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# Curcumin Attenuates The Effects Of Atherogenic Diet In Aged Male Brown Norway Rats

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**CURCUMIN ATTENUATES THE EFFECTS OF ATHEROGENIC DIET IN  
AGED MALE BROWN NORWAY RATS**

by

**VINDHYAJA SRIRAJAVATSAVAI**

**THESIS**

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

**MASTER OF SCIENCE**

2015

MAJOR: NUTRITION AND FOOD SCIENCE

Approved By:

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Advisor

Date

## **DEDICATION**

I dedicate this thesis to my family and friend's whose support has got me through all ups and downs in my life. A special feeling of gratitude to my loving parents and husband. They have built me up and given me the confidence to achieve my dreams.

## ACKNOWLEDGEMENTS

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## **CHAPTER 1: Introduction**

### **1.1 Cardiovascular Disease**

Cardiovascular disease (CVD) is one of the most common causes of morbidity and mortality worldwide. Its incidence is increasing globally as western lifestyles are adopted and populations age.[1] The World Health Organization (WHO) has predicted an increase in prevalence of CVD, with projected combined death toll of 24 million by 2030.

An increased blood cholesterol level, which is governed by cholesterol absorption, synthesis, storage, and excretion, is one of the major risk factors leading to the development of CVD.[2] It is a disease of the vascular intima, in which all of the vascular system from the aorta to coronary arteries can be involved and is characterized by intimal plaques.[3] Currently, it is a common disease in which fatty deposits called atheromatous plaques appear in the inner layers of arteries.[3] The process is characterized, in its earliest stages, by perturbations in endothelial function. Atherosclerosis is likely initiated when endothelial cells over express adhesion molecules in response to turbulent flow in the setting of an unfavorable blood lipid profile. Increased cellular adhesion and the associated endothelial dysfunction then “sets the stage” for the recruitment of inflammatory cells, release of cytokines and recruitment of lipid into the plaque.[4]

Proinflammatory biomarkers, shear stress and apolipoprotein-B sub-endothelial accumulation in the artery wall have a significant contribution to atherosclerosis development. Among these factors, cholesterol plays a major role in CVD.[5] Formation of plaques starts with the deposition of small cholesterol crystals in the intima and its underlying smooth muscle. Then

the plaques grow with the proliferation of fibrous tissues and the surrounding smooth muscle and bulge inside the arteries and consequently reduce the blood flow. Connective tissue production by fibroblasts and deposition of calcium in the lesion cause sclerosis or hardening of the arteries. Finally, the uneven surface of the arteries results in clot formation and thrombosis, which leads to the sudden obstruction of blood flow.[3] The cholesterol and fatty acid burden to the atherosclerotic plaques, contribute to the formation and rupture of these plaques.[5]

### **1.1.1 Hyperlipidemia:**

Hyperlipidaemia, or more accurately dyslipidaemia, is a major risk factor for CVD and may account for up to 55% of age and gender-independent risk. The dyslipidaemia component that accounts for the epidemiological risk is the ratio of total cholesterol to high density lipoprotein-cholesterol (HDL-C), or alternatively the ratio of apolipoprotein B: A-1 concentrations, which identifies the fraction of lipid particles depositing cholesterol in the vascular wall compared to those removing it. At its simplest, this process can be expressed as non-HDL-C (difference of total and HDL-C) vs HDL-C.[1]

Atherosclerotic lesions contain large amounts of cholesterol, cholesteryl esters, and cholesterol crystals. It is well established that high blood cholesterol levels are linked to the pathogenesis of atherosclerosis. Furthermore, atherosclerosis-like vascular lesions can be experimentally induced in animals fed a high-cholesterol diet. On the contrary, lowering levels of serum cholesterol slows down atherogenesis, which can cause plaque regression and reduces the overall risk of cardiovascular events. In addition to excessive amounts of lipids, atherosclerotic lesions harbor all classes of immune cells and serum levels of acute-phase reactants and inflammatory mediators which are linked to the risk of coronary heart disease.[6]

The plasma level of cholesterol is determined by genetic factors, by the type and amount of fat in the diet, and by other factors such as obesity, physical activity, and disease states. Based on the results of animal studies, epidemiologic data, and interventional studies, there is good evidence for an association between hypercholesterolemia and CVD. The association between serum cholesterol levels and the risk of coronary heart disease is continuous. Familial hypercholesterolemia, a disorder caused by an absent or defective LDL receptor, causes premature coronary heart disease. Reduced levels of HDL cholesterol are associated with an increased risk of coronary heart disease. Both the cholesterol level and the prevalence of coronary heart disease are influenced by environmental factors, including diet. The evidence that decreasing serum cholesterol levels with cholesterol-lowering drugs or dietary modification slows or reverses the progression of coronary atherosclerosis and reduces coronary events comes from many randomized trials that include more than 40,000 subjects. Lowering the cholesterol level with diet or drug therapy also slows the progression of angiographically documented coronary atherosclerosis in patients with arterial bypass grafts.[4] Hyperlipidemia and hyperglycemia are related to increased oxidative damage, which affects antioxidant status and lipoprotein levels. Studies have shown that lipid lowering medicinal herbs can reduce the blood lipids especially after meals in addition to their antioxidant effects. Therefore, they can prevent atherosclerosis and vascular endothelium damage.[3] Modifying several risk factors, such as lowering the serum cholesterol level, the blood pressure, and the levels of LDL cholesterol and by cessation of smoking, reduces the risk of ischemic heart disease. Individuals with several risk factors benefit most from these measures. [4]

### **1.1.2 Genetics:**

Apolipoprotein A-I is the major protein constituent of human high density lipoproteins (HDLs), which play a key role in reverse cholesterol transport (RCT), shuttling excess of cholesterol from the circulation to the liver for catabolism. Even though only 5% of the total circulating apoA-I is found in lipid-free or lipid-poor forms, it is thought that the highly dynamic catabolism of HDL yields this protein conformation which subsequently acquires lipids, enhancing cholesterol removal.[7]

Serum levels of HDL and its major protein apoAI are associated with decreased coronary heart disease (CHD) rates . Anti-atherogenic properties of HDL and apoAI are attributed to their reverse cholesterol transport capacity and anti-inflammatory effects. HDL and apoAI drive cholesterol efflux from peripheral cells in general and macrophages in particular, thus preventing foam cell formation and reducing inflammation. Reduced capacity to efflux cholesterol from macrophages by HDL in serum is a predictor for atherosclerosis in mice and humans. ApoA1 is critical for the unloading of cholesterol from macrophages, with pathologic and clinical consequences like atherosclerosis. Both endogenous and exogenous apoAI were shown to stimulate the secretion of macrophage apoE, another physiologic driver of cholesterol efflux. Removal of cholesterol from the aortic wall was mediated through increasing local apoAI concentration and upregulation of cellular cholesterol transporters. Lipid-free apoAI, HDL-bound apoAI, and apoE act in synergy to extract cholesterol from macrophages, thus influencing macrophage cholesterol accumulation, a hallmark of CVD.[8]

### **1.1.3 Oxidation:**

Oxidative stress is defined as the presence of active oxygen species in excess of the available antioxidant buffering capacity. Oxidative stress is well known to be involved in the

pathogenesis of lifestyle-related diseases, including hypertension, diabetes mellitus, atherosclerosis, ischemic diseases, malignancies, or Alzheimer disease, Parkinson's disease, and amyotrophic lateral sclerosis.[9] Oxidative stress has been identified as critical in most of the key steps in the pathophysiology of atherosclerosis and acute thrombotic events, including dyslipidemia leading to atheroma formation, the oxidation of LDL, endothelial dysfunction, plaque rupture, myocardial ischemic injury, and recurrent thrombosis. The role of oxidative stress in the connection between the various coronary disease risk factors such as elevated blood pressure, diabetes and cigarette smoking, and the clinical sequelae of disease associated with vasoconstriction, thrombosis, plaque rupture, and vascular remodeling has been recognized by Moreno and Fuster. Oxidative stress has been implicated as well in diabetic cardiomyopathy, congestive cardiomyopathy, and hypertensive heart disease. Pathological inflammation, a complex whole-cellular pathway, is a cascade that begins with the production of excess free radicals that frequently arise from mitochondria responding to internal or environmental stress and that trigger several signaling steps that end up producing the substances that actually cause the classical signs of redness, swelling, and pain in inflammation.[10]

One of the initial events in the development of atherosclerosis is the accumulation of cells containing excess lipids within the arterial wall. In addition, it has been demonstrated that increased intracellular generation of reactive oxygen species (ROS) plays an important role in chronic inflammatory responses to atherosclerosis. ROS are generated in aerobic organisms during physiological or physiopathological oxidative metabolism of mitochondria. ROS may react with a variety of biomolecules, including lipids, carbohydrates, proteins, nucleic acids, and macromolecules of connective tissue, thereby interfering with cell function. Under normal physiological conditions, there is a critical balance in the generation of oxygen free radicals and

antioxidant defense systems. Impairment in the oxidant/antioxidant equilibrium provokes a situation of oxidative stress and generally results from hyperproduction of ROS. Oxidative stress is known to be a component of molecular and cellular tissue damage mechanisms in a wide spectrum of human diseases. A lot of oxygenated compounds, particularly aldehydes such as malondialdehyde (MDA) and conjugated dienes, are produced during the attack of free radicals to membrane lipoproteins and polyunsaturated fatty acids. A lot of studies have found that serum MDA are higher in subjects with hyperlipidemia and decrease following dietary supplementation with antioxidants. Similar observations have been reported in animal models of hyperlipidemia.[11]

Lipid peroxidation is a fundamental process in CVD.[12] Currently it is believed that oxidative stress modifies LDL and generates a negatively charged LDL that is supposed to be recognized by macrophage scavenger receptors, leading to lipid accumulation. Conversely Ox-HDL becomes ineffective in removing cholesterol from foam cell macrophages.[13] The important role of free radical oxidation of cellular components in CVD has been recognized since the proposal of the oxidative theory of atherogenesis. Lipids are susceptible targets of oxidation because of their molecular structure abundant with reactive double bonds.[14]

