 ng/ml and 18ng/ml respectively as given in table 3.30 and in figure 3.33.

![Figure 3.33: Overlay presenting LLOD and LLOQ of niacin and pterostilbene in spiked plasma samples.](image-url)
3.5.4.3.6. Sample stability studies

Stability of standard solution of niacin was reduced to 80.66% at room temperature while at 4 °C and -20 °C stability was above 95%. Pterostilbene remained stable at all temperatures. In spiked samples niacin and pterostilbene stability was 73.82% and 90.50% at room temperature respectively. While at 4 °C and -20 °C the stability of niacin was 79.15% and 87.43% and pterostilbene stability was 91.57% and 96.63% respectively throughout analysis period.

3.5.5. Development of formulation of the optimized APIs blend

Three methods were followed to get optimized formulation(s) with desired stability, disintegration time and drug release profile.

- Dry granulation (Slugging method).
- Wet granulation method using de-ionized water.
- Wet granulation method using 2% PVP-K30.

3.5.5.1. In-vitro evaluation studies

All the formulations were evaluated for rheological characteristics including angle of repose, Hausner ratio, Carr’s index, various volumes and densities according to standard guidelines402-403.

3.5.5.1.1. Pre-compression tests for formulations

A quantity of 10 gm samples of pure APIs and all the formulations were subjected to precompresion tests. Pure APIs were observed in order to compare changes in pre-
compression characteristics brought by various formulation techniques. Table 3.31 shows the limits of various characteristics against which all the results were compared to get optimum formulation(s).

<table>
<thead>
<tr>
<th>Flow Property</th>
<th>Angle of Repose</th>
<th>Carr’s Index (%)</th>
<th>Hausner Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excellent</td>
<td>25-30°</td>
<td>≤ 10</td>
<td>1.00-1.11</td>
</tr>
<tr>
<td>Good</td>
<td>31-35°</td>
<td>11-15</td>
<td>1.12-1.18</td>
</tr>
<tr>
<td>Fair</td>
<td>36-40°</td>
<td>16-20</td>
<td>1.19-1.25</td>
</tr>
<tr>
<td>Passable</td>
<td>41-45°</td>
<td>21-25</td>
<td>1.26-1.34</td>
</tr>
<tr>
<td>Poor</td>
<td>46-55°</td>
<td>26-31</td>
<td>1.35-1.45</td>
</tr>
<tr>
<td>Very poor</td>
<td>56-65°</td>
<td>32-37</td>
<td>1.46-1.59</td>
</tr>
<tr>
<td>Very, very poor</td>
<td>≥65°</td>
<td>≥38</td>
<td>≥1.60</td>
</tr>
</tbody>
</table>

Precompresion tests (physical properties) performed for pure APIs showed passable to poor flow properties of all the APIs. Carr’s indexes and Hausner ratios of OPC, NA and PT were 25.76 ± 1.68, 27.36 ± 2.21, 21.05 ± 1.33 and 1.34 ± 0.03, 1.38 ± 0.02, 1.27 ± 0.02 respectively. The blend of APIs did not provided any flow enhancement as shown by Carr’s index 21.13, Hausner ratio 1.27 and angle of repose 38.34 ± 1.26. All these results along with other parameters are arranged in table 3.32 as mean ± SD.
Moreover preliminary study on mixing all the ingredients (APIs + excipients) in powder form enhanced the above mentioned characteristics but failed the disintegration test because of poor penetration of water. OPC hydrates first, making a muddy outer surface. It dissolves slowly but prevents tablet from disintegration and dissolution. Therefore our results confirmed the unsuitability of used APIs for direct compression.

