

1.1 INTRODUCTION

Atherosclerosis, leading to coronary artery disease (CAD), has assumed a pandemic proportion the world over and has become the most common cause of morbidity and mortality (Gian *et al.*, 2007) in developed as well as developing nations (WHO, 2003). In South Africa, it is the second leading cause of death after HIV (Raal, 2009). Despite the development of new drugs to prevent the incidence of atherosclerosis, it was predicted that diseases of cardiovascular system will be the leading worldwide cause of death by 2030 (Mathers and Loncar, 2006; Norman *et al.*, 2006) due to ineffective therapy of cholesterol-lowering drugs (example, statins) that could reduce cardiovascular disease (Ming-Shi *et al.*, 2008). In recent years, the clinical importance of herbal drugs has received considerable attention as statins and synthetic antioxidants which had been earlier employed in the management and treatment of CAD have been shown to have one or more side effects (Bradford *et al.*, 1991; Musk *et al.*, 2001). The expected high incidence of the diseases, coupled with the high cost of Western pharmaceuticals and healthcare remedies, makes it all important to search for safe, effective and cheaper remedies. Before the advent of modern medicine, various plants were employed by man in the management, treatment and the related complications of cardiovascular diseases. In the Eastern Cape Province of South Africa, a number of plants are reported to possess cardio-protective properties, due to their use by traditional healers for treatment of chest complaints, high cholesterol, high and low blood pressure and general heart problems. Scientific evaluation of the therapeutic claims, mechanism(s) of action(s) as well as the toxicological effect of these plants is necessary to justify their folkloric usage (Hansotia and Drucker, 2005).

1.2 ATHEROSCLEROSIS

Atherosclerosis is a multifactorial disease of the large and medium-sized muscular arteries and the leading cause of morbidity and mortality in industrialized countries (Braunwald, 1997). It is characterized by endothelial dysfunction, vascular inflammation, build-up of lipids, cholesterol (Ming-Shi *et al.*, 2008), calcium and cellular debris within the intima of the vessel wall (Anuradha, and Raji, 2009). This build-up result in plaque formation, vascular remodelling, acute and chronic luminal obstruction, abnormalities of blood flow and diminished oxygen supply to target organs (Bibave *et al.*, 2011). Depending on the location of the blocked arteries, atherosclerosis may lead to complications generally referred to as cardiovascular diseases (coronary artery disease, carotid artery disease, peripheral artery disease, aneurysms, heart attack and stroke). Lewis (2009) reported that successful treatment minimizes lifetime chances of cardiovascular events, morbidity, and mortality. It was suggested that risk factors for atherosclerosis should be monitored, beginning in childhood, even in asymptomatic patients. Modifiable factors (e.g., blood pressure, smoking, serum lipids) and non modifiable factors (e.g., age, family history) should be assessed (Lewis, 2009). Improved lifestyle such as dietary choices, increased exercise, and smoking cessation combined with lipid-lowering pharmacotherapy and antihypertensive medications may also be employed in the management and prevention of atherosclerosis (Lewis, 2009). However, the treatment and management of this disease are still a challenge to the medical system (Ali and Ketan, 2012). Though, a large number of allopathic hypolipidemic drugs are currently available in the market, they lack desired properties such as efficacy and safety of long-term use, cost and simplicity of administration (Davidson and Tooth, 2004). These deficiencies have led to an increase in demand for cheap and affordable drugs without any or with fewer adverse side effects. Recently, attention has been focused on

a number of medicinal plants used in the treatment of cardiovascular disease by virtue of their lipid lowering, anti-anginal, antioxidant and cardio-protective effects.

1.2.1 PREVALENCE OF ATHEROSCLEROSIS AND RISK FACTORS

Atherosclerosis is the underlying cause of approximately 50 % of all deaths in the Western world (Lusis, 2000) and it accounts for 16.7 million lives per annum world-wide (Greenland *et al.*, 2001). In the United States of America (USA) alone, it is commonly held that coronary artery diseases (CAD) are the leading cause of death (Greenland *et al.*, 2001). In South Africa, atherosclerosis is second only to HIV as the major cause of death (Raal, 2009). There is a paucity of data regarding atherosclerosis and its prevalence in developing countries especially Africa. However, it is projected that the mortality rate from CAD will double from 1990 to 2020 (Okraïnec *et al.*, 2004). Kotze *et al.* (1995) revealed that certain communities in South Africa have a high potential for atherosclerosis. Findings indicate that the prevalence of atherosclerosis is significantly higher in the coloured population than in the other population groups examined (Kets *et al.*, 2011). The consequences of atherosclerosis can be severe and far-reaching, including stroke and transient ischemic attack and peripheral arterial disease. Therefore, atherosclerosis can be referred to as a major public health problem, as it is associated with significant morbidity and mortality (Gian *et al.*, 2007). Risk factors in the increasing prevalence of atherosclerosis and coronary artery disease according to the American Heart Association are classified into five main categories (Smith *et al.*, 2000). The first category such as smoking, elevated blood pressure, elevated serum cholesterol (or LDL cholesterol or, alternatively, elevated Apo lipoprotein B (Apo B), low high-density lipoprotein cholesterol (HDL) and diabetes mellitus are referred to as causative risk factors. The second category includes conditional risk factors such as triglycerides, small LDL particles, lipoprotein (a), homocysteine, coagulation factors (plasminogen activating factor inhibitor-1 and fibrinogen) and elevated C-reactive protein levels (CRP). All of these factors,

except for CRP, are considered conditional when serum levels are abnormally high. The third group is classified as predisposing risk factors such as overweight and obesity, physical inactivity, male sex, family history of premature CHD, socioeconomic factors, behavioral factors (e.g. mental depression) and insulin resistance. The fourth category includes risk factors that relate to the coronary plaque burden. This category includes age and nonspecific ST-segment changes in the resting electrocardiogram. Age is therefore a definitive risk factor, but its effect is mainly conveyed through the accumulation of other risk factors (Pencina *et al.* 2009, Smith *et al.* 2000). The last risk factor, defined as a separate susceptibility factor, is left ventricular hypertrophy of the heart.

1.2.2 CLASSIFICATION OF ATHEROSCLEROSIS LESIONS

According to American Heart Association recommendaton (Table 1), atherosclerosis lesions can be classified into: initial change lesions (I), minimal change lesions (fatty streak) (II), intermediate lesions, pre-atheromas (III), atheromas (IV), fibroatheromas (V), hemorrhagic/thrombotic lesions (VI), subtypes of type V lesions (Vb and Vc). The updated version of these recommendations divided the subtypes of type V lesions into calcified lesions (VII) and fibrotic lesions (VIII) (Stary *et al.*, 1994; Stary *et al.*, 1995; Stary, 2000;). An alternative classification of atherosclerotic plaques based on autopsy data and extensive description of lesions prone to rupture was suggested by Virmani *et al.*, (2000 a and b) (Table 1). However, Stary classifications remain the accepted standard.

Table 1a: The classification of atherosclerotic lesions according to the American Heart Association

Lesion type	Cellular composition.
I Initial change	Isolated macrophage foam cells.
II Minimal change	Multiple layers of foam cells.
IIa: Progression-prone; Abundant SMCs	Few lymphocytes.
IIb: Progression resistant; Few SMCs	Isolated mast cells.
III Pre-atheroma	Isolated pools of densely packed extracellular lipids SMCs accumulate lipid droplets.
IV Atheroma	The confluent core of extracellular lipids; Increased number of lymphocytes SMCs; decrease in numbers.
V Fibroatheroma	Fibrous tissue and collagen added; Intimal SMCs increase in number.
VI Hemorrhagic/thrombotic lesion	Lesion becomes fissured and/or thrombotic.
VII Calcified lesion (Previously type Vb)	Calcification predominates.
VIII Fibrotic lesion (previously type Vc)	Fibrous tissue changes predominate; Lipid core is nearly absent.

Jian-Ming et al., 2002

Table 1b: Classification of autopsy-verified atherosclerotic lesions

Lesion name	Lesion description by histopathology	Thrombosis
Non-atherosclerotic intimal lesions	Normal accumulation of SMCs in the intima With the absence of lipid or macrophage	Thrombus is absent
1. Intimal Thickening	Foam cells	
2. Intimal xanthoma of Fatty streaks	Subendothelial accumulation of foam cells in the intima with no necrotic core or fibrous Cap; animal and human data show that such Lesions usually regress	Thrombus is absent
Progressive lesions	SMCs in a proteoglycan-rich matrix with	The thrombus is absent
3a. Pathologist Thickening	Internal areas of extracellular lipid accumulation without necrosis.	
3b. With erosion	Luminal thrombosis, plaque the same as above.	Thrombus most Often mural and Infrequently Occlusive
4a. Fibrous cap Atheroma	Well-formed necrotic core with overlying fibrous cap	Thrombus is absent
5. TCFA	A thin fibrous cap infiltrated with macrophages and lymphocytes, rare SMCs, and an underlying necrotic core.	Absent, with intraplaque hemorrhage/fibrin.
a. With rupture	Fibroatheroma with cap disruption; luminal Thrombus communicates with the underlying Necrotic core.	Thrombus usually occlusive
6. Calcified nodule	Eruptive nodular calcification with underlying fibrocalcific plaque	Thrombus usually non-occlusive
7. Fibrocalcific plaque	Collagen-rich plaque usually with Significant stenosis; contains large areas of Calcification with few inflammatory cells; Necrotic core may be present	Thrombus is absent

Abbreviations: SMC = smooth muscle cell; TCFA = thin-cap fibroatheroma (Virmani *et al.* 2000b)

1.2.3 PATHOPHYSIOLOGY OF ATHEROSCLEROSIS

Atherosclerosis (cardiovascular disease) is a multifactorial disease of the large arteries caused by the slow buildup of plaque on the inside of the walls of the arteries (Braunwald, 1997; Sing and Moll, 1999). Arteries most commonly involved are those in the heart, brain, kidney, small intestine and lower limb (Anne *et al.*, 2006). Atherosclerosis is initiated by lipid retention, oxidative modification, endothelial dysfunction which provokes chronic inflammation, ultimately causing thrombosis or stenosis (Stocker and Keaney, 2004; Madamanchi *et al.*, 2005; Grassi *et al.*, 2009). There is ample evidence that hypercholesterolemia (that is, elevated plasma levels of low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) induced by genetic modification or enhanced intake of dietary lipids is a major causative factor in atherogenesis (Blum and Levy, 1987; Steinberg, 2005).

Atherosclerosis develops progressively through continuous development of arterial wall lesions centered on the accumulation of cholesterol-rich lipids and the accompanying inflammatory response (Bibave *et al.*, 2011). The response-to-injury hypothesis postulates that the initiation and progression of occlusive atherosclerotic coronary and peripheral vascular disease involves a nonspecific and stereotypical response incited by endothelial damage or dysfunction (Rose, 1999). According to Insull (2009) atherosclerosis involves four major stages: early fatty streak development, early fibroatheroma, advancing atheroma and complex lesion development (Figure 1).

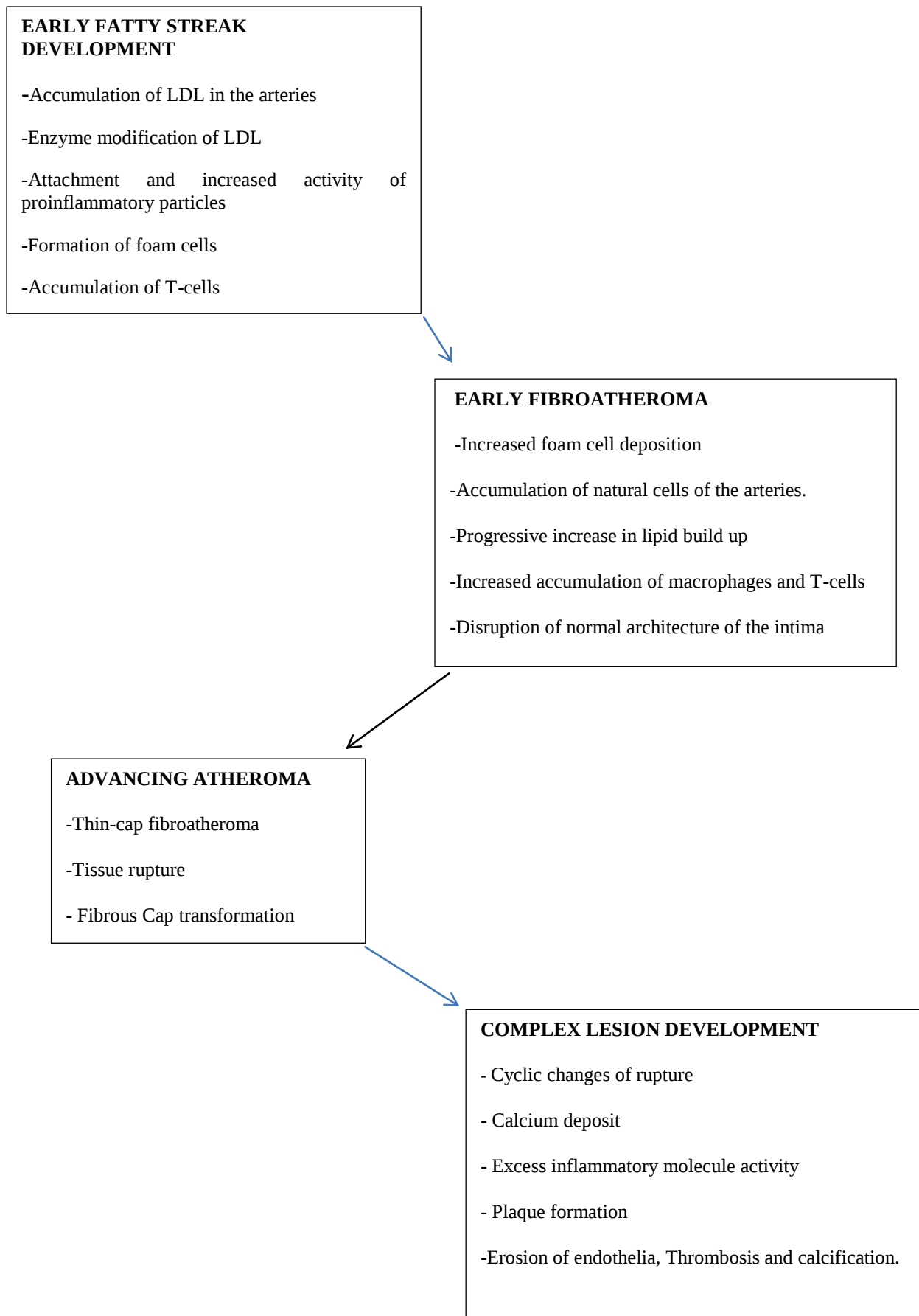


Figure 1: The sequence of events in the development of atherosclerosis (Insull, 2009)

1.2.4 EARLY FATTY STREAK DEVELOPMENT

Early fatty streak development begins in childhood and adolescence (Stary, 2003; Insull, 2009). The initial step involves the accumulation and enzyme modification of LDL to pro-inflammatory particles which provoke innate immunity in the intima (Insull, 2009). The intima is the endothelium layer lining the blood vessel on the lumen side. The enzymes involved in this modification are produced by macrophages and endothelium cells (ECs) in addition to other enzymes found in atherosclerotic lesions, such as myeloperoxidase, sphingomyelinase, and secretory phospholipase (Lamon and Hajjar, 2008). The appearance of oxidized LDL within the intima promotes the attachment, rolling and infiltration of leukocytes and T-cell to the site (Martin-Fuentes *et al.*, 2006; Pavel, 2011;). This creation of foam cells together with the accumulation of T cells stimulates the formation of a fatty streak.