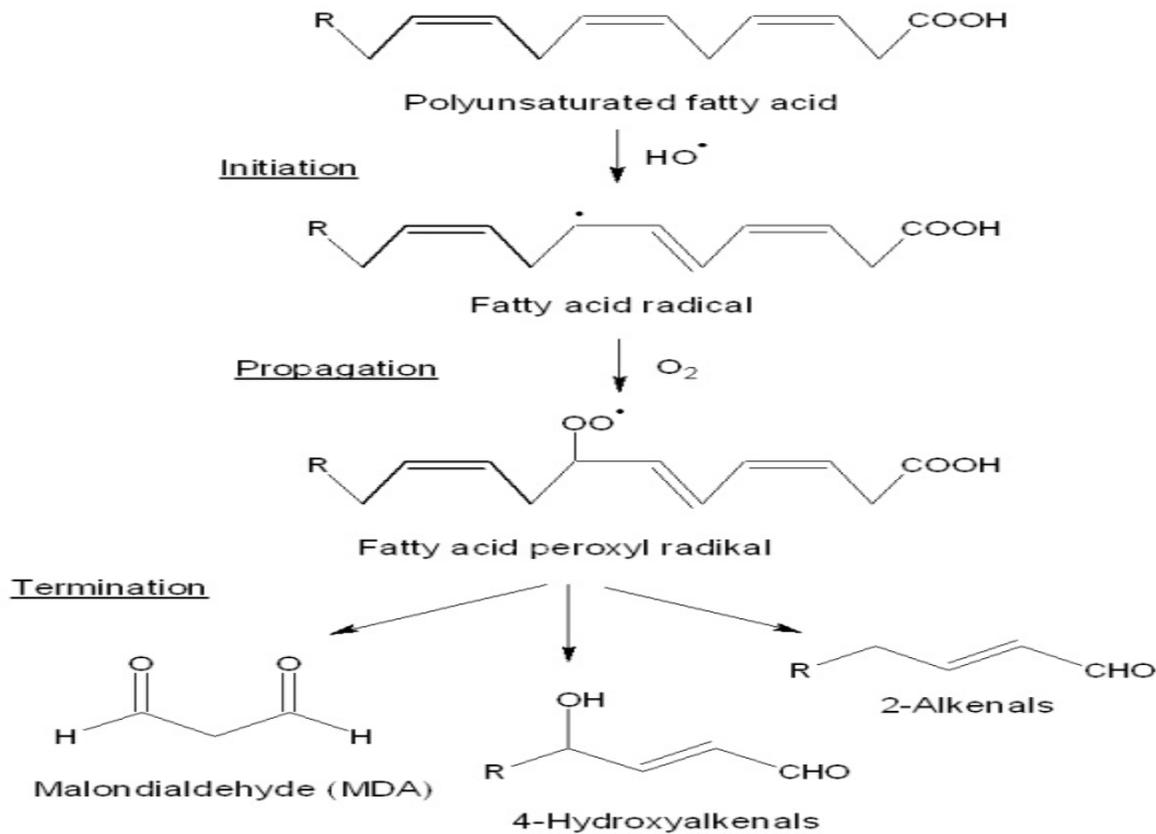


Fig.1. Process of lipid peroxidation[15]

Initially, a free radical takes a hydrogen atom from a poly unsaturated fatty acid. This initial free radical is most likely a lipid hydroperoxide or hydrogen peroxide. The PUFA, now a carbon-centered radical, reacts with oxygen to form a lipid peroxy radical. This lipid peroxy radical takes a hydrogen atom from a nearby PUFA, making a lipid hydroperoxide and another PUFA radical, thus continuing the cycle. These lipid hydroperoxides then decompose into aldehydes, hydrocarbon gases, epoxides, ketones, and alcohols with the help of transition metal ions. The aldehydes that are formed play an important role in oxidizing lipids.[16]

Ketones have been shown to be elevated in humans consuming high-fat diets. Alteration in diet stimulates lipolysis and production of ketone bodies, including 3-hydroxybutyrate which is most abundant in serum and urine.[17] When the accumulation of cholesterol and

phospholipids accelerates, the proportion of ketone bodies incorporated into these lipids increases.[18]

Ketone bodies are produced in the mitochondria of liver cells from acetyl-CoA derived from oxidation of fatty acids, particularly under conditions where high concentration of free fatty acids prevail.[19]

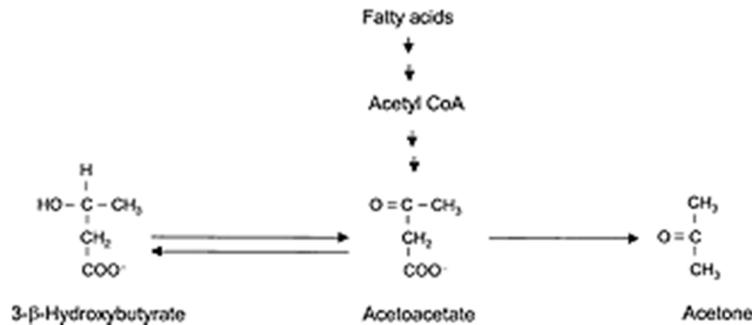


Fig. 2. Major ketone bodies structures[15]

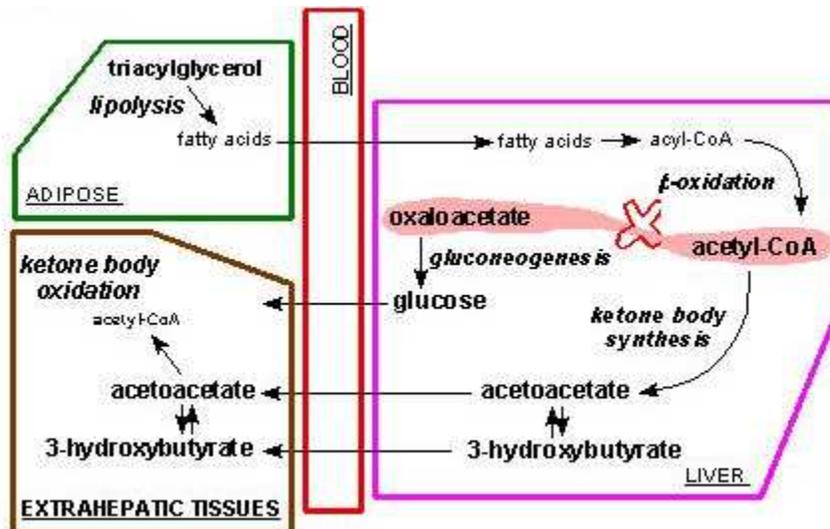


Fig. 3. Major ketone bodies production

Derangements of ketone body metabolism occur in numerous disease states, including types 1 and 2 diabetes and heart failure, and ketone body metabolism changes over the course of normal aging. Studies suggest a therapeutic role for antioxidants in protecting from oxidative

damage by ROS in the higher lipid period of the disease. Thus, in subjects with high risk for developing hyperlipidemia, treatment with antioxidants might reduce the peroxidation rate, restore the body's antioxidant capacity, and possibly prevent or delay development of this disease.[11]

#### **1.1.4 Inflammation:**

While the importance of inflammation in illnesses where the phenomenon is overt, such as following trauma or infection has been recognized since ancient times, its presence and crucial role in the manifestation of many diseases never previously recognized as inflammatory is relatively recent. In such instances, the source of the inflammation is also often imperceptible. This is especially relevant to the many pervasive chronic diseases that are still responsible for so much human suffering. We are currently achieving a major understanding of what is involved in the initiation of the inflammatory signaling cascade as well as the complex signaling pathways themselves that transcribe and counterregulate the molecular messengers (cytokines) that generate the biological combatants such as the inflammatory enzymes associated with the numerous relevant pathologies.[10]

Inflammation has been suggested to be a key mediator of many events during atherosclerosis development. [13] Inflammation resulting from oxidative stress is the cause of much human disease. [10] CVD can generally be viewed as a form of chronic inflammation that is induced and perturbed by lipid accumulation.[11] Proinflammatory cytokines are also involved in cardiac muscle dysfunction and in the complex syndrome of heart failure.[10] Inflammation has been suggested to be a key mediator of many events during atherosclerosis development.[13] It is widely accepted that the earliest stages of the development of CVD are mediated, in large part, by the inflammatory cascade. Expression of adhesion molecules increases recruitment of

monocytes and T-cells to sites of endothelial injury magnifying the inflammatory cascade by recruiting additional leukocytes, activating leukocytes in the media, and causing recruitment and proliferation of smooth muscle cells. In response to signals generated within the early plaque, monocytes adhere to the endothelium and then migrate through the endothelium and basement membrane by elaborating enzymes that degrade the connective tissue matrix. Recruited macrophages both release additional cytokines and begin to migrate through the endothelial surface into media of the vessel. This process is further enhanced by the local release of stimulating factors, which causes monocytic proliferation. Local activation of monocytes leads to both cytokine-mediated progression of atherosclerosis, and oxidation of low-density lipoprotein. Once initiated, many mediators of inflammation have been described to influence the development of the atherosclerotic plaque. Inflammatory mediators expressed by smooth cells within the atherosclerotic plaque include, but are not limited to, interleukin (IL)-1 $\beta$ , tumor necrosis factor (TNF) $\alpha$  and  $\beta$ , IL-6, M-CSF, MCP-1, IL-18 and CD-40L. The impact of these mediators is diverse and includes mitogenesis, intracellular matrix proliferation, angiogenesis and foam cell development.[4]

### **1.1.5 Age:**

The risk for CVD increases with age suggesting age-dependent mechanistic components.[20] CVD is classed as a disease of aging as increasing evidence indicates that aging is also an important risk factor and persists as an independent contributor when all other known factors are controlled.[21] Interestingly, the level of oxidative stress increases during aging and could be either due to increased production of reactive oxygen species (ROS) or reduced ability to scavenge them. Increased oxidative stress can cause damage to cellular structures and can

oxidize LDL to form atherogenic oxLDL, but it also can lead to dysregulation of signal transduction and gene expression.[20]

Premature or accelerated vascular aging can be promoted by cardiovascular risk factors. Vessel aging, even in the absence of atherosclerosis, leads to intimal and medial thickening as well as gradual loss of arterial elasticity, resulting in vascular stiffness. Aged vessels show a number of characteristic pathological processes, many of which are also seen in atherosclerosis. Aged vessels show alterations in matrix and cell composition, elevated expression of a number of proinflammatory molecules and increased uptake of plasma lipoproteins. These effects may be due to increased expression of leukocyte adhesion molecules on endothelial cells in aged vessels, which trigger the familiar processes of monocyte migration followed by increased uptake of atherogenic lipo-proteins with subsequent inflammation, key events that ultimately promote atherosclerosis. Aged ECs and vascular smooth muscle cells also show increased secretion of proinflammatory cytokines, resulting in persistent vascular inflammation. Thus, the effects of atherosclerosis are superimposed on normal aging of the underlying vessel.[21] Since age-associated atherosclerosis correlates with lower expression of antioxidant genes, restoring their expression and activity by curcumin or other phytochemicals or micronutrients could be a strategy to increase stress resistance in vascular cells of the elderly.[20]

Treatment for atherosclerosis includes medical therapy, the mechanical reversal of arterial stenosis, or artery bypass graft surgery. [6] Currently, statins that were first discovered as a natural metabolite in *Aspergillus terreus* are the most widely prescribed drug to lower plasma cholesterol levels. However, there is widespread interest in establishing alternative non-pharmacological ways to manage cholesterol based on natural dietary compounds, which may prove to be more effective for reducing CVD risk. [22]