3.5.5.1.1. Pre-compression evaluation of APIs blend formulations prepared by dry granulation method

All the ingredients were mixed and compressed into slugs. They were further crushed, sieved and after adding the remaining ingredients compressed into 19 mm × 8 mm oblong double convex surfaced tablets. Results showed significant improvement in flow properties and among all formulations F6 and F7 were found with most optimized flowability confirmed by angle of repose 36.45° ± 1.26, 28.72° ± 0.60 respectively as shown in table 3.33.
## Table 3.33: Physical properties of pre-compressed formulations of APIs prepared by dry granulation method

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
<th>F7</th>
<th>F8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
</tr>
<tr>
<td><strong>Bulk Volume</strong> (ml)</td>
<td>15.68 ± 1.21</td>
<td>16.47 ± 0.69</td>
<td>15.23 ± 0.90</td>
<td>15.34 ± 0.39</td>
<td>14.88 ± 0.70</td>
<td>15.34 ± 1.03</td>
<td>14.94 ± 0.59</td>
<td>15.70 ± 1.33</td>
</tr>
<tr>
<td><strong>Tapped Volume</strong> (ml)</td>
<td>12.30 ± 0.55</td>
<td>14.02 ± 0.63</td>
<td>12.40 ± 0.38</td>
<td>13.49 ± 0.80</td>
<td>12.56 ± 0.45</td>
<td>13.88 ± 0.87</td>
<td>13.55 ± 0.82</td>
<td>13.34 ± 1.05</td>
</tr>
<tr>
<td><strong>Bulk Density</strong> (gm/ml)</td>
<td>0.64 ± 0.04</td>
<td>0.61 ± 0.03</td>
<td>0.66 ± 0.05</td>
<td>0.65 ± 0.03</td>
<td>0.67 ± 0.04</td>
<td>0.65 ± 0.06</td>
<td>0.67 ± 0.02</td>
<td>0.64 ± 0.03</td>
</tr>
<tr>
<td><strong>Tapped Density</strong> (gm/ml)</td>
<td>0.81 ± 0.04</td>
<td>0.71 ± 0.03</td>
<td>0.81 ± 0.04</td>
<td>0.74 ± 0.05</td>
<td>0.79 ± 0.04</td>
<td>0.72 ± 0.04</td>
<td>0.74 ± 0.05</td>
<td>0.75 ± 0.04</td>
</tr>
<tr>
<td><em>Carr’s Index</em></td>
<td>20.98</td>
<td>14.08</td>
<td>18.52</td>
<td>12.16</td>
<td>15.19</td>
<td>9.72</td>
<td>9.46</td>
<td>14.67</td>
</tr>
<tr>
<td><em>Hausner Ratio</em></td>
<td>1.27</td>
<td>1.16</td>
<td>1.23</td>
<td>1.14</td>
<td>1.18</td>
<td>1.11</td>
<td>1.10</td>
<td>1.17</td>
</tr>
<tr>
<td>Flow Property</td>
<td>Fair</td>
<td>Good</td>
<td>Fair</td>
<td>Good</td>
<td>Good</td>
<td>Excellent</td>
<td>Excellent</td>
<td>Good</td>
</tr>
<tr>
<td><strong>Angle of Repose</strong> (*)</td>
<td>40.02 ± 1.08</td>
<td>33.15 ± 0.79</td>
<td>37.50 ± 2.02</td>
<td>32.63 ± 0.98</td>
<td>33.09 ± 1.20</td>
<td>28.25 ± 1.26</td>
<td>28.72 ± 0.60</td>
<td>33.53 ± 1.31</td>
</tr>
</tbody>
</table>

*Calculated from mean bulk and tapped densities
3.5.5.1.2. Pre-compression evaluation of APIs blend formulations prepared by wet granulation method using de-ionized water

