EFFECT OF GARLIC (*Allium sativum*) EXTRACT ON SERUM LIPID PROFILE AND ANTIOXIDANT STATUES OF DYSLIPIDAEMIC RAT.

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FEBRUARY, 2021.

DECLARATION

I hereby declare that this work is the product of my research efforts undertaken under the supervision of Prof M.K Atiku and has not been presented anywhere for the award of M.Sc in Biochemistry. All Sources have been duly acknowledged.

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CERTIFICATION

This is to certify that the research work for this dissertation and the subsequent write up of this report by 'YILA ROBERT SPS/16/MBC/00091' were carried out under my supervision.

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List of Abbreviations

Symbol	interpretation
CAT	Catalase
GR	Glutathione reductase
GSH	Glutathione
GSH-Px	Glutathione peroxidases
VLDL-C	Very low density lipoprotein – cholesterol
MDA	Malondialdehyde
ROS	Reactive Oxygen specie
SD	Standard deviation
SOD	Superoxide dismutase
TG	Triglyceride
TC	Total Cholesterol
TAG	Triacylglyceride
HDL-C	High density lipoprotein - cholesterol
CVD	Cardiovascular disease
CHD	Coronary heart disease
HMG-COA	Hydroxy methyl glutaryl-coenzyme A
HFD	High Fat Diet
AI	Artherogenic Index

Abstract

Hyperlipidaemia is a heterogenous disorder involving multiple aetiologies. It is commonly characterised by raised triglycerides, low density lipoprotein cholesterol (LDL-C) and reduced plasma high density lipoprotein cholesterol (HDL-C) concentration as a consequence of metabolic effects, or dietary and lifestyle habits. Oxidative stress occurs when there is imbalance between the production of free radicals and the body's ability to fight them off. The present study is designed to assess the effect of garlic extract on lipid profile and oxidative stress in albino rats fed a high fat diet (HFD). A group of 35 albino rats of both sexes each weighing 50-90g, was divided into seven groups. Group 1 was used as negative control and fed on standard diet and clean water. Group II was used as positive control and fed on high fat diet and clean water for six weeks. Group III was used as standard control and fed on high fat diet and orally administered 10mg/kg of lipid lowering drug (Atorvastatin) for two weeks. Groups IV, V, VI, and VII were fed on high fat diet for 42days and orally administered with garlic extract of different concentration (100, 200, 300, and 400mg/kg/bw/day respectively) for two weeks. Atorvastatin and garlic extract significantly increased ($p<0.05$) plasma HDL-CH and decreased plasma total cholesterol (TC), low density lipoprotein cholesterol (LDL-CH), very low density lipoprotein cholesterol (VLDL-CH), and triglyceride (TG) compared with negative and standard control (group I and III). Significant difference ($p<0.05$) was observed in serum low density lipoprotein cholesterol (LDL-CH), high density lipoprotein cholesterol (HDL-CH), between the rats orally administered with high and low dose of garlic extract. Garlic extract significantly increased ($p<0.05$) superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activities compared to negative and standard control (group I and III). No significant difference was observed in superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activities between the rats orally administered with high and low dose of garlic extract. There was a significant decreased ($p<0.05$) in serum malondialdehyde in rats orally administered with high and low dose of garlic extract as compared to negative or standard control rats. These results suggest that, garlic is effective in improving levels of the antioxidants and coronary heart disease.

CHAPTER ONE

1.1 Introduction

Hyperlipidaemia is a heterogeneous group of disorders characterized by an excess of lipids such as cholesterol, cholesterol esters, phospholipids and triglycerides in the blood stream (Hussein *et al.,* 2014). Hyperlipidaemia is a modifiable risk factor of atherosclerosis and other cardiovascular diseases. It may be primary or secondary type according to the cause of hyperlipidaemia either high intake of fat rich food or as a result of other disease or metabolic disturbances (Watson, 1993).

Jacobson reported that hyperlipidaemia refers to elevated levels of lipids and cholesterol in the blood and it is also identified as dyslipidemia to describe the manifestations of different disorders of lipoprotein metabolism (Xu, 2014).

The major lipids reported to be present in the plasma are fatty acids, triglycerides, cholesterol, cholesterol esters (compounds), and phospholipids. Lipids are transported in the blood as large lipoproteins. Other lipid soluble substances, present in much smaller amounts but of considerable physiological importance, include steroid hormones and fat-soluble vitamins (Bishop *et al.,* 2000).

The major types of lipoproteins are chylomicrons, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Chylomicrons are synthesized by enterocytes from lipids absorbed in the small intestine. Very low density lipoprotein is synthesized in the liver. The function of these lipoproteins is to deliver energy-rich triglycerides to cells in the body (Karam *et al.,* 2015). Triglyceride is stripped from chylomicrons and very low density lipoprotein through the action of lipoprotein lipase, an enzyme that is found on the surface of endothelial cells. The enzyme digests the triglyceride to fatty acids and glycerol, which can then diffuse into the cell to be oxidized, or in the case of an adipose cell, to be re-synthesized into triglyceride and stored in the cell. Low density lipoprotein delivers cholesterol to cells in the body. As very low density lipoprotein particles are stripped of triacylglycerol, they become denser. These particles are remodeled at the liver and transformed into low density lipoprotein. The function of low density lipoprotein is to deliver cholesterol to cells, where it is used in membranes, or for the synthesis of steroid hormones (Karam *et al.,* 2015).

The link between cholesterol and heart disease was recognized through the study of individuals with familial hypercholesterolemia. Individuals with this disorder have several-fold higher levels of circulating low density lipoprotein due to a defect in the function of their low density lipoprotein receptors. Without functioning low density lipoprotein receptors, low density lipoprotein is not cleared from the circulation. As well, because cholesterol cannot get into cells efficiently, there is no negative feedback suppression of cholesterol synthesis in the liver (Xenoulis and Steiner, 2010). A lipid profile typically measures the levels of total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides. Dyslipidemia is the term that is used if lipid levels are outside the normal range. High levels of LDL cholesterol (the so-called "bad cholesterol") greatly increase the risk for atherosclerosis because LDL particles contribute to the formation of atherosclerotic plaques. Low HDL levels ("good cholesterol") are an independent risk factor, because reverse cholesterol transport works to prevent plaque formation, or even cause regression of plaques once they have formed. HDL may also have anti-inflammatory properties that help reduce the risk of atherosclerosis. Fasting triglyceride levels are used to estimate the level of very low density lipoprotein.

Hyperlipidemia also induces oxidative stress, and malondialdehyde (MDA) is one of the products in lipid peroxidation. Plasma MDA levels increased markedly in animals with obesity and diabetes mellitus (Hussein *et al*., 2014). Hyperlipidemia is associated with hepatic fat accumulation. Coronary heart disease (CHD) is caused by the narrowing of artery that supplies the nutrients and oxygen to the heart, the main reason for this narrowing is atherosclerosis. There is a relationship between the elevated plasma lipid and the development of atherosclerotic plaque (Jain *et al.*, 2007).

Cardiovascular diseases (CVD) are the most prevalent cause of death and disability in both developed as well as developing countries (Chaturvedi and Bhargava, 2007). South Asians around the globe have the highest rates of Coronary Artery Disease (CAD) (Enas *et al.*, 2007). According to National Commission on Macroeconomics and Health (NCMH), a government of India undertaking, there would be around 62 million patients with CAD by 2015 in India and of these, 23 million would be patients younger than 40 years of age (Indrayan, 2005). CAD is usually due to atherosclerosis of large and medium sized arteries and dyslipidemia has been found to be one of the most important contributing factors (Executive, 2001). As it has long been

known that lipid abnormalities are major risk factors for premature CAD (Enas *et al.,* 2007 and Executive, 2001).

Many medicinal plants used in ethno medical practices in Nigeria are known or little known by scientific world (Mashi *et al.,* 2018). Many important drugs used in medicine today are directly or indirectly derived from plant (Mashi *et al.,* 2019). Plants and herbs are been tremendous source of food and folk remedies for mankind and have served as starting materials for the development of new synthetic drugs. They have the ability to synthesize a wide variety of chemical compounds which are used to perform biological functions and to depend against attack from predators such as insect, fungi, yeast, bacteria, viruse and other pathogens (Mashi *et al.,* 2019). Chemical compounds in plant mediate their effect on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs (Tapsel et al., 2006).

Garlic (*Allium sativum*) is one of the plant products, traditionally used for its cytotoxic, antitumor, antifungal, antibacterial, antiviral and anti protozoal properties (Sarkar *et al.,* 2006). As a member of the Liliaceous family, garlic (*Allium sativum)* (Ulbricht *et al.,* 2010), contains various substances including minerals, carbohydrates, proteins, fats and vitamins (Ozcan *et al.,* 2002; Cobas *et al.,* 2010; and Block, 1985). Vitamins found in garlic include vitamin A, various kinds of vitamin B, such as riboflavin, thiamine, nicotinic acid, and vitamins C and E. Among many different compounds found in garlic, studies suggest that biological and pharmacological effects of this plant are mainly due to its sulfur compounds (Cobas *et al.,* 2010; Block, 1985; Lanzotti, 2006; and Rahman, 2006). Some of these sulfur compounds are aliin, allicin, agoene, allylpropyl disulfide, diallyltrisulfide, sallylcysteine, vinyldithiines, Sallylmercaptocystein, and others (Sarkar *et al.,* 2006; Lanzotti, 2006; and Rahman, 2006). Because of their high costs, their potential side effects and restrictions of their use, in recent years, there has been a tendency among researchers in attempting to treat disorders by replacing chemical drugs with some natural plant components (Mahmoodi *et al.,* 2011).

The early Egyptians used garlic to treat diarrhea and its medical power was described on the walls of ancient temples and on papyrus dating to 1500 BC (Bradley, 1992). It was used by Greek physicians Hippocrates and Galen to treat intestinal and extra-intestinal diseases; ancient Japanese and Chinese used it to treat headache, flu, sore throat and fever. In Africa, particularly in Nigeria, it is used to treat abdominal discomfort, diarrhea, otitis media and respiratory tract

infections (Jaber and Al- Mossawi, 2007). In Europe and India, it was used to treat common colds, hay fever and asthma. Garlic is nicknamed as Russian penicillin for its widespread use as a topical and systemic antimicrobial agent; it is commonly used in many cultures as an excitement and reputation of healing power (Timbo *et al.,* 2006).

Since ancient time, plants have been used by man to meet various needs, which include food, shelter, fuel, clothing, and medicinal needs.

Medicinal plants are however used frequently by traditional medicine healers in many communities in Nigeria. Herbal medicine products are assuming greater role in the lives of people across the world in the face of global upsurge of drugs resistance, ineffectiveness, toxicity, adverse side effect and escalating costs of synthetic products. It is well accepted that up to 80% of the world's population depends wholly or partly on traditional medicines, which in many cases has been and continues to be a major source of at least 60% of all orthodox medicines (Adewoye, 1998).

1.2 Statement of Problem

Hyperlipidaemia is major risk factor for atherosclerosis. Other complications are coronary heart disease, ischemic cerebrovascular disease, hypertension, obesity and diabetes mellitus (Type II). Although many efficacious lipid-lowering synthetic drugs exist, none is effective for all lipoprotein disorders and are associated with some adverse effects such as hepatic dysfunction, renal insufficiency, hypothyroidism, advanced age and serious infections (Uddin *et al.,* 2016).

Atherosclerosis is an emphatically serious condition where medium and large arteries become clogged up by fatty substances results in formation of plaques (Hussein *et al*., 2014).

This condition is known to increase the production of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radical and singlet molecular oxygen. These ROS are capable of damaging many biological macromolecules such as DNA, RNA, protein and lipids (Fang et al. 2002)

1.3 Justification of the study

Hyperlipidaemia is a major risk factor for atherosclerosis and related diseases such as coronary heart disease, stroke, ischemic cerebrovascular disease, hypertension, obesity and diabetes mellitus (Type II). High dietary cholesterol increases the risk of cardiovascular disease, stroke and increase mortality (Ross, 1999). These diseases are of major public health concern. There

are enough evidences indicated a continuous increase worldwide if appropriate prevention strategies are not put in place. Therefore there is the need to search for other agents from natural sources that are relatively less expensive, safe and more efficacious. Plant-based medicine has been used cost effective worldwide to treat hyperlipidemia (Grijesh *et al.,* 2009). In many parts of the world especially in poor countries, plant-based medicine may be the only form of therapy available for hyperlipidemic complications. Garlic is a well known plant and consumed by human for decades (Bordia, 1981). As such identification and utilization of antihyperlipidemic properties of this plant will be a way forward in order to tackle the problem encountered by people suffering from hyperlipidaemia

1.4 Aim and Objectives of the Study

The aim of the study is to assess the effect of methanolic-aqueous extract of garlic on serum lipid profiles and antioxidant levels of high fat diet induced hyperlipidaemic rats.

Specific Objectives

- To evaluate the oral lethal mean dose (LD_{50}) of garlic methanolic- aqueous extract.
- To assess the serum lipid and antioxidant levels in rats induced hyperlipidemia and treated with atorvastatin and garlic methanolic extract.
- To assess the histopathology of liver and heart of the experimental animals.

CHAPTER TWO

LITERATURE REVIEW

2.1 Lipid Metabolism

Lipid metabolism can be divided into two basic pathways: the exogenous pathway, which is associated with the metabolism of exogenous (dietary) lipids Figure 1, and the endogenous pathway, which is associated with the metabolism of endogenously produced lipids (Ginsberg, 1998; Rifai *et al*., 1999; & Bauer, 2004).

Exogenous pathway is the first step in dietary lipid metabolism is digestion. Dietary lipids that reach the intestine duodenum then undergo emulsification, and then hydrolyzed by the pancreatic and intestinal lipases (Xenoulis and Steiner, 2010; Bauer, 1996). Hydrolysis products (mainly free fatty acids and monoglycerides) are then transferred to the intestinal epithelial cell, where they diffuse through the epithelial cell membranes into the intestinal mucosal cells (Xenoulis and Steiner, 2010; Bauer, 1996). In the intestinal mucosal cell, free fatty acids and monoglycerides reassemble to form triglycerides, which then combine with phospholipids, free and esterified cholesterol (Ginsberg, 1998; Rifai, 1999; Bauer, 2004; Bauer, 1996; & Bauer, 1995). Chylomicrons are the lipoprotein class responsible for transfer of dietary lipids. After formation in the enterocytes, chylomicrons, which mainly contain triglycerides, are secreted into the lacteals and enter first the lymphatic and later the blood circulation (Ginsberg, 1998; Rifai, 1999; Bauer, 2004; Bauer, 1996; & Bauer, 1995). Lipoprotein which is exposed on the chylomicron surface activates the lipoprotein lipase attached to the capillary beds in adipose and skeletal muscle tissues, which then hydrolyzes triglycerides into free fatty acids and glycerol (Ginsberg, 1998; Rifai, 1999; Bauer, 2004; Bauer, 1996; & Bauer, 1995). Free fatty acids enters into the muscle cells (where they are used for energy production) and/or adipocytes (where they are re-esterified into triglycerides for storage). The cholesterol-rich remaining particles (chylomicron remnants), return to HDL and are recognized by specific hepatic receptors that rapidly remove them from the circulation by endocytosis (Ginsberg, 1998; Rifai, 1999; Bauer, 2004; Bauer, 1996; & Bauer, 1995). The cholesterol found in chylomicron remnants can be used for lipoprotein (VLDL) and/or bile acid formation, or stored as cholesteryl esters (Bauer, 1996; Bauer, 1995).

Endogenous pathway, the chylomicrons are responsible for transport of dietary lipids, very low density lipoprotein, low density lipoprotein and high density lipoprotein are mainly involved in the metabolism of endogenously produced lipids (Bauer, 1996). lipoprotein abide with phospholipids to form very low density lipoprotein (VLDL) (Ginsberg, 1998; Rifai, 1999; Bauer, 2004; & Bauer, 1996). After VLDL molecules reach the vasculature (Ginsberg, 1998; Rifai, 1999; Bauer, 2004; and Bauer, 1995). VLDL activates lipoprotein lipase located in the capillary beds, which in turn leads to hydrolysis of VLDL, triglycerides and the production of free fatty acids and glycerol. The VLDL molecules remaining after hydrolysis of VLDL triglycerides (VLDL remnants) are either removed from the circulation by the liver or undergo further transformation by lipoprotein lipase and/or hepatic lipase to form LDL (Johnson, 2005; Ginsberg, 1998; Rifai, 1999; Bauer, 2004; Bauer, 1996; & Bauer, 1995). Low density lipoprotein (LDL) which contains mainly cholesteryl esters and phospholipids circulates in the blood and binds to specific receptors that are widely distributed throughout tissues in order to deliver cholesterol, which can be used for the synthesis of steroid hormones and cell membranes as well as for hepatic metabolism (Ginsberg, 1998; Rifai, 1999; & Bauer, 1996). High density lipoproteins (HDLs) have a critical role in the reverse cholesterol transport pathway; it is well known that a new attempt to reduce the absorption of free fatty acids is by delaying triglyceride digestion with the inhibition of pancreatic lipase. Pancreatic cholesterol esterase plays a pivotal role in hydrolyzing dietary cholesterol esters. The hydrolysis of cholesterol esters in the lumen of the small intestine is catalyzed by pancreatic cholesterol esterase, which liberates free cholesterol (Figure 1).

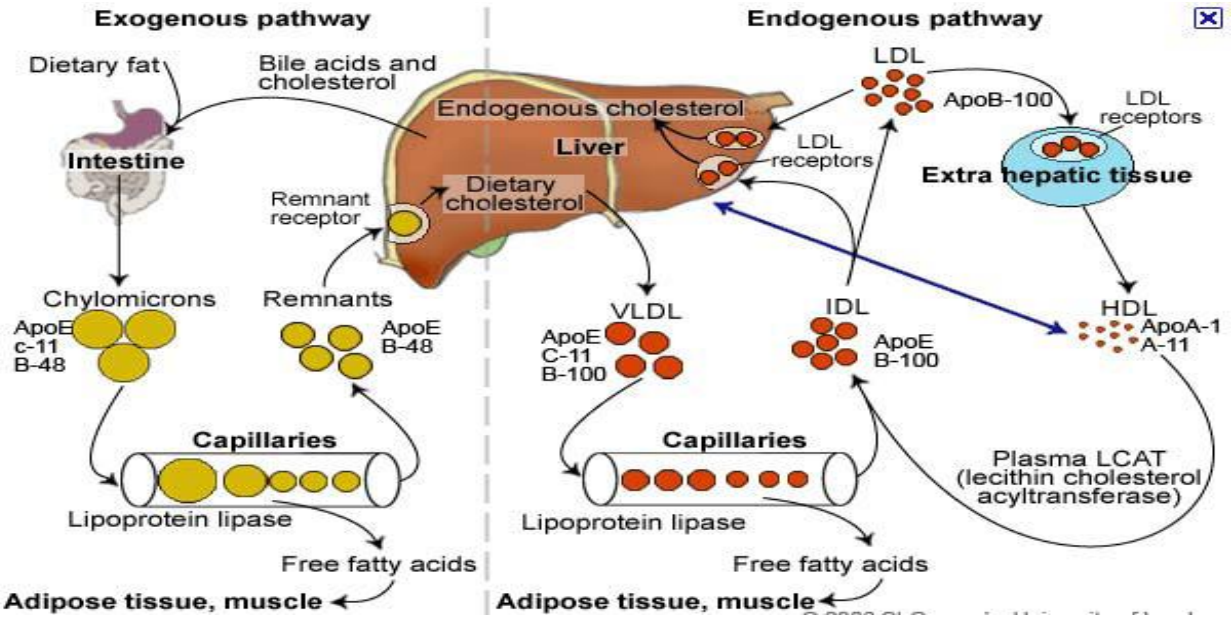


Figure 1: The pathways of lipid metabolism: exogenous pathway and endogenous pathway.