1.2.5 EARLY FIBROATHEROMA

Early fibroatheroma occurs in persons in their teens and 20s (Burke *et al.*, 2001; Stary, 2003). It is characterized by fibrous plaque lesions. Fibroatheroma is caused by a series of biochemical events such as increase in the amount of foam cells deposited within the plaque, accumulation of natural cells of the arteries, increased progressive lipid binding capacity of extracellular proteoglycans secreted by the smooth muscles and death of macrophages, smooth muscle cells (Insull, 2009) and increased inflammatory activity as a results of accumulating macrophages, T lymphocytes, dendritic cells, and necrotic debris (Ross, 1999; Insull, 2009). These events progressively lead to the disruption of the normal architecture of the intima with an enlarged pool of lipid-rich necrotic cores occupying 30 % to 50 % of arterial wall volume (Insull, 2009).

1.2.6 ADVANCED ATHEROMA

Advancing atheroma occurs in persons aged ≥ 55 years. In this stage of plaque development, formation of a thin-cap fibroatheroma (TCFA) may occur and it may proceed with tissue rupture (Virmani et al., 2000; Cheruvu et al., 2007; Moreno et al., 2006). This lesion usually is labelled as vulnerable plaque because of the risk of rupture and life-threatening thrombosis (Insull, 2009). This condition occurs when the fibrous cap transforms into a thin cap most likely due to decreased extracellular matrix (ECM) synthesis by the decreasing number of smooth muscle cells (SMCs) residing in the cap and to matrix degradation by infiltrating macrophages (Thim *et al.*, 2008). Thin-cap atheroma and ruptured plaques account for 1.6 % and 1.2 % of the epicardial portions of the coronary arteries respectively. Most of these lesions are limited to the proximal portions of the major coronary arteries, and 92 % are clustered within ≤ 2 adjacent 20-mm artery segments (Cheruvu *et al.*, 2007).

1.2.7 COMPLEX LESION DEVELOPMENT

This stage of lesion development is characterized by cyclic changes of rupture, calcium deposit, thrombosis and healing which may recur as many as 4 times on a single site in the arterial wall, resulting in multiple layers of healing tissue (Insull, 2009). The exact mechanism of plaque rupture is not known, but it includes cap thinning, excess inflammatory cytokines and proteases that mediate digestion of the matrix, decreased collagen synthesis and the presence of injured or apoptotic cells within the necrotic core. Plaque formation may cause erosion of endothelium resulting in thrombosis. The increasing mass of some plaques alone may become sufficient to form significant stenosis that may cause lethal ischemia.

1.2.8 ROLE OF OXIDATIVE STRESS IN AETIOLOGY OF ATHEROSCLEROSIS

Atherosclerosis is an inflammatory process strongly influenced by oxidative stress. Oxidative stress is characterized by increased generation of reactive oxygen species such as superoxide anion, hydroxyl radical, hydrogen peroxide and peroxidative derivatives of polyunsaturated fatty acids (PUFA) such as conjugated dienes, lipid hydroperoxides and malonyldialdehyde (MDA) and it plays a critical role in tissue and cell injury (Mohammad *et al.*, 2009). The main sources of reactive oxygen species (ROS) in atherosclerotic vessels are macrophages and xanthine oxidase, NADPH oxidase, uncoupled endothelial nitric oxide synthase (eNOS)), smooth muscle cells, and the mitochondrial respiratory chain (Janssen-Heininger *et al.*, 2000).

There are various ways by which oxidative stress induces atherosclerosis:

(1) Free radicals induce endothelial dysfunction which is an initial step in atherogenesis (Georgia *et al.*, 2009). It involves a reduction in nitric oxide (NO) and superoxide ($O_2^{\cdot -}$) produced by the endothelial cells with a resultant decrease in bioavailability of endothelium-derived nitric oxide, platelet aggregation and adhesion of neutrophils to the endothelium (Vepa *et al.*, 1999).

(2) Oxidative stress leads to oxidation of LDL (ox-LDL) in the arterial wall (Georgia *et al.*, 2009). Ox-LDL is a potent inducer of inflammatory molecules. It stimulates inflammatory signaling by endothelial cells, releasing chemotactic proteins such as MCP-1 (Monocyte Chemotactic Protein-1) and growth factors such as MCSF (Monocyte Colony Stimulating Factor), which help in the recruitment of monocytes into the arterial wall (Catapano *et al.*, 2000). Ox-LDL also promotes the differentiation of monocytes into macrophages that take up the oxidized LDL in a process that converts them into foam cells, the hallmark cell of atherosclerosis. Apart from that, Ox-LDL also has other effects, such as inhibiting the

production of NO (Nitric Oxide), an important mediator of vasodilation and expression of endothelial leukocyte adhesion molecules.

(3) ROS such as hydrogen peroxide (H_2O_2) can stimulate vascular smooth muscle cell hypertrophy and hyperplasia, or initiate the development of a vascular proinflammatory state via a variety of mechanisms. Redox-sensitive transcription factors, such as nuclear factor-kappa B (NF- κ B) and leukocyte adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1) or decreased levels of NO and angiotensin II (Ang-II) -dependent pathways may activated pro-inflammatory reactions (Inagi, 2006). Furthermore, oxidative stress by hydrogen peroxide (H_2O_2) increases phosphorylation of tyrosine kinases, which leads to stronger binding of neutrophil cells to the endothelium and alteration of vessel permeability (Bourcier *et al.*, 1997).

1.2.9 ATHEROSCLEROSIS: DIAGNOSIS AND TREATMENT

There is no single test to diagnose atherosclerosis. However, with the help of healthcare providers atherosclerosis can be detected before it becomes symptomatic using some tests such as blood tests, electrocardiogram (ECG or EKG), chest x-ray, stress test, echocardiogram, nuclear scans, ankle/brachial index, ultrasound, computed tomography (CT) scan, angiography, risk factors and family history (Medscape, 2007).

1.2.9.1 Treatment of Atherosclerosis

According to Lewis (2009) successful treatment of atherosclerosis will minimize lifetime chances of cardiovascular events, morbidity, and mortality. It involves monitoring risk factors beginning in childhood, even in asymptomatic patients and assessment of modifiable factors (e.g., blood pressure, smoking, serum lipids) and non-modifiable factors

(e.g., age, family history) (Lewis, 2009). For the effective treatment and management various approaches are employed:

(1) Improved dietary choices, increased exercise, and smoking cessation (Sandra, 2009).

Improved dietary choices combined with increased exercise and smoking cessation play a great role in the control and the development of atherosclerosis. The benefits of this approach include improving cholesterol and fat levels, reducing inflammation in the arteries, helping weight loss programs, and helping to keep blood vessels flexible and open. Physical activity may independently cause a reduction in weight gain, increased HDL cholesterol, myocardial function, and vasodilatory capacity (Leon and Sanchez, 2001).

2) Anti-hyperlipidemia drugs are classified into different groups on the basis of their mechanism of actions.

(a) HMG-CoA reductase inhibitors- HMG-CoA reductase is the rate limiting enzyme in cholesterol synthesis and it catalyses the conversion of HMG-CoA to mevalonic acid. Inhibition of this enzyme will decrease hepatic cholesterol synthesis, up regulates LDL receptor synthesis and increased LDL cholesterol clearance from the plasma to the liver cells. Examples of HMG-CoA reductase inhibitors include lovastatin, simvastatin, pravastatin and atorvastatin; this group of drugs is called statins (Rang and Dale, 2007).

(b) Fibrates are drugs which reduce circulating LDL and triglyceride. The mechanism of action of these drugs is complex but it is assumed that they are agonists for a subset of lipid control gene regulator elements called peroxisome proliferator activator receptors (Jacques, 1994). The example includes bezafibrate, ciprofibrate and fenofibrate.

(c) Bile acid-binding resin is an example of a drug that inhibits absorption of cholesterol. It decreases the absorption of exogenous cholesterol and increases metabolism of endogenous

cholesterol into bile acids in liver (Bibave *et al.*, 2011). The advantage of the action of the drug is that it increases the expression of LDL receptors on hepatocytes; this leads to increased clearance of LDL from the plasma (Bibave *et al.*, 2011). Other anti-hyperlipidemia drugs include nicotinic acid or its derivative and fish oil derivative (Lewis, 2009)..

(3) Surgical treatment

Surgical treatment for atherosclerosis depends on the patterns of the atherosclerosis. According to Mathew *et al.*, 1985 atherosclerotic occlusive disease tends to assume characteristic patterns that may be classified, by predominant site or distribution of the disease, into five major categories: (I) the coronary arterial bed, (II) the major branches of the aortic arch, (III) the visceral arterial branches of the abdominal aorta, (IV) the terminal abdominal aorta and its major branches, and (V) a combination of two or more of these categories occurring simultaneously. Localized or lesion that occurs in the proximal or mid-proximal portions of the arterial bed are easily are amenable to effective surgical treatment directed toward restoration of normal circulation. On the contrary, lesions that occur predominantly in the distal portions of the arterial bed are usually not amenable to effective surgical treatment (Mathew *et al.*, 1985). In all cases, patients with atherosclerotic occlusive disease are at risk of development of new or recurrent critical lesions there is then need for careful and continuing follow-up of such patients after surgery (Mathew *et al.*, 1985).

1.3 MEDICINAL PLANTS AND DISEASE MANAGEMENT

The early success recorded with the use of orthodox medicine for the management of human diseases have suffered a setback worldwide due various reasons such as disease resistance, drug adulteration, and high cost of procurement and negative side effects. As a result, there is a growing interest in herbal medicines all over the world, as they are assumed to have fewer side effects, are cheap and affordable and readily available (Bussmann and

Glenn, 2011). Medicinal plants are potential sources of substances with significant pharmacological and biological activities. The bioactivities and pharmacological properties of most of these plants are attributed to their secondary metabolites; for example, flavonoids, tannins, proanthocyanidins and coumarins are well known polyphenolic compounds with strong antioxidant properties (Sumbul *et al.*, 2011). Hence, they play an important role in protecting food, cells and organs from oxidative damage (Osawa, 1999). They are capable of neutralizing free radicals (e.g., superoxide, nitric oxide, and hydroxyl radicals) and other reactive species (e.g., hydrogen peroxide, peroxynitrite, and hypochlorous acid) that are produced in the body (Oyedemi *et al.*, 2010). The antioxidant activity of the phenolics is determined by the position and number of hydroxyl groups, polarity, solubility and stability of phenolic compounds during processing (Decker, 1998).

Some of the reported pharmacological activities of medicinal plants include anti-diabetic (Olorunnisola *et al.*, 2008), antiviral, anti-inflammatory (Moody *et al.*, 2008), anti-allergic, anti-thrombus, vasodilatory, anti-carcinogenic, anti-immunogenic, anti-aging anticancer, antibacterial and anti-atherosclerosis (Tewtrakul and Subhadhirasakul, 2007).

Tulbaghia violacea Harv. belongs to the family of Alliaceae which is most commonly associated with garlic. *T. violacea* is a bulbous plant with a height of 50 cm. Its leaves are dark green, leathery in texture with strong garlic-like odor (Dyson, 1998). *T. violacea* is indigenous to the Eastern Cape, South Africa. The leaves and bulbs are widely used as an herbal remedy for various ailments (Bungu *et al.*, 2006). Scientific evidence has demonstrated that *T. violacea* and its various preparations possess pharmacological activities. These include anthelmintic activity, anticancer activity, and in vitro growth inhibition and induction of apoptosis in cancer cells (Duncan *et al.*, 1999; McGaw *et al.*, 2000; Bungu *et al.*, 2006). Water and ethanolic extract of *Tulbaghia violacea* appears to have 68% and 71% ACE inhibiting properties, respectively (Ramesar, *et al.*, 2008; Duncan *et al.*, 1999). Despite the

fact that the rhizome of *T. violacea* is closely related to garlic and its reported in traditional medicine, most of the biological activities such as antioxidant, hypolipidemic, anti-hypercholesterolemic properties which have been reported for garlic extract have not been investigated in *T. violacea*.



Plate 1a: *Tulbaghia violacea* Harv. (TV) A: TV in its natural habitat within the environs of University of Fort Hare (Alice).



Plate1b: Rhizomes of *Tulbaghia violacea* Harv.

1.4. Aims and Objectives of the Study

The primary aim of this study was to identify and validate the folkloric uses of plants for the management of hypercholesterolemia induced atherosclerosis.