Results showed that performing wet granulation by de-ionized water, bulk was reduced along with enhanced flow properties. Almost all formulations possessed desired flowability confirmed by measuring indices. Data is presented in table 3.34 showing Carr’s index and Hausner ratios in range of 8.18 to 15.58 and 1.10 to 1.24 respectively. The angles of repose of all the formulations were less than 33.64°. Excipients nature in each formulation produced small variations in bulk and tapped densities. However addition of excipient has positive role in acquiring the optimum flow property by effecting granule hardness and maintenance of their integrity.
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>F1 (mean ± SD)</th>
<th>F2 (mean ± SD)</th>
<th>F3 (mean ± SD)</th>
<th>F4 (mean ± SD)</th>
<th>F5 (mean ± SD)</th>
<th>F6 (mean ± SD)</th>
<th>F7 (mean ± SD)</th>
<th>F8 (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk Volume (ml)</td>
<td>15.48 ± 0.76</td>
<td>15.69 ± 0.65</td>
<td>15.33 ± 0.90</td>
<td>16.04 ± 0.28</td>
<td>14.50 ± 0.53</td>
<td>15.54 ± 0.63</td>
<td>14.70 ± 0.45</td>
<td>15.66 ± 1.13</td>
</tr>
<tr>
<td>Tapped Volume (ml)</td>
<td>13.06 ± 0.50</td>
<td>14.02 ± 0.43</td>
<td>13.20 ± 0.38</td>
<td>14.79 ± 0.83</td>
<td>12.56 ± 0.20</td>
<td>14.02 ± 0.87</td>
<td>12.80 ± 0.37</td>
<td>13.34 ± 0.75</td>
</tr>
<tr>
<td>Bulk Density (gm/ml)</td>
<td>0.65 ± 0.04</td>
<td>0.64 ± 0.03</td>
<td>0.65 ± 0.04</td>
<td>0.62 ± 0.03</td>
<td>0.69 ± 0.04</td>
<td>0.64 ± 0.05</td>
<td>0.68 ± 0.03</td>
<td>0.64 ± 0.04</td>
</tr>
<tr>
<td>Tapped Density (gm/ml)</td>
<td>0.77 ± 0.05</td>
<td>0.71 ± 0.03</td>
<td>0.76 ± 0.04</td>
<td>0.68 ± 0.05</td>
<td>0.80 ± 0.05</td>
<td>0.71 ± 0.04</td>
<td>0.78 ± 0.05</td>
<td>0.75 ± 0.03</td>
</tr>
<tr>
<td>*Hausner Ratio</td>
<td>1.18</td>
<td>1.11</td>
<td>1.12</td>
<td>1.10</td>
<td>1.16</td>
<td>1.11</td>
<td>1.15</td>
<td>1.17</td>
</tr>
<tr>
<td>Flow Property</td>
<td>Good</td>
<td>Excellent</td>
<td>Good</td>
<td>Excellent</td>
<td>Good</td>
<td>Excellent</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>Angle of Repose (*)</td>
<td>33.64 ± 1.01</td>
<td>28.32 ± 0.53</td>
<td>33.50 ± 2.02</td>
<td>27.93 ± 0.65</td>
<td>31.59 ± 1.15</td>
<td>29.45 ± 1.05</td>
<td>33.62 ± 0.40</td>
<td>32.43 ± 1.04</td>
</tr>
</tbody>
</table>

*Calculated from mean bulk and tapped densities.
3.5.5.1.3. Pre-compression evaluation of APIs blend formulations prepared by wet granulation method using aqueous 2% PVP-K30

Results showed that using aqueous 2% PVP-K30 enhanced granulation flowability in all formulations. The resulting granules were slight harder than the other two techniques mentioned before, providing a good flow effect to the granules. The Carr’s index of all the formulations was in good to excellent range 8.11-12.99 and Hausner ratio was in 1.09-1.15 range. Angle of repose was from 28.02° ± 0.30 to 32.50° ± 1.03 signifying optimum flowability characters as shown in table 3.35.
### Table 3.35: Physical properties of pre-compressed formulations of APIs prepared by wet granulation method using 2% PKP-K30