2.2 Hyperlipidaemia

Hyperlipidemia is a medical condition characterized by an elevation of any or all lipid profile and/or lipoproteins in the blood. It is also called hypercholesterolemia/hyperlipoproteinemia (Amit *et al.*, 2011). Although elevated low density lipoprotein cholesterol (LDL) is thought to be the best indicator of atherosclerosis risk, (Amit *et al.*, 2011) dyslipidemia (abnormal amount of lipids in the blood) can also describe elevated total cholesterol (TC) or triglycerides (TG), or low levels of high density lipoprotein cholesterol (HDL). Human body is complex machine for maintaining the homeostasis of various organ and organ system. Any undesirable change will disturb the balance resulting in diseased state (Virchow and Thrombose, 1856). Lipids are fats in the blood stream, commonly divided into cholesterol and triglycerides. Cholesterol circulates in the bloodstream and is involved in the structure and function of cells. Triglycerides (TG) are best viewed as energy that is either used immediately or stored in fat cells. TG is manufactured in the liver from the foods or by being absorbed from the intestine (Ankur *et al.*, 2012). Virchow in 19th century who identified cholesterol crystals in atherosclerotic lesion and stated that endothelial cell injury initiates atherogenesis (Virchow and Thrombose, 1856). In a modification of this hypothesis it was proposed that the endothelium normally influences the behavior of arterial smooth muscle cells by providing a barrier to the passage of plasma proteins, and that the major

effect of hemodynamic or other factors that injure endothelium is to reduce the effectiveness of the barrier (Ross and Glomset, 1976). Arteries are normally smooth and unobstructed on the inside, but in case of increased lipid level, a sticky substance called plaque is formed inside the walls of arteries. This leads to reduced blood flow, leading to stiffening and narrowing of the arteries. It has been proved that elevated plasma levels of cholesterol and of LDL are responsible for atherosclerosis in man, and epidemiological data suggests that elevated plasma levels of HDL have a protective effect (Grundy and Vega, 1998).

2.3 Classification of Hyperlipidaemia

Hyperlipidemia may be classified as either familial (also called primary) caused by specific genetic abnormalities, or acquired (also called secondary) when resulting from another underlying disorder that leads to alterations in plasma lipid and lipoprotein metabolism. Also, hyperlipidemia may be idiopathic, that is without known cause.

2.3.1 Familial (Primary) hyperlipidemia

This usually take place as a result of genetic problems i.e., mutation within receptor protein, which may be due to single (monogonic) gene defect or multiple (polygenic) gene defect. This type may occur as a result of change in dietary and lack of proper physical activities. See table below for summaries the various classes of primary hyperlipidemia based on “Fredricson”

classification.

TYPE	DISORDER	CAUSE	OCCURANCE	ELEVATED PLASMA LIPOPROTEIN
I.	Familial lipoprotein lipase deficiency	Genetic	Very rare	Chylomicrons
IIa	Familial hypercholesterolemia	Genetic	Less common	LDL
IIb	Polygenic hypercholesterolemia	Multifactorial	Commonest	LDL
III	Familial dysbetalipoproteinemia	Genetic	Rare	IDL, Chylomicrons Remnants
IV	Hypertriglyceridemia	Multifactorial Genetic	Common	VLDL
V	Familial combined hyperlipidemia	Genetic	Less common	VLDL,LDL

2.3.2 Acquired (Secondary) Hyperlipidemia

Acquired hyperlipidemias (also called secondary dyslipoproteinemias) may mimic primary forms of hyperlipidemia and can have similar consequences. They may result in increased risk of premature atherosclerosis or, when associated with marked hypertriglyceridemia, may lead to pancreatitis and other complications of the chylomicronemia syndrome. The most common causes of acquired hyperlipidemia are: diabetes Mellitus, myxoedema, nephritic syndrome, chronic alcoholism, use of drugs such as diuretics, beta blockers, and estrogens (Joseph, 2005).

2.4 Causes of Hyperlipidemia

The main cause of hyperlipidemia includes changes in lifestyle habits in which risk factor is mainly poor diet i.e. with a fat intake greater than 40 percent of total calories, saturated fat intake greater than 10 percent of total calories; and cholesterol intake greater than 300 milligrams per day or treatable medical conditions (Durrington, 1995).The abnormal cholesterol levels are the result of an unhealthy lifestyle including taking high-fat diet and other lifestyle factors like being overweight, smoking heavy alcohol use and lack of exercise. Other factors include diabetes, kidney disease, pregnancy, and an underactive thyroid gland (Kelly, 2010). Other illnesses that may elevate cholesterol levels include polycystic ovarian syndrome and kidney disease.

The higher levels of female hormones like estrogen, have been noted to increase or change cholesterol levels. In addition, drugs like diuretics, beta-blockers and medicines used to treat

depression have also been reported to raise cholesterol levels (Lipman et al., 2000). Another modifying factors in the development and progression of hyperlipidemia are age and gender. It has been shown that cholesterol levels rise as the person gets older (Lipman et al., 2000). Heredity has also been a modifying factor for the progression of hyperlipidemia as it has been noted that the genes partly determine the amount of cholesterol body makes (Durrington, 1995). Durrington, (1995), described other factors that cause hyperlipdemia without any prevalence information which are presented in the table below. It has also been noted that chronic renal failure, metabolic syndrome and nephrotic syndrome can predispose to hyperlipidemia are (Durrington, 1995).

S/N	Other Causes of Hyperlipidemia	Description and characteristics
1	**Berardinelli-Seip congenital lipodystrophy- hyperlipidemia**	A rare genetic disorder having heptomegaly, genetic disorder characterized by diabetes mellitus, loss of body fat, enlarged genitals, increased skeletal growth and other abnormalities.
2	**Berardinelli-Seip congenital lipodystrophy, type 1 - hyperlipidemia**	A rare genetic disorder caused by a defect on the AGPAT2 gene on chromosome 9q34.326 characterized by early-onset diabetes mellitus, loss of body fat, serious insulin resistance, high blood triglycerides and fatty liver (Durrington, 1995).
3	**Berardinelli-Seip congenital lipodystrophy, type 2 - hyperlipidemia**	A rare genetic disorder caused by a defect on the BSCL2 gene on chromosome 11q13 by early-onset diabetes mellitus, loss of body fat, serious insulin resistance, high blood triglycerides and fatty liver (Seip, 1959).
4	**Cholestasis**	A condition the bile flow from the liver to the duodenum is blocked. It is of two types first one is caused by mechanical blockage in the duct system which occur from a gallstone or malignancy and other type is metabolic cholestasis, in which disturbances in bile formation occur because of genetic defects or acquired as a side effect of many medications (Trauner *et al.*, 1998).
5	**Chromosome 15q, deletion**	A rare chromosomal disorder which occurs because of deletion of genetic material from the long arm of chromosome 15 (Chaer *et al.*, 2004)
6	**Neuropathy, hereditary motor and sensory, Okinawa type**	This is a dominantly inherited, slow-progressing motor and sensory nerve disease which primarily involves the proximal muscles (i.e. the muscles closest to the trunk of the body) (Takashima, 1997).

2.5 Pathophysiology of Hyperlipidemia

The pathophysiology of hyperlipidemia can be studied under the two basic classification of hyperlipidemia. The pathophysiology of primary hyperlipidemia involve the idiopathic hyperchylomicronemia in which defect in lipid metabolism leads to hypertriglyceridemia and hyperchylomicronemia caused by a defect in lipoprotein lipase activity or the absence of the surface apoprotein CII31. Moreover, hyperchylomicronemia in cats with autosomal recessive defect in lipoprotein lipase (LPL) activity showed the occurrence of primary hyperlipidemia (Gotto and Moon, 2010).

In secondary hyperlipidemia, the postprandial absorption of chylomicrons from the gastrointestinal tract occurs 30- 60 min after ingestion of a meal containing fat that may increase serum triglycerides for 3-10 hours (Bennett, 2005). The diabetes mellitus patients have been noted to possess low LPL activity which further caused high synthesis of VLDL cholesterol by the liver ultimately leading to hyperlipidemia. Moreover, hypothyroidism- induced low LPL activity and lipolytic activity has been noted to reduce hepatic degradation of cholesterol to bile acids. Furthermore, hyperadrenocorticism increased the synthesis of VLDL by the liver causing both hypercholesterolemia and hypertriglyceridemia (Stone, 1994 and Baron, 2005). Liver disease hypercholesterolemia has been noted to be caused by reduced excretion of cholesterol in the bile. Furthermore, in nephrotic syndrome, the common synthetic pathway for albumin and cholesterol causes low oncotic pressure ultimately leading to enhanced cholesterol synthesis (Castilla-Guerra *et al.,* 2009). The response-to-injury hypothesis states that risk factors such as oxidized LDL, mechanical injury to the endothelium, excessive homocysteine, immunologic attack, or infection-induced changes in endothelial and intimal function lead to endothelial dysfunction and a series of cellular interactions that culminate in atherosclerosis. The eventual clinical outcomes may include angina, myocardial infarction, arrhythmias, stroke, peripheral arterial disease, abdominal aortic aneurysm, and sudden death (Castilla-Guerra et al., 2009).

Atherosclerotic lesions are thought to arise from transport and retention of plasma LDL through the endothelial cell layer into the extracellular matrix of the subendothelial space.

Once in the artery wall, LDL is chemically modified through oxidation and nonenzymatic glycation. Mildly oxidized LDL then recruits monocytes into the artery wall. These monocytes then become transformed into macrophages that accelerate LDL oxidation (Castilla-Guerra *et al.,* 2009). Oxidized LDL provokes an inflammatory response mediated by a number of

chemoattractants and cytokines (e.g., monocyte colony-stimulating factor, intercellular adhesion molecule, platelet-derived growth factor, transforming growth factors, interleukin-1, interleukin-6) (Castilla-Guerra et al., 2009). Repeated injury and repair within an atherosclerotic plaque eventually leads to a fibrous cap protecting the underlying core of lipids, collagen, calcium, and inflammatory cells such as T lymphocytes. Maintenance of the fibrous plaque is critical to prevent plaque rupture and subsequent coronary thrombosis (Stone, 1994 and Baron, 2005). The extent of oxidation and the inflammatory response are under genetic control, and primary or genetic lipoprotein disorders are classified into six categories for the phenotypic description of hyperlipidemia. The types and corresponding lipoprotein elevations include the following: (chylomicrons), (LDL), (LDL + very low density lipoprotein, or VLDL), (intermediate-density lipoprotein, or IDL); (VLDL), and (VLDL + chylomicrons). Secondary forms of hyperlipidemia also exist, and several drug classes may elevate lipid levels (e.g., progestins, thiazide diuretics, glucocorticoids, Î² blockers, isotretinoin, protease inhibitors, cyclosporine, mirtazapine, sirolimus) (Stone, 1994 and Baron, 2005). The primary defect in familial hypercholesterolemia is the inability to bind LDL to the LDL receptor (LDL-R) or, rarely, a defect of internalizing the LDL-R complex into the cell after normal binding. This leads to lack of LDL degradation by cells and unregulated biosynthesis of cholesterol, with total cholesterol and LDL-C being inversely proportional to the deficit in LDL receptors (Barbara *et al.,* 2005).

2.6 Clinical Manifestations

The clinical manifestations of hyperlipidemia are as follows;

1. Familial hypercholesterolemia is characterized by a selective elevation in plasma LDL and deposition of LDL- derived cholesterol in tendons (xanthomas) and arteries (atheromas) (Barbara *et al.,* 2005).

2. Familial lipoprotein lipase deficiency is characterized by a massive accumulation of chylomicrons and a corresponding increase in plasma triglycerides or a type I lipoprotein pattern. Presenting manifestations include repeated attacks of pancreatitis and abdominal pain, eruptive cutaneous xanthomatosis, and hepatosplenomegaly beginning in childhood. Symptom severity is proportional to dietary fat intake, and consequently to the elevation of chylomicrons. Accelerated atherosclerosis is not associated with this disease (Barbara *et al.,* 2005).

3. Patients with familial type III hyperlipoproteinemia develop the following clinical features after age 20: xanthoma striata palmaris (yellow discolorations of the palmar and digital creases); tuberous or tuberoeruptive xanthomas (bulbous cutaneous xanthomas); and severe atherosclerosis involving the coronary arteries, internal carotids, and abdominal aorta (Barbara *et al.,* 2005).

4. Type IV hyperlipoproteinemia is common and occurs in adulthood primarily in patients who are obese, diabetic, and hyperuricemic and do not have xanthomas. It may be secondary to alcohol ingestion and can be aggravated by stress, progestins, oral contraceptives, thiazides, or Î² blockers (Barbara *et al.,* 2005).

5. Type V is characterized by abdominal pain, pancreatitis, eruptive xanthomas, and peripheral polyneuropathy. These patients are commonly obese, hyperuricemic, and diabetic; alcohol intake, exogenous estrogens, and renal insufficiency tend to be exacerbating factors. The risk of atherosclerosis is increased with this disorder (Barbara *et al.,* 2005).

2.7 Hyperlipidemia in Different Animals

Hyperlipidemia can be the result of an inherited disease in some animals. Hyperlipidemia in dogs and cats can be physiological (postprandial) or pathological. Increased serum triglyceride and/or cholesterol concentrations have been observed in obese dogs (Chikamune *et al.,* 1995; Bailhache et al., 2003; and Jensette et al., 2005). The most profound changes were associated with severe chronic obesity (Jensette *et al.,* 2005). Weight loss in obese dogs leads to significant decreases in both serum triglyceride and cholesterol concentrations (Jensette *et al.,* 2005; and Diez *et al.,* 2004).

In pets, hyperlipidemia most often occurs as a consequence of some other disorder, such as diabetes mellitus (sugar diabetes), hypothyroidism (low levels of circulating thyroid hormones), Cushing's disease (excessively high cortisone levels in the body), certain liver diseases, and protein-losing nephropathy (a disease of the kidneys resulting in protein loss in the urine). However, hyperlipidemia can also occur spontaneously after a meal of high-fat foods, particularly table scraps.

After eating a meal, the nutrients in an animal's body pass into the small intestine, from which chylomicrons, micro particles of liquid fat, are absorbed 30-60 minutes later. Chylomicrons are

in the classes of lipids, which includes both triglycerides and cholesterol, and which are formed during the digestion of fats from food.

Normally, the absorption of chylomicrons increases serum triglycerides for 3-10 hours, but some animals will have high cholesterol and high triglyceride levels for more than twelve hours after a meal - one of the main indications of hyperlipidemia.

In Equine Poor feed quality or decrease in feed intake, particularly during a period of high-energy requirement (e g, pregnancy, systemic disease), may result in hyperlipidemia syndrome (Karam *et al.,* 2015). Hyperlipidemia is seen most commonly in ponies, miniature horses, and donkeys, and less frequently in standard-size adult horses.

A number of studies have shown that the feeding of fat supplements to ruminant's raises the cholesterol concentration in the serum but not in the tissues or milk in non-ruminants, including primates and man, hypercholesterolemia may be increased by dietary manipulations such as feeding excessive cholesterol or fats with a high saturated fatty acid content. The serum cholesterol concentration does not rise uniformly and hyper-responsiveness has been variously attributed to excessive absorption of cholesterol, (Nestel *et al.,* 1978). In some species of monkey, to diminished re-excretion of cholesterol or bile acids, or to the failure of absorbed cholesterol to exert appropriate feedback inhibition on cholesterol synthesis. As ruminants normally derive all of their cholesterol from endogenous biosynthesis (Nestel *et al.,* 1978), it is reasonable to suppose that the fat-induced hypercholesterolemia in ruminants is due to either an increased synthesis of cholesterol and/ or a decreased fecal excretion of cholesterol or bile acids.

A great number of animal models, such pigeons, chickens, swine, cats, dogs, non-human primates, mice, rabbits and rats, have been tested for hyperlipidemia (Moghadasian, 2002; and Moghadasian, 2001). Consequently, it has been tried to provoke hyperlipidemia in laboratory animals, in order to understand better the relationship between disorders in cholesterol metabolism and atherogenesis and to test possible treatments for the reduction of circulating cholesterol level.

For inducing hypercholesterolemia in rats triglycerides-rich diets containing cholesterol, with or without cholic acid have been used (Lichtman *et al.,* 1999), the level of cholesterol varies substantially as well.

2.8 Garlic (Allium sativum)

Genus Allium is formally classified in the family Liliaceae, represented by 280 separate genera and 4000 species. However, recent taxonomic revisions have seen members of this genus placed in the family Alliaceae of approximately 700 species of Allium. The edible members, including onion (A. *cepa* L.), garlic (A. *sativum* L.), chives (A. *schoenoprasum* L.) leek (A. porrum L.), and welsh onion (A. *fistilulosum* L.*)* are highly prized (Fenwick and Hanley, 1985). Among them, garlic is one of the oldest cultivate plants. It's possible ancestor appears to be A. longicuspis, a native in the mountainous regions of central Asia, which later spread to China, the near east, and the Mediterranean regions before moving west to central and southern Europe, Northern Africa (Egypt) and Mexico (Lutomski, 1987). Today, garlic cultivation is distributed throughout most regions of the temperate world.

Garlic has been used as spice and food ingredient in cooking all over the world because of its combines well with an enormous range of foods, adding its own aroma and flavor as well as enhancing the flavors of the foods with which it is mixed (Woodward, 1996). Besides to be used like food, garlic has long been used in folk medicine with protective and curative purposes.

The earliest indication of the use of garlic is in clay models in Egyptians cemeteries, dated to as early as 3,750 B.C. (Woodward, 1996). It was part of the staple diet of the Egyptian pyramid builders and several cloves of garlic were found in the tomb of Tutankamen. The pharaohs believed that by taking garlic to the afterlife, the food there would always be well seasoned. The Codex Ebers, an Egyptian medical papyrus dated to about 1550 B.C. and translated in 1937, contains over 800 therapeutic formulas of which 22 mention garlic as an effective remedy for a variety of ailments including heart problems, headache, bites, worms and tumors (Block, 1985). Garlic is also mentioned in the literature of Ancient Israel (The Talmud) and in the Bible during the time of Exodus. The Romans also extolled the virtues of garlic. Pliny the elder, a Roman naturalist, described in his Historian Naturalist how garlic could be used for gastrointestinal disorder, dog and snake bites, scorpion stings, asthma, madness, convulsion, tumors and constipation. Dioscorides, a chief physician to the Roman army in the first century A.D., prescribed garlic as a vermifuge or expeller of intestinal worms. Likewise, in Babylonian and Greek civilization, use of garlic has been recorded by Hipocrates, "the father of medicine", as an effective laxative and diuretic, by Aristophanes and Galen as excellent for treatment of uterine tumors, and by Aristotle as a cure for rabies. During the first Olympic Games in Greece in 776

B.C., athletes ingested garlic as stimulant (Fenwick and Hanley, 1985; Block, 1985). In China, garlic tea has long been recommended for fever, headache, cholera, dysentery and prolonging longetivity (Srivastava *et al.,* 1995) and in India, garlic has been used for centuries for the treatment of hemorrhoids, rheumatism, dermatitis, abdominal pain, cough and as an antiseptic lotion for washing wounds and ulcers, due to its antibacterial properties. Indeed, the realization in 1858 by the French Louis Pasteur that garlic had potent antibacterial properties later led to its use in the first and second World Wars, when penicillin and sulfa drugs were scarce, as an antiseptic to disinfect open wounds and prevent gangrene.

Nowadays, garlic is being still employed in folk medicine for over the world for the treatment of various ailments such as cardiovascular diseases, cancer and microbial infections (Ali *et al.,* 2000).