1.4.1 Specific Objectives

1.4.1.1 Ethnobotanical Survey of Plants

An ethnobotanical survey was undertaken to record information on medicinal plants from herbalists, traditional healers and rural dwellers in Nkonkobe Municipality, Eastern Cape Province to identify the medicinal plants used for managing cardiovascular diseases.

1.4.1.2 *In vitro* and *In vivo* Antioxidant Activities of Extract of *T. violacea*

The present study was designed using different *in vitro* and *in vivo* bioactivity techniques to give scientific credence to the antioxidant activity of the plant.

1.4.1.3 *In vitro* and *In vivo* Toxicity Evaluation of Extract of *T. violacea*

This study was designed to give information on the safety/toxicity risk of extract of the rhizomes of *T. violacea* using the brine shrimp lethality assay and albino rats as a model.

1.4.1.4 Anti-hyperlipidemia and Clinical Significance of *T. violacea* Extract

In view of the reported uses of *T. violacea* in the management of heart diseases, the present study was designed to investigate anti-hypercholesterolemia effect of extract of rhizomes of *T. violacea* in diet induced hyperlipidemic rats.

1.4.1.5 *In vivo* Anti-atherosclerotic Properties of *Tulbaghia Violacea* Rhizomes in South African Herbal Medicine

The present study was undertaken to understand the anti-atherosclerotic activity of methanolic extract of rhizomes of *T. violacea* and its possible mechanisms in diet induce atherosclerosis.

CHAPTER 2

ETHNOBOTANICAL SURVEY OF MEDICINAL PLANTS USED FOR THE MANAGEMENT OF HEART DISEASES IN THE NKONKOBÉ MUNICIPALITY SOUTH AFRICA

Published in Journal of Medicinal Plants Research Vol. 5 (17), pp. 4256-4260, 2011

2.0 INTRODUCTION

Cardiovascular diseases refer to any disease of the heart and blood vessels. The most common ones are diseases of the heart muscle, stroke, heart attack, heart failure and those caused by high blood pressure. Worldwide, cardiovascular diseases are assuming an increasing role as a major cause of morbidity and mortality (Krisela, 2007). According to World Health Organization report (WHO, 2003), approximately 16.7 million lives are lost annually worldwide. Between 1990 and 2020, the proportion of deaths from cardiovascular disease is projected to increase from 28.9 to 36.3% (Gowri *et al.*, 2011). Moreover, in terms of number of years of life lost, cardiovascular disease is expected to jump in ranking from fourth to first, while as a cause of premature death and disability, it will rise from fifth to first (Hennekens, 2000). The predisposing factors to cardiovascular diseases include cigarette smoking, elevated cholesterol, hypertension, obesity, physical inactivity and diabetes.

The prevalence and treatment status of common heart conditions, such as ischaemia heart disease, heart failure, rheumatic heart problem, diseases of the heart muscle, the heart valves, heart disease caused by hypercholesterolemia, high blood pressure, cigarette smoking, physical inactivity and alcohol use is unknown in South Africa (Sliwa *et al.*, 2005; Commerford, 2005; Mayosi *et al.*, 2006). The few available data suggest that these conditions are poorly managed and it is predicted that by 2020 cardiovascular problems will be among the top five causes of death and diseases in South Africa. The expected high incidence of the diseases, coupled with high cost of western pharmaceuticals and healthcare remedies, makes it all important to search for safe, effective and cheaper remedies.

Before the advent of modern medicine, various plants have been employed by man in the management of cardiovascular diseases. In the Eastern Cape Province of South Africa, a number of plants are reputed to possess cardio-protective properties, resulting in their use by

traditional healers for treatment of chest complaints, high cholesterol, high and low blood pressure and general heart problems. Considering the rate at which the vegetation is getting depleted in this part of the world, coupled with increasing demand for effective, cheap and less toxic drugs, it is necessary and important to document the precious knowledge of these plants and to search for more plants with cardio-protective potential. In this chapter, the information gathered from traditional and elderly rural dwellers, on plants used in Nkonkobe Municipality for treatment of the cardiovascular disease and some of their predisposing factors were reported.

2.1 MATERIALS AND METHODS

2.1.1 Study Area

Nkonkobe Municipality is an area in South Africa that is situated between 32° 47' S and 26° 50' E. The area is bounded by the sea in the east and drier Karroo in the west. The altitude is approximately 1300 m above sea level and the vegetation is veld type 7 (Masika and Afolayan, 2003).

2.1.2 Methodology

The data was collected from traditional healers, herbalists and rural dwellers using scientifically guided questionnaires, interviews and general conversations. Although informants were not scientifically literate, they were born in the region and had lived there for most of their lives. Plants used in each individual case were collected with the help of actual users. The reported plants with cardio-protective potential were identified by Prof D.S. Grierson of Botany Department, University of Fort Hare and deposited (Sin 2010/1–Sin 2010/19) at the Giffen Herbarium.

2.2 Intellectual Property Agreement Statement

All the elderly and the traditional healers who contributed information during ethnobotanical survey, were financially rewarded with further verbal agreement that this research shall not be for commercial purposes but to serve as information to the community and the entire Eastern Cape.

2.3 Compliance Statement

No part of this study in any form has been or will be commercialized; instead the entire thesis is meant to be used as a tool for information dissemination on the medicinal plants used for the treatment of cardiovascular diseases in Eastern Cape Province of South Africa.

2.4 RESULTS AND DISCUSSION

The results of this study have revealed that 19 plant species belonging to 16 families are frequently used for the treatment of cardiovascular diseases and their predisposing factors in the study site (Table 2). The informants consulted in this investigation claimed that the diagnoses of cardiovascular diseases in their patients is determined by symptoms such as chest pains, palpitation, short breath, swollen eyes, severe back pain, profuse sweating and in most cases patients that have been diagnosed with high blood pressure.

Two species of each of the Hypoxidaceae, Asteraceae and Fabaceae families are the most commonly mentioned plants during the survey. Previous reports have also linked some of these plants to remedies for common cold, hypertension, psoriasis, urinary tract infections, prostate diseases, gastrointestinal complaints, and even mental disorders (Mac *et al.*, 2004; Laporta *et al.*, 2007). Fifty percent (50 %) of the methods of preparation are by infusion while 15 % are by decoctions (Table 2). The leaves are the most used for the treatment of the

diseases. Seventy-eight percent (38%) of herbal preparations used in the management of cardiovascular disease are made from leaf extracts followed by the bulb (22 %) and root (19 %), while the stem and the corm contributed 16 % and 5 % respectively. Extracts are mainly taken orally twice per day (morning and afternoon) for a long period of time depending on the severity of the disease.

Fifty-three (33 %) of the plants reported were used in treatment of chest pain complaints, 27 % for the treatment of high blood pressure, 12 % for heart disease, 17 % for stroke and 11 % for the management of high blood cholesterol. During this investigation, *T. violaecea* Harv, was repeatedly mentioned as the plant used in the treatment of cardiovascular diseases and their associated risk factors.

In addition to the results obtained on ethnobotanical survey, literature search also showed that *T. violaecea* is an important plant used locally and found throughout the Eastern Cape and southern KwaZulu- Natal (Wyk *et al.*, 1997). The bulbs and leaves are commonly used as decoctions for the treatment of various ailments (Buwa and Afolayan, 2009). The Zulus also grow *T. violacea* around their homes to repel snakes (Hutchings *et al.*, 1996; Wyk and Gericke, 2000). The active ingredient, alliin, appears to be antiseptic and anti-hypertensive (George *et al.*, 2001). Based on this observation, work was initiated in our laboratory on the scientific evaluation of the therapeutic claims; mechanism(s) of action(s) as well as toxicological properties of this plant.

Table 2: The plants used for the treatment of cardiovascular diseases and their predisposing factors in Nkonkobe Municipality of South Africa

Scientific name	Family name	Local name	Therapeutic indications	Parts used	Preparation
<i>Agathosma betulina</i> (P.J.Bergium) Pillans.	Rutaceae	iBuchu	High blood pressure and chest complaints.	Leaves and stem	Infusion of both parts of plant is taken orally for several weeks
<i>Cannabis sativa</i> L.	Cannabaceae	UmYa	Stroke, high blood pressure.	Leaves Fresh leaf	infusion and decoction is taken orally until symptoms disappear
<i>Cissampelos capensis</i> L.f	Minespermaceae	uMayisake or idabulitye	Heart problems and high and low blood pressure.	Root	Root infusion warmed gently and taken orally for several weeks.
<i>Dodonaea angustifolia</i> L.f	Sapindaceae	Ysterhouttoppe	Chest complaints	Leaves	Decoction of the leaves is taken orally twice a day
<i>Elephantorrhiza elephantina</i> (Burch) Skeels	Fabaceae	Intolwane or igwejobmvu	High blood pressure	Leaves	Infusion of leaf taken orally until symptom disappears.
<i>Gunnera perpensa</i> L.	Gunneraceae	iPhuzi lomlambo	High cholesterol.	Bulb and leaves	Aqueous infusion or decoctions of both parts are taken orally for several days
<i>Geranium incanum</i> (Burm .F)	Geranaiceae	Tlako	Heart problem and chest Complaints	Leaves and stem	Both plant parts are Boiled together and rub on the chest.
<i>Helichrysum odoratissimum</i> L.	Asteraceae	imphepho	Heart problems, high blood pressure, stroke and chest pain complaints.	Leaves and root	Leaves are burnt and the smoke is inhaled or infusion of root taken orally.
<i>Hypoxis sp.c.filifolia.</i>	Hypoxidaceae	Ikhubalo	High blood pressure	Bulb	The bulb is chewed twice a day for several weeks
<i>Hypoxis hemerocallidea.</i> Fisch. Mey. & Ave-Lall.	Hypoxidaceae	INonqwe	Stroke, high blood pressure and heart weakness.	Corm and root	Both parts are boiled and administered orally until the patient is cured.
<i>Leonotis leonurus</i> (L.). R.Br.	Lamiaceae	umfincafincane	High blood pressure and chest complaints.	Bulb and leaves	Infusion of the leaf and bulb is taken orally twice a day for

<i>Lichtensteina lacera</i> cham. & Schltdl	Apiaceae	iQwili	Chest complaints	Leaves and bulb	several weeks Both parts boiled together to wash and rubbed on the chest
<i>Ocimum basilicum</i> L.	Lamiaceae	Timie (Africaana) or basil.	Heart problems and chest complaints.	Leaves and stem	Infusion of both parts of plant is taken orally for many days
<i>Olea europea</i> L. subsp. <i>Africana</i> . Mill. P.S. Green	Oleaceae	uMquma	Heart problems.	Leaves and root	Both plant parts are boiled together to wash and rub on the chest until symptoms disappeared
<i>Osteospermum imbricatum</i> Subsp. <i>nervatum</i> (DC) T.Norl	Asteraceae	inkhupuhlana	Chest complaints	Bulb and leaves	Parts are boiled to wash and rubbed on the chest.
<i>Phylsalis peruviana</i> L.	Scophulariaaeae	igquzu	Chest complaints	Leaves and bulb	Both parts are boiled together to wash and rubbed on the chest
<i>Rhoicissus digitata</i> (L.f.) Gilg & M.Brandt	Vitaceae	Uchithithibuna	High blood pressure	Bulb	Infusion of bulb is taken orally for several weeks
<i>Ruta graveolens</i> L.	Rutaceae	Gwabeni iVendrit (Africaans	Heart disease, cardiac arrest and asthma.	Leaves	Leaf infusion is taken orally for several weeks.
<i>Sutherlandia frutescesnce</i> (L.) R.Br.	Fabaceae	UmNwele	High blood pressure	Leaves	Leaves are boiled with imphepho leaves and taken orally for several weeks
<i>Tulbaghia violaecea</i> Harv.	Alliaceae	itswele lomlambo	High blood pressure, heart problems, chest complaints and high cholesterol.	Bulbs	Fresh bulb is boiled in water and infusion taken orally for several weeks.

CHAPTER 3

IN VITRO AND IN VIVO TOXICITY EVALUATION OF METHANOLIC EXTRACT OF TULBAGHIA VIOLACEA RHIZOMES IN WISTAR RATS

**Part of this chapter is published in African Journal of Pharmacy and Pharmacology Vol. 5(22),
pp. 2490-2497, 15 December, 2011,**

Journal of Medicinal Plants Research Vol. 6(12), pp. 2340-2347, 30 March, 2012

and

African Journal of Biotechnology Vol. 11(83), pp. 14934-14940, 16 October, 2012

3.0 INTRODUCTION

The majority of the world population rely on medicinal plants for the treatment and management of diseases (Rodrigues and Casali, 2002). Although, about 5000 plants have been reportedly used for medicinal purposes in Africa, a few have been described or studied (Adebayo *et al.*, 2010). The use of herbal remedies could be attributed to its advantages of being efficacious and also a cheap source of medical care (Ogbonnia *et al.*, 2010). There is also a strong belief that herbal remedies being natural may be devoid of adverse and toxic effects associated with conventional orthodox medicine (Pushpalatha *et al.*, 2010). However, herbal preparations assumed to be safe may contain contaminants such as microbes (Kneifel *et al.*, 2002) heavy metals (Abou-Arab and Abou-Donia, 2000) and aflatoxins (Dusanee *et al.*, 2007) due to the methods of their production. In addition, they are administered for a long period of time during disease management without consideration for toxic effect (Ogbonnia *et al.*, 2009). Although, medicinal preparations have been reported to be relatively safe, a lot of them still demonstrate a considerably high level of toxicity (Rodrigues and Casali, 2002).

Tulbaghia violacea is a very important plant used in the Eastern Cape of South Africa for the treatment of various ailments (Bungu *et al.*, 2008). An ethnobotanical survey showed that *T. violacea* is the most frequently used plant in the management of cardiovascular diseases in the Nkonkobe Municipality, South Africa (Olorunnisola *et al.*, 2011a). Remedies prepared with *T. violacea* leaves and roots are used locally to treat fever and colds, asthma, tuberculosis, stomach problems and esophageal cancer; the plant may also be eaten as a vegetable (Bungu *et al.*, 2009) or used as a snake repellent (Wyk *et al.*, 1997; Wyk and Gericke, 2000).