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>F1 (mean ± SD)</th>
<th>F2 (mean ± SD)</th>
<th>F3 (mean ± SD)</th>
<th>F4 (mean ± SD)</th>
<th>F5 (mean ± SD)</th>
<th>F6 (mean ± SD)</th>
<th>F7 (mean ± SD)</th>
<th>F8 (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk Volume (ml)</td>
<td>14.90 ± 0.45</td>
<td>15.50 ± 0.28</td>
<td>15.43 ± 0.65</td>
<td>15.34 ± 0.44</td>
<td>14.80 ± 0.50</td>
<td>15.87 ± 0.39</td>
<td>14.85 ± 0.30</td>
<td>15.26 ± 0.93</td>
</tr>
<tr>
<td>Tapped Volume (ml)</td>
<td>13.05 ± 0.52</td>
<td>14.12 ± 0.40</td>
<td>14.03 ± 0.34</td>
<td>13.79 ± 0.80</td>
<td>13.56 ± 0.34</td>
<td>14.22 ± 0.66</td>
<td>13.20 ± 0.55</td>
<td>13.80 ± 0.73</td>
</tr>
<tr>
<td>Bulk Density (gm/ml)</td>
<td>0.67 ± 0.03</td>
<td>0.65 ± 0.03</td>
<td>0.65 ± 0.04</td>
<td>0.65 ± 0.05</td>
<td>0.68 ± 0.04</td>
<td>0.63 ± 0.05</td>
<td>0.67 ± 0.03</td>
<td>0.66 ± 0.04</td>
</tr>
<tr>
<td>Tapped Density (gm/ml)</td>
<td>0.77 ± 0.05</td>
<td>0.71 ± 0.04</td>
<td>0.71 ± 0.05</td>
<td>0.73 ± 0.04</td>
<td>0.74 ± 0.04</td>
<td>0.70 ± 0.05</td>
<td>0.76 ± 0.04</td>
<td>0.72 ± 0.03</td>
</tr>
<tr>
<td>*Carr’s Index</td>
<td>12.99</td>
<td>8.45</td>
<td>8.45</td>
<td>10.96</td>
<td>8.11</td>
<td>10.0</td>
<td>11.84</td>
<td>8.33</td>
</tr>
<tr>
<td>*Hausner Ratio</td>
<td>1.15</td>
<td>1.09</td>
<td>1.09</td>
<td>1.12</td>
<td>1.09</td>
<td>1.11</td>
<td>1.13</td>
<td>1.09</td>
</tr>
<tr>
<td>Flow Property</td>
<td>Good</td>
<td>Excellent</td>
<td>Excellent</td>
<td>Good</td>
<td>Excellent</td>
<td>Excellent</td>
<td>Good</td>
<td>Excellent</td>
</tr>
<tr>
<td>Angle of Repose (º)</td>
<td>32.50 ± 1.03</td>
<td>28.02 ± 0.30</td>
<td>28.50 ± 1.25</td>
<td>31.66 ± 0.60</td>
<td>29.09 ± 1.02</td>
<td>29.20 ± 0.85</td>
<td>32.40 ± 0.48</td>
<td>26.30 ± 0.64</td>
</tr>
</tbody>
</table>

*Calculated from mean bulk and tapped densities.
3.5.5.1.2. Post compression studies of formulations

Physical characteristics that were evaluated of compressed tablet dosage forms were categorized in the following sub sections:

- Physical and mechanical characteristics including tablet thickness, weight variation, friability and crushing strength (tablet hardness)\(^{404}\).
- Tablet disintegration time test.
- *In-vitro* drug release profiling.

3.5.5.1.2.1. Evaluation of tablet dosage form formulated by dry granulation method

The total theoretical weight of all the formulations was 880 mg, in which ingredients were added in percentage manner.

3.5.5.1.2.1.1. Physical and mechanical properties of formulations prepared by dry granulation method

Results showed that percent weight variations and crushing strength were within acceptable limits and were in range of 2.30 to 3.25\% and 5.38-7.04 kg respectively. The physical appearance of the prepared tablets was brown shiny surfaces which increased with compression force. Taking care of friability the compression force of 4-7 kg was selected for all the formulations. The tablet friability and thickness of all formulations were in range of 0.34-0.55 and 5.72-6.02 mm including the convex curved surfaces respectively as shown in table 3.36.
### Table 3.36: Physical and mechanical properties of formulations prepared by dry granulation method

<table>
<thead>
<tr>
<th>Formulation</th>
<th>† Tablet Thickness (mm)</th>
<th>Weight Variation (%)</th>
<th>Friability (%)</th>
<th>† Crushing Strength (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5.91 ± 0.24</td>
<td>± 2.30</td>
<td>0.42</td>
<td>6.77 ± 0.59</td>
</tr>
<tr>
<td>F2</td>
<td>6.02 ± 0.18</td>
<td>± 2.57</td>
<td>0.55</td>
<td>5.38 ± 0.70</td>
</tr>
<tr>
<td>F3</td>
<td>5.72 ± 0.20</td>
<td>± 3.16</td>
<td>0.36</td>
<td>7.04 ± 0.35</td>
</tr>
<tr>
<td>F4</td>
<td>5.87 ± 0.21</td>
<td>± 3.70</td>
<td>0.40</td>
<td>6.38 ± 0.83</td>
</tr>
<tr>
<td>F5</td>
<td>5.76 ± 0.32</td>
<td>± 2.66</td>
<td>0.37</td>
<td>6.94 ± 0.55</td>
</tr>
<tr>
<td>F6</td>
<td>5.95 ± 0.15</td>
<td>± 3.25</td>
<td>0.51</td>
<td>5.48 ± 0.45</td>
</tr>
<tr>
<td>F7</td>
<td>5.81 ± 0.26</td>
<td>± 2.89</td>
<td>0.34</td>
<td>6.60 ± 0.42</td>
</tr>
<tr>
<td>F8</td>
<td>5.83 ± 0.30</td>
<td>± 2.50</td>
<td>0.38</td>
<td>6.82 ± 0.38</td>
</tr>
</tbody>
</table>