2.9 Chemistry of Garlic

Some of the nutritional and chemical properties of garlic bulbs are given in the table below. Garlic has been analyzed for moisture, carbohydrates, protein, fat, minerals, vitamins, energy, ash, pH, acidity and essential oil contents (Haciseferogullari *et al.,* 2005). Protein content was found to be considerably higher than that in other vegetables such as bean and pea (Cemeroglu and Acar, 1986). But crude oil content was considerable lower. Garlic moisture was also low as compared to caper bud and capperberries fruits (Ozan and Akgul, 1998; Ozan, 1999) and other vegatables (Cemeroglu and Acar, 1986). Among the minerals, garlic is known to contain high levels of potassium (21 g/kg), phosphorus (6 g/kg) followed by magnesium (1 g/kg), sodium (532.78 mg/kg) and iron (52.92 mg/kg). In addition, garlic contains the minerals selenium and germanium. The amount of these minerals in the bulb depends on the content of the respective minerals in the soil where the bulb is grown. Vitamins like riboflavin, thiamin, nicotinic acid, vitamin C and vitamin E are other important chemical constituents.

The biological effects of some of these constituents in intact garlic, such as lectins (the most abundant proteins in garlic), prostanglandins, fructan, pectin, adenosine, vitamin B1, B2, B6, C, and E, biotin, nicotinic acid, fatty acids, glycolipids, phospholipids and essential amino acids, have been studied for over several decades (Fenwick and Hanley, 1985). Recently, special attention has been given to certain steroid saponins and sapogenins such as β-chlorogenin. Several studies have demonstrated the importance of their biological and pharmacological

activities such as antifungal, antibacterial, anttumor, anti-inflammatory, antithrombotic and hypocholesterolaemic properties (Matsuura, 2001 and Lanzotti, 2006). Since β-chlorogenic is bioavailable in vivo and detected in blood, this indicates that β-chlorogenic may be a bioactive compound in garlic. Other characteristics chemical constituents of garlic include allixin and organo-selenium compounds. These chemical compounds are reported to exhibit several biological effects, including cholesterol reduction, cancer prevention and others (Amagase, 2006).

However, despite the fact that the above mentioned compounds contribute in part to garlic bioactivity, evidence from several investigations suggests that the biological and medical functions of garlic are mainly due to their high content in organo-sulphur compounds (Augusti and Mathew, 1974; Wargovich et al., 1988), which likely work synergistically with other compounds such as organo-selenium compounds.

Properties	values	Minerals	values	Vitamins	values
Energy	119kcal	Potassium	446mg	Thiamin	0.16mg
Moisture	70%	Phosphorus	134mg	Riboflavin	0.02mg
Protein	4.3g	Magnesium	24.1mg	Niacin	1.02mg
Carbohydrate	24.3	Sodium	19mg	Pirridoxin	0.32mg
Fiber	1.2g	Calcium	17.8mg	Folic acid	4.8mg
Fat	0.23g	Iron	1.2mg	Vitamin C	14mg
Alcohol	0g	Zinc	1.1mg	Carotenoid	5µg
Ash	2.3%	Iodine	4.7µg	Vitamin A	traces
pH	6.05	Selenium	2µg	Vitamin E	0.011µg
Acidity	0.172%				

Intact garlic cloves contain only a few medicinally active compounds (Block, 1992; Lawson, 1993). The primary sulphur-containing constituents in whole garlic are the S-alk(en)yl-L-cystein sulfoxides (CSs, 1.8%) and Ɣ-glutamyl-S-alk(en)yl-L-cystein peptides (0.9%), both non-volatile and odour-free sulphur compounds. It has been stimated that S-allyl-L-cystein sulphoxide (1)

and S-methyl-L-cystein sulphoxide (methiin), the major CSs in garlic, together with S-(2-carboxypropyl) glutathione, Ɣ-glutamyl-S-allyl-L-cysteine, Ɣ-glutamyl-S-(trans-1-propenyl)-L-cysteine and Ɣ-glutamyl-S-allyl-mercapto-L-cysteine, make up more than 82% of the total sulphur content of whole garlic (Sugii et al., 1964; Fenwick and Hanley, 1985; Sendl, 1995). The glutamylcysteine peptides are biosynthetic intermediates for corresponding CSs (Lancaster and Shaw, 1989). On prolong storage or during germination, the enzyme Ɣ-glutamyl transpeptidase acts on Ɣ-glutamylcysteine peptides to form thiosulfinates (Sendl, 1995) such as S-allyl-cysteine (SAC) (2), which is also present in intact garlic and contributes heavily to the health benefits of some garlic preparation (Amagase *et al.,* 2001). The thiosulfinates other than SAC (e.g. allicin) as well as other oil-soluble components such as ajoenes (e.g. E-ajoene and Z-ajoene), vinyldithiines e.g 2-vinyl-(4H)-1, 3-dithiin and 3-vinyl-(4H)-1,2-dithiin, and sulfides (e.g. diallyl sulphides, DAS, diallyl disulphides, DADS, and diallyl trisulphides, DATS), provide to garlic its characteristic odour and flavor as well as most of their biological properties (Lanzotti, 2006), but they are not naturally occurring compounds in intact garlic. When garlic is cut, crushed, chewed, dehydrated or otherwise processed, the vacuolar enzyme, allinase, is released and rapidly lyses the cytosolic CSs (mainly alliin), which are converted into hundreds of organo-sulphur compounds in a short period of time. First, it is formed the reactive intermediate allylsulfenic acid (R-SOH), which immediately condenses to form the odoriferous alkyl alkane-thiosulfinates, among which allicin represents 70-80% of total. Then allicin (allyl 2-propene thiosulfinate) and other thiosulfinates such as allyl methane thiosulfinate, which are very unstable products, instantly undergo a number of transformations, giving rise to other sulphur-compounds derivatives (e.g. ajoene, dithiins, DAS, DADS etc.), depending on environmental and processing conditions (as temperature, PH, and solvent polarity) (Block, 1985; Reuter and Sendl, 1995; Amagase, 2001) sulphur containing compounds in commercial garlic preparation vary depending on their manufacturing processes. Likewise, the variety of garlic determines the composition and quantity of each CS identified in garlic, which, in turn, determine the odour, flavor variation and biological activities observed for garlic.

In addition to odoriferous oil-soluble compounds, less odorous water-soluble organosulphur compounds such as SAC and S-allylmercaptocysteine (SAMC) have shown to be biologically active in several areas. The non-volatile sulphur-containing compounds SAC and SAMC are

present in several garlic preparations, although the content varies considerably (Lawson, 1993; Imai, *et al.*, 1994).

Given such chemical diversity, garlic has received considerable attention from both chemist and biologist alike as new source of bioactive compounds.

Below is the chemical reaction showing the formation of allicin from alliin by the action of an enzyme called allinase.

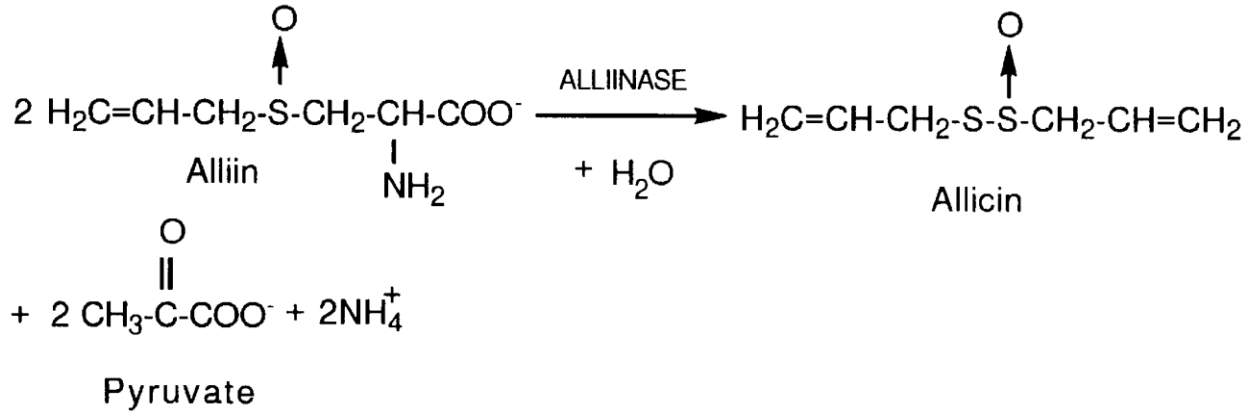


Figure 2. Generation of allicin in garlic (Ellmore, 1994).

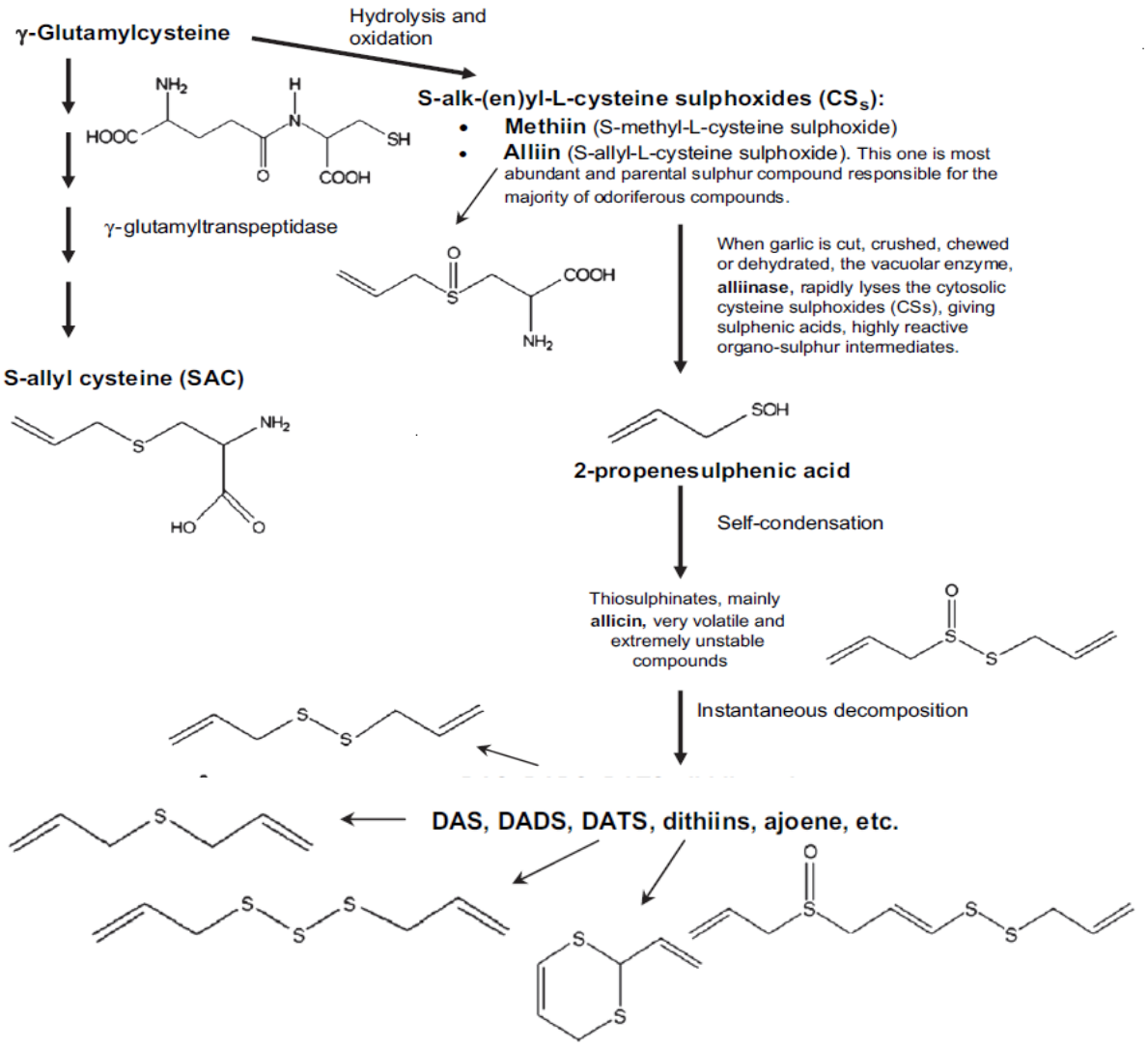


Figure 3. Formation of organo-sulphur compounds during metabolic pathways in processed garlic (*Allium sativum*) (Truswel *et al.*, 1991).

2.10 Role of Garlic in Health

Garlic can rightfully be called one of nature's wonderful plants with healing power. It can inhibit and kill bacteria, fungi, lower (blood pressure, blood cholesterol and blood sugar), prevent blood clotting, and contains anti-tumor properties. It can also boost the immune system to fight off potential disease and maintain health (Abdullah *et al.*, 1988). It has the ability to stimulate the lymphatic system which expedites the removal of waste products from the body. It is also considered an effective antioxidant to protect cells against free radical damage. It can help to prevent some forms of cancer, heart disease, strokes and viral infections. Garlic alone can

provide us with over two hundred unusual chemicals that have the capability of protecting the human body from a wide variety of diseases. The sulfur containing compounds found in garlic afford the human body with protection by stimulating the production of certain beneficial enzymes (Mansell and Reckless, 1991).

2.10.1 Treat Cardiovascular Disease

Disorders of the heart and the circulatory system claim more lives than any other diseases. It is the obstruction or clogging of the coronary arteries which causes more deaths than any other factors. The arteries, which supply the heart with blood and oxygen, become increasingly narrower as plaque builds up over time. When blood supply becomes restricted, a certain portion of the heart is deprived of oxygen and leads to heart attack. The two greatest means of heart disease are high blood pressure and high blood serum cholesterol levels; which are directly impacted by the therapeutic action of garlic. The relevant role of garlic in coronary heart disease was done on rabbits and found that even pre-existing athero-sclerotic deposits and lesions could actually be reversed if garlic was consistently consumed (Bordia, 1981).

From a study conducted in India, 432 coronary artery patients were randomly grouped into two groups and half of them were supplied with garlic juice in milk, whereas the other group patients were not supplied with garlic juice. The result showed that within the three years of the study time, nearly twice as many patients had died in the group not supplied with garlic juice (Yeh *et al.,* 2006). It is well reported to scavenge oxidants, increase superoxide dismutase, catalase, glutathione peroxidase, glutathione levels, inhibit lipid peroxidation as well as it reduces cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA. It has been shown to reduce platelet aggregation, arterial plaque formation, decrease homocysteine, lower blood pressure, and increase microcirculation. It may also help prevent cognitive decline by protecting neurons from neurotoxicity and apoptosis, thereby preventing ischaemia or reperfusion-related neuronal death and by improving learning and memory retention (Borek, 2006)

2.10.2 Reduces High blood Pressure

Garlic has probably been most popularized as a com-plementary therapy for blood pressure control (Capraz *et al.,* 2006). A recent *in vitro* study has confirmed that, the vasoactive ability of garlic sulfur compounds whereby red blood cells convert garlic organic polysulfides into hydrogen sulfide, a known endogenous cardio-protective vascular cell signaling molecule

(Benavides *et al.,* 2007). Using 2400 mg garlic tablet containing 31.2 mg allicin has high dose reduced diastolic pressure by 16 mmHg after 5 h of administration (McMahon and Vargas, 1993). A meta-analysis made on pooled data from 415 patients showed also reduction of 7.7 mmHg diastolic pressure (Silagy and Neil, 1994).

2.10.3 As Natural Blood Thinner

Platelets and fibrin play great role in blood clotting and higher amount of fibrin in blood can cause heart attack. Garlic constituents can reduce fibrin formation and also help reduce the fibrin existing in the blood even better than aspirin (Fukao *et al.,* 2007). Ajoene, a sulfur compound found in garlic seems to be responsible for its anti-clotting effect; but ajoene is only viable at room temperature or above, it is not present in raw or freeze-dried garlic. It is believed that the addition of garlic to a diet can help to increase the breakdown of fibrin from 24 to 30% in people (Ernst, 1994).

2.10.4 As Natural Immunity Booster

With the arrival of frightening viral diseases like HIV/AIDS, boosting immunity system is receiving a new attention. Because these types of diseases have no effective cures or treatments, strengthening the body's ability to fight off infection has become even more important. Garlic has abundant sulfur containing amino acids and other compounds that seem to initiate increased activity in the immune system (Lau *et al.,* 1991). It is one of the impressive conductors of the body's immune system; which stimulates immune function by making macrophages or killer cells more active. We are constantly beaten by inadequate nutrition, cigarette smoke, physical injury, mental tension and chemical pollution. In light of the enormous pressures, which our immune systems sustain, supplemental nutrients like garlic are clearly needed (Salman *et al.,* 1999). Its remarkable content of germanium alone offers excellent immune stimulation. In addition to germanium, garlic contains thiamine, sulfur, niacin, phosphorous, and selenium (Morioka *et al.,* 1993).

Preliminary studies in humans, using an alliin standar-dized garlic powder preparation, have demonstrated positive effects on immunoreactions and phagocytosis. In aged subjects, the administration of 600 mg garlic powder per day for 3 months induced significant ($p<0.01$) increases in the percentage of phagocytosing peripheral granulocytes and monocytes when tested *ex vivo* for their ability to engulf *Escherichia coli* bacteria. Another human study was conducted

with an unrefined garlic extract (5 to 10 g/day) which was given to HIV/AIDS patients. For the seven patients who completed the 12 weeks study, there was a major increase in the natural killer cells activity from a seriously low mean value (Abdullah *et al.*, 1988).

In USA, trials in HIV/AIDS patients have demonstrated the enhancement of natural killer cells activity using garlic extracts; and Chinese studies with viral infections in bone marrow transplant patients have demonstrated a "potent antiviral activity". A double blind placebo controlled survey using a 100% allicin yielding supplement has reported that allicin can reduce the occurrence of the common cold and recovered from symptoms (Josling, 2001).

2.10.5 Prevents Diabetes

A number of animal studies support the effectiveness of garlic in reducing blood glucose in streptozotocin-induced as well as alloxan-induced diabetes mellitus in mice. Most of the studies showed that garlic can reduce blood glucose level in diabetic mice and rabbits (Ohaeri, 2001). A study was conducted to evaluate oral administration of garlic extract for 14 days on the level of serum glucose, total cholesterol, triglycerides, urea and uric acid, in normal and streptozotocin-induced diabetic mice. The result of the study showed significant decrease ($p<0.05$) in serum glucose, total cholesterol, triglycerides, urea, uric acid, aspartate amino transferase and alanine amino transferase levels, while increased serum insulin in diabetic mice, but not in normal mice. From a comparison study made between the action of garlic extract and glibenclamide, it was shown that the antidiabetic effect of the garlic was more effective than the glibenclamide (Eidi *et al.*, 2006).

2.10.6 Anticancer

The favorable action of garlic, inhibition of the growth of cancer is perhaps the most prominent. It has several synergistic effects that either prevent or possibly may fight cancer. The action of garlic has been attributed to stimulate immune effector cells including T-cell and natural killer cells. Numerous epidemiological, clinical and laboratory studies have demonstrated that, garlic has a great role in cancer prevention especially in relation to digestive tract cancers. Human population studies have shown that, regular intake of garlic reduces the risk of esophageal, stomach and colon cancer. This was thought to be due to the antioxidant effect of allicin in reducing the formation of carcinogenic compounds in the gastro-intestinal tract (Galeone *et al.*, 2006).

Dutch research in the Netherlands cohort study found a significant decrease in the development of stomach cancer in those consuming garlic close relatives of onions (Dorant *et al.,* 1996). Garlic reduces the risk of patients with prostate cancer, especially those with localized disease. Men in the higher of two intake categories of total *Allium* vegetables (>10.0 g/day) had a statistically significant lower risk of prostate cancer than those in the lowest category (<2.2 g/day). Similar comparisons between categories showed reductions in risk for men in the highest intake categories for garlic specifically. The reduced risk of prostate cancer was independent of body size, intake of other foods and total calorie intake and was more pronounced for men with localized prostate cancer than with advanced prostate cancer (Hsing *et al.,* 2002). Prostate specific antigen serum markers had significant decreases during short term ingestion, but returned to baseline after 4 weeks (Mehraban *et al.,* 2006).