Scientific evidence shows that various parts of *T. violacea* possess biological activities. It was reported that the aqueous and ethanolic extracts of the plant tubers possess *in*

vitro anthelmintic activity (McGaw *et al.*, 2000). Other reported biological activities of *T. violacea* include *in vitro* antibacterial (Gaidamashvili and Staden, 2000), antihypertensive effects (Motsei *et al.*, 2003; Thanaboripat *et al.*, 1999) and *in vitro* anticoagulant and antithrombotic activity (Bungu *et al.*, 2009). Recently, Davison *et al.* (2012) reported that the organic bulb extract of the plant possesses anticoagulant and anti-platelet properties. Water and ethanolic extract of *Tulbaghia violacea* was also reported to have 68% and 71% ACE inhibiting properties, respectively (Ramesar *et al.*, 2008; Thanaboripat *et al.*, 1999).

In spite of long a record of usage of different parts of *T. violacea* for various purposes, to the best of our knowledge, information regarding safety following repeated exposure to extracts of *T. violacea* rhizomes to experimental animals is sparse. *In vitro* toxicity evaluation of the dried and fresh methanolic extract shows that the plant is highly toxic (Olorunnisola *et al.*, 2011b). Therefore, it is important to investigate the *in vivo* toxicity of the plant. The present study was designed to investigate the possible toxic effect of methanolic extract from rhizomes of *T. violacea* related to different doses in order to find the acceptable safety level of the plant extract in rats, using histopathological and hematological examinations as well as biochemical parameters.

3.1 MATERIALS AND METHODS

3.2 Collection of Plant Material

Whole fresh rhizomes of *T. violacea* Harv. were collected from Alice, Eastern Cape, South Africa. They were collected in April, 2011 and authenticated by Professor D. S. Grierson of the Botany Department, University of Fort Hare. A voucher specimen of the plant (Sin 2010/2) was deposited at the Giffen herbarium, UFH. Plant material was separated into two groups; fresh and oven dried (40°C for 3 days)

3.3 Preparation and Extraction of Plant Material

A sample of 327.4 g of chopped *T. violacea* rhizome was homogenized in a blender (Torrington Conn, U.S.A) with 1.6 L of 100 % methanol at 4 °C. The crude extract was allowed to stand at 37 °C for 15 min, followed by centrifugation at 1500 g for 10 min at 4 °C (Mohammad and Woodward, 1986). The supernatant was filtered through Whatman No. 1 filter paper and concentrated in *vacuo* at 65 °C using a rotary evaporator. The concentrated aqueous solution was freeze dried and stored at 4 °C in the dark. Plant yield was 9.6 g dry powder /327.4 kg fresh rhizomes. The oven dried plant was powdered using a laboratory blender machine. Fifty grams of the powdered plant was extracted with 100 % methanol (200 ml) by shaking for 48 h in an orbital shaker (Labotec, 201, VervardiG, SA). The extract was filtered using a Buchner funnel and Whatman No.1 filter paper. The filtrate was concentrated to dryness under reduced pressure at 40 °C using a rotary evaporator (Laborota 4000-efficient, Heidolph, Germany). Plant yield was 3 g dry powder.

3.4 Extraction of Essential Oils

Rhizomes were hydro-distilled for 3 h in a Clevenger-type apparatus in accordance with the British pharmacopoeia specifications (1980). The essential oil was collected and analysed immediately.

3.5 Brine Shrimp Lethality Test

Shrimp eggs were allowed to hatch and mature as nauplii in two days in a hatching tank filled with seawater. The free-swimming nauplii were attracted by a light to a compartment from which they could be collected for the assay. Vials containing 4 to 20 µg/ml of fresh and dried samples of RTV and 2.5 to 20 µg/ml of essential oil of the same plant were prepared by dissolving the extracts and the oil in dimethyl sulfoxide (DMSO) and then

serially diluted in artificial sea water to the desired concentrations (Ali *et al.*, 2010). The final DMSO concentration did not exceed 1 %, which was shown not to have any harmful effects on the larvae (Ali *et al.*, 2010). The solvent was evaporated at room temperature for 72 h and sea water was added to achieve the correct concentration. Fifteen shrimps were added to each vial via a disposable pipette. The number of deaths out of 15 shrimps per dose was recorded after 24 h and LC₅₀ values obtained from the best-fit line slope. The control solutions consisted of 15 nauplii in the sea water (1 % DMSO) without the extracts or the oil. For acceptable readings; the LC₅₀ for the toxicant should fall within 27 to 35 µg/ ml range (Sam *et al.*, 1988).

3.6 *In vivo* Toxicity Evaluation

3.6.1 Animals

Adult Wistar rats (155.25 ± 2.11) g. Animals of both sexes (males and females and no pregnant subjects) were used for the assessment of acute toxicity, while the female (nulliparous and no pregnant) adult Wistar rats (160.33 ± 3.12) g were employed for the subchronic study. Animals were randomly assigned to control and treated groups (10 females per group). They were maintained under standard environmental conditions (22 ± 2 °C, 12:12 h dark/light cycle, frequent air change) and had free access to tap water and standard animal feed. All animals were obtained from the animal house of the laboratory of the School of Biological Sciences, University of Fort Hare Alice, 5700, South Africa. All procedures used in the present study followed the “Principles of Laboratory Animal Care” (NIH Publication No.85-23, revised 1985) and were approved by the Animal Ethics Committee of our university.

3.6.2 Acute Oral Toxicity

The acute toxicity study was conducted following the OECD guidelines 420 (OECD, 2001) where the limit test dose of 5000 mg/kg was used (OECD, 2003). Rats (six weeks old) were divided into two groups of 3 animals each (males and females). The control group received distilled water that did not contain *T. violacea* extract. The extract when applied was suspended in a vehicle (distilled water). Following an overnight fasting period, body weight of the rats was determined and the administered dose was calculated in reference to the body weight. The volume of the extract solution given (oral gavage) to the rats is 0.5 g/ml. Body weight, signs of toxicity and mortality were observed after the administration of the first, second, fourth and sixth hour and once daily for next 14 days. On the 15th day, all rats were subjected to fasting for 16-18 h, and then sacrificed prior to examination. The internal organs (heart, liver, kidney and pancreases) were excised and weighed. The gross pathological features of the tissues were observed and recorded.

3.6.3 Subchronic Toxicity

Subchronic toxicity tests were conducted according to OECD guidelines (OECD, 2008). Rats were assigned into 4 groups of 10 animals each (5 females and 5 males). In the present study, the doses of methanolic *T. violacea* rhizome extracts were 125, 250 and 500 mg/kg/day. The extracts were dissolved in distilled water and orally given to each group of rats daily for 28 days, while the control group received the water vehicle. Rats were fasted overnight, anesthetized using halothane and sacrificed after the 29th day. Paired blood samples, heparinised and non-heparinised, were collected for hematological and serum biochemical assays.

3.6.4 Hematological and Biochemical Analysis

The hematological parameters were determined using Beckman Coulter (Beckman Coulter Inc. Brea CA, USA) while serum biochemical parameters were analysed using Piccolo an automated chemistry analyser (Abaxis, Inc. Union City. CA, USA). Hematological parameters assayed included white blood cell (WBC) count, red blood cell (RBC) count, differential leukocyte counts, red cell distribution width (RCDW), platelets, haematocrit, hemoglobin estimation, mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Sera were assayed for glucose, creatinine, blood urea nitrogen, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (γ GT), calcium, magnesium, inorganic phosphorus, protein and albumin.

3.6.5 Histopathological Studies

The liver and aorta excised from each group of the animals were subjected to histopathological examinations. After fixing the tissues in 10 % formaldehyde, were dehydrated and mounted in paraffin blocks. Sectioning was done at 5-7 μ M. Routine histopathology was performed using the Haematoxylin stain.

3.7 Statistical Analysis

Data were expressed as mean $\pm$ SD of six replicates and were subjected to one way analysis of variance (ANOVA) followed by Duncan multiple range tests to determine significant differences in all the parameters. Values were considered statistically significant at $p < 0.05$.

3.8 RESULTS AND DISCUSSION

3.8.1 Brine Shrimp Cytotoxicity Bioassay

The brine shrimp lethality assay is frequently used as a model system to measure cytotoxic effects of a variety of toxic substances and plant extracts against brine shrimp nauplii (Morshed *et al.*, 2011). The method provides a simple and inexpensive screening test for cytotoxic compounds and possesses the advantages of requiring only small amounts (0.6 mg) of compound for investigation. In the present study, the results obtained for the IC_{50} of the fresh and dried (9.2 and 10.0 $\mu\text{g/ml}$) methanolic extract of the rhizomes (Figure 2 and 3) and the IC_{50} (Figure 4) obtained for the oil (9.8 $\mu\text{g/ml}$) suggested that the plant may be highly toxic. This significant lethality of the crude plant extracts (IC_{50} values less than 100 ppm or $\mu\text{g/ml}$) to brine shrimp is indicative of the presence of potent cytotoxic and probably insecticidal compounds (Morshed *et al.*, 2011). These results also revealed that the oil extract is more toxic when compared with the fresh and dried extracts.

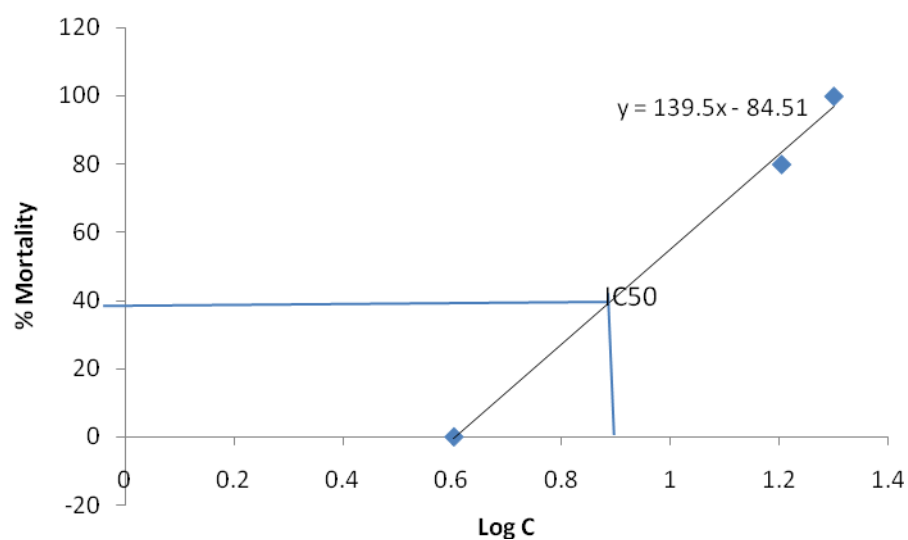


Figure 2: Determination of IC_{50} of fresh methanolic extract of rhizome (FMRTV) of *T. violacea* against brine shrimp nauplii. N=3, results are represented as mean $\pm$ SD.

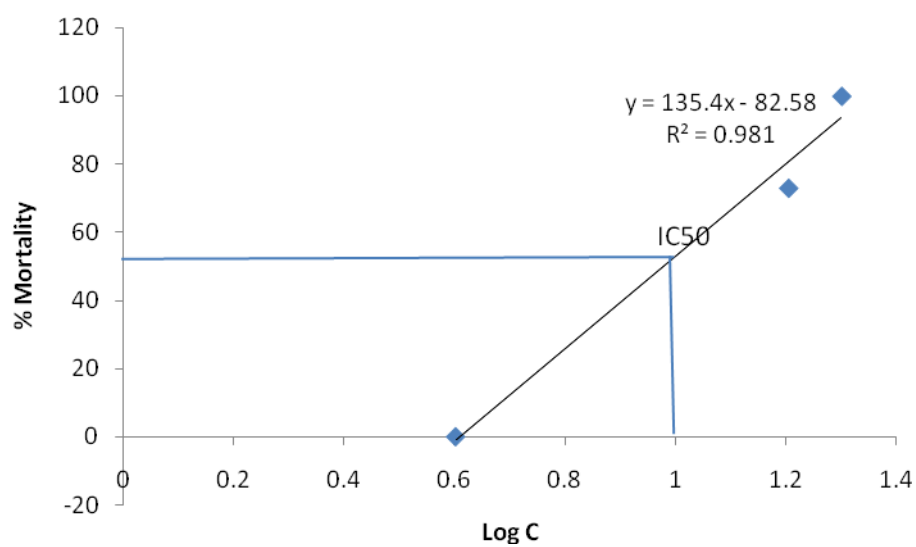


Figure 3: Determination of IC_{50} of dried methanolic extract of rhizome (DMRTV) of *T. violacea* against brine shrimp nauplii. N=3, results are represented as mean $\pm$ SD.

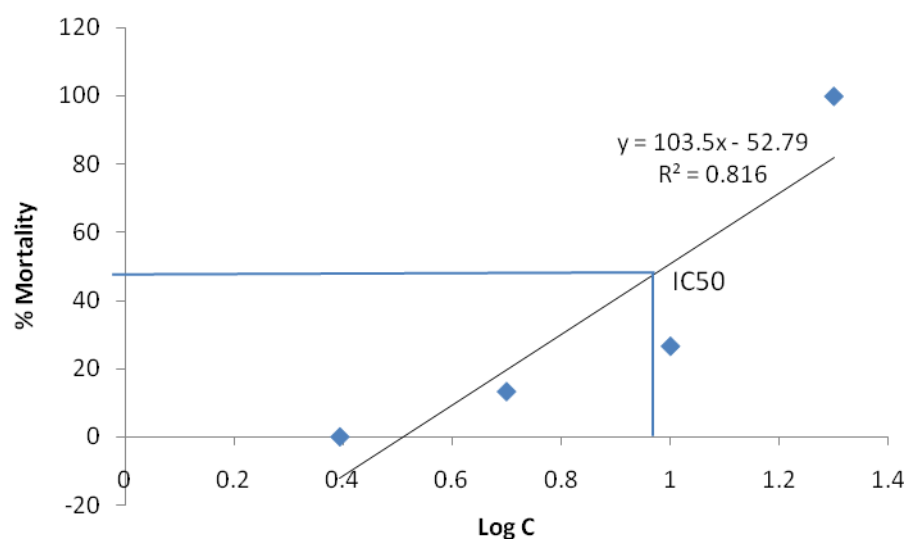


Figure 4: Determination of IC₅₀ of essential oil of rhizome of *T. violacea* against brine shrimp nauplii. Results are represented as mean $\pm$ SD (n=3).