† Data presented as mean ± SD (n=10).

#### 3.5.5.1.2.1.2. Disintegration behavior evaluation of tablets prepared by dry granulation method

Disintegration time (DT) test was performed on n= 12 at 37 ± 2 °C. Results showed variations based on excipients added. As per standards for uncoated tablets; only F1 and F2 were failed to disintegrate completely within time limit, however F3, F6 and F8 showed higher DT (13 minutes average) as shown in figure 3.34.
3.5.5.1.2.1.3. In-vitro dissolution profile of formulations prepared by dry granulation method

Drug release profile from formulations were performed according to British Pharmacopoeia 2009\textsuperscript{405}, using 900 ml of 0.1 N HCl using apparatus II (paddle method) operated at 60 rpm at 37 ± 2 °C. Dissolution test was performed on F3-F8 formulations. Drug release was increased with the quantities of micro crystalline cellulose and cross carmellose sodium. Using cross carmellose sodium with sodium starch glycolate further increased the release rate confirmed from initial release of OPC 68.34%, NA 69.09% and PT 57.42% in first 20 minutes. Sodium starch glycolate alone showed less effect (OPC 60.20%, NA 58.43%, PT 42.19%). Overall drugs release after 60 minutes from all formulations was above 80% with some variations as shown in figure 3.35.
Figure 3.35: *In-vitro* drug release profile from tablet dosage form prepared by dry compression method.
3.5.5.1.2.2. Evaluation of tablet dosage form formulated by wet granulation method using de-ionized water

The weight variation of all the formulations were in 2.25-3.09% range of 880 mg tablet weight.

3.5.5.1.2.2.1. Physical and mechanical properties of formulations prepared by wet granulation method using de-ionized water

Using water for wet granulation resulted in harder granules with reduced bulk compared to dry granulation and so as the tablet thickness. The crushing force was in range of 4.69-6.68 kg and friability was 0.20-0.51%. Thickness was within 5.53 ± 0.21-5.95 ± 0.25 mm range at selected compression force (4-7 kg) as shown in table 3.37.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>† Tablet Thickness (mm)</th>
<th>Weight Variation (%)</th>
<th>Friability (%)</th>
<th>† Crushing Strength (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5.80 ± 0.21</td>
<td>± 2.28</td>
<td>0.45</td>
<td>5.58 ± 0.52</td>
</tr>
<tr>
<td>F2</td>
<td>5.95 ± 0.25</td>
<td>± 2.44</td>
<td>0.28</td>
<td>6.68 ± 0.68</td>
</tr>
<tr>
<td>F3</td>
<td>5.68 ± 0.20</td>
<td>± 2.57</td>
<td>0.33</td>
<td>6.32 ± 0.44</td>
</tr>
<tr>
<td>F4</td>
<td>5.88 ± 0.17</td>
<td>± 3.20</td>
<td>0.39</td>
<td>5.88 ± 0.63</td>
</tr>
<tr>
<td>F5</td>
<td>5.57 ± 0.31</td>
<td>± 3.06</td>
<td>0.34</td>
<td>6.36 ± 0.49</td>
</tr>
<tr>
<td>F6</td>
<td>5.53 ± 0.21</td>
<td>± 2.75</td>
<td>0.20</td>
<td>6.68 ± 0.73</td>
</tr>
<tr>
<td>F7</td>
<td>5.70 ± 0.27</td>
<td>± 3.09</td>
<td>0.39</td>
<td>5.70 ± 0.72</td>
</tr>
<tr>
<td>F8</td>
<td>5.62 ± 0.25</td>
<td>± 2.25</td>
<td>0.51</td>
<td>4.69 ± 0.55</td>
</tr>
</tbody>
</table>