A very important epidemiological study for Americans has been published in which the intake of 127 foods (including 44 vegetables and fruits) was determined in 41,387 women (ages 55 to 69) followed by a five year monitoring of colon cancer incidence. The most striking result of this "Iowa Women's Health Study" was the finding that garlic was the only food which showed a statistically significant association with decreased colon cancer risk. For cancers anywhere in the colon, the modest consumption of one or more servings of garlic (fresh or powdered) per week resulted in a 35% lower risk, while a 50% lower risk was found for cancer of the distal colon (Steinmetz *et al.,* 1994).

2.10.7 Dermatologic Applications

A study examined 43 persons for their topical use of two different garlic extracts for wart and corn treatment. Of these persons, 15 volunteers utilized a water extract of garlic, while 23 volunteers applied lipid extract to appropriate areas twice a day. Five controls applied only a neutral solvent. All lipid extract volunteers experienced complete resolution of wart and 80% of corn within one to two weeks. The water extract seemed to be less potent, with complete dissolution of smaller warts and corns, and only partial dissolution of larger ones. Controls showed no improvement from baseline. The lipid extract did cause some burning, redness, blistering and skin darken-ing, which was resolved after conclusion of use (Dehghani *et al.,* 2005).

2.10.8 Antimicrobial Activity

The antimicrobial properties of garlic were first described by Pasteur (1958), and since then, many researches had demonstrated its effectiveness and broad spectrum antimicrobial activity against many species of bacteria, viruses, parasites, protozoan and fungi (Jaber and Al-Mossawi, 2007). Garlic is more effective with least side effects as compared to commercial antibiotics; as a result, they are used as an alternative remedy for treatment of various infections (Tepe *et al.,* 2004). Out of the many medicinal plants, garlic has an antimicrobial property which protects the host from other pathogens highlighting the importance of search for natural antimicrobial drugs (Bajpai *et al.,* 2005; Wojdylo *et al.,* 2007). Previously conducted researches confirmed that garlic is not only effective against Gram positive and Gram negative bacteria but also possess antiviral and antifungal activities (Tsao and Yin, 2001).

2.10.9 Antiviral

Garlic and its sulfur constituents verified antiviral activity against coxsackievirus species, herpes simplex virus types 1 and 2, influenza B, para-influenza virus type 3, vaccinia virus, vesicular stomatitis virus, human immunodeficiency virus type 1 and human rhinovirus type 2. The order of compounds found in garlic for virucidal activity was, ajoene > allicin > allyl methyl thiosulfanate > methyl allyl thiosulfanate; no activity was found for the polar fractions, alliin, deoxyalliin, diallyl disulfide, or diallyl trisulfide. Several laboratory tests have shown that garlic is an effectual treatment for both the influenza B virus and herpes simplex virus. Two independent researchers in Japan and Romania have found that garlic is able to protect living organisms from the influenza virus (Tsai *et al.,* 1985). Most recently, a double blind placebo con-trolled study has shown significant protection from the common cold virus. As conducted by The Garlic Centre, published in Advances in Therapy, this is the first serious work to show prevention, treatment and reduction of re-infection benefits from taking Allimax Powder capsules once daily (Josling, 2001).

2.10.10 Antibacterial

Garlic extract inhibits the growth of Gram positive and Gram negative bacteria, such as *Staphylococcus, Streptococcus, Micrococcus, Enterobacter, Escherichia, Klebsiella, Lactobacillus, Pseudomonas, Shigella, Salmonella, Proteus,* and *Helicobacter pylori* (Tsao and

Yin, 2001). Its antibacterial activity is mainly due to the presence of allicin produced by the enzymatic activity of allinase on alliin. Allicin is considered to be the most potent antibacterial agent in crushed garlic extracts, but it can be unstable, breaking down within 16 h at 23°C (Hahn, 1996). However, the use of a water-based extract of allicin stabilizes the allicin molecule due to the hydrogen bonding of water to the reactive oxygen atom in allicin or there may be water soluble components in crushed garlic that destabilize the molecule (Lawson, 1996). The disadvantage of this approach is that allicin can react with water to form diallyl disulphide, which does not exhibit the same level of antibacterial activity of allicin (Lawson and Wang, 1996).

2.10.11 Antifungal

Ajoene is an active compound found in garlic which plays a great role as topical antifungal agent (Ledezma and Apitz-Castro, 2006). Garlic has been shown to inhibit growth of fungal diseases as equally as the drug ketoconazole, when tested on the fungi *Malassezia furfur*, *Candida albicans*, *Aspergillus*, *Cryptococcus* and other *Candida* species (Shams-Ghahfarokhi et al., 2006). A report from a Chinese medical journal delineates the use of intravenous garlic to treat a potentially fatal and rare fungal infection of the brain called *Cryptococcus meningitis*. In the report, the Chinese compared the effec-tiveness of the garlic with standard medical treatment which involved a very toxic antibiotic called Amphotericin-B. The study revealed that, intravenous garlic was more effective than the drug and was not toxic regardless of its dosage (Lemar *et al.,* 2007).

A study found that *Candida* colonies were substantially reduced in mice that had been treated using liquid garlic extract. The study also revealed that garlic stimulated phagocytic activity. This implies that infections such as *Candida* may be controlled because garlic stimulates the body's own defenses. Garlic oil can be used to treat ring-worm, skin parasites and warts if it is applied externally. Lesions that were caused by skin fungi in rabbits and guinea pigs were treated with external applications of garlic extract and began to heal after seven days (Sabitha *et al.,* 2005).

2.10.12 Antiparasitic

Many herbalists worldwide recommend garlic as a treat-ment for intestinal parasites. In some cultures, children infested with helminthes are treated with enemas containing crushed garlic. One of the traditional Chinese medical treatments for intestinal diseases is an alcoholic extract of

crushed garlic cloves. Allicin exhibits anti-parasitic activity against major human intestinal parasites such as *Entamoeba histolytica*, *Ascaris lumbricoides* and *Giardia lamblia* (Kalyesa *et al.,* 1975). *Entamoeba histolytica,* the human intestinal protozoan parasite, is very sensitive to allicin, as only 30 µg/ml of allicin totally inhibits the growth of amoeba cultures (Mirelman *et al.,* 1987). Moreover, researchers have found that at lower concentrations (5 µg/ml), allicin inhibited 90% the virulence of trophozoites of *E. histolytica* as determined by their inability to destroy mono-layers of tissue-cultured mammalian cells *in vitro* (Ankri *et al.,* 1997).

2.10.13 Antioxidant

Whole garlic and aged garlic extract exhibit direct antioxidant effects and enhance the serum levels of two antioxidant enzymes, catalase and glutathione peroxidase (Prasad *et al.,* 1995). Garlic extract, allicin is efficiently scavenged exogenously generated hydroxyl radicals in a dose dependent fashion, but their effective-ness was reduced about 10% by heating to 100°C for 20 min. Other garlic constituents, such as S-allyl cysteine, also confirmed significant antioxidant effects. The sulfur compounds found in fresh garlic appear to be nearly 1000 times more potent as antioxidants than crude, aged garlic extract. Garlic (both the homogenate of 10% in physiological saline solution and its supernatant) was able to reduce the radicals present in cigarette smoke (Torok *et al.,* 1994).

2.10.14 Reduces Stress

Among the many uses of garlic, it appears to have the fortunate capacity for protecting against the negative effects of stress that affects the autonomic nervous and neuroendocrine system. Rats that were trained with endurance exercises to physical fatigue enjoyed improved parameters of aerobic glucose metabolism, attenuated oxidative stress, and vasodilations, when given garlic at a dosage of 2.86 g/kg for 30 min before exercise (Morihara *et al.,* 2006). In rats exposed to psychologically stressful situations, aged garlic extracts significantly prevented the decreases in spleen weight seen in control animals. Additionally, the garlic significantly prevented the reduc tion of hemolytic plaque forming cells in spleen cells.

Moreover, garlic was able to block the lipopolysaccharide induced immune cytokine and plasma corticosterone and catecholamine changes following cold water immersion stress (Nance *et al.,* 2006). Aged garlic extract is also effective to prevent adrenal hypertrophy, hyperglycemia and elevation of corticosterone in hyperglycemic mice induced by immobilization stress. Given the

extreme chronic stress many people now face in their daily life, garlic may prove useful to counter the negative impact of this stress on human physiology (Kasuga *et al.,* 1999).

2.11 Adverse Effects of Garlic

The main adverse effect commonly associated with garlic intake is breath odor, especially when raw forms of the herb are used. Nausea and vomiting are other major adverse effects and care should be taken in consuming high quantities. Although an entire bulb produces little juice, it is potent and can act as a strong emetic, even in small quantities. Although garlic generally poses little in terms of safety issues, there are isolated cases of topical garlic burns (Friedman *et al.,* 2006) and anaphylaxis (Yin and Li, 2007). Rare garlic allergy has been attributed to the protein allinase, which has induced immunoglobulin E (IgE) mediated hypersensitivity responses from skin prick testing (Kao *et al.,* 2004). As a result, the literature has generally cautioned against using garlic while using anticoagulant therapy. There is a reported case of spontaneous spinal or epidural hematoma in an 87 years old man, with associated platelet dysfunction related to excessive garlic ingestion (Saw *et al.,* 2006).

2.11.1 Role of Garlic against Multi-Drug Resistant Bacteria

Garlic is active against microorganisms that are resistant to antibiotics and the combination of garlic extracts with antibiotics leads to partial and total synergism (Didry *et al.,* 1992). The emergence of multi-drug resistant strains of Gram negative (*Pseudomonas*, *Klebsiella*, *Entero-bacter*, *Acinetobacter*, *Salmonella* species, etc) and Gram positive (*Staphylococcus*, *Enterococcus*, *Strepto-coccus* species, etc) bacteria is troubling for human and animals. The emergence of epidemic methicillin resistant *Staphylococcus aureus* (MRSA) resistant to mupirocin has led many authors to suggest that the use of mupirocin should be controlled more strictly, especially as there is a lack of alternative agents. Consequently, garlic is an alternative agent for the treatment of MRSA and in a great demand (Sharma *et al.,* 2005).

2.11.2 Role of Garlic against Multi-drug Resistant Tuberculosis (MDR-TB)

Scientific evidence from randomized clinical trials supports the use of garlic and enhances access for MDR-TB infected people, through the public health system. Its use can allow an effective MDR-TB management, due to its affordability and the absence of toxic effects (Catia *et al.,* 2011). In view of the increased incidence of MDR-TB, the research of new anti-tubercular drugs

based on affordable and more effective treatments has already begun. Studies on innovative alternative plant extracts of medicinal values need to be emphasized, as plants are an important source of new antimicrobial agents, with little toxicity, able to replace drugs to which *Mycobacterium* resistance has occurred (Amin *et al.*, 2009).

As garlic is concerned, the *in vitro* tests undertaken about the inhibitory effect on MDR-TB are at an advanced stage whereas few researches *in vivo* have been conducted. The concentration of garlic extract re-quired was in the range of 1.34 to 3.35 mg/ml suggesting that there is only a slight variation in the susceptibility of the strains to allicin (Delaha and Garagusi, 1985). The anti-tuberculosis activity *in vivo* of garlic oil preparation was demonstrated in a study of guinea pigs which were given an intra-peritoneal dose of 0.5 mg/kg. However, when garlic oil was used, a reduced causative process was noted in the organs involved, indicating that garlic oil administration causes less marked lesions in the viscera of the animals inoculated with tubercle bacilli (Jain, 1998). The high potential of garlic extract was revealed to inhibit the growth of *Mycobacterium tuberculosis* H37Rv and *M. tuberculosis* TRC-C1193, susceptible and resis-tant to isoniazid (first-line anti-tuberculosis medication), respectively. The minimum inhibitory concentration (MIC) of garlic was between 80 and 160 μg/ml for the susceptible strain and 100 and 200 μg/ml for the resistant strain. In addition, water extract of garlic was proven to inhibit the incorporation of 14C glycine into the whole cells, indicating that the primary mechanism of action is by inhibition of protein synthesis (Ratnakar and Murthy, 1996).

An interesting *in vitro* test about the anti-tubercular activity of garlic was performed in Nigeria using disc diffusion method and compared with standard antibiotics. The anti-tubercular activity of garlic on multiple-drug re-sistant *Mycobacterium* was investigated among Nigerian HIV-infected-persons and it exhibited maximal activity against all isolates even at reduced concentrations. Only two of the standard anti-tubercular antibiotics used, streptomycin and rifampicin, showed significant activity against isolates tested (Dibua, 2010).

2.11.3 Effect on Levels of Serum Lipids (Cholesterol and Triglycerides)

Cholesterol is an extremely important biological molecule that has role in membrane structure as well as being a precursor for the synthesis of the steroid hormones and bile acids. Both, dietary cholesterol and that synthesized *de novo* are transported through the circulation in lipoprotein particles, being stored as cholesteryl esters in cell.

The synthesis and utilization of cholesterol must be tightly regulated in order to prevent over accumulation and abnormal deposition within the body. Slightly less than half of the cholesterol in the body derives from biosynthesis *de novo*. Biosynthesis in the liver accounts for approximately 10% and in intestine approximately 15%, of the amount produced each day. Cholesterol synthesis occurs in the cytoplasm and microsomes from the two-carbon acetate group of acetyl-CoA (King and Marchesini, 2007).

2.11.4 Active Compounds and Anti-cholesterolaemic Pathway by Garlic Derivatives

Organo-sulphur compounds are the main active substances responsible for the hypolipidaemic and hypocholesterolaemic effects of garlic, as much in humans as in experimentation animals (Yeh *et al.,* 1997; Liu and Yeh, 2002). Several decades ago, Gebhardt (1993) reported the multiple inhibitory effects of garlic extracts in several different steps in cholesterol biosynthesis pathway in human hepatic cells. According to him, defined compounds (allicin) present in water-soluble extracts of garlic inhibit the biosynthesis of cholesterol in hepatocytes, thus contributing to the reduction of serum cholesterol. Thus, it was demonstrated that allicin extracted from garlic decreases total serum lipids, cholesterol and phospholipids contents in rats fed allicin as compared to control animals (Augusti and Mathew, 1974). Some allicin-derived compounds in garlic that have demonstrated to possess a beneficial effect on cardiovascular variables are ajoene, methyl ajoene, DAS, DATS, 2-vinyl-4H-1,3-dithiin and SAC. Methiin and flavonoid quercetin (Glasser *et al.,* 2002) have also shown to have the ability to reduce serum cholesterol levels and arteriosclerosis severity. Moreover, other no sulphur components of garlic, such as steroid saponins, have also demonstrated to be able to reduce serum cholesterol concentrations (Koch, 1993).

All these compounds may exert their hypocholesterolaemic effect by three different mechanism; by inhibiting hepatic cholesterol biosynthesis (Gebhardt *et al.,* 1994; Gupta and Porter, 2001; Singh and Porter, 2006), by enhancing cholesterol turnover to bile acids and its excretion through gastrointestinal tract (Srinivasan and Sambaiah, 1991), or in the case of plant saponins, and by inhibiting cholesterol absorption from intestinal lumen without changing HDL cholesterol levels in hypercholesterolaemic animal models (Matsuura, 2001; Slowing *et al.,* 2001).

2.12 Antioxidants and its Functions in Human Body

Antioxidants are believed to play a very important role in the body defense system against ROS Boxin *et al.* (2002), Vivek and Surendra (2006). In another term antioxidant is "any substance that when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate Halliwell and Gutteridge (1995). Halliwell (2007) reported that an antioxidant is "any substance that delays, prevents or removes oxidative damage to a target molecule. Antioxidants are an inhibitor of the process of oxidation, even at relatively small concentration and thus have diverse physiological role in the body. Antioxidant constituents of the plant material act as radical scavengers, and helps in converting the radicals to less reactive species. A variety of free radical scavenging antioxidants is found in dietary sources like fruits, vegetables and tea, etc. This review presents some information about the antioxidant/antiradicals and their role in our body and also their presence in spices and herbs Nema *et al.* (2009). Mark Perciva (1998) Antioxidants are our first line of defense against free radical damage, and are critical for maintaining optimum health and well being. Regular consumption of anti-oxidative vegetables and fruits has been recognized as reducing the risk of chronic diseases Dembinska-Kiec et al. (2008). Studies demonstrate that an antioxidantrich diet has a very positive health impact in the long run Sin *et al.* (2013) and Willis *et al.* (2009). It is a well-known fact that citrus fruits (oranges, lemons, etc.) contain a high amount of natural antioxidants, such as vitamin C. Blueberries, strawberries, grapes, plums, prunes, red beans, spinach, kale, broccoli flowers, alfalfa sprouts, and more have been proven to contain a high amount of antioxidants and have been incorporated into many dietary menus Cao *et al.* (1998) and Grossman *et al.* (1994). Recent studies also suggested that fruit-like jackfruit, araticu-domato, pindo palm, and mandacaru-de-trêsquinas are good sources of vitamins C and A and phenolic compounds (Swami *et al.* (2012) and Pereira *et al*. (2013). In addition, there are studies that research genetic, chemical, or biological modification in order to increase the antioxidant potency of fruits (Gomes *et al*. (2013).

2.13 History of Antioxidants

Antioxidants have been used for the first time in the nineteenth century in the rubber industry, when it was observed that some molecules, identified empirically, could slow the degradation and allow optimization of the process of vulcanization. Today we know that the production and

use of rubber reactions take place involving free radicals and oxygen and antioxidants is still a useful tool in the hands of those who need to optimize the performance of our tires. In the twentieth century antioxidants are then entered in the arsenal of the emerging food industry, as a key tool to curb the oxidative degradation of stored food**.** In that regard, it must be said that, since at that time known mechanisms of oxidation nor antioxidant effect, the connotation of "antioxidant" could only be empirical, grouping any compound or procedure that led to the result of slow degradation and rancidity (Entian, 1980). This has resulted in an inaccuracy semantics which often leads today to a different definition of antioxidant between chemists, biologists and food technologists. If also did not know the free radicals, was known already at the end of the eighteenth century that oxygen, had an extremely important role for life, but also for degradation of biological material. The most significant demonstration of the role in modern biology radical oxidation is given in the early 50's when in Buenos Aires Gerchman Rebecca and Daniel Gilbert found that the toxic effect of radiation was greatly enhanced by the presence of oxygen. In terms of antioxidants, a milestone in the evolution of knowledge is the discovery of Albert Szent Györgyi, in the thirties, which, starting from the study of the reasons dell'imbrunimento apples, discovered vitamin C. The same researchers also studied the antioxidant effect of polyphenols found in plants and proposed a function vitamin (vitamin P) but could not withstand the criticism of the absence of deficiency syndrome, as fundamental to the definition of a function vitamin. The cultural boundary was beginning to be quite defined in 50-60 years: it was known that breathing (which is a form of controlled combustion) required oxygen activation and that breathing could generate free radicals. On this basis, supplemented by observations of acute medical and patho physiological, Denim Harman proposed the free radical theory of aging: if we use oxygen to convert our energy, we cannot escape its toxicity which slowly deteriorates there. The 'sharpness of the proposal has been confirmed in experimental biology, it is now generally accepted that the maximum health potential of a species is determined largely (Bhide, 1995), though not entirely, the ability to defend against oxidation**.** The 'scientific information is then available today to interpret phenomena from the cellular level to that of quantum mechanics. But what is the medical and nutritional aspect of it all? The first answers cannot be that epidemiology in recent years has seen an explosion of meaning, quality and therefore of relevance. In practice, epidemiological studies of various kinds agree that "taking antioxidants in the diet is good" with respect to the reduction of risk of chronic degenerative diseases-mainly atherosclerosis and

possibly reduce the risk of cancer (Entian, 1980). The syllogism that were due to the mechanisms of chemical reactivity of oxide-reductive antioxidants was then supported by a significant number of studies in vitro or cellular models showed different effects of different molecules-generally-polyphenols.

A more careful examination, however, concentrations obtainable in the body compared to those necessary to obtain an increase in antioxidant defense seems to exclude any realistic in vivo antioxidant effect of different dietary polyphenols. In practice, the only food antioxidants that reasonably can have an effect in vivo antioxidant are Vitamin C and Vitamin E. Their deficiency syndrome, however, is typical dell'avitaminosi and certainly not what you would expect from a deficiency of antioxidant defense (Raghavarao, 1998).