## 1.2 CURCUMIN

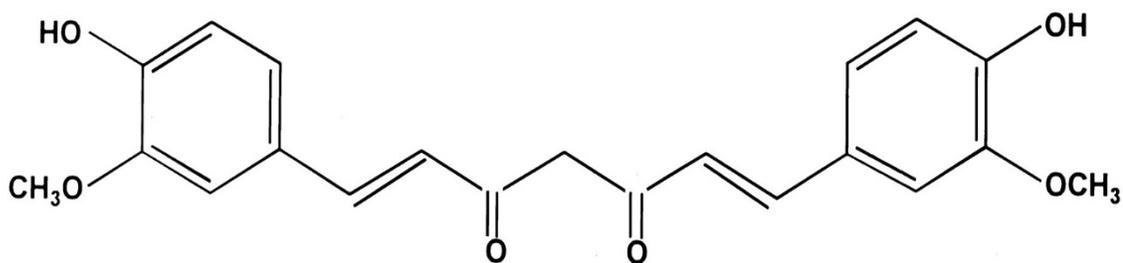
Diet plays an important role in modulating the level of oxidative stress, as certain components of diet (e.g., high glucose, fructose, and fat) have been associated with increased levels of ROS, whereas antioxidants can chemically neutralize and prevent their damages. Free fatty acids increase lipid-mediated oxidative and endoplasmic reticulum (ER) stress by various mechanisms. On the other hand, reducing the amount of food intake by, for example, caloric restriction has been linked to a lower level of oxidative stress and to an increased maximum lifespan. Thus, the composition, quantity, and quality of diet are all important for regulating the level of oxidative stress.[20]

Despite considerable efforts, the prevalences of complex multigenic human diseases such as cardiovascular diseases, metabolic diseases, cancer, and neurological diseases have not decreased significantly in recent years. A number of mono targeted drugs have emerged over the past decade; however, the aforementioned diseases are caused by perturbations of multiple signaling pathways. Thus, attacking only one of these multiple pathways is highly unlikely to be effective. In addition, such monotargeted drugs are often very expensive and can produce numerous adverse effects. These features of monotargeted drugs underscore the importance of multitargeted, inexpensive, and readily available dietary agents or nutraceuticals for the prevention and treatment of human diseases.

Curcumin is one such widely studied nutraceutical that was first purified about two centuries ago by Harvard College laboratory scientists Vogel and Pelletier from the rhizomes of *Curcuma longa* (turmeric).[23]

The therapeutic benefits of dietary constituents of plant origin have been the focus of many extensive studies. Curcumin is a pigment obtained from the rhizomes of *Curcuma*

longa.[24] The plant has a rhizome of bright orange color under a fine light brown cell layer. It is in common use as a spice in Asian cultures, where it is considered to be a magical plant because of its organoleptic properties and undoubted therapeutic and protective effects, especially for the skin and liver. Since ancient times, many properties have been ascribed to extracts of *Curcuma longa*. [12] Curcumin is also commonly used as a spice and a food-coloring agent.[24] The plant has been applied for the prevention and cure of skin and hepatic conditions and of ulcers and digestive disorders. It has also been used in the treatment of intestinal parasites and as a remedy for poisoning, snakebites, and various other complaints. [12]



### CURCUMIN

Fig.4. Chemical structure of curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione]. [25]

Curcumin is a highly pleiotropic molecule that was first shown to exhibit antibacterial activity in 1949. Since then, this polyphenol has been shown to possess anti-inflammatory, hypoglycemic, antioxidant, wound-healing, and antimicrobial activities. Extensive preclinical studies over the past three decades have indicated curcumin's therapeutic potential against a wide range of human diseases. In addition, curcumin has been shown to directly interact with numerous signaling molecules. [23]

In addition to a direct chemical action, recent studies suggest that phytochemicals and micronutrient can also prevent oxidative stress by influencing signal transduction and gene expression in non-antioxidant manners, for example by reducing the expression of enzymes

producing free radicals, by inducing the expression of enzymes scavenging free radicals or possibly as a secondary effect by influencing glucose and lipid homeostasis and thus reducing systemic glucose and lipid mediated oxidative and ER stress.[20] For diabetes, a limited number of studies indicate that curcumin is hypoglycemic and improves glucose tolerance. In a mouse model of diabetes, curcumin increased the expression of FOXO1/FOXO3a in white adipose tissue.[20] The capacity of curcumin to stabilize membranes has also been demonstrated.[12]

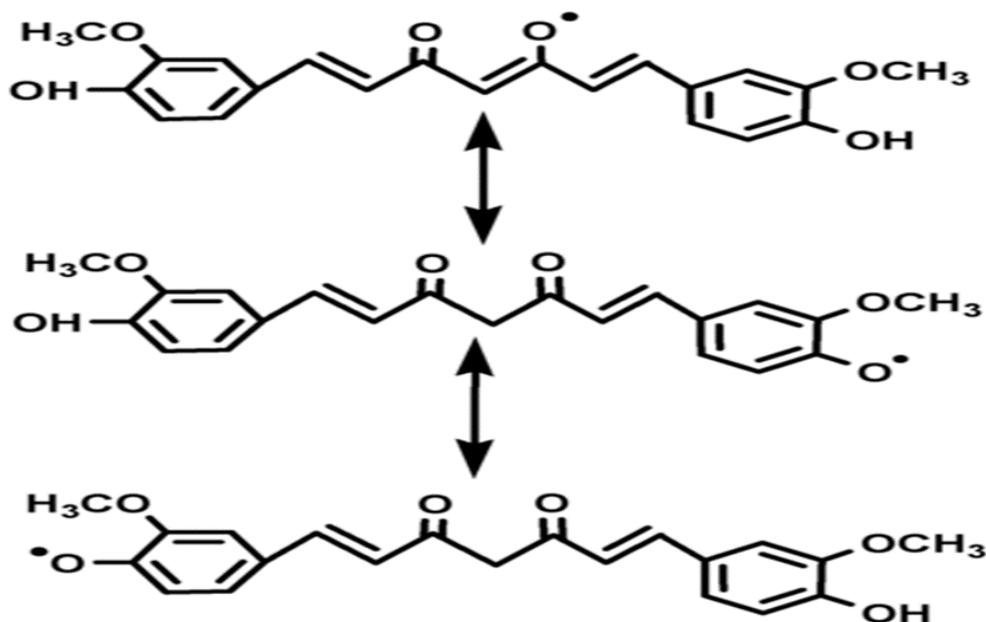


Fig.5 Possible sites of attack of free radical oxidants with curcumin[26]

Curcumin has been found to be an excellent scavenger of most ROS, a property that bestows curcumin with antioxidant activity in normal cells. ROS consists of both free radical oxidants and molecular oxidants. Free radical oxidants participate in hydrogen abstraction and also in electron transfer reactions. All three active sites of curcumin can undergo oxidation by electron transfer and hydrogen abstraction. Detailed investigations by different groups have confirmed that during free radical reactions, the most easily abstractable hydrogen from

curcumin is from the phenol-OH group, resulting in formation of phenoxyl radicals, which are resonance stabilized across the keto-enol structure.[26]

Diet-induced hyper-cholesterolemia has long been useful for the assessment of agents interfering with the absorption, degradation and excretion of cholesterol, rather than interfering with cholesterol biosynthesis. Indeed, many animal species have been used in experiments to evaluate the hypercholesterolemic effect of high-cholesterol diet, including rabbits, normal rats, diabetic rats, diabetic and non-diabetic hamsters, Guinea pigs, and monkeys. [24]

### **1.3 HYPOTHESIS**

In this study we have hypothesised that curcumin would help in attenuating the affects of atherogenic diet in aged rats. This hypothesis was tested using the following specific aims:

#### **Specific aim 1A**

To determine the effects of curcumin on the lipid profiles in aged rats fed an atherogenic diet ( 2% cholesterol and 2% cholesterol+ 2% curcumin)

#### **Specific aim 1B**

To evaluate effect of curcumin on HDL levels through gene expression analysis in aged rats fed an atherogenic diet ( 2% cholesterol and 2% cholesterol+ 2% curcumin)

#### **Specific aim 2A**

To investigate effects of curcumin on lipid peroxidation in aged rats fed an atherogenic diet ( 2% cholesterol and 2% cholesterol+ 2% curcumin)

#### **Specific aim 2B**

To determine the effects of curcumin on inflammatory response in aged rats fed an atherogenic diet ( 2% cholesterol and 2% cholesterol+ 2% curcumin)

We assessed the possible hypolipidemic and antiatherogenic effect of the active component of turmeric, curcumin, in male norway brown rats fed a high cholesterol diet. The lipid profile was assesed as the major marker of hypercholesterolemia. Accordingly, total cholesterol(TC), and high-density lipoprotein-cholesterol (HDL-C), Non- HDL, TC/HDL Ratio were among the parameters investigated. To evaluate the effects of curcumin on lipid oxidation MDA as TBARS and 3 Hydroxybutyrate were quantified while IL-6 was measured to evaluate its effect on the inflammatory response.

## **CHAPTER 2: Materials and Methods**

### **2.1 Animals**

24 in-bred Brown Norway (BN) rats were obtained from the aged rodent colonies of the National Institute of Aging (Bethesda, MD). Upon arrival, 12 rats were 22 weeks old and 12 rats are 24 weeks old.

### **2.2 Housing and husbandry**

All animals were housed in individual cage at Wayne State University Division of Laboratory Animal Resources (DLAR) facility under standard conditions as approved by the Wayne State University Animal Investigation Committee (AIC). All of them were kept in the same room with alternating 12 hours light alternating with 12 hours darkness under normal humidity and at room temperature. Cage bedding and water were replaced weekly and their health were monitored regularly.

### **2.3 Experimental protocol and diets**

Upon arrival at the facility, all animals were allowed to acclimatize for one week prior to start of the experiment. Following acclimatization period, they were assigned into 3 groups on a random basis with the constraint that all diet groups had the same mean body weight. The groups were named by their diets: Control Diet (C) (n = 8), High Cholesterol Diet (H) (n=8), and High Cholesterol with Curcumin Diet (HC) (n=8). The diet compositions are summarized in Table 1. Diets for all the groups were isocaloric. The purified diets were obtained in pellets from Dyets Inc. (Bethlehem, PA), and sufficient diet was obtained for the entire duration of the study. The diets were kept at -20°C, and diet was taken weekly as needed and kept refrigerated at 4°C. Animals were fed ad libitum and had free access to tap water.