† Data presented as mean ± SD (n=10).
3.5.5.1.2.2.2. Disintegration behavior evaluation of tablets prepared by wet granulation method using de-ionized water

Granulation with de-ionized water reduced DT in all formulations ranging 7.1-12.15 min. DT of Tablettose-80 containing formulations (F1 & F2) were also within limits i.e. 12.15 ± 1.03 and 11.15 ± 1.23 respectively. The minimum DT was observed with formulation F7 of 7.1 minutes due to cross carmellose sodium and sodium starch glycolate combination that provided strong wicking, expulsion and tablet breaking effect as shown in figure 3.36.

![Disintegration time data of formulations prepared by wet granulation method using de-ionized water.](image)

**Figure 3.36:** Disintegration time data of formulations prepared by wet granulation method using de-ionized water.
3.5.5.1.2.2.3. *In-vitro* dissolution profile of formulations prepared by wet granulation method using de-ionized water

Drug release was found maximum in formulations F2- F4, F6 and F7 ≥ 90%, as compared to other formulations. Release of APIs in first 20 minutes was above 50% in all formulations, indicating the fast dispersibility effect of disintegrants added both pre and post granulation. Increasing MCC and cross carmellose sodium percentage in pregranulation step enhanced dissolution rate in F4 and F6 up to 70% of each drug compared to F3 (64% each drug) and F5 (68, 69, 66%) respectively. Despite of hard tablets the dissolution rate of all the formulations were within standard limits as shown in figure 3.37.
Figure 3.37: *In-vitro* drug release profile from tablet dosage form prepared by wet granulation method using de-ionized water.
3.5.5.1.2.3. Evaluation of tablet dosage form formulated by wet granulation method using aqueous 2% PVP –K30

Weight of compressed tablets prepared showed fewer variations up to 3.23% of theoretical weight 880 mg.

3.5.5.1.2.3.1. Physical and mechanical properties of formulations prepared by wet granulation method using aqueous 2% PVP-K30

Results showed controlled friability 0.14-0.31% since slight harder granules are compressed into tablets with the crushing strength of 5.72-6.54 kg. Tablet thickness was in similar to tablets prepared by de-ionized water and was in range of 5.55 ± 0.24 to 5.90 ± 0.27 mm. The physical and mechanical properties of tablets are shown in table 3.38.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>† Tablet Thickness (mm)</th>
<th>Weight Variation (%)</th>
<th>Friability (%)</th>
<th>† Crushing Strength (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5.73 ± 0.21</td>
<td>± 2.28</td>
<td>0.27</td>
<td>5.78 ± 0.47</td>
</tr>
<tr>
<td>F2</td>
<td>5.90 ± 0.27</td>
<td>± 2.35</td>
<td>0.19</td>
<td>6.25 ± 0.58</td>
</tr>
<tr>
<td>F3</td>
<td>5.65 ± 0.22</td>
<td>± 3.23</td>
<td>0.28</td>
<td>5.72 ± 0.49</td>
</tr>
<tr>
<td>F4</td>
<td>5.57 ± 0.21</td>
<td>± 1.89</td>
<td>0.20</td>
<td>5.95 ± 0.23</td>
</tr>
<tr>
<td>F5</td>
<td>5.75 ± 0.18</td>
<td>± 3.16</td>
<td>0.14</td>
<td>6.54 ± 0.41</td>
</tr>
<tr>
<td>F6</td>
<td>5.50 ± 0.18</td>
<td>± 3.10</td>
<td>0.31</td>
<td>5.73 ± 0.54</td>
</tr>
<tr>
<td>F7</td>
<td>5.68 ± 0.22</td>
<td>± 2.69</td>
<td>0.22</td>
<td>5.95 ± 0.45</td>
</tr>
<tr>
<td>F8</td>
<td>5.55 ± 0.24</td>
<td>± 1.95</td>
<td>0.21</td>
<td>6.25 ± 0.63</td>
</tr>
</tbody>
</table>