On other side, it is now shown that many natural substances of polyphenolic nature**,** and functionally antioxidants, may act by modulating the expression of genes**,** a mechanism that little or nothing to do with transitions oxide-reductive typical antioxidant effect. The acquisition of this type of information is in its infancy, being supported by complex expression analysis today only possible as a result dell'elucidazione genome. In other words phenolic antioxidants food regulatory elements are configured as "nutrigenomics," to use a practical how ugly neologism (Yanishlieva, 2000).

2.14 Reactive Oxygen Species

Reactive oxygen species (ROS) is a term which encompasses all highly reactive, oxygen containing molecules, including free radicals. Types of ROS include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides. All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage (Rhopde *et al.*, 2001).

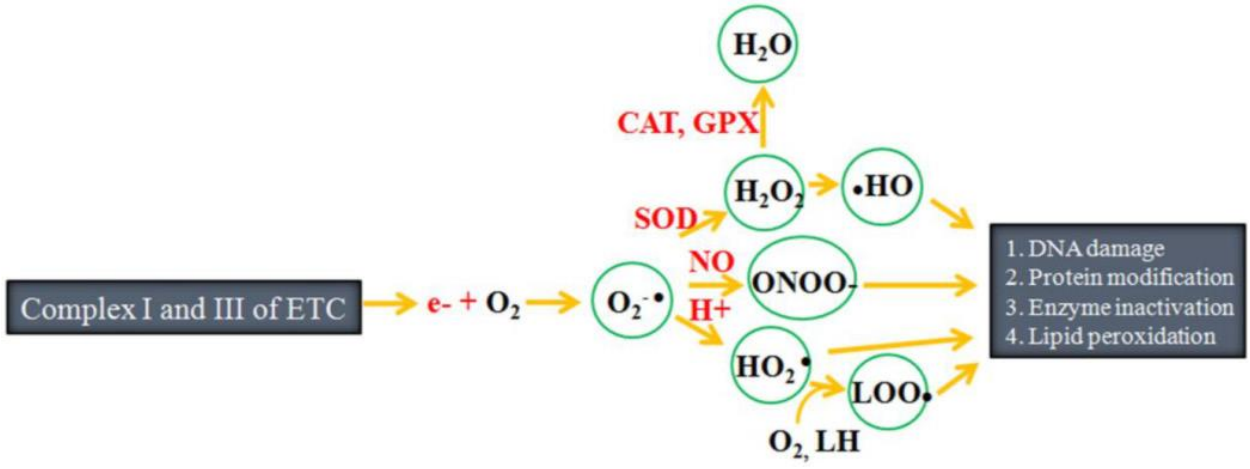


Figure 4. Outline of free radical production:

During electron transfer, approximately 1–2% of the electrons slip from complex I and III of the electron transfer chain (ETC), after which they react with molecular oxygen to form free radicals such as superoxide anion ($O_2^{-\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$), hydroxyl radical, hydrogen peroxide (H_2O_2), hydroperoxyl radical ($^{\bullet}OH$), peroxynitrite ($ONOO^-$) and lipid peroxyl radical ($LOO^{\bullet}$). These free radicals target biomolecules such as DNA, protein and lipids, ultimately damaging them.

2.15 Various Types of Antioxidants

In present time various antioxidant found in food viz. natural antioxidants, synthetic antioxidants, dietary antioxidant, endogenous antioxidant which play an important role in preservation of food.

2.15.1 Dietary Antioxidants

The dietary antioxidants such as ascorbates, tocopherols and carotenoids are well known and there is a surplus of publications related to their role in health Boskou *et al.* (2005). Vitamin C, vitamin E, and beta carotene, Beta carotene and other carotenoids and oxycarotenoids, e.g., lycopene and luteinare among the most widely studied dietary antioxidants. In extracellular fluids vitamin C is considered the most important water-soluble antioxidant. It is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated. Vitamin E, a major lipid-soluble antioxidant, is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation. Vitamin C has been cited as being capable of regenerating vitamin E Sies (1992). Beta carotene and other carotenoids

are also believed to provide antioxidant protection to lipid-rich tissues. Research suggests beta carotene may work synergistically with vitamin E Jocab (1995). In plants, flavonoids serve as protectors against a wide variety of environmental stresses while, in humans, flavonoids appear to function as "biological response modifiers." Flavonoids have been demonstrated to have anti-inflammatory, antiallergenic, anti-viral, anti-aging, and anti carcinogenic activity Cody *et al.* (1986); Kuhnau *et al.* (1976); Havsteen (1983) and Middleton (1984).

2.15.1.1 Basic Characteristics of Dietary Antioxidants

Dietary antioxidants are mainly secondary metabolites that plants synthesize to protect themselves against oxidative stress. According to their chemistry, they may be grouped into four classes: vitamin C (ascorbic acid); vitamin E (tocopherols); carotenoids (e.g. α and β-carotenes, lycopene, lutein) and polyphenolic antioxidants. The latter are a very diverse group among which phenolic acids and flavonoids such as anthocyanins and quercetin are the most important antioxidants. The four classes of dietary antioxidants differ dramatically in their mean antioxidant potency, absorbability and environmental availability. Flavonoids and phenolic acids are the antioxidants with the highest environmental availability, being most concentrated in all food categories Flavonoids also have the highest antioxidant potency in vitro, followed by carotenoids, vitamin E and vitamin C (Ruberto *et al.*, 2000).

2.15.1.2 Mechanisms of Action

Dietary antioxidants reduce oxidative stress by scavenging free radicals by three main mechanisms (variations are possible depending on the reactive species involved). Tocopherols and most polyphenols donate a hydrogen ion, carotenoids quench oxygen singlets and ascorbate transfers electrons (reviewed in Vertuani *et al.* 2004; Halliwell and Gutteridge 2006). The end result of these actions is that the free radical is neutralized. The efficiency with which an antioxidant destroys free radicals is called anti- oxidant potency and is measured in TEAC nM Trolox equivalent antioxidant capacity; Trolox is a synthetic analogue of tocopherol After reducing the free radicals, antioxidants are oxidized. At this stage, most antioxidants are fairly stable and relatively innocuous molecules that are subsequently catabolized and excreted. In contrast, carotenoid radicals are rather noxious pro- oxidants that may oxidize other biologically important molecules. However, if other antioxidants are present (usually vitamin C or E), they usually reduce the oxidized carotenoids, which are thereby recycled Therefore, the function of

carotenoids as antioxidants is debatable and always contingent on the presence of other antioxidants (Sudhir *et al.*, 1986; Rhopde *et al.*, 2001 and Bisson, 1983). According to modern theory of free radical biology and medicine, reactive oxygen species are involved in several disorders. The harmful action of the free radicals can, however, be blocked by antioxidant substances which scavenge the free radicals and detoxify the organism. Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases and cancers and neurodegenerative diseases. Therefore, plant derived antioxidants are now receiving a special attention. A large number of phenolic compounds present in vegetable foods, such as fruits and nuts, have been reported to possess good antioxidant properties. Moreover, the essential oils and various extracts of aromatic plants have been of great interest for their potential antioxidative effects for the preservation of the foods from the toxic effects of the oxidants (Sas, 2007).

2.15.2 Synthetic Antioxidant

Synthetic antioxidants are chemically synthesized since they do not occur in nature and are added to food as preservatives to help prevent lipid oxidation Shahidi *et al.* (1992). These antioxidants fall into two major categories depending on their mode of action Primary antioxidants and Secondary antioxidants. The primary antioxidants, which prevent the formation of free radicals during oxidation, can further include three major categories.

2.15.3 Natural Antioxidant

Natural antioxidants are constituents of many fruits and vegetables and they have attracted a great deal of public and scientific attention Diwani *et al.* (2009). Natural antioxidants occur in all parts of plants. Food tissues, because they are (or were) living, are under constant oxidative stress from free radicals, reactive oxygen species, and prooxidants generated both exogenously (heat and light) and endogenously (H_2O_2 and transition metals). For this reason, many of these tissues have developed antioxidant systems to control free radicals, lipid oxidation catalysts, oxidation intermediates, and secondary breakdown products Nakatani (2003), Agati and others (2007), Brown and Kelly (2007), Chen (2008), Iacopini and others (2008). These antioxidant compounds include flavonoids, phenolic acids, carotenoids, and tocopherols that can inhibit Fe^3 induced oxidation, scavenge free radicals, and act as reductants Khanduja (2003), Ozsoy and others (2009). Spices and herbs, used in foods for their flavor and in medicinal mixtures for their

physiological effects, often contain high concentrations of phenolic compounds that have strong H-donating activity Lugasi and others (1995), Muchuweti and others (2007). Natural antioxidants are those oxidants that are found in natural sources, such as fruits, vegetables and meats. There are several common natural antioxidants which are found in everyday foods, the most common of which being Vitamin C (ascorbic acid), Vitamin E (tocopherols), Vitamin A (carotenoids), various polyphenols including flavonoids, and Anthocyanins (a type of flavonoid), Lycopene (a type of carotenoid), And Coenzyme Q10, also known as Ubiquitin, which is a type of protein.

2.16 Endogenous Antioxidants

In addition to dietary antioxidants, the body relies on several endogenous defense mechanisms to help protect against free radical-induced cell damage. The antioxidant enzymes – glutathione peroxidase, catalase, and superoxide dismutase (SOD) – metabolize oxidative toxic intermediates and require micronutrient cofactors such as selenium, iron, copper, zinc, and manganese for optimum catalytic activity. It has been suggested that an inadequate dietary intake of these trace minerals may compromise the effectiveness of these antioxidant defense mechanisms Duthie and Brown (1994). Glutathione, an important water-soluble antioxidant, is synthesized from the amino acids glycine, glutamate, and cysteine. Glutathione directly quenches ROS such as lipid peroxides, and also plays a major role in xenobiotic metabolism. Exposure of the liver to xenobiotic substances induces oxidative reactions through the upregulation of detoxification enzymes, i.e., cytochrome P-450 mixedfunction oxidase. When an individual is exposed to high levels of xenobiotics, more glutathione is utilized for conjugation (a key step in the body's detoxification process) making it less available to serve as an antioxidant. Research suggests that glutathione and vitamin C work interactively to quench free radicals and that they have a sparing effect upon each other Jocab (1995). Lipoic acid, yet another important endogenous antioxidant, categorized as a "thiol" or "biothiol," is a sulfur-containing molecule that is known for its involvement in the reaction that catalyzes the oxidative decarboxylation of alpha-keto acids, such as pyruvate and alphaketoglutarate, in the Krebs cycle. Lipoic acid may also exert its antioxidant effect by chelating with prooxidant metals. Research further suggests that lipoic acid has a sparing effect on other antioxidants Kagen (1992).

2.17 Exogenous Antioxidant

Exogenous antioxidants can derive from natural sources (vitamins, flavonoids, anthocyanins, some mineral compounds), but can also be synthetic compounds, like butylhydroxyanisole, butylhydroxytoluene, gallates, etc. Litescu *et al.* (2011). There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, as well as the deterioration of fats and other constituents of foodstuffs Molyneux (2004).

2.18 Sources of Antioxidants

Vitamin C, Vitamin E, a-carotene, Licopein, Selenium, Polyphenol, Glutathione, Proxidase, Cystine are main sources of antioxidants. Fruit juices, beverages and hot drinks contain high amounts of antioxidants, like polyphenols, vitamin C, vitamin E, Maillard reaction products, â-carotene, and lycopene Ramadan-Hassanien (2008). The consumption of fruit juices, beverages and hot drinks was found to reduce the morbidity and mortality caused by degenerative diseases Gillman *et al.* (1995); Rimm *et al.* (1996); Cohen *et al.* (2000); La *et al.* (2001); Terry *et al.* (2001); Rodriguez and Costa (2006). The recommendations based on epidemiological studies are such, that fruits, vegetables and less processed staple foods ensure the best protection against the development of diseases caused by oxidative stress, such as cancer, coronary heart disease, obesity, type 2 diabetes, hypertension and cataract Halvorsen *et al.* (2002). The explanation consists in the beneficial health effect, due to antioxidants present in fruit and vegetables Halvorsen *et al.* (2006).

2.19 Function of Antioxidants

The Food and Drug Administration (FDA) defines antioxidants only as dietary supplements to be taken in addition to normal food consumption in an effort to prevent diseases Ohlsson and Bengston (2002). Antioxidants are known to play a key role in the protective influence exerted by plant foods Gey K.F (1990), Gey KF *et al.* (1991) Willett W.C (1991), Liyana *et al.* 2006). Regular consumption of vegetables and fruits has been recognized as reducing the risk of chronic diseases Dembinska *et al*. (2008). Studies demonstrate that an antioxidant-rich diet has a very positive health impact in the long run Sin *et al.* (2013) and Wills *et al.* (2009). Recently, antioxidants have attracted considerable attention in relation to radicals and oxidative stress,

cancer prophylaxis and therapy, and longevity Kalcher *et al.* (2009). All antioxidants are working in concert as a team, the (antioxidant system), responsible for prevention of the damaging effects of free radicals and toxic products of their metabolism. However, the antioxidant (team) acts to control levels of free radical formation as a coordinated system where deficiencies in one component impact the efficiency of others Peter (2007). Four possible mechanisms have been suggested John (1989) by which antioxidants function to reduce the rate of oxidation of fats and oils. These are hydrogen donation by antioxidants, electron donation by antioxidants, addition of lipid to the antioxidants and formation of a complex between lipid and antioxidants. Among food components fighting against chronic diseases, great attention has been paid to phytochemicals, plant derived molecules endowed with steady antioxidant power. The cumulative and synergistic activities of the bioactive molecules present in plant food are responsible for their enhanced antioxidant properties.

2.19.1 Function of Vitamin C

Vitamin C intake is inversely related to cancer, with protective effects shown for cancer of the lung, breast, pancreas, stomach, cervix, rectum and oral cavity Simon et al. (2001). In stressful situations adrenal glands react by releasing hormones that trigger the "fight or flight" reaction. It has been indicated that 200mg of vitamin C a day may reduce the levels of stress hormones. Stress suppresses the immune system. Mega doses of vitamin C increase the levels of antibody that fights against germs and viruses in both stressed and unstressed rats, with greater antibody increase in the unstressed rats Block (1999).

2.19.2 Vitamin E

Vitamin E is one of the most important lipid-soluble primary defense antioxidants Handan et al. (2007); Paul and Sumit (2002); Abdalla (2009). It is a generic term used for several naturally occurring tocopherols and tocotrienols. In its function as a chain-breaking antioxidant, vitamin E rapidly transfers its phenolic H-atom to a lipid peroxyl radical, converting it into a lipid hydroperoxide and a vitamin E radical Bashir *et al.* (2004). Tocopherols (vitamin E) and tocotrienols (provitamin E) are powerful antioxidants that confer oxidative stability to red palm olein (RPO) as well as help to keep the carotenoids and other quality parameters of the oil stable (Nesma *et al.*, 2010). Vitamin E scavenges peroxyl radical intermediates in lipid peroxidation and responsible for protecting Poly Unsaturated Fatty Acid (PUFA) present in cell membrane

and density lipoprotein (LDL), against lipid peroxidation Vivek and Surendra (2006). A fat-soluble vitamin that can be stored with fat in the liver and other tissues, vitamin E (tocopherols, tocotrienols) is promoted for a range of purposes from delaying aging to healing sun burn. The various function are maintains normal conditions of cells, and healthy skin and tissues, Protects red blood cells, antioxidation, enhance immunity. The important sources of vitamin E include wheat germ, nuts, seeds, whole grains, green leafy vegetables, vegetable oil and fish-liver oil.

2.19.3 β-Carotene

Beta-carotene has antioxidant properties that can help neutralize free radicals – reactive oxygen molecules potentially damaging lipids in cell membranes and genetic material, which may lead to the development of cardiovascular disease and cancer Pavia *et al.* (1999). At present, it is unclear whether some beneficial effects of beta-carotene and other carotenoids in humans are a result of their antioxidant activity or other non-antioxidant mechanisms. The relevance of deactivating reactive oxygen species to human health, potentially preventing diseases such as cancer and coronary heart disease, is not clear. In vitro studies indicate that carotenoids can also inhibit the oxidation of fats under certain conditions. They may have anti-atherosclerotic potential, but their effects in humans appear to be more complex Young *et al.* (2001).

2.19.4 Selenium

Selenium is mostly known for its potential antioxidant properties. Indeed, it is a required oligo element for the synthesis and function of about 20-40 enzymes, among which most of them help prevent cellular damage from natural by-products of oxygen metabolism, called reactive oxygen species (ROS) or free radicals Hawkes and Alkan (2010); Higuchi *et al.* (2010). Selenium is also essential for the proper function of the immune system and is known to have antiviral properties Mckenzie *et al.* (1998); Levander (1997). Effects on inflammatory responses are among the other key activities identified for selenoproteins Curran *et al.* (2005).

2.19.5 Polyphenol Antioxidant

Current evidence strongly supports a contribution of polyphenols to the prevention of cardiovascular diseases, cancers and osteoporosis and suggests a role in the prevention of neurodegenerative diseases and diabetes mellitus Scalbert et al. (2005). Significant progress has been made in the field of cardiovascular diseases, and today it is well established that some

polyphenols, administered as supplements or with food, do improve health status, as indicated by several biomarkers closely associated with cardiovascular risk Vita (2005). Arts *et al.* (2005) reported that epidemiologic studies tend to confirm the protective effects of polyphenol consumption against cardiovascular diseases.

2.19.6 Glutathione

Dolas and Gotmare (2015) reported that Glutathione protects cells from toxins such as free radicals. The human body produces glutathione from the synthesis of three key amino acids cysteine, glycine and glutamic acid. Food sources with the highest amounts of naturally occurring glutathione include; asparagus, avocado, grapefruit, squash, potato, cantaloupe, peach, zucchini, spinach, broccoli, watermelon, and strawberries. Fish, meat, and foods which yield sulfur containing amino acids (e.g. eggs) are the preferred sources for maintaining and increasing bodily glutathione levels.

2.19.7 Peroxidase

Dolas and Gotmare (2015) reported that an enzyme occurring especially in plants, milk, and leukocytes and consisting of a protein complex with hematin groups that catalyzes the oxidation of various substances. Food sources of peroxidase include horseradish root, soybean, mango fruit, and turnip.

2.19.8 Flavonoids

Sunil Kumar (2014) reported that Flavonoids promote antioxidant activity, cellular health and normal tissue growth and renewal throughout the body. They also work with vitamin C to reduce oxidative stress for the water based portion of the cell and may slow down some of the effects of aging. There are more than 4,000 unique flavonoids and they are most effective when several types are consumed together. Food sources include: cranberries, kale, beets, berries, red and black grapes, oranges, lemons, grapefruits and green tea Banerjee *et al.* (1993).

Antioxidants are present in foods as vitamins, minerals, carotenoids, and polyphenols, among others. Natural antioxidant, Synthetic antioxidant and Dietary antioxidant play a vital role in our body. Endogenous and Exogenous are also play an important role in human body. The main function of antioxidants is to prevent oxidation in various contexts. The human body is protected from cardiovascular, neurological and carcinogenic diseases, delaying chronic health problems

like cataracts by the use of antioxidants. The recommendations based on epidemiological studies are such that fruits and vegetables ensure the best protection against the development of diseases caused by oxidative stress, such as cancer, coronary heart disease, obesity, type 2 diabetes, hypertension and cataract.

2.20 An Overview of Statins as Hypolipidaemic Drugs

Statins are the treatment of choice for the management of Dyslipidemia because of their proven efficacy and safety profile. They also have an increasing role in managing cardiovascular risk in patients with relatively normal levels of plasma cholesterol. Although all statins act by blocking the HMG-CoA reductase enzyme, which catalyzes the rate-limiting step in *de novo* cholesterol synthesis, they differ in terms of their chemical structures, pharmacokinetic profiles, and lipid-modifying efficacy. Lovastatin, Pravastatin and Simvastatin are derived from fungal metabolites and have elimination half-lives of 1–3h. Atorvastatin, Cerivastatin (withdrawn from clinical use in 2001), Fluvastatin, Pitavastatin and Rosuvastatin are fully synthetic compounds, with elimination half-lives ranging from 1h for Fluvastatin to 19h for Rosuvastatin. As a class, statins are generally well tolerated and serious adverse events, including muscle toxicity leading to rhabdomyolysis, are rare. Consideration of the differences between the statins helps to provide a rational basis for their use in clinical practice.