Ingredient	Isocaloric Control	High Cholesterol (2% Cholesterol)	High Cholesterol + 2% Curcumin
Casein	140	140	140
L-Cystine	1.8	1.8	1.8
Sucrose	100	77.5	77.5
Cornstarch	465.692	465.692	445.692
Dyetrose	155	155	155
Soyabean oil	40	40	40
t-butylhydroquinone	0.008	0.008	0.008
Cellulose	50	50	50
Mineral Mix#210050	35	35	35
Vitamin Mix#310025	10	10	10
Choline Bitartrate	2.5	2.5	2.5
Cholesterol	–	20	20
Cholic Acid	–	2.5	2.5
Curcumin	–	–	20
Total	1000	1000	1000
Calorie	3602	3512	3440

Table 1: Composition of purified diets (*G/kg*)

## 2.4 Experimental Procedures

Experimental procedures carried out for this study as summarized in Table 2. All mice were provided with their respective diets for 23 weeks and had free access to water. Body weight and food intake were recorded twice a week throughout the duration of the study. Urine was collected once a month for urinary metabolomic profiling. Upon completion of the experiment at Week 23, each animal was anesthetized using carbon dioxide chamber and decapitated followed by exsanguination and tissue collection. Tissues were flash-frozen in liquid nitrogen then stored at -80°C until ready to be used for analysis. All procedures and protocols were in accordance with and ratified by the Animal Investigation Committee of Wayne State University.

Procedures	Frequency of Measurement
Body weight, diet intake, water intake	Twice weekly (Week 1-6)
Urine collection	Once weekly (Week 2,4,6)
Blood and tissue collection	End of study (Week 6)

Table 2: Study timeline based on the overall study

## **2.5 Plasma Total Cholesterol**

Total cholesterol (TC) cholesterol were determined using enzymatic kits (TC; Pointe Scientific Inc. Canton, MI.). Plasma was isolated by centrifugation of blood at 4000 rpm for 20 minutes at 4°C and stored in -80°C until use. Samples and standards were taken and the test tubes were labeled accordingly. 1ml of reagent was pipetted into each tube and pre-warmed at 37°C for five minutes. 10µL of sample was added into respective tubes, mixed and returned to 37°C for incubation for five minutes. Spectrophotometer was zeroed with blank at 500nm and absorbances of all test tubes were recorded. The concentration of unknown samples was calculated by using standard curve.

## **2.6 Liver Total Cholesterol**

### **2.6.1 Folch Assay**

Folch method [27] was used to extract lipids from liver tissues. 0.5g of liver tissue was taken and homogenized in 10mL chloroform-methanol solution 2:1(v/v). The samples were left overnight in a shaking water bath. 3mL of 0.5% H<sub>2</sub>SO<sub>4</sub> was added for separating the phases. The lower phase was taken and the volume was brought to 10ml by addition of chloroform-methanol mixture. Aliquots of 50µL were made and dried overnight. Reconstituted the aliquots with 50µL EtOH.

### **2.6.2 TC Assay**

TC was determined using enzymatic kit and same protocol as plasma was followed.

## **2.7 HDL Plasma**

Extracted plasma from -80 °C was used. Liver lipids were extracted using Folch's method described above.

### **2.7.1 Separation of HDL Cholesterol:**

Samples were taken and the test tubes were labeled accordingly. 0.5 ml (500 $\mu$ L) sample was pipetted into the respective tubes and 0.5 ml (500 $\mu$ L) reagent was added into each tube and mixed using vortex. The tubes were centrifuged at 2000g for 10 minutes.

### **2.7.2 HDL Cholesterol Determination:**

Samples and standards were taken and the test tubes were labeled accordingly. 1ml enzymatic cholesterol reagent, which was prepared according to package insert instructions, was pipetted into each tube. Then 0.05 ml (50 $\mu$ L) standard or clear supernatants from above step were pipetted into their respective tubes. All tubes were incubated for 10 minutes at 37°C. The spectrophotometer was zeroed at 500nm with reagent blank and absorbance's of all test tubes were recorded. The concentration of unknown samples was calculated by using standard curve.

## **2.8 PCR- ApoA1**

### **2.8.1 RNA Extraction**

Liver tissue samples were taken from each group for gene expression analysis. The total RNA extraction assay was performed using commercial kit (RNeasy Mini Kit, Qiagen Valencia, CA) following the manufacturer's instructions. Tissue samples from liquid nitrogen were taken and approximately 30mg of frozen liver tissue was excised, weighed and then placed into 700 $\mu$ L QIAzol Lysis Reagent in a vessel which is suitable for disruption and homogenization using tissue homogenizer. The tube which contains the homogenate was then placed at room temperature (15-25°C) for 5 minutes. Added 140 $\mu$ L of chloroform to the tube, capped securely

and the tube was shaken vigorously for 15 seconds. The tube was kept at room temperature for 3 minutes followed by centrifugation for 15 minutes at 12,000rcf at 4°C. After centrifugation, the upper aqueous phase was transferred to a new collection tube. Then 525µL of 100% ethanol was added to the tube and mixed thoroughly by pipetting. Next, 700µL of sample including any precipitate was pipetted into RNeasy Mini spin column in 2 ml collection tube and centrifuged at 10,000 rpm for 15 seconds. Discarded the flow-through and the same step as above was repeated for the remainder of sample. Added 700µL of Buffer RWT to RNeasy Mini spin column, centrifuged at 10,000 rpm for 15 seconds and flow-through was discarded. 500µL of Buffer RPE was then pipetted onto RNeasy Mini spin column and again and centrifuged at 10,000 rpm for 15 seconds. The flow-through was discarded and 500µL of Buffer RPE was added to RNeasy Mini column and then centrifuged at 10,000 rpm for 2 minutes. RNeasy Mini spin column was then placed into a new 2 ml collection tube and centrifuged at full speed for 1 minute. The RNeasy Mini spin column was transferred to a new 1.5 ml collection tube after the old collection tube was discarded with the flow through. 40µL of RNase-free water was then pipetted directly on the RNeasy Mini spin column membrane and centrifuged for 1 minute at 10,000 rpm to elute the RNA. Then using the Nanodrop spectrophotometer quantity measurement and the spectrophotometric quality assessment (A260/280 and A260/230 ratios) of RNA were carried out.

### **2.8.2 cDNA Preparation**

Reverse transcription of the liver RNA was performed using High Capacity RNA to cDNA Master Mix kit (Applied Biosystems, Carlsbad, CA). 20 µL of RT buffer mix, 2µL of RT enzyme mix, 8µL of RNA sample and 10µL of nuclease-free water were taken and mixed into 0.2mL PCR tube and centrifuged for few seconds. The prepared samples were then loaded into

Eppendorf mastercycler realplex 4 (Eppendorf, Hauppauge, NY) for reverse transcription process with the following temperature setting; 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and was then transferred to a -20°C freezer until use for qRT-PCR analysis.

### 2.8.3 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR was performed as part of gene expression analysis. ApoA1 gene which encodes apolipoprotein A-I, the major protein component of HDL was tested in liver samples. The primer sequence of the gene is listed in Table 2. The final reaction volume of 25 uL was made using 12.5µL SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 1µL of 20 µM reverse and forward primer mixture, 9.5µL nuclease-free water and 2µL of cDNA. qRT-PCR was carried out on the Eppendorf mastercycler realplex 4 instrument (Eppendorf, Hauppauge, NY) in Mx3000P 96-Well Plates (Agilent Technologies) with the following settings; initial denaturation: 95°C for 10 minutes, 45 repetitions of denaturation: 95°C for 15 seconds and elongation: 60°C for 1 minute, dissociation curve: 95°C for 1 minute, 60°C for 30 seconds followed by gradual temperature increase from 60°C to 95°C in 20 minutes and finally at 95°C for 30 seconds. Each gene was analyzed in triplicate with single NTC. mRNA expression levels in the samples were calculated relative to Control, High cholesterol diet and High cholesterol with curcumin diet using the comparative CT method:  $\Delta\Delta CT = \Delta CT_{\text{sample}} - \Delta CT_{\text{control}}$ , fold change =  $2^{-\Delta\Delta CT}$ . GAPDH was used to normalize the expression values ( $\Delta CT$ ).

Primer's Name	Forward Sequence	Reverse Sequence
ApoA1	5'- AGGAGCAGACCCAGCAGATA- 3'	5'- AACCCAGAGTGTCCCAGTTG-3'

Table 3: Primer sequence of the ApoA1 gene

### 2.8.4 Data Analysis

A fold change cut-off of more than 2 was used in determining any significant change of gene expression relative to control

## **2.9 Liver TBARS**

### **2.9.1 Liver tissue homogenization**

25mg of tissue was weighed and transferred into a tube. 250 $\mu$ L of RIPA buffer containing protease inhibitor was added to the tube. The tissue was sonicated on ice for 15 seconds and vial tube was centrifuged at 1600 $\times$ g for 10 minutes at 4 $^{\circ}$ C. The supernatant was stored at -80 $^{\circ}$ C until used for analysis.

### **2.9.2 BCA Protein Quantification**

Pierce BCA Protein Assay Kit was purchased from Thermo Fisher Scientific Inc. Grand Island, NY. The standards and working reagents were prepared according to manufacturer's protocol. 25 $\mu$ L of each standard or unknown sample replicates were pipetted into a microplate well. 200 $\mu$ L of the WR added to each well and mixed the plate thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37 $^{\circ}$ C for 30 minutes. Cooled the plate to RT and measured the absorbance at 562nm on a plate reader.

### **2.9.3 TBARS Assay**

TCA TBARS Assay kit was purchased from Cayman Chemical Ann Arbor, Michigan. All the reagents were brought to room temperature before starting the assay. The standards and samples were prepared according to manufacturer's instructions and the vials were labeled accordingly. 100 $\mu$ L of sample or standard was taken into the vial and 100 $\mu$ L of TCA Assay Reagent was added to each vial. Then 800 $\mu$ L of color reagent was added to each vial and

vortexed. The vials were capped and placed in foam holder to keep the vials upright during boiling. The vials were then added to vigorously boiling water and left for an hour. After one hour the vials were taken and placed in ice bath immediately to stop the reaction and incubated on ice for 10 minutes. The vials were then centrifuged for 10 minutes at  $1600\times g$  at  $4^{\circ}C$ .  $200\mu L$  from each vial was taken and transferred to assigned slots in plate an absorbance was read at 540nm. The concentration of unknown samples was calculated by using standard curve. The samples were normalized using BCA protein quantification.

## **2.10 Plasma TBARS**

Stored plasma from  $-80^{\circ}C$  and thawed on ice. Plasma TBARS was performed by using the same method as liver tissues following the manufacturers protocol.