3.8.2 Acute Toxicity

The oral administration of fresh methanolic extracts of *T. violacea* rhizomes (5000 mg/kg bw/day) did not cause rat mortality during the 72 h and 14 days observation. The rats did not show any signs of toxicity, behavioral or physiological changes or distress. The extract did not cause any significant difference in water and food intake in both male and female during 2 weeks of the experiment. Further, body weight gain during the observation period among the treated animals was comparable to their respective controls, and no sex-related differences were evident. The results of the *in vivo* acute toxicity study indicate that the LD₅₀ of the extract of rhizomes of *T. violacea* is more than 5000 mg dry powder/kg. Therefore, the methanolic extract of rhizomes of *T. violacea* is devoid of acute toxicity at the levels used in this study.

3.8.3 Sub-Chronic Toxicity

Administration of extracts of *T. violacea* rhizomes for 28 days produced no signs of toxicity or mortality during the 28 day experimental period, in both sexes. The treated animals did not show any significant alteration in water or food consumption. Similarly, no significant differences in percentage body weight gain were recorded between control and treated groups during this period (Table 3). Assessment of the effect of plant extracts on body weight and organs of experimental animals is an important test in toxicity evaluation. The alteration in body weight or organ-body weight ratio is an indication of impairment in the normal functioning of the organs (Amresh *et al.*, 2008). As shown in Table 3, both control and the animals (both males and females) treated with different doses of methanolic extracts of *T. violacea* rhizomes showed progressive increase in body weight at the end of the four weeks of treatment.

The observed increase in body weight could be attributed to the nutritive components in the plant (Duke, 1997; Ezeonwumelu *et al.*, 2011). According to Rao and Alice (2001), plant extracts can cause changes in the general metabolic status, affecting the body or organ weight of these animals. Gauthaman *et al.* (2003), reported that the increase in the body weight in the extract treated animals may be due to appetite stimulation. However; there was a general insignificant reduction in mean percentage body weight gain of rats administered with the rhizomes of *T. violacea* extract as compared with control. The observed reduction in body weight gain among the rats down the groups was relatively dose dependent (Figure 5 and 6) and this suggested that the extract could be used in weight control management. Since there were no significant reduction in relative body and organ weight of the treated animals at all doses (Table 3 and 4), the extract did not show any structural toxicity.

Table 3: Body weights of male and female rats after 28 days of oral administration of methanolic extracts of *T. violacea* rhizomes

Female	Body weight (g $\pm$ SD) per week				
	0	1	2	3	4
Control	138.01 $\pm$ 1.32 ^a	143.01 $\pm$ 1.21 ^b	146.11 $\pm$ 1.42 ^c	149.10 $\pm$ 1.03 ^d	152.10 $\pm$ 1.25 ^e
125 (mg/kg)	138.00 $\pm$ 1.21 ^a	141.01 $\pm$ 1.25 ^b	145.10 $\pm$ 1.11 ^c	148.11 $\pm$ 1.20 ^d	151.11 $\pm$ 1.30 ^e
250 (mg/kg)	138.02 $\pm$ 1.23 ^a	142.02 $\pm$ 1.19 ^b	146.10 $\pm$ 1.18 ^c	148.12 $\pm$ 1.16 ^d	152.10 $\pm$ 1.08 ^e
500 (mg/kg)	135.02 $\pm$ 1.20 ^a	140.01 $\pm$ 1.15 ^b	141.10 $\pm$ 1.16 ^c	144.11 $\pm$ 1.21 ^d	149.11 $\pm$ 1.25 ^e
Male					
Control	127.03 $\pm$ 1.44 ^a	132.11 $\pm$ 1.11 ^b	137.13 $\pm$ 1.23 ^c	141.12 $\pm$ 1.14 ^d	144.11 $\pm$ 1.05 ^e
125 (mg/kg)	128.02 $\pm$ 1.30 ^a	133.10 $\pm$ 1.23 ^b	138.11 $\pm$ 1.20 ^c	142.11 $\pm$ 1.32 ^d	143.12 $\pm$ 1.25 ^e
250 (mg/kg)	130.11 $\pm$ 1.01 ^a	135.11 $\pm$ 1.31 ^b	140.12 $\pm$ 1.31 ^c	144.10 $\pm$ 1.20 ^d	145.10 $\pm$ 1.03 ^e
500 (mg/kg)	129.12 $\pm$ 1.34 ^a	134.10 $\pm$ 1.17 ^b	139.11 $\pm$ 1.05 ^c	143.10 $\pm$ 1.38 ^d	144.10 $\pm$ 1.14 ^e

Mean $\pm$ SD (n= 5) .Values in same row with different alphabet are significantly different (p <

0.05).

Table 4: Relative organ weight of rats (Males and Females) after 28 days treatment with methanolic rhizome extracts of *T. violacea*.

Female		Relative weight of Organ (g $\pm$ SD)			
GP	Extract (mg/kg)	Heart			
1	Control	0.64 $\pm$ 0.03 ^a	6.79 $\pm$ 0.02 ^b	0.64 $\pm$ 0.02 ^c	0.44 $\pm$ 0.01 ^d
2	125	0.66 $\pm$ 0.02 ^a	6.98 $\pm$ 0.01 ^b	0.66 $\pm$ 0.02 ^c	0.48 $\pm$ 0.01 ^d
3	250	0.65 $\pm$ 0.03 ^a	6.12 $\pm$ 0.01 ^b	0.62 $\pm$ 0.02 ^c	0.48 $\pm$ 0.02 ^d
4	500	0.67 $\pm$ 0.10 ^a	6.17 $\pm$ 0.03 ^b	0.65 $\pm$ 0.02 ^c	0.45 $\pm$ 0.01 ^d
Male					
1	Control	0.61 $\pm$ 0.01 ^a	6.50 $\pm$ 0.02 ^b	0.58 $\pm$ 0.12 ^c	0.39 $\pm$ 0.08 ^d
2	125	0.59 $\pm$ 0.05 ^a	6.21 $\pm$ 0.11 ^b	0.61 $\pm$ 0.10 ^c	0.42 $\pm$ 0.12 ^d
3	250	0.60 $\pm$ 0.02 ^a	5.99 $\pm$ 0.21 ^b	0.59 $\pm$ 0.08 ^c	0.40 $\pm$ 0.10 ^d
4	500	0.58 $\pm$ 0.03 ^a	6.07 $\pm$ 0.13 ^b	0.60 $\pm$ 0.10 ^c	0.38 $\pm$ 0.11 ^d

Mean $\pm$ SD (n= 5). Values in same row with different superscripts are significantly different

(p < 0.05).

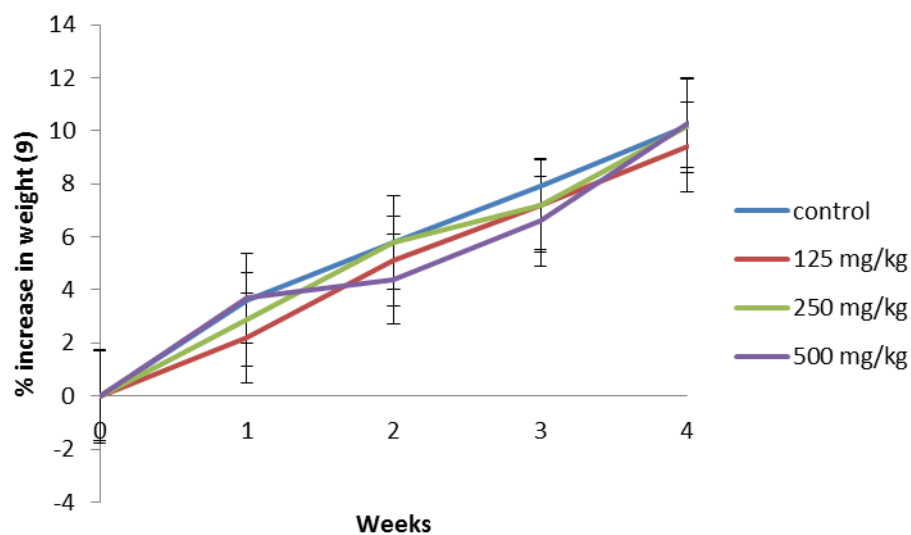


Figure 5: Mean percentage change weight of the control and treated female in the subchronic toxicity study. Results indicate the mean $\pm$ SD of n= 5 replicates

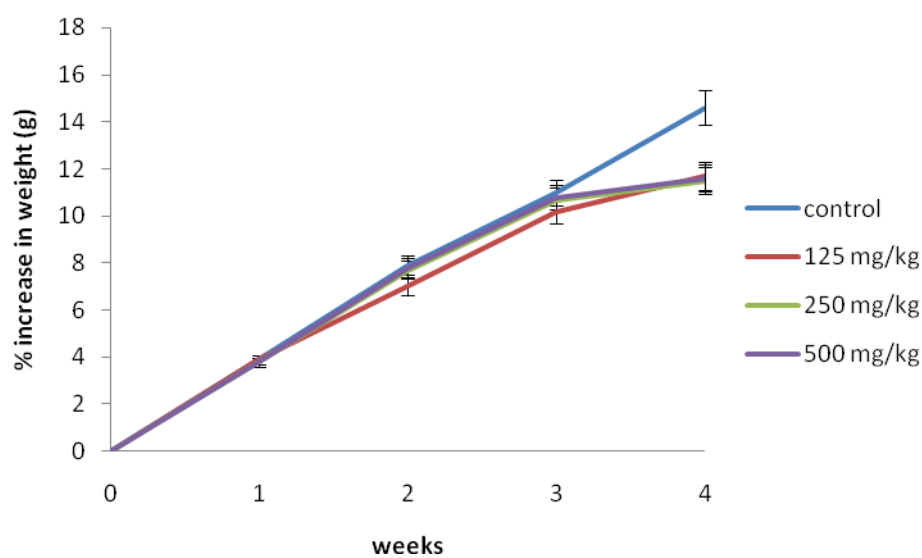


Figure 6: Mean percentage change weight of the control and treated male in the subchronic toxicity study. Results indicate the mean $\pm$ SD of n= 5 replicates

3.8.4.1 Biochemical Parameters

Assessment of liver and kidney function is very important in toxicity evaluation of drugs and plant extracts as both organs are necessary for the survival of an organism. Aspartate transaminase (AST) and Alanine transaminase (ALT) activities are commonly measured to monitor liver damage. A mild or higher activity of AST indicates liver injury or myocardial infarction (Feldman *et al.*, 2000; Crook, 2006) and the ratio of AST/ALT may be employed in disease diagnosis. AST/ALT ratio greater than 1 suggest myocardial infarction while a ratio less than 1 may be due to release of ALT from the affected liver (Sacher *et al.*, 1991) and AST /ALT of more than 2 indicates alcoholic hepatitis or cirrhosis (Varadarasou *et al.*, 2010). In the present study, the extract did not induce any damage to the liver or kidney as revealed by the results of clinical blood chemistry (Table 5). The *in vivo* acute toxicity study conducted using the OECD guidelines 420 (OECD, 2001) where the limit test dose of 5000 mg/kg/bwt/day of methanolic extract of rhizome of *T. violacea* was used, indicated that a single oral administration of 5000 mg/kg/bwt dose does not produce mortality or significant behavioral changes during 14 of days observation. Also the extract administered at a dose of 125, 250 and 500 mg/kg/bwt respectively for a period of 28 days does not cause mortality, morbidity or change in relative weight of organs. Indicators of liver damage alanine amino transferase (ALT), aspartate amino transferase (AST) as well as total protein and albumin were not negatively affected although, the extracts caused an insignificant increase in the level of AST and a significant ($p < 0.05$) decreases in Alkaline phosphatase (ALP) and gamma glutamic transferase (gamma-GT) (ALP). The reason for this decreased observation in ALP activity may be attributed to antioxidant activity (Olorunnisola *et al.*, 2011b). Alo *et al.*, 2012, reported that plant extracts contain chemical compounds which enhanced the suppression of liver enzymes to significant amounts could be explained by the enhanced suppressive effect thereby, preventing over-sensitization of

enzymes to the metabolism. The results suggested that the plant may possess hepatoprotective properties.

The extract did not show any significant effect on markers of kidney functions such as albumin, creatinine and urea. The lack of significant alterations in the level indicators of liver (ALT, AST, total protein, albumin and GGT) damage (Table 5), suggests that sub-chronic administration of extract of *T. violacea* rhizomes do not affect hepatocyte function in the animals. Although, a slight insignificant dose dependant increase in the activity of AST was observed in the extract treated groups, the increase may not be connected to liver damage as the extract produced a significant reduction ($p < 0.05$) in the activity of ALP and ALT which are the main markers of liver injury. The results (Table 5) also showed an insignificant decrease in levels of gamma GT at all doses compared to the control after 28 days of administration. However, the values obtained are within the normal range (Barry, 1995). The secretory ability of the liver was assessed by changes in albumin, total bilirubin and globulin concentration (Guyton and Hall, 2000). Reduction in total serum protein, albumin, and globulin are indications of diminished synthetic function of the liver or might be due to impaired hepatocellular function. Low serum albumin content may suggest infection or continuous loss of albumin (Tietz and Prude, 1994; Yakubu *et al.*, 2003). The insignificant change in serum total protein concentration, albumin and globulin in the treated and control group further suggested that the extract does not impair hepatocellular or secretory functions of the liver at all doses considered as confirmed. Results of the histopathological studies of the tissue sections of the liver of the rats administered methanolic extract of RTV (500 mg/kg/bwt) showed no gross tissue damage when compared to those of control rats. The incisions from the liver tissue of central veins, portal areas and sinusoids appeared (figure 7a and b). According to this information, it could be said that the extract of RTV does not have toxic effect on hepatocytes. Kidney functions were evaluated by means of serum urea,

creatinine, and electrolytes in blood. Increased blood creatinine is a good indicator of compromised kidney functions. In the present study, serum creatinine, urea and some electrolytes such as sodium and calcium were not affected by the extract treatment when compared with the normal. However, a slight insignificant increase was observed in the levels of urea, sodium and potassium when compared with control but they are still within the normal range. These results suggested that the extract does not alter the kidney functions.