2.21 3-Hydroxy-3-methylglutaryl-CoA Reductase Inhibitor (statins)

Mevastatin was the first HMG-CoA reductase inhibitor and was isolated from *Penicillum citrinum*. Other statins Simvastatin, Lovastatin and Pravastatin are also fungal derivatives, while Atorvastatin, Cerivastatin, Fluvastatin, Pitavastatin and Rosuvastatin are fully synthetic compounds (Wierzbicki, 2001). The use of statins (Simvastatin, Pravastatin, Lovastatin, Fluvastatin, Rosuvastatin and Atorvastatin) has become the preferred method for treating elevated LDL-C levels in children and adolescents who meet the criteria for drug therapy. In fact, their use is generally safe and well tolerated. However, it must be remembered that cholesterol is an essential structural component of cells, a precursor for steroid hormones, vitamin D metabolites and bile acids, and an important factor in neural myelinization and brain growth. Concerns of possible side effects of statins on growth, pubertal development and endocrinologic functions have restricted their use in children during the prepubertal stage.

Furthermore, since fat-soluble vitamins are transported by lipoproteins, their reduction by statins has been suspected to lead to vitamin deficiencies (Arambepola *et al.*, 2007)

The chemical structures of the different statins are shown below these structures can be broadly divided into three parts (Gaw, 2000): an analogue of the target enzyme substrate, HMG-CoA; a complex hydrophobic ring structure that is covalently linked to the substrate analogue and is involved in binding of the statin to the reductase enzyme; side groups on the rings that define the solubility properties of the drugs and therefore many of their pharmacokinetic properties. Atorvastatin, Fluvastatin, Lovastatin and Simvastatin are relatively lipophilic compounds, while Pravastatin and Rosuvastatin are more hydrophilic as a result of a polar hydroxyl group and methane sulphonamide group, respectively. (McTavish, 1991 and McTaggart, 2001).

2.22 Mechanism of Action

3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors (statins) act by blocking the HMG-CoA reductase enzyme, which catalyzes the rate-limiting step in *de novo* cholesterol synthesis. All statins are competitive inhibitors of HMG-CoA reductase with respect to the binding of the substrate, HMG-CoA, but not for that of the co-enzyme NADPH, suggesting that their HMG-CoA-like moieties bind to the HMG-CoA-binding portion of the enzyme active site. Comparison of the six statin–enzyme complexes revealed subtle differences in their modes of binding. An additional hydrogen bond was demonstrated in the Atorvastatin– and Rosuvastatin enzyme complexes along with a polar interaction unique to Rosuvastatin, such that Rosuvastatin has the most binding interactions with HMG-CoA reductase of all the statins (Istvan, 2001).

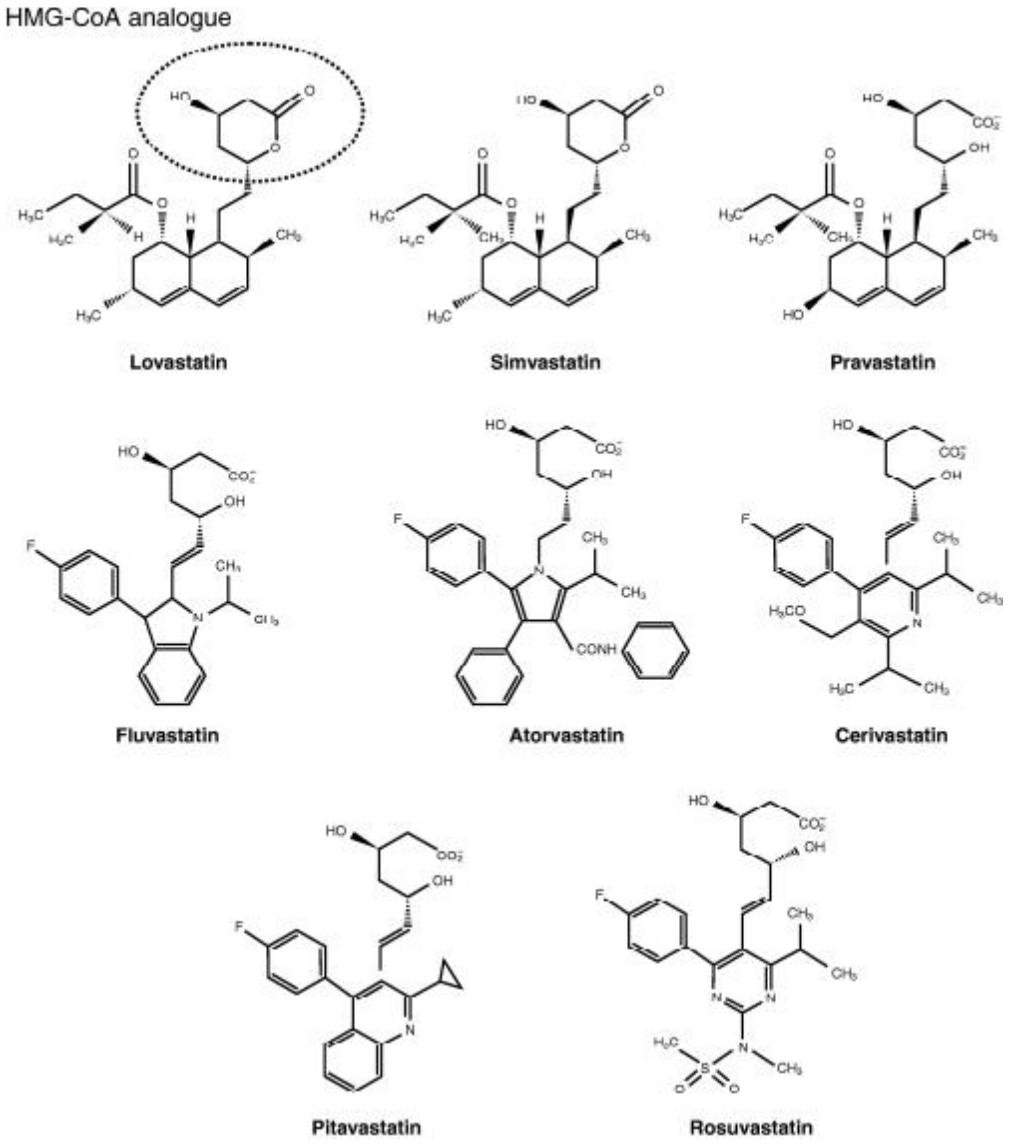
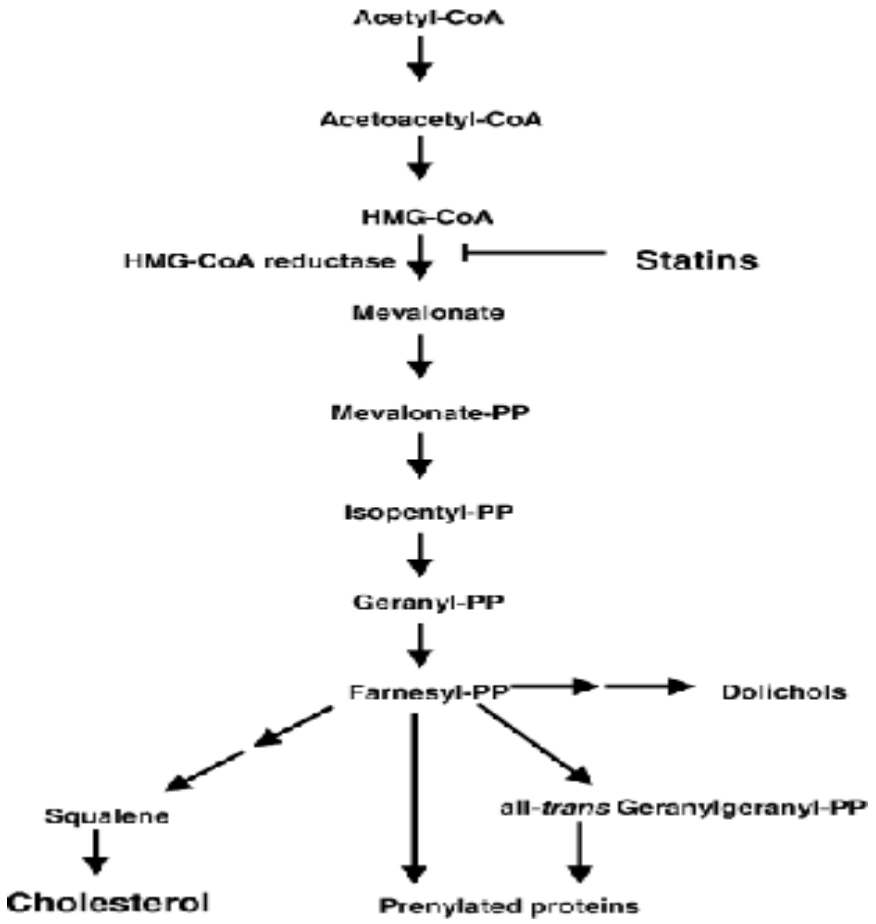


Figure 5: Chemical structures of the statins



2.22.1 Pharmacokinetics

Lovastatin and Simvastatin are administered as lactone prodrugs, and are enzymatically hydrolysed in vivo to their active, hydroxy-acid form (Corsini, 1995). The other statins are administered as the active hydroxy acid (Corsini *et al.*, 1999; Kajinami, 2000). All statins are absorbed rapidly following administration, reaching peak plasma concentration (Tmax) within 4h (Cilia *et al.*, 1996; Tse, 1992; Pan *et al.*, 1990; and Warwick *et al.*, 2000). The rate and extent of absorption of Atorvastatin is affected by time-of day administration (Cilia *et al.*, 1996)**,** while pharmacokinetic properties of Rosuvastatin are unaffected (Martin, 2002), however, for both drugs, the lipid-lowering effects are similar whether administered in the morning or evening (Cilia *et al.*, 1996 and Martin, 2002). Food intake has a variable effect on statin absorption; Lovastatin is more effectively absorbed when taken along with food, (Garnett, 1995), whereas the bioavailability of Atorvastatin, Fluvastatin and Pravastatin is decreased (Radulovic *et al.*,

1995; mith *et al.,* 1993; and Pan *et al.,* 1993). No such effect is apparent for Simvastatin or Rosuvastatin. (Corsini *et al.,* 1999 and Davidson, 2002). Statins are predominantly metabolized by the cytochrome P450 (CYP450) family of enzymes, composed of over 30 isoenzymes (Bottorff, 2000). The CYP3A4 isoenzyme metabolizes the greatest number of drugs in humans, (Michalets, 1998) including Lovastatin, simvastatin and Atorvastatin (Bottorff, 2000). A proportion of the circulating inhibitory activity of these three agents for HMG-CoA reductase is attributable to active metabolites. For Atorvastatin, the major active metabolites are 2-hydroxy- and 4-hydroxy-atorvastatin acids (Jacobson, 2000) while for simvastatin the *β*-hydroxy acid and its 6′-hydroxy, 6′-hydroxymethyl and 6′-exomethylene derivatives are the major active metabolites (Vickers, 1990; and Lennernas, 1997). Fluvastatin is chiefly metabolized by the CYP2C9 isoenzyme, while Pravastatin, Pitavastatin and Rosuvastatin do not undergo substantial metabolism by CYP450 pathways. (Bottorff, 2000; Fujino *et al.,* 1999; and McComick, 2000) Lipophilic drugs are known to be much more susceptible to oxidative metabolism by the CYP450 system (Schachter, 2001). It is now recognized that the statins metabolized by the CYP450 system are more likely to produce muscle toxicity because of the risk of drug interactions with many drugs that inhibit CYP450, notably the CYP3A4 isoform (Sica, 2002; and Muscari, 2002); drug interactions may increase plasma levels of statins, with a consequent increased risk of toxic effects The predominant route of elimination for the majority of statins is via the bile after metabolism by the liver (Knoff, 2002). Consequently, hepatic dysfunction is a risk factor for statin induced myopathy, (Maron, 2002) and all manufacturers recommend caution when prescribing statins to patients with a history of liver disease. Pravastatin is eliminated by both the kidney and liver, mostly as unchanged drug. (Singhavi *et al.,* 1990 and Quion, 1994) However, as with some of the other currently available statins, its pharmacokinetics is altered in patients with hepatic (Garnett, 1995) Rosuvastatin is also eliminated, largely unchanged, by both the kidney and liver (Martin *et al.,* 2003 and Martin, 2003) and its pharmacokinetic properties are not altered in patients with mild to moderate hepatic impairment (Simonson *et al.,* 2003).

2.22.2 Phamarcodynamics

Statins are highly efficacious at lowering LDL-C, although there are differences in the extent of LDL-C lowering at therapeutic doses and in the maximal reduction achieved with each agent. Of the statins currently available, Rosuvastatin is the most effective at lowering LDL-C, with

reductions of up to 63% reported with a daily dose of 40 mg (Olsson et al., 2001). Data from comparative trials confirm that on a milligram basis, Rosuvastatin is the most efficacious statin for lowering LDL-C, followed by Atorvastatin, simvastatin and Pravastatin (Jones *et al.*, 2003 and Jones, 2003) In general, statins are well tolerated and serious adverse events are rare. The most serious adverse effect associated with statin therapy is myopathy, which may progress to fatal or nonfatal rhabdomyolysis. The withdrawal of Cerivastatin from clinical use in 2001 heightened scrutiny of these effects, although all available data indicate that the increased incidence of rhabdomyolysis reported for Cerivastatin appears to be specific to this agent (Furberg, 2001). The incidence of myopathy is low (approximately one in 1000 patients treated), is dose-related, and is increased when statins are used in combination with agents that share common metabolic pathways.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents and chemicals

All the reagents used are of analytical grade and sourced from spectrum and manufactured by Egyptian company for Biotechnology (S.A.E) as listed below:

3.1.2 Cholesterol

Standard cholesterol

Pipes Buffer pH 7.0

Phenol

Sodium cholate

Cholesterol esterase

Cholesterol oxidase

Peroxidase

4-amino-antipyrine

Sodium Azide

3.1.2.1 HDL-Cholesterol

Phosphotungstate

Magnesium chloride

3.1.3 Triglycerides

Reagent 1 (Buffer)

Good pH 7.5

P-chlorophenol

Lipoprotein lipase

Glycerolkinase

Glycerol-3-oxidase

Reagent 2 (Enzymes)

Peroxidase (POD)

4-Aminophenazone

ATP

3.2 experimental Animals

The experimental animals used were obtained from the animal house, department of biological sciences, Bayero University Kano. The animals were kept in cages and fed with animal feed and water one week for acclimatisation prior to the experiment.

3.3 Formulation of High Fat Diet (HFD)

The earliest method of Vesselinvitch *et al.* (1980) was 2% cholesterol, 20% palm oil and 78% grower marsh. The method was modified to induce hyperlipidaemia and was formulated by adding 5% egg yolk, 20% palm oil to 75% pelletized super starter feed.

3.4 Induction of Hyperlipidaemia in the Experimental Rats

The experimental animals weighed 50-90g were induced by high fat diet formulated above for six weeks.

3.5 Collection/Preparation of Garlic Extract

Fresh garlic bulbs (*Allium Sativum)* was purchased from Gombe main market (July, 2018) and was peeled and air dried under shade and powdered. Powdered garlic bulbs (200g) was weighed and soaked in 80% methanol for 72 hours (i.e. 3 days). The mixture was filtered using Muslin cloth; the filtrate was put in a water bath at 50^{o}C and dried. The dried extract was stored at -20^{o}C until it is required for use. The extract when required was suspended in distilled water and volume of the extract determined based on the weight of the rats and required dose, using the relation below.

$$\text{Dose in mg} = \frac{\text{Body weight of animal (g)}}{1000\text{g}} \text{ X dose (mg)}$$

3.6 Acute Toxicity (LD_{50})

The method used to determine acute toxicity was that described by Lorke (1983). The study was conducted in two phases. In the first phase, three groups of three albino rats each were administered with the extract at respective oral doses of 10mg, 100mg, and 1000mg per kg body weight. The albino rats were observed for signs of toxicity and possible deaths for 24 h, 2weeks and for 4weeks. In the second phase, another three groups of three albino rats each were administered respective doses of 1500mg, 2900mg, and 5000mg per kg body weight of the extract. They were equally monitored as in phase one for toxicity signs and deaths. From data obtained, LD_{50} was determined, and experimental doses were selected as 100mg, 200mg, 300mg, and 400mg

3.7 Experimental Design

Thirty five (35) albino rats were divided into seven groups of five rats each according to their body weighted. Hyperlipidaemia was induced by feeding hyperlipidaemic diet (high fat) for 42 days. The rats were orally administered the extract and Atorvastatin (antihyperlipidemic drug, 10mg/kg/b.w) once daily in the morning.

Group I Negative control, no extract was administered, only standard diet and clean water.

Group II Positive control (HFD), no extract was administered.

Group III Standard control HFD + 10mg/kg of Atorvastatin.

Group IV High fat diet with 100mg/kg of the methanolic-aqueous garlic extract.

Group V High fat diet with 200mg/kg of the methanolic-aqueous garlic extract.

Group VI High fat diet with 300mg/kg of the methanolic-aqueous garlic extract.

Group VII High fat diet with 400mg/kg of the methanolic-aqueous garlic extract.

3.8 Collection of Blood Samples and Biochemical Analysis from Serum

Blood sample were collected by sacrificing the experimental animals on 42nd day after 12 hours of last dose. Samples were collected separately into heparin anticoagulant container. The clear serum was separated at 3500rpm for 10min using centrifuge machine. The biochemical investigation was carried out to assess the effect of the extract on serum levels of total cholesterol, triglyceride, high density lipoprotein cholesterol, low density lipoprotein cholesterol,

and very low density lipoprotein cholesterol (Friedewald, 1972). The effect on serum levels of malondialdehyde, superoxide dismutase, glutathione peroxidase and catalase were also determined.

3.8.1 Determination of Total Cholesterol

Method

CHOD-PAP-enzymatic colorimetric method

Principle

1. Cholesterol esters are enzymatically hydrolysed by cholesterol esterase (CE) to cholesterol and free fatty acids.

$$\text{Cholesterol Esters} \xrightarrow{\text{CE}} \text{Cholesterol + fatty acids}$$

2. Free cholesterol is then oxidized by cholesterol oxidase (CO) to cholest-4-en-3-one and hydrogen peroxide.

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHOD}} \text{Cholest-4-en-3-one} + H_2O_2$$

3. The hydrogen peroxide combines with phenol and 4-amino-antipyrine (4AAP) in the presence of peroxidase (POD) to form a chromophore (quinoneimine dye) which can be quantitated at 500-550nm (Ellefson and Caraway, 1976).

$$2H_2O_2 + \text{phenol} + (4AAP) \xrightarrow{\text{POD}} \text{Quinoneimine Dye} + 4H_2O$$

Procedure:

Test tubes were labeled blank, standard and sample. 1.0ml of the reagent was added to each test tube, 1.0ml of standard was added to standard test tube and 10µl of sample was added into the sample test tube. The contents of each tube were then mixed and incubated for 5 minutes at 37^0C. The absorbance of specimen ($A_{specimen}$) and standard ($A_{standard}$) against reagent blank was measured within 30minutes in a spectrophotometer at 546nm.