## **2.11 Urine 3-Hydroxybutyrate**

### **2.11.1 Creatinine Quantification**

The Colorimetric Assay kit was purchased from Cayman Chemical Ann Arbor, Michigan. Stored urine samples from  $-80^{\circ}C$  were taken and thawed on ice. Urine was diluted 1:5 with assay buffer and the standards, and reagents were prepared, according to manufacturer instructions.  $15\mu L$  of samples and standards are added to the wells. The reactions were initiated by adding  $150\mu L$  of alkaline pictrate solution to each well. The plate was covered and incubated at room temperature for 10 minutes. The initial absorbance was measured at 500nm after removing the cover. Then  $5\mu L$  of acid solution was added to each well and the plate was covered followed by incubation for 20 minutes at room temperature on a shaker. The cover was removed and the final absorbance was read at 500nm. The average final absorbance was subtracted from the average initial absorbance to get the corrected absorbance. The corrected absorbance of standard A was subtracted from itself and all other standards and samples to get adjusted

absorbance. This adjusted absorbance of standards was plotted on standard curve to obtain the concentration of creatinine of unknown samples.

### **2.11.2 3-hydroxybutyrate Assay**

Urine samples were used for measuring the ketone body 3-hydroxybutyrate. The Colorimetric Assay kit was purchased from Cayman Chemical Ann Arbor, Michigan. Stored urine samples from -80°C were taken and thawed on ice. Urine was diluted 1:5 with assay buffer and the standards, and reagents were prepared, according to manufacturer instructions. 50µL of standards and samples were added to the wells in triplicates. The reaction was initiated by adding 50µL of developer solution to each well. The plate was incubated in dark at 25°C for 30 minutes. The absorbance was read at 455nm and the values were recorded. The concentration of unknown samples was calculated by using standard curve. The urine samples were normalized using creatinine.

### **2.12 IL-6 ELISA**

IL-6 ELISA Immunoassay kit was purchased from R&D Systems Inc. Minneapolis, MN. All the reagents, standard dilutions, control, and samples were reconstituted according to manufacturer's protocol. Plasma was taken from -80 °C and thawed on ice. 50 µL of Assay Diluent RD1-54 was added into each well and then 50 µL of standards or samples were added into their respective wells. The samples were mixed by gently tapping the plate frame for 1 minute. The plates was covered with the adhesive strip provided and incubate for 2 hours at room temperature. Aspirated each well and washed, repeating the process four times for a total of five washes. The wells were washed by filling each well with Wash Buffer (400 µL) using a squirt bottle. Plate was inverted and blotted against clean paper towels. After this 100 µL of Rat IL-6 Conjugate was added to each well and covered with a new adhesive strip. Incubated for 2

hours at room temperature and repeated the aspiration/wash as explained above. Then 100  $\mu\text{L}$  of Substrate Solution to each well and incubated for 30 minutes at room temperature taking care it is protected from light. After 30 minutes 100  $\mu\text{L}$  of Stop Solution was added to each well and gently tapped the plate to ensure thorough mixing. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm. The concentration of unknown samples was calculated by using standard curve.

## CHAPTER 3: Results

### Body weight and food intake:

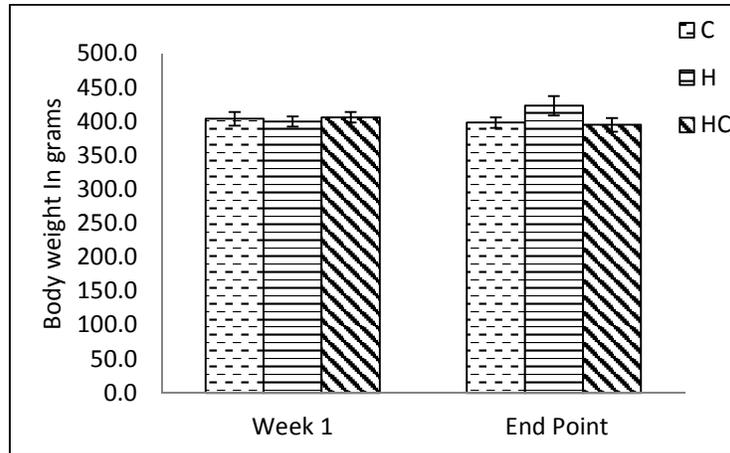


Fig. 6. Mean body weight of rat at week 1 and Week 23 is shown. Results represent mean + SE. Comparisons were achieved using one way ANOVA followed by LSD as post-hoc test. No significant difference was between group in week 1 and Week 23.

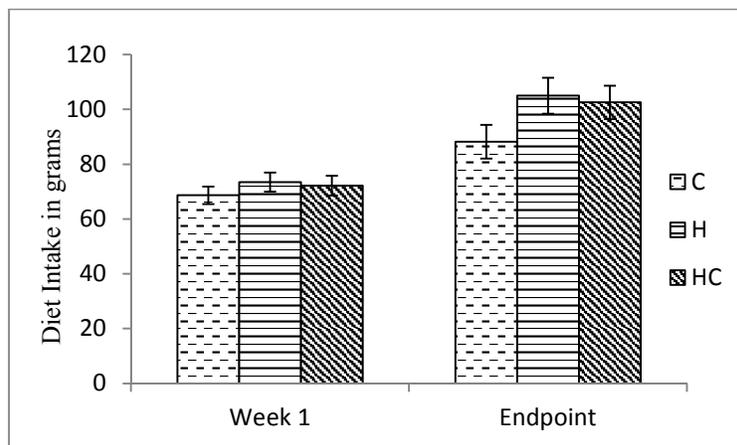


Fig. 7. Mean food intake of rats at week 1 and week 23 is shown. Results represent mean diet consumption. Comparisons were achieved using one way ANOVA followed by LSD as post-hoc test. No significant difference was between group in week 1 and Week 23.

### Specific aim 1A

To determine the effects of curcumin on the lipid profiles in aged rats fed an atherogenic diet (2% cholesterol and 2% cholesterol+ 2% curcumin)

### Plasma Total Cholesterol:

The effects of curcumin on total cholesterol content of plasma has been shown in the figure 8. The groups Control (C), high cholesterol (H) and high cholesterol with curcumin (HC)

have been compared. TC was measured in C, H, and HC group's rats using enzymatic assay kits. Significant difference was observed between the groups.

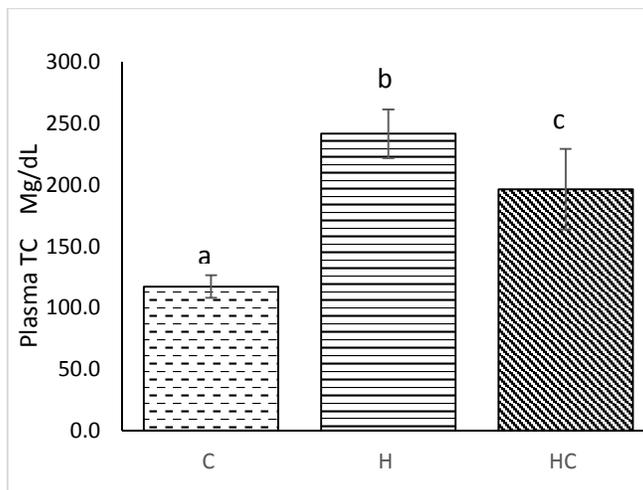


Fig. 8. Effect of curcumin on the Plasma TC in aged rats. Results represent mean + SE. Comparisons were achieved using one way ANOVA followed by LSD as post-hoc test. a-H is significantly different from C, at  $p < 0.05$ . b-H is significantly different from HC at  $p < 0.05$ . c- HC is significantly different from C.

### Plasma High Density Cholesterol:

The effects of curcumin on HDL cholesterol content of plasma has been shown in the figure 9. The groups Control (C), high cholesterol (H) and high cholesterol with curcumin (HC) have been compared. HDL was measured in C, H, and HC group's rats using enzymatic assay kits. Significant difference was observed between the groups.

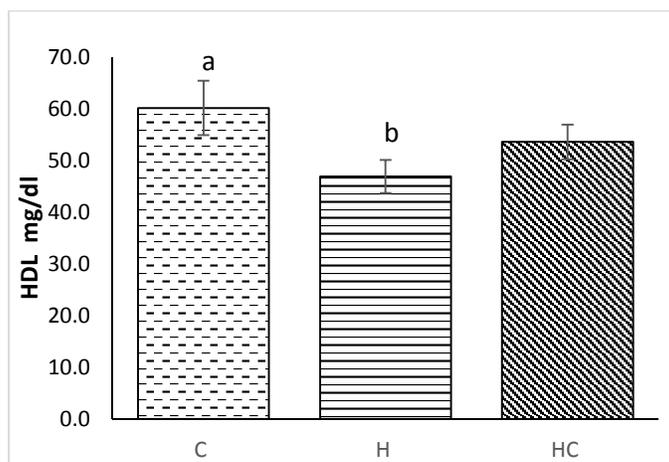


Fig. 9. Effect of curcumin on the Plasma HDL in rats. Results represent mean + SE. Comparisons were achieved using one way ANOVA followed by LSD as post-hoc test. a - H, Significantly different from C at  $p < 0.05$ .

### Plasma Non-HDL:

The plasma Non-HDL cholesterol content has been shown in the figure 10. It is measured as difference of TC and HDL. The groups Control (C), high cholesterol (H) and high cholesterol with curcumin (HC) have been compared. Significant difference was observed between the groups.

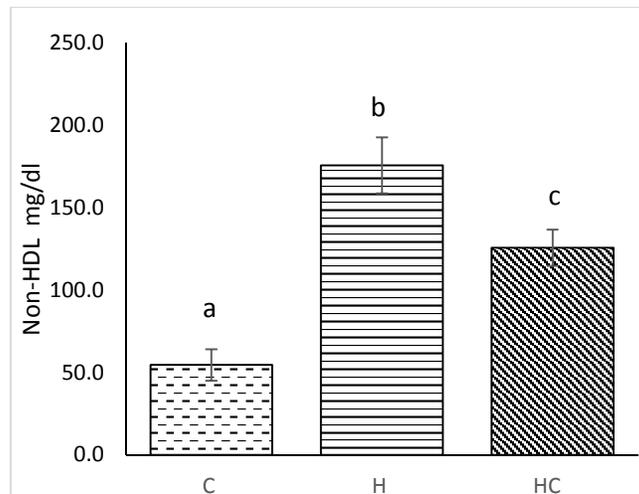


Fig. 10. Effect of curcumin on the Plasma Non-HDL measured as TC-HDL in rats. Results represent mean + SE. Comparisons were achieved using one way ANOVA followed by LSD as post-hoc test. a- H is significantly different from C, at  $p < 0.05$ . b -H is significantly different from HC at  $p < 0.05$ . c- HC is significantly different from C at  $p < 0.05$ .