† Data presented as mean ± SD (n= 10).
3.5.5.1.2.3.2. Disintegration behavior evaluation of tablets prepared by wet granulation method using 2% PVP-K30

Granulation with 2% PVP-K30 produced harder granules with small voids between granules in compressed form resulted in quick wetting and disintegration. Overall the DT time was in range of 5.8-12 minutes with major effect observed in F7 (5.8 minutes) as compared to compressed batch prepared by deionized water (7.1 minutes in F7). Negligible variations were observed in F1, F2, F4 and F8. Application of blend disintegrants produced synergistic effects in reducing DT. The DT graph of F1-F8 formulations is given in figure 3.38.

![Disintegration Time Data](image)

**Figure 3.38:** Disintegration time data of formulations prepared by wet granulation method using 2% PVP-K30.
3.5.5.1.2.3.3. *In-vitro* dissolution profile of formulations prepared by wet granulation method using aqueous 2% PVP-K30

Results showed that granulation with 2% PVP-K30 enhanced dissolution rate by acting as solubility enhancer $^{407}$. The release rates of APIs were between 60-80% in initial 20 minutes which is much higher compared to de-ionized water based formulations as shown in figure 3.39. Addition of PVP-K30 enhanced total drug release $\geq 90\%$ in all formulations and addition of disintegrants in pregranulation step provided synergistic effect in dissolution fluid diffusion.
Figure 3.39: *In-vitro* dissolution profile of formulations prepared by wet granulation method using 2% PVP-K30.
3.5.6. Selection of optimum formulation(s)

Optimized formulations were selected on the basis of highest CSFR/DT ratio. Formulations F7 (A), F6 (B), and F7 (C) were selected as optimum formulations with CSFR/DT ratios of 1.76, 3.77 and 4.69 respectively as shown in table 3.39. PVP-K30 was evaluated previously for enhancing dissolution rate in-vitro and confirmed that it enhances the dissolution rate of poor water soluble drugs. Similar effects were observed in our experiments performed on blended drug formulations. It is evident from the results that polymers like PVP-K30 should be incorporated in formulations containing lipophilic drugs and for quick drug release.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Dry granulation</th>
<th>Wet granulation by de-ionized</th>
<th>Wet granulation by 2% PVP-K30 aqueous solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>Failed</td>
<td>1.02</td>
<td>1.78</td>
</tr>
<tr>
<td>F2</td>
<td>Failed</td>
<td>2.14</td>
<td>2.95</td>
</tr>
<tr>
<td>F3</td>
<td>1.59</td>
<td>1.62</td>
<td>1.87</td>
</tr>
<tr>
<td>F4</td>
<td>1.38</td>
<td>1.53</td>
<td>3.13</td>
</tr>
<tr>
<td>F5</td>
<td>1.66</td>
<td>1.66</td>
<td>4.47</td>
</tr>
<tr>
<td>F6</td>
<td>0.83</td>
<td><strong>3.77</strong></td>
<td>2.26</td>
</tr>
<tr>
<td>F7</td>
<td><strong>1.76</strong></td>
<td>2.06</td>
<td><strong>4.69</strong></td>
</tr>
<tr>
<td>F8</td>
<td>1.38</td>
<td>1.05</td>
<td>3.39</td>
</tr>
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</table>
3.5.7. Stability studies of optimized formulations

The selected optimized formulations showed optimum stabilities in 3 months, with mild increase in DT in two formulations however within limits. The friability, DT and degradation half life ($t_{1/2}$) of antioxidant blends in these formulations are mentioned in percent and mean ± SD in the table 3.40. From the degradation half life of each drug it can be concluded that drug excipient mixtures used in current formulation studies are stable even at accelerated conditions of temperature and relative humidity.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Friability (%)</th>
<th>Disintegration (min) (mean ± SD)</th>
<th>Degradation half life ($t_{1/2}$) (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>OPC (mean ± SD)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>NA (mean ± SD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PT (mean ± SD)</td>
</tr>
<tr>
<td>F7 (dry granulation)</td>
<td>0.67</td>
<td>11.6 ± 0.86</td>
<td>98.11 ± 1.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37.72 ± 2.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>81.19 ± 2.38</td>
</tr>
<tr>
<td>F6 (wet granulation)</td>
<td>0.54</td>
<td>8.50 ± 1.03</td>
<td>92.42 ± 1.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>38.25 ± 1.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>68.96 ± 3.15</td>
</tr>
<tr>
<td>F7 (2% PVP-K30)</td>
<td>0.50</td>
<td>6.38 ± 0.79</td>
<td>94.35 ± 2.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>34.66 ± 2.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>76.67 ± 2.53</td>
</tr>
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</table>