Calculation

$$\text{Serum cholesterol conc. (mg/dL)} = \frac{(A_{specimen})}{(A_{standard})} \text{ X } 200$$

3.8.2 Determination of Serum Triglycerides

Principle

Sample triglycerides incubated with lipoprotein lipase (LPL), liberate glycerol and free fatty acids. Glycerol is converted to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP) by glycerol kinase and ATP. Glycerol-3-phosphate is then converted by glycerol phosphate dehydrogenase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). In the last reaction, hydrogen peroxide reacts with 4-aminophenazone (4-AP) and p-chlorophenol in presence of peroxidase to give a red colored dye (Tietz, 1995).

$$\text{Triglycerides} + H_2O \xrightarrow{\text{LPL}} \text{Glycerol} + \text{free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GLYCEROL KINASE}} \text{DAP} + H_2O_2$$

$$\text{G3P} + O_2 \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{4-AP} + \text{p-chlorophenol} \xrightarrow{\text{POD}} \text{Quinine} + H_2O$$

Procedure:

Into three test tubes, labeled blank, standard and sample, 1.0ml of the reagent was added to each tube, 10µl of standard was added to standard test tube and 10µl of sample was added into the sample test tube. The contents of each tube were then mixed and incubated for 5 minutes at 37^0C. The absorbance of specimen ($A_{specimen}$) and standard ($A_{standard}$) against reagent blank was measured within 30minutes, in a spectrophotometer.

3.9 HDL- Cholesterol

Precipitation method

Principle

Low density lipoprotein cholesterol (LDL) and very low density lipoprotein cholesterol (VLDL) in sample precipitate with phosphotungstate and magnesium ions. After centrifugation, the

cholesterol concentration in the high density lipoprotein cholesterol (HDL) fraction, which remains in the supernatant, is determined (Friedwald *et al*, 1972).

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Chol. esterase}} \text{Cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} + {}^{1}/_{2}O_2 + H_2O \xrightarrow{\text{Chol. Oxidase}} \text{Cholesteronone} + H_2O_2$$

$$2H_2O + \text{4-Aminoantipyrine} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

Procedure

1- Precipitation:

Into a single test tube, 0.5ml of the reagent and 0.2ml of specimen were added. The contents in each tube were then mixed and incubated for 10 minutes at room temperature, then was centrifuged for 10 minutes at 4000rpm. The supernatant was carefully collected.

2- Cholesterol-liquizyme

Then, two test tubes were labeled as blank and specimen, 50µl of distilled water was added into the blank test tube, 50µl specimen supernatant from 1 above was added into specimen test tube and 1.0ml of cholesterol reagent was added into each test tube. The contents in each tube were then mixed and incubated for 10 minutes at 20-25^{0}C. The absorbance of the specimen (A_{sample}) against reagent blank at 546nm was determined within 60 minutes, in a spectrophotometer.

Calculation

HDL cholesterol concentration (mg/dl) = A_{sample} x 570

3.10 LDL Cholesterol (mg/dl)

To determine or calculate low density lipoprotein cholesterol, the relation below can be used (Friedwald *et al*, 1972).

LDL Cholesterol = Total CHO – Trig/5 – HDL Cholesterol.

3.11 Antioxidants and Oxidative Stress Levels

3.11.1 Estimation of Reduced Glutathione (GSH) Levels

The method of Beutler *et al*., (1963) was followed in estimating the level of reduced glutathione.

The principle

The reduced form of glutathione comprises in most instances the bulk of cellular non protein sulfhydryl groups. This method is therefore based upon the development of a relatively stable yellow color when 5', 5'- dithios – (2-nitrobenzoic acid) (Ellman's reagent) is added to sulfhydryl compounds. The chromophoric product resulting from the reaction of Ellman reagent with the reduced GSH, 2-nitro 5-thiobenzoic acid possess a molar absorption at 412nm. Reduced GSH is proportional to the absorbance at 412nm.

Determination of GSH Concentration in the Samples

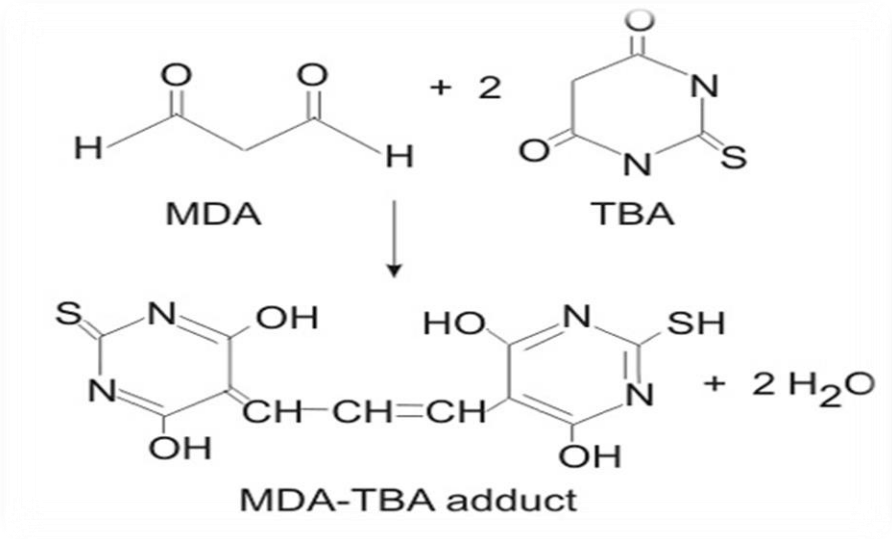
An aliquot of the homogenate was deproteinated by the addition of an equal volume of 4% sulfosalicyclic acid. This was centrifuged at 4,000 xg for 5 minutes. Thereafter, 0.5 ml of the supernatant was added to 4.5 ml of Ellman reagent. A blank was prepared with 0.5 ml of the diluted precipitating agent and 4.5ml of Ellman reagent. Reduced GSH level is proportional to the absorbance at 412 nm.

3.11.2 Assessment of Lipid Peroxidation (LPO)

Lipid peroxidation was determined by measuring the levels of malondialdehyde produced during lipid peroxidation according to the method described by Varshney and Kale (1990).

Principle

This method is based on the reaction between 2-thiobarbituric acid (TBA) and MDA: an end product of lipid peroxide during peroxidation. On heating in acidic pH, the product is a pink complex which absorbs maximally at 532nm and which is extractable into organic solvents such as butanol. Malondialdehyde (MDA) is often used to calibrate this test and thus the results are expressed as the amount of the free MDA produced.



Structure of TBA + MDA--MDA-TBA (pink coloured complex)

Procedure

An aliquot of 400µl of the sample was mixed with 1.6ml of tris-KCl buffer to which 500 µl of 30% TCA was added. Then 500 µl of 0.75% TBA was added and placed in a water bath for 45 minutes at 80^0C. This was then cooled in ice and centrifuged at 3000 g for 5 minutes. The clear supernatant was collected and absorbance measured against a reference blank of distlled water at 532 nm. Lipid peroxidation expressed as MDA formed/mg protein or gram tissue was computed with a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ Cm^{-1}$

$$\text{LPO (MDA formed/mg protein)} = \frac{\text{Absorbance x volume of mixture}}{E_{532nm} \text{ x volume of sample x mg protein}}$$

MDA FORMED = mmol/mg protein

3.11.3 Determination of Catalase Activity

Catalase activity was determined according to the method of Claiborne (1985)

Principle

This method is based on the loss of absorbance observed at 240 nm as catalase splits hydrogen peroxide. Despite the fact that hydrogen peroxide has no absorbance maximum at this wavelength, its absorbance correlates well enough with concentration to allow its use for a quantitative assay. An extinction coefficient of 0.0041 mM^{-1} cm^{-1} (Noble and Gibson, 1970) was used.

Procedure

Hydrogen peroxide (2.95 ml of 19 mM solution) was pipette into a 1 cm quartz cuvette and 50 µl of sample added (as shown in the table below). This was done to reduce the dilution of the samples (done according to the other protocols whereby H_2O_2 was prepared separately in distilled water (100mls) and the buffer was also prepared separately.

The mixture was rapidly inverted to mix and placed in a spectrophotometer. Change in absorbance was read at 240 nm every minute for 5 minutes. Optical density was read at 240 nm at 1 min, 2, 3, 3:30, 4, 4:30, 5 mins

Calculation

$$\textbf{Catalase activity} = \frac{(\Delta OD/min \times volume\ of\ assay\ system)}{(0.0041 \times Volume\ of\ Sample \times mg} = \mu mole\ H_2O_2/min/mg\ protein$$

3.11.4 Determination of Superoxide Dismutase (SOD) Activity

The level of SOD activity was determined by the method of Misra and Fridovich (1972).

Principle

The ability of superoxide dismutase to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide ($O_2\bullet^-$) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per $O_2\bullet^-$ introduced increased with increasing pH (Valerino and

McCormack, 1971) and also increased with increasing concentration of epinephrine. These results led to the proposal that autoxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving superoxide ($O_2^{\bullet -}$) radical and hence inhabitable by superoxide dismutase.

Procedure

0.2 ml of sample was diluted in 0.8 ml of distilled water to make a 1 in 5 dilution. An aliquot of 0.2 ml of the diluted sample was added to 2.5 ml of 0.05 M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3 ml of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5 ml buffer, 0.3 ml of substrate (adrenaline) and 0.2 ml of water. The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds.

Calculation

$$\text{Increase in absorbance per minute} = \frac{A_3 - A_0}{2.5}$$

Where A_0 = absorbance after 30 seconds

A_3=absorbance after 150 seconds

$$\% \text{ inhibition} = 100 \times \frac{\text{Increase in absorbance for substrate}}{\text{Increase in absorbance for blank}}$$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline.

3.12 Atherogenic Index (AI)

The atherogenic index of plasma (AIP) is defined as the base 10 logarithm of the ratio of the concentration of TG to HDL-C, where each concentration is expressed in mmol/L. the non-HDL-C is defined as TC minus HDL-C, the atherogenic index (AI) is defined as the ratio of non-HDL-C to LDL-C.

$$AIP = \text{Log}_{10}\left(\frac{TG}{HDL\text{-}C}\right) \qquad \text{Or} \quad AI = \frac{HDL\text{-}C}{LDL\text{-}C}$$

3.13 Histopathological Studies of Liver and Heart

Procedure

The biopses of liver and heart of albino rats were fixed with 10% formal saline, dehydrated with ascending grade of alcohol, cleared with toluene, infiltrated with molten paraffin wax. The microtone section were stained with haematoxylin and eosin staining technique and examined with Leica DM 750 microscope and photographed with Leica LCC 50HD camera (Auwioro, 2010).

3.14 Statistical Analysis:

Data analysis was performed using the statistical package for the social sciences (SPSS) software. Descriptive statistics were adopted to display data in means±SD. The statistical method of one way analysis of variance (ANOVA) was used to compare the mean values obtained among the different groups. Differences were considered significant whenever the p-value is $p<0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Acute Toxicity (LD_{50})

In the first phase of LD_{50} determination, no mortality was observed in the groups including the group orally administered the highest dose of the extract (1000mg/kg). In the second phase, there was no mortality including the group orally administered with the highest dose of the extract (5000mg/kg).

Table 1. Record of mortality in phase 1.

Extract Dose (mg/kg body weight)	Mortality
10	**0/3**
100	**0/3**
1000	**0/3**

Number of deaths per group = 0, number of rats per group = 3

Table 2. Record of mortality in phase 2.

Extract Dose (mg/kg/body weight)	Mortality
1600	**0/3**
2900	**0/3**
5000	**0/3**

Number of deaths per group = 0, number of rats per group = 3

The effect of administration of methanolic-aqueous extract of garlic on lipid profile of high fat diet induced hyperlipidaemia rats is presented in the table 3. There was significant ($p<0.05$) increase in serum total cholesterol, low density lipoprotein cholesterol and triglyceride in hyperlipidaemic control group (II) compared to negative control. A significant ($p<0.05$) decrease in serum total cholesterol, low density lipoprotein and triglyceride was observed in the groups administered with the extract compared with the hyperlipidaemic group.

Table 3: Effect of methanolic-aqueous extract of garlic on lipid profile (mg/dl) of high fat diet induced hyperlipidaemic rats.

		Serum Lipid Parameters (mg/kg)				
Groups	**T-CH**	**TG**	**HDL-CH**	**AI**	**LDL-CH**	**VLDL-CH**
G 1	84.24±2.14[a]	84.95±2.84[a]	37.05±3.25[a]	0.36±0.02	29.10±2.98[a]	17.2±0.60[a]
G 11	203.00±2.20[*]	165.00±3.03[*]	26.40±1.47[*]	0.66±0.31	133.10±4.47[*]	33.20±5.89[*]
G 111	67.30±13.40[a]	43.80±2.30*[a]	44.90±2.58[*a]	0.25±0.05	33.60±10.6[a]	8.76±0.50*[a]
G 1V	134.60±10.9*[a]	138.10±8.21*[a]	42.60±1.10[*]	0.51±0.91	64.80±10.9[*a]	27.50±1.79[*]
G V	122.10±26.00[*a]	89.10±4.66[a]	39.90±2.20[*a]	0.47±0.33	74.10±26.20[*a]	17.90±0.90[a]
G V1	95.52±2.26[a]	86.19±4.00[a]	43.9±0.74[a]	0.39±0.73	44.58±2.75[a]	15.20±0.80[a]
G VII	88.00±4.80[a]	75.50±2.50*[a]	40.90±2.10[*a]	0.39±0.08	38.60±9.40[a]	18.10±0.5[a]

Key: Results are expressed as mean ± standard deviation (n = 5). Values indicated by asterisk (*) down the group are significantly different compared to negative control (p>0.05), values with superscript (a) down the group are statistically different compared to the positive control.

Group I: Negative control **Group II:** Positive control (HFD)

Group III: Standard control (10mg/kg atorvastatin)

Group IV: HFD + 100mg/kg methanolic-aqueous garlic extract

Group V: HFD + 200mg/kg methanolic-aqueous garlic extract

Group VI: HFD + 300mg/kg methanolic-aqueous garlic extract **Group VII:** HFD + 400mg/kg methanolic-aqueous garlic extract

Table 4 shows the effect of administration of methanolic-aqueous garlic extract on antioxidant enzymes. There was significant ($p<0.05$) decrease in malondialdehyde in the positive control group. No significant ($p<0.05$) difference in superoxide dismutase, glutathione peroxidase and catalase were observed in the positive control group.

Table 4. Effect of oral administration of methanolic-aqueous extract of garlic on oxidative stress and antioxidant levels in high fat diet induced hyperlipidaemic rats.

Groups	MDA(mmol/mL)	SOD (U/mL)	GPx (U/ml)	CAT (µmole)
G I	21.09±3.76	6.94±0.48[a]	4.75±0.00[a]	17.85±4.25
G II	26.25±0.12	4.57±0.01[*]	2.20±0.27[*]	18.41±0.78
G III	16.75±7.55	7.74±0.03[a]	8.35±0.55[*]	17.04±2.62
G IV	18.05±8.58	9.45±0.56[a]	5.15±0.55[*]	17.99±4.98
G V	17.18±1.91[*a]	9.70±0.22*[a]	5.75±0.00[*]	29.10±5.69[*a]
G VI	17.81±2.55[*a]	11.07±0.62[a]	6.10±0.14[*]	31.35±3.41[*a]
G VII	16.26±2.50[a]	13.20±0.07[a]	7.30±0.27[*]	33.64±7.l1[*a]

Key: Results are expressed as mean ± standard deviation (n = 5). Values indicated by asterisk down the group are statistically different compared to the negative control (G I) at ($P<0.05$) whereas all values indicated by the superscript (a) down the group are statistically different compared to the positive control (G II).

Group I: Negative control **Group II:** Positive control (HFD)

Group III: Standard control (10mg/kg atorvastatin)

Group IV: HFD + 100mg/kg methanolic-aqueous garlic extract

Group V: HFD + 200mg/kg methanolic-aqueous garlic extract

Group VI: HFD + 300mg/kg methanolic-aqueous garlic extract

Group VII: HFD + 400mg/kg methanolic-aqueous garlic extract

4.1.2 Results of histopathological analysis of hyperlipidaemic-induced rats treated with atorvastatin (statin) drug and methanolic-aqueous garlic extract.

The results of liver and heart histopathological analysis of induced hyperlipidaemic rats for six weeks treated with statin drug (atorvastatin) and methanolic-aqueous garlic extract for two weeks are presented in I to XIV

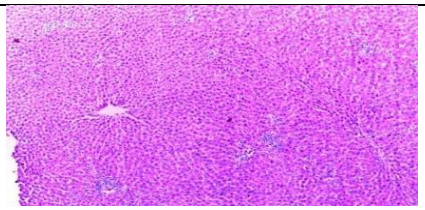
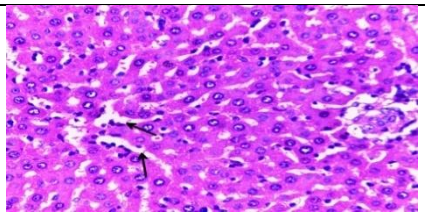
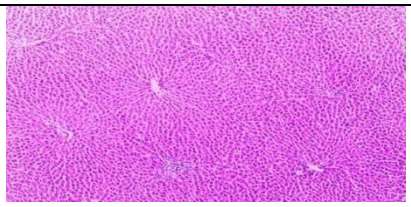
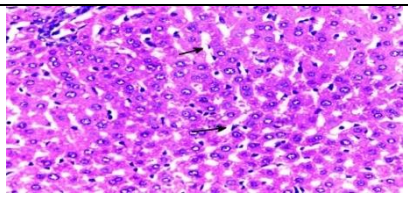
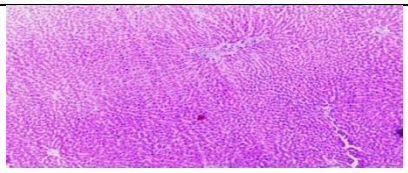
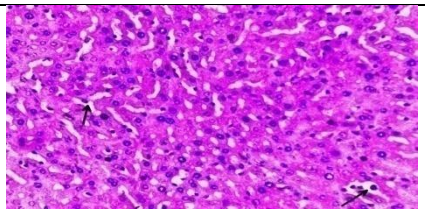
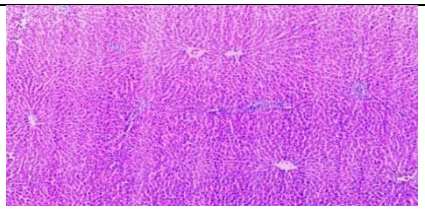
Group	Tissues	Observation
Group 1. Negative control		The liver section of the normal control shows unremarkable liver tissues (H&E, magx100)
G II, positive control. Induced hyperlipidaemic not treated		The liver Section of the rat induced hyperlipidemia shows areas of fat storing cells (Ito cells) in the perinusoidal space (H&E, magx 400).
G III, standard control. HFD +10mg/kg Atorvastatin		The liver section shows unremarkable liver tissue (H&E, magx 100).
G IV, HFD +100mg/kg of methanolic-aqueous extract of garlic		The liver section shows areas of fat storing cells (Ito cells) in the perinusoidal space (magx 400).
G V: HFD +200mg/kg of methanolic-aqueous garlic extract		The liver section shows unremarkable liver tissue (H&E, magx 100).
G VI: HFD + 300mg/kg of methanolic-aqueous garlic extract		The liver section shows areas of fat storing cells (Ito cells) in the perinusoidal space (H&E, magx 400).
GVII: Induced hyperlipidemic and treated with 400mg/kg of methanolic-aqueous garlic extract		The liver section shows unremarkable tissue (H&E, magx 100).