### Plasma TC/HDL Ratio:

The effects of curcumin on TC/HDL cholesterol ratio of plasma has been shown in the figure 11. The groups Control (C), high cholesterol (H) and high cholesterol with curcumin (HC) have been compared. Significant difference was observed between the groups.

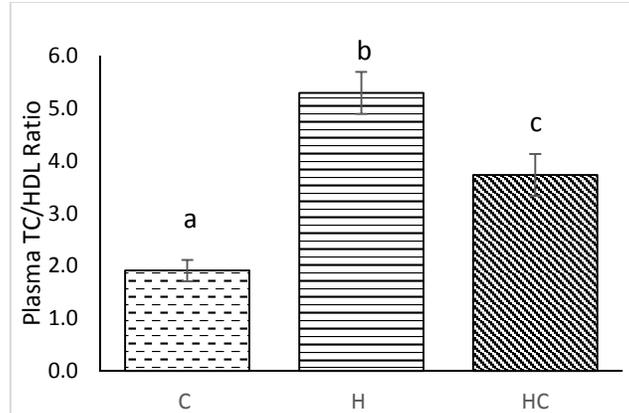


Fig. 11. Effect of curcumin on the Plasma TC/HDL ratio in rats. Results represent mean + SE. Comparisons were achieved using one way ANOVA followed by LSD as post-hoc test. a – H is significantly different from C, at  $p < 0.05$ . b – H is significantly different from HC at  $p < 0.05$ . c – HC is significantly different from C at  $p < 0.05$ .

### Liver TC:

The effects of curcumin on total cholesterol of liver has been shown in the figure 12. The groups Control (C), high cholesterol (H) and high cholesterol with curcumin (HC) have been compared. TC was measured in C, H, and HC group's rats using enzymatic assay kits. Significant difference was observed between the groups.

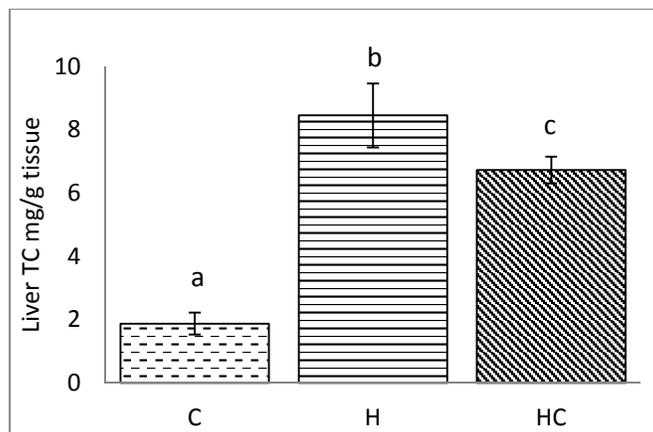


Fig. 12. Effect of curcumin on the liver TC in rats. Results represent mean + SE. Comparisons were achieved using one way ANOVA followed by LSD as post-hoc test. a – H, Significantly different from C at  $p < 0.05$ . b – H, Significantly different from HC at  $p < 0.05$ .

### Specific aim 1B

To evaluate effect of curcumin on HDL levels through gene expression analysis in aged rats fed an atherogenic diet ( 2% cholesterol and 2% cholesterol+ 2% curcumin)

## RT-PCR:

The effects of curcumin on gene expression of liver has been shown in the figure 14. The groups Control (C), high cholesterol (H) and high cholesterol with curcumin (HC) have been compared PCR was done in C, H and HC group's rats using kits. Significant difference was observed between the groups.

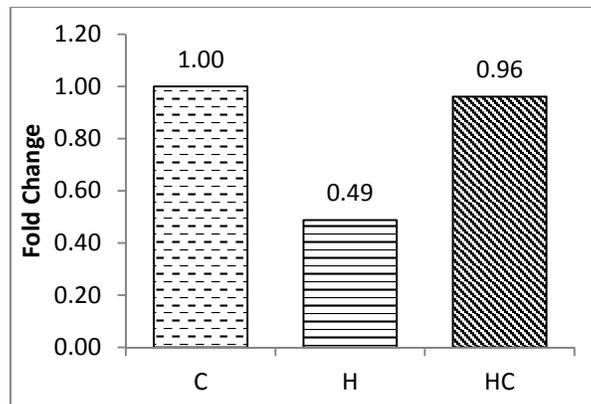


Fig. 14. Effect of curcumin on the mRNA expression of ApoA1 in rat liver. The expression is presented as fold change with respect to KC. a is significantly different from b.

## Specific aim 2A

To investigate effects of curcumin on lipid peroxidation in aged rats fed an atherogenic diet ( 2% cholesterol and 2% cholesterol+ 2% curcumin)

## Liver TBARS:

The effects of curcumin on lipid peroxidation of liver has been shown in the figure 15. The groups Control (C), high cholesterol (H) and high cholesterol with curcumin (HC) have been compared. TBARS was performed in C, H, and HC group's rats using kits. Values are expressed per mg of protein. Significant difference was observed between the groups.

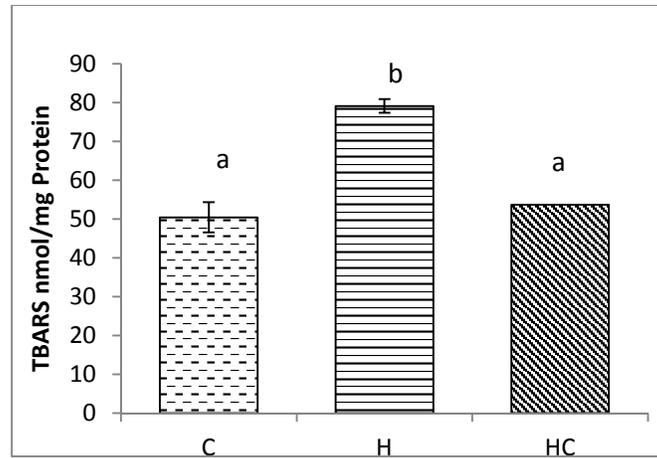


Fig. 15. Effect of curcumin on the the lipid peroxidation in rat liver. Results represent mean + SE. Comparisons were achieved using one way ANOVA followed by LSD as post-hoc test. a – H is significantly different from C, at  $p < 0.05$ . b –H is significantly different from HC at  $p < 0.05$ . c- HC is significantly different from C at  $p < 0.05$ .

### Plasma TBARS:

The effects of curcumin on lipid peroxiation of plasma has been shown in the figure 16. The groups Control (C), high cholesterol (H) and high cholesterol with curcumin (HC) have been compared. TBARS was perofromed in C, H, and HC group's rats using kits. Significant difference was observed between the groups

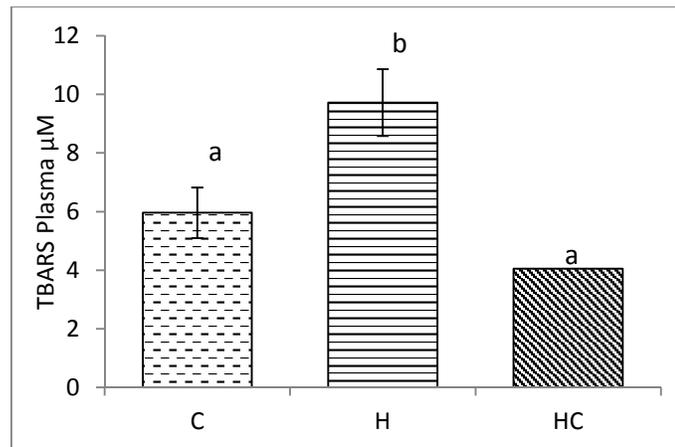


Fig. 16. Effect of curcumin on the lipid peroxidation in rat Plasma. Results represent mean + SE. Comparisons were achieved using one way ANOVA followed by LSD as post-hoc test. b– H is significantly different from C at  $p < 0.05$ . b –H is significantly different from HC at  $p < 0.05$ .

### Urine 3-hydroxybutyrate:

The effects of curcumin on ketone body production through oxidation of fatty acids has

been shown in the figure 17. The groups Control (C), high cholesterol (H) and high cholesterol with curcumin (HC) have been compared. Assay was performed in C, H, and HC group's rats using kits. Values are represented per mg of creatinine. Significant difference was observed between the groups.

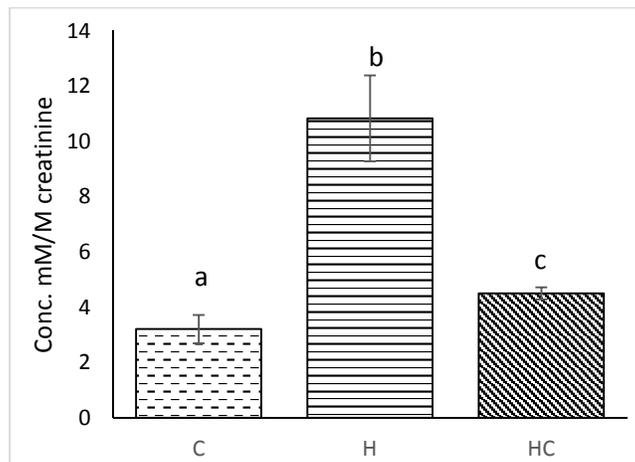


Fig. 17. Effect of curcumin on the urine 3-hydroxybutyrate levels in rats. Results represent mean + SE. Comparisons were achieved using one way ANOVA followed by LSD as post-hoc test. a – H is significantly different from C, at  $p < 0.05$ . b – H is significantly different from HC at  $p < 0.05$ . c- HC is significantly different from C at  $p < 0.05$ .

## Specific aim 2B

To determine the effects of curcumin on inflammatory response in aged rats fed an atherogenic diet ( 2% cholesterol and 2% cholesterol+ 2% curcumin)

## Interleukin-6:

The effects of curcumin on plasma inflammatory cytokine IL-6 has been shown in the figure 18. The groups Control (C), high cholesterol (H) and high cholesterol with curcumin (HC) have been compared. Assay was performed in C, H, and HC group's rats using ELISA kit. Values are represented per mg of creatinine. Significant difference was observed between the groups.

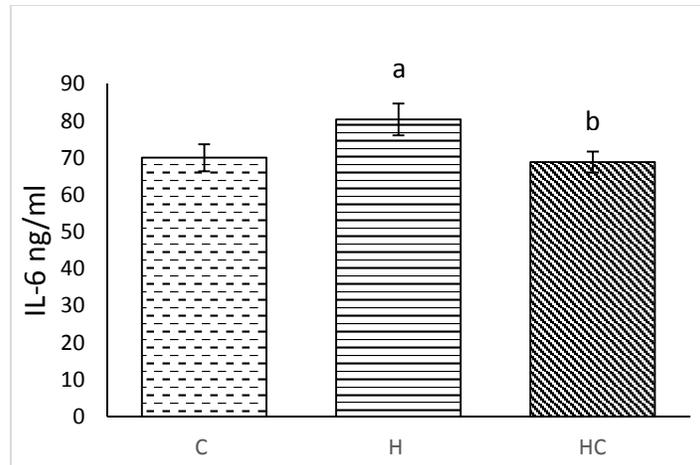


Fig. 18. Effect of curcumin on the Plasma IL-6 levels in rats. Results represent mean + SD. Comparisons were achieved using one way ANOVA followed by LSD as post-hoc test. a – H, Significantly different from HC at  $p < 0.05$ .