Table 5: Effect of daily administration of rhizomes of *T. violacea* extracts for 28 days on biochemical profiles of the control and treated rats in the sub-chronic toxicity study. Data are presented as mean $\pm$ SD (n = 5)

Female	Control	administered (mg/kg of body weight/day)		
		125	250	500
ALP (U/L)	237.2 $\pm$ 0.01 ^a	169.0 $\pm$ 0.05 ^b	180.2 $\pm$ 0.05 ^b	184.3 $\pm$ 0.02 ^b
AST (U/L)	112.1 $\pm$ 0.12 ^b	113.0 $\pm$ 0.02 ^b	115.10 $\pm$ 0.02 ^b	121.10 $\pm$ 0.01 ^b
ALT (U/L)	42.30 $\pm$ 0.31 ^b	45.25 $\pm$ 0.03 ^b	43.10 $\pm$ 0.03 ^b	42.10 $\pm$ 0.03 ^b
AST/ALT (U/L)	2.65 $\pm$ 0.22 ^b	2.50 $\pm$ 0.03 ^b	2.67 $\pm$ 0.03 ^b	2.88 $\pm$ 0.02 ^b
γGT (U/L)	5	< 5	< 5	< 5
Total protein (mg/dl)	6.70 $\pm$ 0.05 ^b	6.95 $\pm$ 0.04 ^b	6.50 $\pm$ 0.03 ^b	6.90 $\pm$ 0.01 ^b
Albumin (mg/dl)	2.10 $\pm$ 0.09	2.35 $\pm$ 0.10 ^b	2.30 $\pm$ 0.03 ^b	2.40 $\pm$ 0.03 ^b
Total bilirubin (mg/dl)	0.30 $\pm$ 0.11 ^b	0.30 $\pm$ 0.02 ^b	0.30 $\pm$ 0.01 ^b	0.30 $\pm$ 0.02 ^b
Urea (mg/dl)	2.10 $\pm$ 0.04 ^b	3.01 $\pm$ 0.50 ^b	2.40 $\pm$ 0.50 ^b	2.70 $\pm$ 0.20 ^b
Creatinine (mg/dl)	0.02 $\pm$ 0.07 ^b	0.02 $\pm$ 0.05 ^b	0.02 $\pm$ 0.01 ^b	0.02 $\pm$ 0.10 ^b
Potassium (mmol/L)	6.62 $\pm$ 0.11 ^b	6.80 $\pm$ 0.01 ^b	6.86 $\pm$ 0.02 ^b	6.90 $\pm$ 0.13 ^b
Sodium (mmol/L)	140.0 $\pm$ 0.12 ^b	143.1 $\pm$ 0.11 ^b	141.2 $\pm$ 0.12 ^b	144.0 $\pm$ 0.12 ^b
Calcium (mg/dl)	10.9 $\pm$ 0.01 ^b	9.80 $\pm$ 0.02 ^b	10.3 $\pm$ 0.01 ^b	10.2 $\pm$ 0.03 ^b

Values in same row with different alphabet are significantly different (p < 0.05).

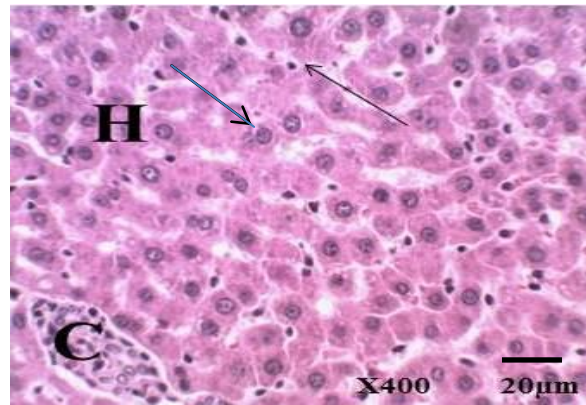


Figure 7a: Photomicrograph of liver section from a normal rats (H&E, X400).

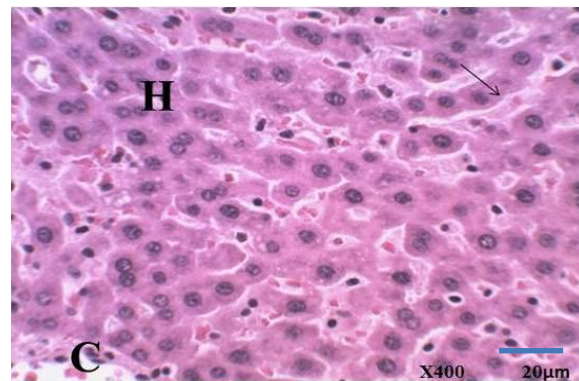


Figure 7b: Photomicrograph of liver section from rats treated with 500 mg/kg/bwt (H&E, X400).

3.8.4.2 Hematological parameters

Evaluation of hematological parameters is important in toxicity assessment as changes in haematological index have a higher predictive value for human toxicity (Adebayo *et al.*, 2005). Subchronic administration of rhizome *T. violacea* (RTV) daily for 28 days did not cause any significant change in most of hematological parameters assessed. As observed in Table 6, the extract significantly reduced ($P < 0.05$) Hb, RBC and PCV only at dose of 250 and 500mg/kg body weight, while other doses had no significant effect ($P > 0.05$) when compared with controls. However, MCH, MCHC and MCV were not significantly altered ($P > 0.05$) by the extract at all doses administered when compared with controls. Since MCHC, MCH and MCV relate to individual red blood cells while Hb, RBC and PCV relate to the total population of red blood cells in the blood, it thus imply that the extract may neither affect the incorporation of haemoglobin into red blood cells nor the morphology and osmotic fragility of red blood cells produced. However, the reduction in Hb, RBC and PCV implies that the extract may reduce the population of red blood cells produced from the bone marrow. Since MCH, MCHC and MCV were not affected, the extract may not affect the oxygen – carrying capacity of each red blood cell but may reduce the oxygen- carrying capacity of the whole blood because of the reduced population of red blood cells in the blood. Reduction in Hb, RBC and PCV observed in this study suggests anaemia which may result from impaired red blood cell production (Malomo *et al.*, 2002). However, significant ($P < 0.05$) increase was observed for lymphocytes and white blood cell (WBC) in the group treated with 500 mg/kg dose compared to the control (Table 6). The values obtained are also within the normal range (Andrew *et al.*, 1965; Archer *et al.*, 1977; Mohammad and Woodward, 1986; and Birgel *et al.*, 1986; Olson *et al.*, 2000;). The significant dose dependant increase in lymphocytes and WBC suggested that the extract may contain some bioactive agents that could boost immune system (WHO, 2003) or it may be due to an

imbalance in the rate of hematological parameter synthesis and catabolism (OECD, 2001). It has been reported that granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor, interleukins IL-2, IL-4 and IL-5 regulate the proliferation, differentiation, and maturation of committed stem cells responsible for the production of white blood cells (Guyton and Hall, 2000; Ganong, 2001). It may be that some components of the extract increased the production of these regulatory factors or interfered with the sensitivity of the committed stem cells (responsible for the production of white blood cells) to these factors. The results also showed that the extract caused a significant dose dependent reduction in neutrophils and monocytes population. (Table 6). Monocytes have been shown to increase in cases of infection (Mohammad and Woodward, 1986; Barry, 1995) and do not provoke tissue damage, hence, the reduction in monocytes could imply that there was little or no infection caused by the extract. Thus administration of the extract may protect against infection. Since monocytes have been shown to increase in cases of infection the alterations produced by the extract of *T. violacea* rhizomes on the lymphocytes, white blood cell, neutrophils and monocytes as well as the red blood cells suggest a dose selective localized toxicity and stimulatory effect on the bone marrow (Andrew, 1965). Therefore, the plant may not be completely safe as an oral remedy at the doses investigated.

Table 6: Mean hematological values in rats treated with the *T. violacea* extract for 28 days.

Parameters	Control	Dosage Dose administered (mg/kg of body weight/day)		
		125	250	500
Red cell count/L	7.26×10^{12a}	6.73×10^{12b}	6.82×10^{12b}	6.80×10^{12b}
Hemoglobin g/dl	14.9 ^a	13.9 ^b	13.6 ^b	13.8 ^b
PCV %	37 ^a	37 ^a	36.9 ^b	36.8 ^b
Haematocritic	0.42 ^a	0.40 ^a	0.40 ^a	0.40 ^a
MCV	58 ^a	59 ^a	58 ^a	58 ^a
MCH	21 ^a	21 ^a	20 ^a	20 ^a
MCHC	35 ^a	35 ^a	35 ^a	35 ^a
RDW	12.3 ^a	14.2 ^b	13.7 ^b	12.8 ^a
White cell count/L	6.90×10^9a	6.50×10^9a	6.3×10^9a	7.25×10^9b
Neutrophils/L	1.79×10^9a	1.40×10^9b	1.37×10^9b	1.45×10^9b
Lymphocytes/L	4.97×10^9b	5.07×10^9b	5.67×10^9b	6.10×10^9a
Monocytes/L	0.14×10^9a	0.26×10^9b	0.10×10^9c	0.19×10^9c
Platelet count/L	949×10^9a	942×10^9a	941×10^9a	939×10^9a

Data are represents as mean $\pm$ SD. Values in same row with different alphabet (a, b or c) are significantly different ($p < 0.05$).

3.9 Conclusion

Although, the *in vitro* toxicity evaluation of *Tulbaghia violacea* rhizome (Oil, fresh and dry methanolic extracts) revealed that the plant may be cytotoxic, the results of the *in vivo* acute and subchronic toxicity evaluation of the fresh methanolic extract of the plant in rats suggested that the plant may be relatively safe. The discrepancy observed in the two methods of toxicity evaluation may be due to the ability of higher organism such as rats or human to metabolize toxic compound present in the extracts. The alterations produced by the extract of *T. violacea* rhizomes on the haematological parameters suggested a dose selective localized toxicity and stimulatory effect on the bone marrow. Therefore, the plant may not be completely safe as an oral remedy at the doses investigated..

CHAPTER 4

IN VITRO AND IN VIVO ANTIOXIDANT EVALUATION OF EXTRACTS OF *TULBAGHIA* *VIOLACEA RHIZOMEA*

Part of this chapter was published in African Journal of Pharmacy and Pharmacology Vol.
5(22), pp. 2490-2497, 15 December, 2011

and

Journal of Medicinal Plants Research Vol. 6(12), pp. 2340-2347, 30 March, 201

4.0 INTRODUCTION

The maintenance of pro-oxidant and antioxidant homoeostasis status in living cells has been the primary focus of research in recent years. Imbalance in pro-oxidant and antioxidant homeostasis occasioned by excessive free radical generation or insufficient antioxidants has been implicated in the development of several human disease conditions, such as atherosclerosis, hypertension, ischemic diseases, Alzheimer's disease, Parkinsonism and cancer (Narendhirakannam and Rageswari, 2010). Pro-oxidants are free radicals (superoxide, nitric oxide and hydroxyl radicals) produced in normal or pathological cell metabolism (Jadeja *et al.*, 2009; Ponmari *et al.*, 2011) and through exogenous sources, such as human exposure to ionizing radiation, injury, oxidative drugs and pollutants (Erasto and Mbwambo, 2009).

The human body constantly quenches and/or scavenges, activates a battery of detoxifying enzymes or inhibits the generation of ROS (Ayoola *et al.*, 2011) through various mechanisms, such as antioxidant enzymes and molecules (Erasto and Mbwambo, 2009). Several synthetic substances, such as vitamins, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) (Vinay *et al.*, 2010) are used as antioxidants, but are suspected to be toxic to human and experimental animals (Anagnostopoulou *et al.*, 2006). Therefore, the development and utilization of more effective antioxidants from natural origin is very important (Kolar *et al.*, 2011). In addition, antioxidant evaluation of medicinal plants might give an insight to the mechanism (s) of their pharmacological activities. *Tulbaghia violacea* belongs to the family of *Alliaceae* which is most commonly associated with garlic. *T. violacea* is indigenous to the Eastern Cape, South Africa. The leaves and bulbs are widely used as an herbal remedy for various ailments (Bungu *et al.*, 2006). Scientific evidence has demonstrated that *T. violacea* and its various preparations possess pharmacological activities such as anthelmintic activity, anticancer and

in vitro growth inhibition and induction of apoptosis in cancer cells (Bungu *et al.*, 2006; Duncan *et al.*, 1999; McGaw *et al.*, 2000). Despite the fact that the rhizomes of *T. violacea* are used for the treatment of many diseases, information on antioxidant activity, cytotoxicity or phytochemical content is relatively scarce.

The present study was designed to investigate phytochemical constituents, free radical scavenging activities of fresh, dried methanolic and oil extracts of *T. violacea* using different *in vitro* experimental models. The effect of the fresh extract of the plant on *in vivo* antioxidant enzymes in normal and diet induced hypercholesterolemic rats will also be assessed.

4.1 Materials and Methods

4.1.1 Chemicals

The compounds 2, 2-Diphenyl-2-picrylhydrazyl (DPPH), 2, 4-6-tripyridyl-s-tyrosine (TPTZ) were purchased from Sigma Chemicals Co Ltd; ascorbic acid and butylated hydroxytoluene (BHT) were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Folin-Ciocalteu reagent, hydrogen peroxide (H₂O₂) was obtained from Merck Limited-India, (Mumbai, India), methanol (HPLC grade) from Merck, (Darmstadt, Germany), ascorbic acid from Sigma Chemicals. Brine shrimps for cytotoxicity were obtained from Pet shop King William South Africa. The other chemicals and solvents used in the present study were of analytical grade obtained from local suppliers.