Plate 1: histology of Liver of high fat diet induced dyslipidaemic rats treated with methanolic-aqueous garlic extract

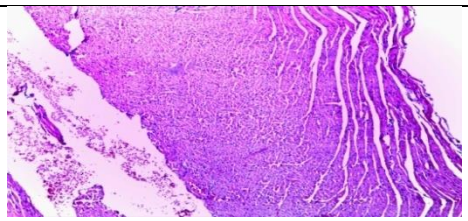
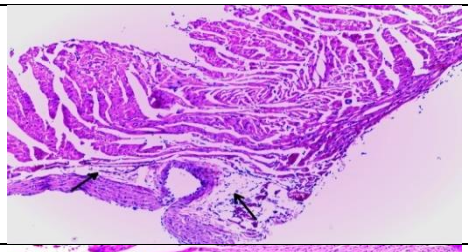
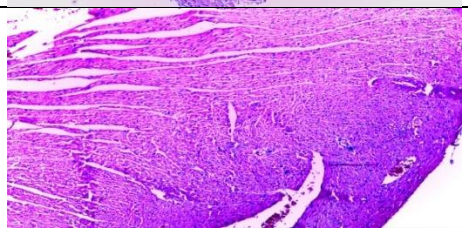
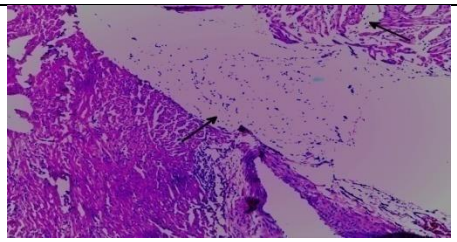
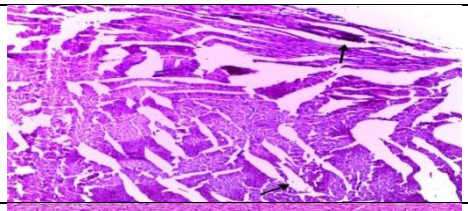
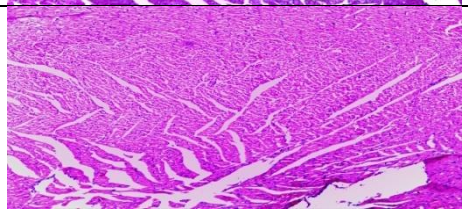
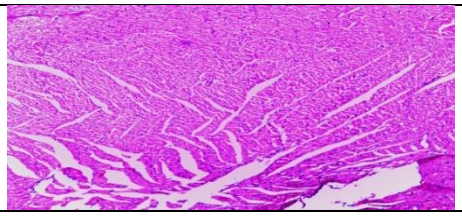
Group	Tissues	Observation
G I: Negative control		The heart Section of the normal control group Section shows unremarkable myocardium (H&E, magx100)
G II: Positive control. Induced hyperlipidaemic and not treated		The heart section of group two induced hyperlipidemic shows areas of adipose tissue (H&E, magx100).
G III: Standard control induced hyperlipidemic and treated with 10mg/kg of antihyperlipidemic drug (atorvastatin)		The heart section shows unremarkable myocardium (H&E, magx 100).
GIV: Induced hyperlipidemic and treated with 100mg/kg of methanolic-aqueous garlic extract		The heart section shows areas of adipose tissue (H&E, magx 100).
GV: Induced hyperlipidemic and treated with 200mg/kg of methanolic-aqueous garlic extract		The heart section of shows areas of adipose tissue (H&E, magx 100).
GVI: Induced hyperlipidemic rat and treated with 300mg/kg of methanolic-aqueous garlic extract		The heart section shows unremarkable myocardium (H&E, magx 100).
GVII: Induced hyperlipidemic and treated with 400mg/kg of methanolic-aqueous garlic extract		The heart section shows unremarkable myocardium (H&E, magx 100).

Plate 2: histology of heart of high fat diet induced dyslipidaemic rats treated with methanolic-aqueous garlic extract

4.2 DISCUSSION

In the present study the hypolipidemic and antioxidant activities of methanolic-aqueous garlic extract was evaluated. The obtained data presented in table (1 and 2) showed the results of acute toxicity (LD_{50}) of methanolic-aqueous extract of garlic according to the method of Lorke (1983). The results of the toxicity study (Table 1 and 2) showed that there were no mortalities with the highest dose of the extract at both phases and therefore the result revealed that methanolic-aqueous garlic extract may be safe at doses up to 5000mg/kg. Accordingly 2, 4, 6, and 8% of the highest doses (100, 200, 300 and 400mg/kg/b.w) of garlic extract were chosen.

Feeding or inducing rats with high fat diet for the period of six weeks has led to the increased in lipid levels of low density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and triglyceride (TG) and have significantly decreased plasma HDL-cholesterol and significantly increased antioxidant levels such as malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT). This observation is in agreement with results reported previously by Zhang *et al.,* (2007) who has reported that a high fat diet (HFD) results in significant increase in body weight, serum lipid levels and release of free fatty acids (FFAs). Free fatty acids also produce oxidative stress. The release of excessive free fatty acids provokes lipotoxicity, as lipids and their metabolites create oxidative stress. This affects adipose as well as non-adipose tissue, accounting for its pathophysiology in many organs, such as the heart, kidney liver and pancreas, and resulting in the metabolic syndrome (Gordon, 1997). Treatment with garlic to high fat diet induced hyperlipidemia in albino rats resulted in significant decrease ($p<0.05$) in serum level of lipids in the hyperlipidaemic rat.

Oral administration of lipid lowering drug (Atorvastatin 10mg/kg) to group III, and garlic extract 100, 200, 300 and 400mg/kg in rats fed high fat diet has caused a significant reduction ($p<0.05$) in serum total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C) and significantly increased serum high density lipoprotein cholesterol (HDL-C) when compared with the positive control (group II), these effects have been more pronounced with the high dose of garlic extract, the higher the dose the lower the lipid levels.

Alhassan *et al.,* (2018) reported that, the levels of total cholesterol, triglyceride, low density lipoprotein cholesterol, very low density lipoprotein cholesterol decrease in hyperlipidemic rats when administered with ginger and cinnamon extract which correlated to this study, where

hyperlipidemic-induced rats lipid levels significantly decreased when administered with garlic extract. The data obtained presented in Tables 3 showed that, a significant increase in serum total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL)-cholesterol, very low density lipoprotein (VLDL)-cholesterol was observed in high fat diet-induced hyperlipidaemic rats**.** A large body of evidence has shown that high-fat diet rich in saturated fatty acid results in hyperlipidemia. The pronounced increase in serum cholesterol, triglyceride (TG) and low density lipoprotein cholesterol (LDL-C) levels in hyperlipidaemic rats in the current study is in agreement with results reported previously by Xu *et al.*, (2012) who demonstrated that serum lipids were significantly increase in rats fed a high-fat diet. The increased serum levels of total cholesterol, low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) as well as lowered levels of high density lipoprotein cholesterol (HDL-C) have been identified in development of hypercholesterolemia (Ross, 1999). Excessive dietary intake of fat cause serum cholesterol to rise by down regulating LDL receptor synthesis as a result of which the uptake of LDL-C via LDL receptor is reduced which result in an increase of blood cholesterol level (Dietschy *et al.,* 1993). Therefore, the increased serum levels in the lipid rich lipoproteins (LDL-C and VLDL-C) indicate that more cholesterol and triglyceride are been transported from the liver to the extra-hepatic tissues to be taken up by those tissues. The increase in the serum high density lipoprotein cholesterol may be due to the boost of high density lipoprotein (HDL)-Cholesterol biosynthesis majorly in the liver and partly in the small intestine. High Density Lipoprotein (HDL)-Cholesterol particles are formed basically from apoprotein (ApoAI) and apoprotein (ApoAII). Apo lipoproteins, whose expression were shown to be influenced by nutritional interventions, such as a switch from high carbohydrate to a high-fat diet to lipid rich diet that was reported to increase the production rate of apoprotein (ApoA-I) rather than its clearance (Jiang et al., 2006).

The obtained data presented in Table 4 revealed that significant increase in liver malondialdehyde (MDA) in the group fed with high fat diet. On the other hand, a significant decrease in glutathione peroxidase (GSH-Px), Catalase (CAT) and superoxide dismutase (SOD) were observed in high fat diet induced hyperlipidemia. Oxidative stress has emerged as an important pathogenic factor in the development of hyperlipidemia and also most of the complications related to hyperlipidemia are associated with oxidative stress, induced by the generation of free radicals (hydrogen peroxide, superoxide anion, hydroxyl, etc) (Soanker, 2012).

The result provides a perfect correlation between lipid peroxidation products and decreased activities of CAT and SOD, which play an important role in scavenging the toxic intermediate products of incomplete lipid peroxidation. A decrease in the activity of these enzymes, as seen in liver of high fat diet induced hyperlipidemia in rats, can lead to the excessive availability of superoxide and peroxyl radicals, which in turn generate hydroxyl radicals, resulting in the initiation and propagation of more lipid peroxidation products (Sacks et al; 1978). As seen in the present study, garlic extract have lowered the blood cholesterol level and improve blood lipid profile to a significant extent. Bello (2013) reported that free radicals formed either by the reaction of metabolites with oxygen or by the interaction of superoxide radicals with H_2O_2, which is seem to initiate peroxidative degradation of membrane lipids rich in polyunsaturated fatty acids. This leads to formation of lipid peroxides which in turn give products like MDA that cause loss of integrity of cell membrane and damage to hepatic tissue. It also increases the serum antioxidant enzymes activities (superoxide dismutase and glutathione peroxidase) and decreasing the plasma malondialdehyde level, which is an important indicator of lipid peroxidation.

The significant lower concentration of malondialdehyde in rats fed a high cholesterol diet and orally administered garlic extract (groups IV, V, VI, and VII), suggest that the addition of methanolic-aqueous garlic extract increased the antioxidant enzyme activities (superoxide dismutase and glutathione peroxidase) as a protective mechanism against oxidative stress (Arivazhagan et al; 2000).

Histological assessment of liver and heart sections of rats showed normal tissue from the negative control [group I] (plate 1 and 2), while group II (plate 3 and 4) induced hyperlipidemia but not treated which serve as positive control showed areas where fat get deposited, in the perinusoidal space and adipose tissue in the liver and heart respectively which might likely resulted to damage organs. Group III, (plate 4 and 5) were induced hyperlipidemia and treated with antihyperlipidemic drug (10mg/kg atorvastatin), the sections showed unremarkable or normal tissues. Groups IV, V, VI, and VII were induced hyperlipidemia for six weeks and treated with different concentration of the extract 100, 200, 300, and 400mg/kg for two weeks respectively, so group IV plate 7 and 8, group V plate 10 and group VI plate 11 showed areas of necrosis where fat stored (Ito cells) in the perinusoidal space of the liver and adipose tissue of the heart. While plate 9 group V, plate 12 group VI and plate 14 group VII showed area of repair or

unremarkable or normal tissue and myocardium when compared to negative and standard control.

Hyperlipidemia, including hypercholesterolemia and hypertriglyceridemia, is a major risk factor for the development of cardiovascular diseases (Makni *et al.,* 2008). Elevated levels of plasma total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and triglyceride (TG) as well as reduced levels of plasma high density lipoprotein cholesterol (HDL-C) are often associated with an increased risk of coronary heart disease (Smith et al., 2004). In addition, hyperlipidemia can induce oxidative stress in liver (Bolkent *et al.,* 2005). Lipids have been noted to perform important functions in the body, but may cause various health problems if present in excess amounts. The term hyperlipidemia refers to the elevated lipid levels in the body including high cholesterol and high triglyceride levels (Braamskamp *et al.,* 2012). Lipids have been considered as "fats" in the bloodstream, which is commonly divided into cholesterol and triglycerides. However, the cholesterol circulates in the bloodstream and is involved in the structure and function of cells, whereas, triglycerides are either used immediately or stored in the fat cells (Iughetti *et al.,* 2010). When plasma cholesterol exceeds the level required, it results in the development of atherosclerosis and stroke (Inoue *et al.,* 2002). Atherosclerosis is an emphatically serious condition where medium and large arteries become clogged up by fatty substances results in formation of plaques. Disorders of lipid and lipoprotein metabolism i.e. dyslipidemia is a risk factors for atherosclerosis. Accumulations of cholesterol and low density lipoprotein (LDL)-cholesterol are main cause for formation of atherosclerotic plaque, which results in strokes, heart attack and eventually death (Turner *et al.,* 1998).

Natural remedies have been investigated for centuries for a wide variety of ailment. Garlic has received special attention for its beneficial effects (Auer *et al.,* 1990; Santos and Grunwals, 1993), but until recently there has been little scientific support for its therapeutic and pharmacological properties. There is no satisfactory data from randomized controlled test linking supplementation of garlic in diet with a reduction in cardiovascular morbidity and mortality (Silagy and Neil, 1994), thought significant reductions in blood cholesterol and triglyceride levels were observed in some studies when garlic extract or powder were used. Thus, the current study was conducted to assess the hypocholesterolaemic and antioxidant effect of garlic extract on rats fed a high cholesterol diet. In this experiment, high cholesterol diet fed to rats (group II and not treated) has significantly increased ($p<0.05$) their plasma total cholesterol (TC),

triglyceride (TG), Low Density Lipoprotein-cholesterol (LDL-CH) and has significantly decreased plasma High Density Lipoprotein-cholesterol (HDL-CH).

Result of an earlier study has shown that ingestion of garlic appears to inhibit hepatic fatty acid synthesis (Gebhardh, 1991) by lowering key enzymes activities in supplying substrates, thus reducing lipid accumulation in the liver and triglyceride level in plasma.

With respect to the cholesterol lowering property of garlic, it has been suggested that some constituents of garlic may act as inhibitors for some enzymes such as hydroxy methyl glutaryl - COA (HMG-COA) reductase which participates in cholesterol synthesis (Gebhardh and Beck, 1996; Durak *et al*; 2004).

Garlic (*Allium sativum*) components have been reported to elevate the levels of malondialdehyde (MDA) superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) (Pinto et al; 1997; Wei and Lau, 1998). Other beneficial effects of garlic can be attributed to the presence of non-enzymatic antioxidant such as selenium, and copper metals, vitamin C and other phytochemicals such as organosulphur compounds (Prasad et al; 1995).

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATION

5.1 Summary

The aim of the study is to assess the effect of oral administration of methanolic-aqueous garlic extract on serum lipid and antioxidant levels in fat diet induced hyperlipidaemic rats in comparison with negative and positive controls (group I and II). Garlic with its constituent plays a significant role in the reduction of serum lipid and antioxidant levels. Treatment with garlic to high fat diet induced hyperlipidemia in albino rats showed significant reduction in serum lipid and antioxidant levels.

The acute oral toxicity test (LD_{50}) garlic methanolic-aqueous extract showed no behavioral changes. There was no mortality up to 5000mg/kg/b.w of the extract. Therefore, the result of acute toxicity test ($LD_{50)}$ of garlic methanolic-aqueous extract revealed to be safe.

The results of hyperlipidaemic and antioxidant showed a significant ($p>0.05$) decrease in lipid and antioxidant levels of the extract treated groups compared to hyperlipidaemic group (group II) and also standard control (group III), which is showing the hypolipidaemic and antioxidant effect of the plant.

The histological study of liver and heart showed necrosis in some groups of the experimental animals but when treated with 10mg/kg Atorvastatin and different concentration of garlic extract, the organs were treated most especially the one given the highest dose (group 7) when compared to negative and standard control.

The effect of the garlic extract compared to lipid lowering drug (Atorvastatin 10mg/kg), showed that the drug gave the better result than the higher dose (400mg/kg/b.w) of the extract and this may be suggested that further higher doses of the extract may achieve comparable result to standard treatment of Atorvastatin.

5.2 Conclusion

In conclusion the result of the present study demonstrates that methanolic-aqueous garlic extract possess antioxidant and antihyperlipidemic effect on induced hyperlipidaemic rats. This suggests that the extract may have a beneficial effect on the blood lipid profile and antioxidant status by improving lipid and antioxidant levels. The presence of allicin and organosulphur compounds in the extract may be responsible for its medicinal effect. The histological study showed some organs (liver and heart) of different groups of rats affected by high fat diet but when administered the extract, there was reversal of the effect in some groups. The result of acute toxicity study of garlic methanolic-aqueous extract revealed to be safe at <5000mg/kg.

5.3 Recommendation

It is recommended that, administration of diet rich in garlic as natural dietary product is very important and suitable attenuate metabolic disorders of different body tissues and protection against hyperlipidaemic complications.

Further research should be carried out on the effect of high dose of garlic extract on both hyperlipidaemic and normolipidaemic rats for a long period of time.

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APPENDIX I

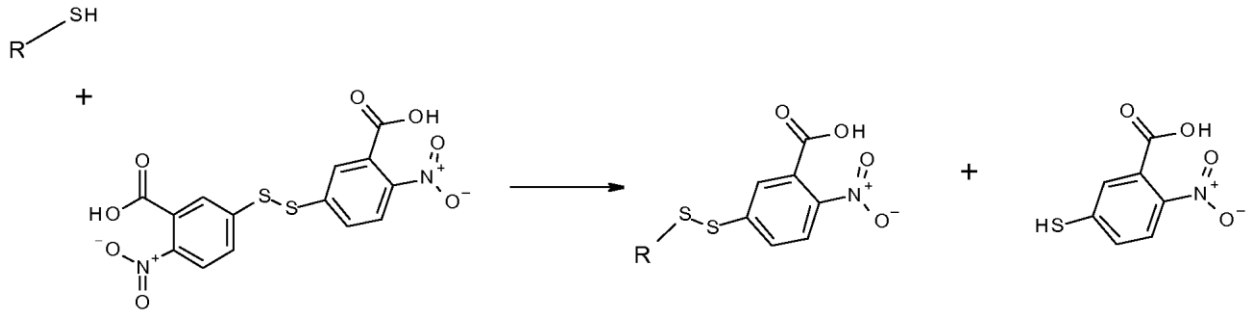
Preparation of serial dilutions of the GSH working Standard

Stock ml	Phosphate buffer	Ellman's reagent	Abs (412nm)	GSH conc (µg/ml)
0.01	0.24	2.25	0.04	8
0.025	0.225	2.25	0.101	20
0.05	0.20	2.25	0.194	40
0.10	0.15	2.25	0.38	80
0.15	0.10	2.25	0.572	120
0.20	0.05	2.25	0.749	160

Total reaction mixture: 2.25ml

GSH is proportional to absorbance at 412nm. All readings were taken within 5 minutes, as colour developed is not stable after that duration, following addition of Ellman's reagent.

Mechanism of action of reduced GSH with Ellman's Reagent (Beutler *et al*., 1963)



Ellman's reagent **Nitro-5-thiobenzoate**

APPENDIX II

PREPARATON OF REAGENTS FOR GSH

1 GSH Working Standard

40 mg GSH (Sigma Chemical Co., London, Mol. Wt 307.3g) was dissolved in 100 ml of 0.1M phosphate buffer, pH 7.4, and then stored in the refrigerator.

2 Phosphate buffer (0.1M, pH 7.4)

a. 7.16 g of $K_2HPO_4\,12H_2O$ (Hopkins and Williams, Ltd, Mol. Wt. 358.22) was dissolved in 200ml of distilled water.
b. 1.56 g of $KH_2PO_4.\,2H_2O$ (MW. 156.03) was dissolved in 100 ml of distilled water.

Finally, solution (a) and (b) were added together and the pH adjusted to 7.4

3 Ellman Reagent [5', 5'-Dithiobis- (2-nitrobenzoate) DTNB]

40 mg of DTNB was dissolved in 0.1M phosphate buffer of pH 7.4 and made up to 100 ml

3 Precipitating Agent

APPENDIX III

Standard Curve for Reduced Glutathione

APPENDIX IV

Preparation of Reagents for Lipid Peroxidation (LPO)

1. **30% Trichloroacetic acid (TCA)**

   4.5 g of TCA was dissolved in distilled water and made up to 15 ml with same

2. **0.75% Thiobarbituric acid (TBA)**

   This was prepared by dissolving 0.1125 g of TBA in 0.1M HCl and made up to 15ml with same.

3. **0.15 M Tris-KCl buffer (pH 7.4)**

   1.12 g of KCl and 1.817 g of Tris base were dissolved in 100 mls of distilled water and the pH was then adjusted to 7.4

4. **0.1M HCl**

   0.124 ml conc HCl was diluted with 14.876 ml of distilled H_2O to make 15 mls.