Parameter	C	H	HC
Plasma TC	<sup>a</sup> 117.2 ± 20.5	<sup>b</sup> 221.1 ± 35.5	<sup>c</sup> 179.2 ± 23.0
Plasma HDL			
Plasma TC/HDL	<sup>a</sup> 62.6 ± 11.8	<sup>b</sup> 45.3 ± 9.6	53.5 ± 9.2
Plasma Non-HDL	<sup>a</sup> 1.9 ± 0.4	<sup>b</sup> 5.0 ± 1.3	<sup>c</sup> 3.4 ± 0.7
Liver TC			
Liver HDL	<sup>a</sup> 54.6 ± 21.4	<sup>b</sup> 175.8 ± 38.1	<sup>c</sup> 125.7 ± 24.7
Plasma TBARS			
Liver TBARS	<sup>a</sup> 1.8 ± 0.7	<sup>b</sup> 8.4 ± 2.26	<sup>b</sup> 6.7 ± 1.8
Urine 3-Hydroxybutyrate	0.76 ± 0.2	0.44 ± 0.19	0.61 ± 0.3
Plasma IL-6	<sup>a</sup> 5.96 ± 1.9	<sup>b</sup> 9.7 ± 2.58	<sup>a</sup> 4.1 ± 1.3
	<sup>a</sup> 50.5 ± 8.7	<sup>b</sup> 80.3 ± 19.15	<sup>a</sup> 53.8 ± 8.17
	<sup>a</sup> 3.2 ± 0.5	<sup>b</sup> 10.8 ± 1.6	<sup>c</sup> 4.4 ± 0.2
	70.1 ± 8.3	<sup>a</sup> 80.5 ± 9.6	<sup>b</sup> 68.9 ± 6.4

Values are expressed as the means ± SE. Groups were compared by one-way analysis of variance, followed by LSD.

Table 4: Effect of curcumin on plasma and liver lipids, oxidation and inflammatory cytokines in aged Brown Norway rats.

## CHAPTER 4: Discussion

Cardiovascular disease(CVD) is a leading cause of global mortality, accounting for almost 17 million deaths annually.[28] For many years, it has been recognized prolonged elevation of blood cholesterol levels, is an established cardiovascular disease risk factor in humans.[29] Therefore, reduction of circulating TGs, TC and LDL-C, increasing HDL-C is a primary step in the prevention of vascular disease.[28] The induction of hyperlipidemia, in particular hypercholesterolemia, by feeding experimental animals a high cholesterol diet, has been suggested by many scientists as a reliable model for CVD in humans.[24] Therefore, our main objective in the current study was to induce hypercholesterolemia in rats after feeding the animals a high cholesterol diet, which was earlier described by Fillios et al. as an atherogenic diet. In this context, the plasma lipid profile, liver lipid profile, and some anti-oxidant and anti-inflammatory parameters were investigated. Feeding rats with a high cholesterol diet for 23 consecutive weeks resulted in marked hypercholesterolemia, as the plasma total cholesterol (TC) level was much greater than that of control animals. This is in accord with previous findings reported by Beynen et al. , who showed that feeding four different strains of rats a diet containing 2% cholesterol and 0.5% cholate for 13 days induced a pronounced hypercholesterolemia. Also, Monte and Jimenez have demonstrated that feeding Wistar rats for 20 days a hypercholesterolemic diet increased the plasma cholesterol and hepatic content of total, free and esterified cholesterol. Similar results have been reported by Sakuma et al. following one week feeding of a 1% cholesterol, 0.5% cholic acid diet to normal and diabetic rats.[24]

We addressed the possible hypolipidemic effects of curcumin. Curcumin is extracted from *Curcumae Longae*, and it has been demonstrated that curcumin has a variety of pharmacological effects, such as antitumor, anti-inflammatory, as well as antioxidative

effects.[30] The present study investigated curcumin's ability to reduce effects of atherogenic diet in aged rats. Using this hypercholesterolemic rat model, we evaluated the lipid-lowering effect of curcumin, and our results showed that rats fed a high cholesterol diet supplemented with curcumin for 23 weeks had a reduced plasma TC, Non-HDL Cholesterol, TC/HDL Ratio, and liver TC, and improved plasma HDL Cholesterol levels, which are sensitive biomarkers of coronary heart disease, compared with rats fed a high cholesterol diet only. Increased plasma HDL-C levels are widely reported to be associated with decreased risk of cardiovascular disease.[22] These results are in agreement with studies by Yuan et al. [31] and Um et al. [29] suggesting that curcumin consumption significantly decreased plasma lipid levels in animals. Similar results have been observed by Hossam et al.[24] in rats fed high cholesterol diet. Soni and Kuttan have shown a significant increase in serum HDL-C and a decrease in serum TC in human volunteers ingesting turmeric spice, suggesting a protective role of curcumin in arterial diseases. Similarly, Soudamini et al. have reported that oral administration of curcumin to mice could significantly lower serum and tissue cholesterol levels. In rabbits with experimental atherosclerosis, an ethanol extract of turmeric was found to induce a hypocholesterolemic effect. Using an adriamycin-induced nephrotic hyperlipidemia model in rats, Venkatesan et al. proved that curcumin had a lipid-lowering effect. Furthermore, Asai and Miyazawa have demonstrated that dietary curcuminoids had lipid-lowering potency in rat liver and epididymal adipose tissues. [24] Hasan et al. [32] also reported that curcumin administration has reduced the liver and plasma lipids significantly in mice fed high fat diet.

ApolipoproteinAI (apoAI), a major component of the high-density lipoprotein (HDL) particle, is necessary for the efficient transport and clearance of cholesterol from peripheral tissues to the liver for metabolism and secretion through a process called reverse cholesterol

transport. Epidemiologic studies suggest that low levels of this protein are associated with an increased risk of heart disease.[33] We found the expression of the gene ApoA-1, which encodes the apolipoprotein A-I was significantly upregulated by curcumin. This is in accordance with the study by shin et al.[22] in LDLR<sup>-/-</sup> mice fed a high-cholesterol diet.

Free radicals and associated reactive species have been implicated in atherosclerosis and its complications. A cholesterol rich diet also induces free radical production, followed by oxidative stress.[28] Oxidative stress represents a situation where there is an imbalance between the reactive oxygen species (ROS) and the availability and the activity of antioxidants. This balance is disturbed by increased generation of free radicals or decreased antioxidant activity.[9] Lipids are very susceptible to attack by free radicals and oxidized LDL (Ox-LDL) species appear to contribute to the atherosclerosis pathobiology. Hypercholesterolemia leads to increased production of ROS which exert their cytotoxic effect by causing lipid peroxidation.[28] Numerous studies have reported that dietary cholesterol consumption by animals elevates lipid peroxidation, as measured by levels of a lipid oxidation marker thiobarbituric acid reactive substances (TBARS).[28, 34] The TBARS assay has been applied as an indicator of oxidative stress in a number of cardiovascular disease animal models.[14] TBARS levels were seen to be strongly associated with cardiovascular risk factors, such as hyperlipidemia, cigarette smoking, hypertension, and diabetes. [28, 34] TBARS concentrations are elevated in the plasma of rats, which are streptozotocin induced diabetic models. TBARS were also increased in the serum of cigarette smokers. A study of TBARS in 634 patients with documented coronary artery disease found that serum levels of TBARS could predict major cardiovascular events and the need for a major vascular procedure in a 3-year follow-up period independently of traditional risk factors and inflammatory markers. Moreover, elevated TBARS levels predicted carotid atherosclerotic

plaque progression over 3 years as assessed by carotid wall thickness on ultrasound. Animal and human studies therefore support a potential role of lipid oxidation in predicting the progression of CVD and response to therapies.[14] In our study curcumin administration the levels of TBARS in plasma and liver were significantly lower in the HC and C groups when compared to H group. This is in accordance with the study by Quiles et al.[12] who showed that supplementation with *Curcuma longa* reduced levels of TBARS significantly in rabbits fed atherogenic diet. Kuo et al. [35] also reported similar results in obese mice with hepatic steatosis fed curcumin diet.

Studies have shown that fatty acid administration leads to elevated rates of fat oxidation.[36] Alteration in diet stimulates lipolysis and production of ketone bodies, including 3-hydroxybutyrate which is most abundant in serum and urine.[17] When the accumulation of cholesterol and phospholipids accelerates, the proportion of ketone bodies incorporated into these lipids increases. 3-Hydroxybutyric acid (or beta-hydroxybutyrate) is a ketone body. Like the other ketone bodies (acetoacetate and acetone), levels of 3-hydroxybutyrate in blood and urine are raised in ketosis. In humans, 3-hydroxybutyrate is synthesized in the liver from acetyl-CoA, and can be used as an energy source by the brain when blood glucose is low. Diabetic subjects who experience frequent episodes of hyperketonemia also experience increased incidences of vascular disease, morbidity, and mortality. The mechanisms by which hyperketonemia influences the development of vascular disease remains unknown.[18] Cholesterol is an important biomarker of hyperlipidemia, and its level significantly decreased after administering curcumin. Endogenous cholesterol synthesis by acetyl-CoA should be lower, but the  $\beta$ -oxidation of fatty acids should be higher in the diet-induced hyperlipidemia rats because of the feedback regulation during excessive lipid metabolism. These processes should

result in an accumulation of acetyl-CoA in vivo. The excess of acetyl-CoA may have been converted to acetone bodies, as indicated by the high level of  $\beta$ -hydroxybutyric acid in the high cholesterol group.[37] Previous studies have reported increased production of ketone body 3-hydroxybutyrate in hyperlipidemic animal models.[38] In our study significant difference was observed between the groups following treatment. The lipid-lowering effect of curcumin might have led to a decrease in the  $\beta$ -hydroxybutyric acid level in the urine following its administration. This is accordance with another study by Li et al. [39] who showed that curcumin administration significantly reduced ketone body levels in mice fed high fat diet. In conclusion, supplementation with *Curcuma longa* extract reduces oxidative stress and attenuates the development of CVD events in rats fed a high cholesterol diet.[12]