4.1.2 Collection of Plant Materials was as described in section 3.2

4.1.2.1 Preparation and Extraction of Plant Materials was as described in section 3.3

4.1.3 Extraction of Essential Oil was done as described in section 3.4

4.1.3.1 GC-MS Analysis of the Oil

GC-MS analyses of the oil were carried out using a Hewlett-Packard HP 5973 mass spectrometer interfaced with an HP-6890 gas chromatograph with an HP5 column. The following conditions were used: initial temperature 70°C, maximum temperature 325°C, equilibration time 3 min, ramp 4°C/min, final temperature 240°C; inlet: split less, initial temperature 220°C, pressure 8.27 psi, purge flow 30 ml/min, purge time 0.02 min, gas type helium; column: capillary, 30 m × 0.25 mm i.d., film thickness 0.25 µm, initial flow 0.7 ml/min, average velocity 32 cm/s; MS: EI method at 70 eV.

4.1.3.2 Identification of Components

The individual constituents of the oil were identified by matching their mass spectra and retention indices with those of Wiley 275 library (Wiley, New York) in the computer library (Kovats 1958; Adams, 1995; Joulain *et al.*, 2001; Joulain and König, 1998). The yield of each component was calculated per g of the plant material, while the composition was calculated from the summation of the peak areas of the total oil composition. The whole experiment was done in triplicate.

4.1. 4 Quantification of Phytochemical Content

4.1.4.1 Total Phenolics Determination

Total phenol content in the extracts was determined by the modified Folin-Ciocalteu method (Wolfe *et al.*, 2003). An aliquot of the extract was mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water 1:10, v/v) and 4 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 15 s and left undisturbed for 30 min at 40°C for colour development. Absorbance was then measured at 765 nm using the Hewlett Packard UV-Vis spectrophotometer. Samples of the extract were evaluated at a final concentration of 1.0

mg/ml. Total phenolic content was expressed as mg/g tannic acid equivalent using the following equation based on the calibration curve: $y = 0.1216x$, $R^2 = 0.9365$, where x was the absorbance and y was the tannic acid equivalent (mg/g).

4.1.4.2 Total Flavonoid Determination

Flavonoid content was determined by aluminum chloride colorimetric assay (Marinova, and Ribarova, 2005). 1 ml of the extracts or standard solution of catechin was added to a 10 ml volumetric flask containing 4 ml of distilled water. To the above mixture, 0.3 ml of 5 % NaNO_2 was added. After 5 min, 0.3 ml of 10 % AlCl_3 was added. At 6 min, 2 ml of 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid content of the extract was expressed as mg catechin equivalents (CE/100) g fresh mass. Sample were analysed in triplicates.

4.1.4.3 Total Tannin Determination

Tannins were determined using the Folin Phenol reagent as reported by Folin and Ciocalteu (Folin, 1927). Briefly, 0.1 ml of the sample extract was added to 7.5 ml of distilled water and mixed with 0.5 ml of Folin Phenol reagent, 1 ml of 35 % sodium carbonate solution and diluted to 10 ml with distilled water. The mixture was shaken well, kept at room temperature for 30 min and absorbance was measured at 725 nm. A blank was prepared with water instead of the sample. A set of standard solutions of tannic acid were treated in the same manner as described above and read against water blank. The results of tannins are expressed in terms of tannic acid equivalents in mg/g of extract.

4.1.5 *In vitro* Antioxidant Activity

4.1.5.1 DPPH Radical Scavenging Activity

The method of Liyana-Pathiranan and Shahidi (2005) was used for the determination of scavenging activity of free radicals in the essential oil. A solution of 0.135 mM 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) in methanol was prepared. 1.0 ml of this solution was mixed with 1.0 ml of oil (prepared in methanol containing 0.1 to 0.5 mg/ml of the oil) or fresh and dried extracts (0.02 - 0.1 mg) and standard drugs (BHT (0.02 - 0.1 mg) and ascorbic acid (0.1- 0.5 mg/ml)). The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorption of the mixture was measured spectrophotometrically at 517 nm. The actual decrease in absorption was measured against that of the control. All tests and analysis were run in triplicates and the results obtained were averaged. The activities were also determined as a function of their % Inhibition which was calculated using the formula; % scavenging activity = $[Ac - As / Ac] \times 100$

Ac = Absorbance of the control; As = Absorbance of the sample.

4.1.5.2 Nitric oxide Scavenging Activity

Nitric oxide scavenging activity of plant extracts was determined by the method of Govindarajan et al. (2003). Sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with different concentrations of the extract (0.1 to 0.5 mg/ml prepared in methanol) and incubated at 25 °C for 30 min. After 30 min, 1.5 ml of the incubated solution was removed and diluted with 1.5 ml of Griess reagent (1 % sulphanilamide, 2 % phosphoric acid and 0.1 % N-1- naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-1-naphthylethylene diamine dihydrochloride was measured at 546 nm. BHT

and ascorbic acid were used as standards to compare results. A blank solution (without extract) was also prepared and used as control. Blank and standards solutions were also run in similar way. All tests were repeated thrice and results were averaged. Results were reported in term of percentage inhibition of nitric oxide radical by plant extracts and it was calculated as follows: Percentage inhibition of nitric oxide = $[1 - A_1/A_0] \times 100$ Where; A_1 = Absorbance of sample. A_0 = Absorbance of control

4.1.5.3 Lipid Peroxidation and Thiobarbituric Acid Reactions

A modified thiobarbituric acid reactive species (TBARS) assay (Ohkawa *et al.*, 1979) was used to measure the lipid peroxide formed using egg yolk homogenate as lipid rich media (Ruberto *et al.*, 2000). Egg homogenate (0.5 ml of 10 %, v/v) and 0.1 ml of the oil extract (0.1- 0.5 mg/ml) were added to a test tube and made up to 1 ml with distilled water; 0.05 ml of FeSO_4 (0.07 M) was added to induce lipid peroxidation and the mixture incubated for 30 min. A control without the extract was prepared in the same way. Then, 1.5 ml of 20 % acetic acid (pH 3.5) and 1.5 ml of 0.8 % (w/v) thiobarbituric acid in 1.1 % sodium dodecyl sulfate was added and the resulting mixture vortexed and heated at 95 °C for 60 min. After cooling, 5.0 ml of Butan-1-ol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the upper organic layer was measured at 532 nm. Inhibition of lipid peroxidation percent of the oil extract was calculated as:

$$[(1-E) / C] \times 100$$

Where C is the absorbance value of the fully oxidized control; E is the absorbance in presence of the extract.

4.1.5.4.1 Reducing Power of Oil Extract

The reducing power of the oil was determined according to the method of Yen and Chen (1995). 2.5 ml of extract (0.1 to 0.5 mg/ml) in water were mixed with a phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3 Fe (CN)_6$] (2.5 ml, 1 %). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10 %) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 %), and the absorbance was measured at 700 nm. The increased absorbance of the reaction mixture indicated increased in reducing power. Vitamin C was used as a positive control at concentrations of 0.1- 05 mg/ml.

4.1.5.4.2 Reducing Power of Fresh and Dried Extracts of *T. violacea* Rhizomes

The Fe^{3+} reducing power of the extract was determined by the method of Oyaizu (1986) with a slight modification. Different concentrations (0.02–1.0 mg/ml) of extract (0.5 ml) were mixed with 0.5-ml 0.2M phosphate buffer (pH 6.6) and 0.5 ml 0.1 % potassium hexa-cyanoferrate, followed by incubation at 50 °C in a water bath for 20 min. After incubation, 0.5 ml 10 % TCA was added to terminate the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml of distilled water and 0.1 ml 0.01 % $FeCl_3$ solution was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against the appropriate blank solution. All tests were performed six times. A higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a positive control at a concentration of 0.02–1.0 mg/ml.

4.1.5.5 Determination of H₂O₂ Inhibitory Activities of Fresh and Dried Extracts *T. violacea*

The H₂O₂ inhibition activity of the extracts was assessed by the method of Ilhami (Ilhami *et al.*, 2005). Briefly, a solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). One ml of sample (0.01- 0.05 mg/ml) was added to a 0.6 ml of hydrogen peroxide solution (40 Mm). The absorbance of the hydrogen peroxide at 230 nm was determined after 10 min. against a blank solution containing phosphate buffer solution without hydrogen peroxide. BHT (0.01 – 0.05 mg/ml) and ascorbic acid (0.01 – 0.05 mg/ml) were used as positive controls. The percentage scavenging of hydrogen peroxide of samples was calculated using the following formula:

$$\text{H}_2\text{O}_2 \text{ inhibition capacity (\%)} = [1 - (\text{H}_2\text{O}_2 \text{ cons. of sample} / \text{H}_2\text{O}_2 \text{ cons. of blank})] \times 100.$$

4.2 *In vivo* Antioxidant Evaluation

4.2.1 Animals

Healthy, male, Wister albino rats (130-160 g) were randomly assigned to control and treated groups (6 animals per group/cage). They were maintained in standard environmental conditions (22 ± 2 °C, 12:12 h dark/light cycle, humidity: 45-50 %) frequent air change) and had free access to tap water and food. All animals were obtained from the animal house of the laboratory of the School of Biological Sciences, University of Fort Hare, Alice, South Africa. All procedures used in the present study followed the “Principles of Laboratory Animal Care” from NIH Publication No.85-23 and were approved by the Animal Ethics Committee of our university.

4.2.2 Experimental Design

After a 2 - week acclimation period, the experimental animals were divided into the following:

Group 1: Experimental animals fed with a standard diet and orally administered 1ml distilled water served as control

Group II: Positive control fed with a high cholesterol diet and normal diet [standard diet+ pure cholesterol (1% w/w)].

Group III and IV: Fed with a high cholesterol diet orally [standard diet+ pure cholesterol (1 % w/w)] but also supplemented with an extract of *T. violacea* (250 and 500 mg/kg body weight, respectively) by oral gavage once daily for 4 weeks

Group V: fed with high cholesterol diet [standard diet + cholesterol (1 % w/w)] also supplemented with Atorvastatin (50 mg/kg body weight) by oral gavage once for 4 weeks.

4.2.3 Preparation of Liver Homogenate

Liver homogenate was prepared according to the method described by El-Demerdash *et al.* (2005). Dissected livers were excised, washed with ice cold 0.9% NaCl (w/v) to remove the blood, cut into small pieces by fine scissors, and then homogenized (10%w/v) separately in ice-cold 1.15% KCl-0.01M sodium phosphate buffer, pH 7.4 with a Potter–Elvehjem glass homogenizer. The homogenate was centrifuged at 10,000 xg 20 min at 4 °C. Supernatant of the liver homogenate was collected into sterilized tubes and stored at -20°C.

4. 3 Biochemical Analysis

4.3.1 Total Protein and Albumin

Total protein was measured using the biuret reaction while albumin was measured by colorimetric estimation using the Sigma diagnostics albumin reagent (Sigma Diagnostic, U.K.) which contained bromocresol green (BCG). Globulin was obtained from the difference between total protein and albumin.

4.3.2 Malondialdehyde (MDA)

The MDA content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS) (Ohkawa et al., 1979). Briefly, the reaction mixture consisted of 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid was added to 0.2 ml of 10% (w/v) of PMS. The mixture was brought up to 4.0 ml with distilled water and heated at 95°C for 60 min. After cooling with tap water, 1.0 ml distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v) was added and centrifuged. The organic layer was taken out and its activity was measured at 532 nm and was compared with those obtained from MDA standards. The concentration values were calculated from absorption measurements. 1, 1, 3, 3-tetraethoxypropane as standard in terms of nmol/mg protein.

4.3.3 Superoxide Dismutase activity

Levels of SOD in the cell free supernatant were measured by the method Kakkar *et al.* (1984). Briefly, 1.3 ml of solution A (0.1 mM ethylenediaminetetraacetic acid (EDTA) containing 50 mM Na₂CO₃, pH 10.5), 0.5 ml of solution B (90 mM nitro blue tetrazolium (NBT) dye), 0.1 ml of solution C (0.6% TritonX-100 in solution A) and 0.1 ml of solution D

(20 mM hydroxylamine hydrochloride, pH 6.0) was mixed and the rate of NBT reduction was recorded for 1 min at 560 nm. 0.1 ml of the supernatant was added to the test cuvette as well as reference cuvette, which do not contain solution D. Finally, the percentage inhibition in the rate of reduction of NBT was recorded as described earlier. One enzyme unit was expressed as inverse of the amount of protein (mg) required inhibiting the reduction rate by 50% in 1 min.

4.3.4 Catalase activity (CAT)

Catalase was assayed colorimetrically at 620 nm and expressed as moles of H_2O_2 consumed/ min/ mg protein as described by Sinha (1972). The reaction mixture (1.5 ml) contained 1.0 ml of 0.01M phosphate buffer (pH 7), 0.1 ml of tissue homogenate and 0.4 ml of 2M H_2O_2 . The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5 % potassium dichromate and glacial acid were mixed in 1:3 ratio) and absorbance read at 620 nm.

4.3.5 Glutathione Peroxidase (GPx)

Glutathione peroxidase was measured by the method described by Rotruck *et al.* (1973). To 0.2 ml Tris buffer (0.4 M, pH 7.0), 0.2 ml of ethylene diamine tetraacetic acid (EDTA), 0.1 ml of sodium azide and 0.5 ml of tissue homogenate (Tris buffer 0.4 M, pH 7.0) was added. To the mixture, 0.2 ml of glutathione (GSH) followed by 0.1 ml of H_2O_2 was added. The contents were mixed well and incubated at 37 °C for 10 minutes, along with a control containing all reagents except tissue homogenate. After 10 minutes, the reaction was stopped by the addition of 0.5 ml of 10 % trichloroacetic acid (TCA) and centrifuged. Supernatant was assayed for glutathione content by using Ellmans reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate). The activity was expressed as mg of GSH consumed/ min.