3.5.8. Discussion

Immediate release formulations are suitable for antioxidants because for pharmacological response, they should be administered in sufficient doses until and unless modifications are made in their molecular structure. Despite of using them in combinations in which the dose of each candidate is reduced compared to its individual dose, still the total dosage form weight is high enough to be incorporated in modified release dosage form. This co-administration will reduce the adverse effects such as in case of niacin facial flushing and hepatic related effects which is most common with higher doses. Studies on using superdisintegrants individually and in combinations reported their effectiveness in tablet disintegration and drug release especially in case of
antioxidants and other natural products \(^{409-411}\). Insufficient permeability was observed with some polymeric natural compounds preventing fluid entrance towards tablet inner core and may affect disintegration \(^{412-413}\). Several formulations are available not only for lipid lowering but for other chronic conditions in which antioxidants are blended in matrix solid dosage form(s) \(^{301,305,413-414}\). Moreover in most reported formulations either the doses of antioxidant were kept high enough or recommended in high dose up to 2000 mg/day. Such formulations if used for longer time may produce side effects and or may peak above the ceiling line causing system saturation, wastage of drug and excessive therapy cost. In our study we selected 100 mg/kg animal dose which in human equivalent dose is equal to 1500 mg per body weight. We further evaluated the lipid lowering effects by incorporating three antioxidants in aforementioned doses. This quantity can be easily incorporated in solid and liquid dosage form. In solid dosage form conventional tablet, effervescent granules, minitabs and capsules are very suitable for incorporating antioxidants. Liquids may face stability problems. Our formulations offer three antioxidants in such dose rationale that can produce comparable effects on lipid profile and blood endogenous antioxidants in humans to that produced in animal studies.

Stability study in terms of degradation half life is an effective tool determining the durability of finished dosage form \(^{336}\). Stability checking under accelerated conditions is necessary to observe the effects of formulation steps and excipients used on the drug stability \(^{415}\). The results of finished dosage form were found parallel with the stability results of drug-excipient binary admixtures performed in the very first step of our study.
Chapter 4

CONCLUSION
4. CONCLUSION

Current work was carried out to evaluate the effects of natural antioxidants in combination therapies on lipid profile and blood antioxidants in hyperlipidemia. Various bio-parameters were observed in normal, disease control and treated animal groups. Moreover the blend delivered best results was formulated into tablet dosage forms. Various analytical techniques were carried out on UV/visible spectrophotometer and HPLC-UV-ECD in pursue of findings. Our lab findings showed that, with the rise of blood lipid profiles there was equal rise of endogenous antioxidants in initial cholesterol dosing. This is because of over activation of antioxidant producing enzymes which declined to the bottom line in disease control. Natural antioxidant treatments were able to control the depletion of blood antioxidants by preventing rise in blood lipid levels and scavenging free radicals. The lower antioxidant levels and higher peroxidative biomarker in disease group with respect to normal group provided evidence of hyperlipidemic associated oxidative stress which can propagate towards other pathological anomalies.

Antioxidant interventions should be incorporated while dealing with chronic diseases and their beneficial effects can be maximized if used in proper dose rationale. It is evident from the current study that their combinations can provide synergistic effects in maintaining the redox state. Cocktail of dietary antioxidants including polyphenols, vitamins or the nutriments enriched of it should be added to the daily diet to prevent the initiation of oxidative stress.
REFERENCES
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<table>
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<tr>
<th>Reference</th>
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<td>Nutrition reviews 2012, 70, 257.</td>
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<td>(45)</td>
<td>Manea, A.</td>
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<td>Catala, A.</td>
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APPENDIX
FTIR spectra of various admixtures

Sample 1

Sample 2
Sample 3

Sample 4
Sample 7