Inflammation is another important contributor to CVD, as cytokines amplify the local inflammatory response and promote the progression of atherosclerotic lesions.[29] Interleukin-6 (IL-6), a circulating cytokine, has been identified as a marker of inflammation in coronary atherosclerotic plaques.[40] In previous studies the levels of TBARS measured in CVD patients also correlated directly with levels of the proinflammatory cytokine, IL-6. Production of IL-6 from vascular cells is triggered by the proatherogenic factor, angiotensin II, which also stimulates reactive oxygenspecies involved in lipid oxidation. Cytokine production from smooth muscle cells is also stimulated directly by oxidized lipids. The correlation between levels of this cytokine and TBARS results from common early events in atherogenesis. [34] IL-6 also regulates the expression of other inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor- $\alpha$  (TNF-  $\alpha$ ). [40] In addition, previous in vitro and animal studies indicate that curcumin has widespread anti-inflammatory effects. Therefore, attenuation of inflammatory cytokines may contribute to the decreased inflammatory response in rats receiving curcumin

supplementation.[29] For this reason, we investigated whether curcumin prevents CVD risk by reducing cytokine levels. In high cholesterol + curcumin fed rats, our results showed that curcumin reduced plasma levels of IL-6. This is in accordance with another study by Singh and Vinayak [41] suggesting that curcumin administration significantly decreased IL-6 levels in rats skin. Also Um et al. [29] have shown similar results in rabbits fed high cholesterol diet. Similar results have been reported by Belcaro et al. [42] in serum of human patients with diabetes when treated with a curcumin for 8 months. Usharani et al. [43] have shown that IL-6 levels were reduced in human osteoarthritis patients when treated with a curcumin supplementation for 8 weeks. Also Hasan et al.[32] reported similar results in plasma of mice fed high fat diet with curcumin supplementation when compared to control group.

Cholesterol levels in the body result from two sources: absorption from the gastrointestinal tract and endogenous de novo synthesis.[24] The hypolipidemic effect of curcumin observed in the current study could possibly be ascribed to an effect on the absorption of cholesterol in the gut, especially curcumin was admixed with the HCD. Many previous reports could lend support to this view. Ammon and Wahl[44] have reported that after oral administration only traces of curcumin were found in the blood, and that most of the curcumin was excreted via feces, indicating poor absorption of curcumin from the gut. Another plausible explanation for the hypolipidemic effect of curcumin, though speculative at present, is that it may have increased the rate of cholesterol catabolism by increasing the activity of hepatic cholesterol 7- $\alpha$ -hydroxylase enzyme. This enzyme is the rate-limiting enzyme of bile acid biosynthesis, thus suggesting that curcumin could stimulate the conversion of cholesterol to bile acids, an important pathway of elimination of cholesterol from the body as reported by Kim et al..[2]

Curcumin has two o-methoxy phenolic OH groups attached to the  $\beta$ -diketone moiety having methylene  $\text{CH}_2$  group. It is believed that the H abstraction from these groups are responsible for the remarkable antioxidant activity of curcumin. Some methods attributed the H abstraction from the methylene  $\text{CH}_2$  group but others attributed to the phenolic OH groups. It has been illustrated the mechanism of probable two sites of free radical reaction with curcumin to produce a phenoxyl radicals or at the methylene  $\text{CH}_2$  group to produce the carbon-centered radical. These radicals are resonance stabilized and can be interconverted through the conjugation.[45]

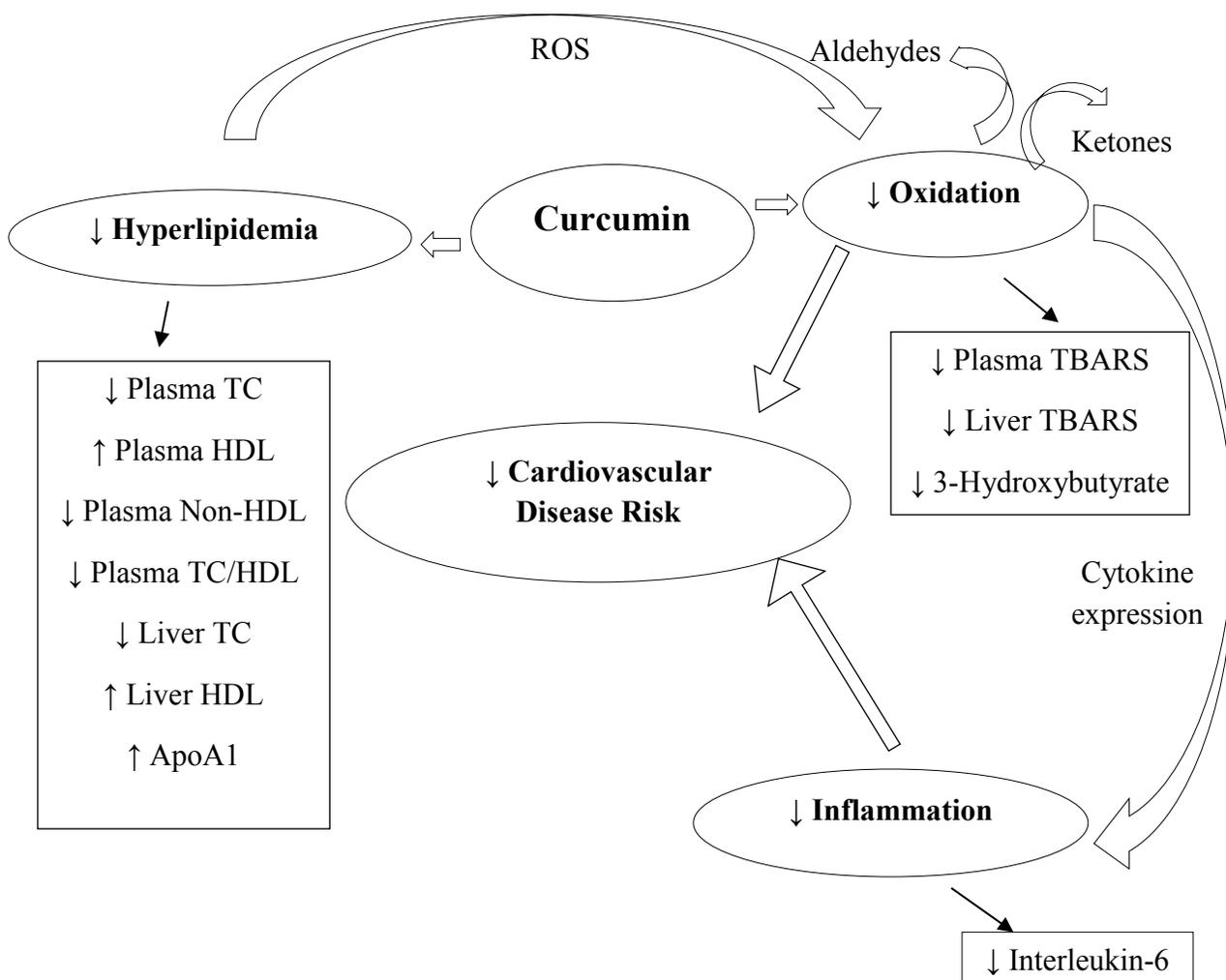


Fig 19: Effect of Curcumin on atherosclerotic diet and reduced risk of CVD

## CHAPTER 5: Conclusion and future directions

In summary, these results support our hypothesis and indicate that curcumin may prevent the progression of CVD events by reducing atherogenic diet effects in rat model. This beneficial effect may be mediated by a reduction in plasma and liver TC, Non-HDL, TC/HDL levels, oxidation and proinflammatory cytokine production, and increasing plasma and liver HDL levels. These findings demonstrate the protective mechanisms of curcumin in an experimental model of CVD and suggest its potential role in treating CVD. [29]

*Measuring HDL particle size and functionality-* Epidemiological studies have shown that low plasma levels of high density lipoprotein cholesterol (HDL-C) represent a cardiovascular disease (CVD) risk factor. HDL particles are very heterogeneous in terms of size, structure, composition and metabolism. [46] HDL is comprised of number of particles of different size. It is believed that lipid poor HDL particles are better acceptors than large HDL particles.[47] Additionally, recent studies challenge the concept that an increase of plasma HDL-C will uniformly translate into a reduction in CVD risk. Certain patients with atherosclerosis may have “dysfunctional” HDL despite normal HDL-C levels.[46] Researchers have proposed that in some people HDL was dysfunctional, or malformed, and unable to do its job properly. HDL particles have several functions related to trafficking cholesterol and proteins. If HDL particles perform these biologic tasks they are termed, “functional”. If they do not, they are termed “dysfunctional”. [48] These characteristics may play divergent roles and result in different clinical outcomes. Hence, the association of the structure of HDL particle with its functionality and metabolism should be clarified and accordingly used in the clinical setting.[46]

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**ABSTRACT****CURCUMIN ATTENUATES THE EFFECTS OF ATHEROGENIC DIET IN AGED MALE BROWN NORWAY RATS**

by

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Cardiovascular disease (CVD) is a universal problem in modern society. Hyperlipidemia is a major risk factor for the development of cardiovascular disease. Hyperlipidemia further leads to increased oxidation and inflammation. Curcumin has long been used as a spice and food-coloring agent. In experimental animals, curcumin has shown anti-diabetic, anti-inflammatory, cytotoxic and anti-oxidant properties. In this study, Aged Brown Norway Rats were fed 1 of 3 experimental diets: a normal control diet (C), a normal diet enriched with 2% cholesterol (H), or a High Cholesterol Diet supplemented with 2% curcumin. Feeding the animals a high cholesterol diet for 23 weeks resulted in marked hypercholesterolemia, increased Plasma Total Cholesterol (TC), but decreased plasma high-density lipoprotein cholesterol (HDL). Curcumin admixed with the diet significantly decreased plasma and liver total cholesterol (TC) and increased HDL. The atherogenic indices TC/HDL was significantly reduced. Curcumin also decreased the mRNA expression of ApoA1 gene, oxidative and inflammatory responses which were increased in High

cholesterol group. Our results demonstrate that curcumin improved CVD risk by lowering detrimental plasma total cholesterol, Non- HDL cholesterol, TC/HDL, and increasing beneficial HDL cholesterol, possibly by modulating the oxidation and proinflammatory biochemical pathways.

## **AUTOBIOGRAPHICAL STATEMENT**

Vindhyaja Sri Raja Vatsavai was born in Hyderabad, Telangana, India on September 29, 1991. She received her early education in public schools in Hyderabad. In 2013 she earned her Bachelor of Technology degree from TKR College of Engineering and Technology, Jawaharlal Nehru Technological University, Hyderabad, India in the field of Biotechnology. The same year she was accepted to pursue Master of Science degree in Nutrition and Food Science at Wayne State University, Detroit, MI and would be completing her degree in December 2015. Upon completion of Masters the author intends to work in Food industry.