4.3.6 Determination of Reduced Glutathione (GSH).

Reduced glutathione in liver homogenate was estimated as described by Ellman (1959). Briefly, 1.0 ml of supernatant was treated with 0.5 ml of Ellmans reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1 % sodium nitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm.

4.4 Statistical Evaluation

Statistical evaluation was done using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Data were expressed as mean $\pm$ SD of three replicates. Statistical significance was set at ($p < 0.05$).

4.5 Results and Discussion

4.5.1 GC-MS Analysis of the Essential Oil of RTV

Essential oils from freshly collected rhizome of *T. violacea* were obtained by hydro-distillation. The yield from 250 g of the rhizomes after hydro-distillation was 0.05 % (v/w). A light yellowish oily liquid with a pungent garlic-like odor was produced. The result of GC-MS analysis of the oil shows that dimethyl disulfide, methyl (methylthiol) methyl sulfide and 2, 4-dithiapentane (11.35 % peak area), (Methylthio) acetic acid, 2-(Methylthio) ethanol and (3-methylthio) - (7.20 %) are main the components (Table 7). The results (Table 7) obtained from the analysis of the oil compared favorably with compounds identified earlier from methanolic extracts of the rhizomes using ion-exchange chromatography (Kubec *et al.*, 1999) and from garlic oil (Pino *et al.*, 1991; Münchberg *et al.*, 2007). It was believed that the polysulfides obtained are suggested to be 'second generation' biologically active sulfur species, formed by decomposition of non-volatile sulfur compounds called S-alkylcysteine sulfoxides or allicin and are responsible for the characteristic flavour and biological

properties of the plant (Kubec et al., 2002; Ute *et al.*, 2007). Diallyltetrasulfide and higher polysulfides such as the diallylpenta-, hexa- and hepta sulfide reported in garlic oil extracts were not found in oil extract of *T. violacea* rhizome. The absence of these polysulfides may be due to differences in processing procedure, time of plant collection, species and variation in techniques of extraction and analysis (Pino *et al.*, 1991; Kubec *et al.*, 2002; Ute *et al.*, 2007). The GC-MS protocol was not particularly sensitive to nitrogen compounds (Amy *et al.*, 2000). The single nitrogen compound revealed by the GC-MS analysis, propane nitrile (7.20 % peak area), was described by Ellison (1999) as having an unpleasant odour.

Table 7: Compounds obtained from GC-MS analysis of *T. violacea* rhizome essential oil.

S/N	Retention time (min)	Chemical composition	% Area
1	21.94	Dimethyl trisulfide,	0.57
2	26.77	Dimethyl disulfide, methyl(methylthio)methyl sulfide	11.35
		2,4-dithiapentane	
3	33.92	(Methylthio) acetic acid	2.58
4	38.00	(Methylthio) acetic acid, (2-methylthiol)ethanol, propanitrile, 3-(methylthio)-	7.20
5	44.99	2,4-dithiapentane,bis- (methlythio), disulfide	0.78
		Total %	22.48

4.5.2 Total phenolics, flavonoid, flavonol, tannin and proanthocyanidin Content

Phenolic compounds have been reported to be crucial for bioactivities in plants (Nagavani *et al.*, 2010). They serve as antioxidant and exhibit a wide spectrum of medicinal properties, such as anti-cancer, anti-allergic and cardio-protective (Banerjee and Bonde, 2011). Analysis of phenolic compounds in the fresh and dried methanolic extract of rhizome of *T. violacea* (RTV) revealed that the extracts possess high concentrations of total flavonoid (38.9 to 67.9 mg/g quercetin equivalent) followed by total phenolics (18.3 to 38.2 mg/g

tannic acid equivalent, flavones (25.3 to 11.5 mg/g quercetin equivalent), tannin (14.6 to 37.4 mg/g tannic acid equivalent) and proanthocyanidin (17.2 to 8.4 mg/g quercetin equivalent), respectively (Table 8). Our results showed that the concentration of polyphenolic compounds in the dry extract was significantly ($p < 0.05$) lower than the fresh extract. The concentration of the aforementioned phytochemicals present in *T. violacea* followed similar trends reported in three varieties of *Allium sativum* by Narendhirakannan and Rajeswari (2010). However, the amounts of phenolic compounds are lesser than what was obtained in the three varieties of *A. sativum*. The differences in the content of metabolites may be due to the nature of the soil, microclimate variations (Millogo-Kone, 2008) and processing methods (Choi *et al.*, 2008), since the same method was used by Narendhirakannan *et al.* (2010) in the extraction of the three varieties of *A. sativum* as was employed in this study. The high levels of phytochemicals in the extracts indicates that rhizomes of *T. violacea* could be a good source of anti-inflammatory, anti-clotting, antioxidant, immune enhancing and hormone modulating agents (Okwu and Emenike, 2006); This may therefore, explain the medicinal value of the plant in management and treatment of oxidative stress induced disorders.

Table 8: Polyphenol contents of methanolic extracts of fresh and dried rhizomes of *T. violacea*

Phenolics	Fresh	Dried
Phenol ^a	38.2 ± 0.01	18.3 ± 0.04*
Tannin ^b	37.4 ± 0.13	14.6 ± 0.09*
Flavonoid ^c	67.9 ± 0.11	38.9 ± 0.15*
Flavonol ^d	25.3 ± 0.00	11.5 ± 0.02*
Proanthocyanidins ^e	17.3 ± 0.21	8.40 ± 0.16*

^{a,b}Expressed as mg tannic acid/g of dry plant material. ^{c,d,e}Expressed as mg quercetin/g of dry plant material. * $p < 0.05$

4.5.3 *In vitro* Scavenging Activities of the extracts of *T. violacea*

The antioxidant activities of extracts of rhizomes *T. violacea* (essential oil, dried and fresh methanolic extracts) were assessed by using DPPH, nitric oxide free radical-scavenging, reducing power and lipid peroxidation inhibition assay.

4.5.3.1 DPPH Radical Scavenging Activity

DPPH free radical scavenging assay is considered as a simple and very fast method for determining antioxidant activity. The effect of antioxidant on DPPH radical scavenging was due to their hydrogen donating ability or radical scavenging activity (Viuda- Martos *et al.*, 2010). The antioxidant ability of plant products to donate hydrogen to DPPH radical, thus converting it into stable molecules has been attributed to phenolic compounds, such as flavonoid, polyphenol, tannins and terpenes (Diouf *et al.*, 2009; Rahman and Moon, 2007). Phenolics compounds due to their redox properties, play an important role in adsorbing and neutralising free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Hasan *et al.*, 2008) The results of DPPH radical scavenging activity (% inhibition) of the oil, dried and fresh methanolic extracts showed a concentration dependent radical scavenging activity pattern (Fig. 8 and 9). The oil extract demonstrated increased scavenging effect reaching as high as 89.2 ± 1.5 % at 0.5 mg/ml. This value was very close to the activity of synthetic antioxidants BHT (92.4 ± 3.20 %) and vitamin C (98.1 ± 1.30 %) at the same concentration. The results obtained for the calculated fifty percent effective inhibition concentration of the oil extract ($EC_{50} = 83.0$ μ g/ml) also correspond favourably well with what has been reported for other members family of *Alliaceae* such *Petroselinum sativum* (Parsley) herb oil ($EC_{50} = 82.1$ μ g/ml), *Cuminum cyminum* (Cumin) oil ($EC_{50} = 81.5$ μ g/ml) and *Allium cepa* L. (Onion) oil ($EC_{50} = 80.0$ μ g/ml) at the same concentrations (Shalaby *et al.*, 2011). However, the oil extract of rhizome of *T. violacea* demonstrate higher EC_{50} value

when compared with vitamin C ($EC_{50} = 52.0 \mu\text{g/ml}$) and BHT ($EC_{50} = 67.0 \mu\text{g/ml}$) reference compounds. These values revealed that the antioxidant activity of *T. violacea* rhizome oil was still less active than butylated hydroxytoluene (BHT) and vitamin C. It was observed that the antioxidant activity of the oil extract is higher than for the individual components and this might be as a result of synergetic effects of multiple components of the oil (Misharina *et al.*, 2009).

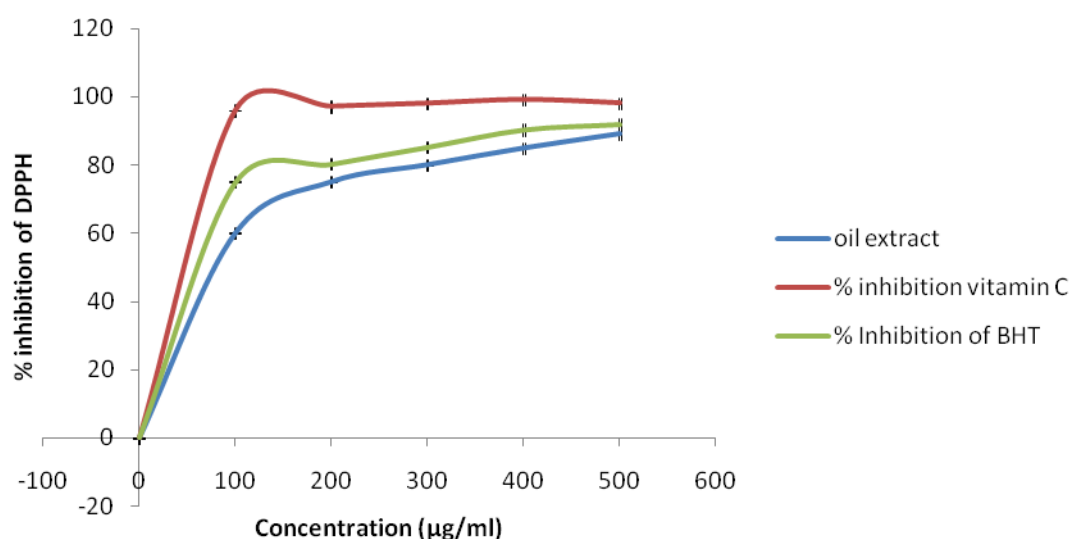


Figure 8: Scavenging effects of oil extracts from rhizomes of *T. violacea*, vitamin C and BHT on DPPH.

The methanolic extracts of dry and fresh rhizomes of *T. violacea* (RTV) were also able to reduce the stable DPPH, thus changing the colour from purple to yellow. The fresh extract of *T. violacea* demonstrated a higher percentage (65.3 %) DPPH scavenging activity (Figure 9) when compared with dried extract (51.4 %) at a concentration of 50 $\mu\text{g/ml}$ or other *Allium* species like green onion and yellow onion (Noureddine, 2005), but exhibited lower percentage DPPH inhibition when compared with red onion, purple onion, garlic (Noureddine, 2005) and ascorbic acid standard (70.2 %) at a concentration of 50 $\mu\text{g/ml}$. The strong DPPH activity (50 % inhibition concentration of $36.0 \pm 0.12 \mu\text{g/ml}$) of the fresh

extract rhizome of RTV (Table 9) agreed favourably with the report of Drużyńska and Wojda (2007) in which they reported that the DPPH radical scavenging activities of fresh extracts of garlic, oregano and rosemary are higher than those of the dried material. The results of the present study indicated that oil, dried and fresh extracts of RTV have good antioxidant activity.

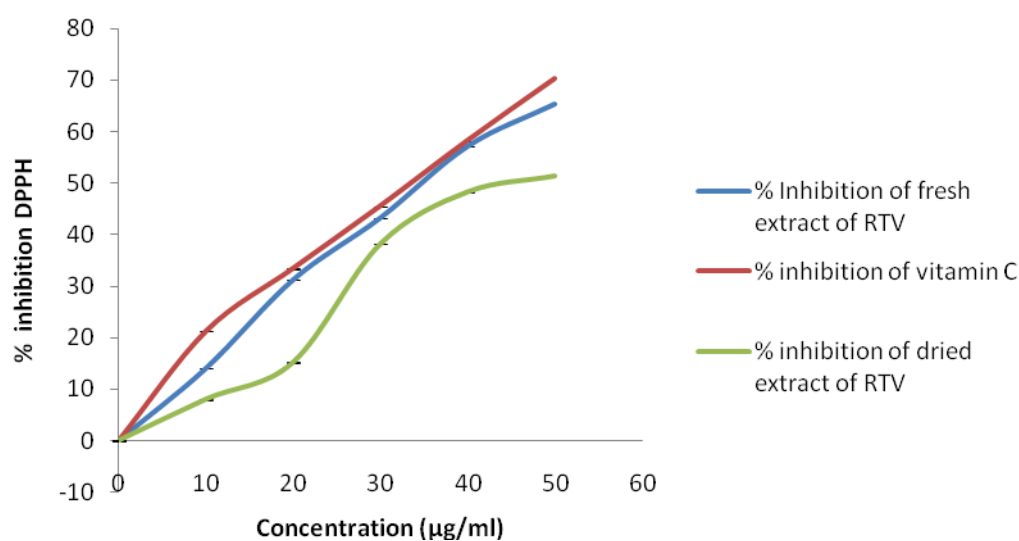


Figure 9: Scavenging effects of fresh and dried extracts from rhizomes of *T violacea* and vitamin C on DPPH.

Table 9: Effective inhibitory concentration for nitric oxide and lipid peroxidation of oil extract of rhizomes of *T. violacea*

Parameters	FRTV	Ascorbic acids	DRTV
DPPH			
Linear equation	$y = 1.34x + 1.72$	$y = 1.35x + 4.219$	$y = 1.15x - 1.77$
R ²	0.99	0.99	0.96
IC ₅₀ (µg/ml)	36.0	33.9	45.1*
H ₂ O ₂ (µg/ml)			
Linear equation	$y = 12.23 + 5$	$y = 13.11x + 7.66$	$y = 9.51x - 4.4$
R ²	0.80	0.76	0.96
IC ₅₀ (µg/ml)	3.6	3.2	5.72*
Lipid peroxidation			
Linear equation	$y = 1.28x + 28.11$	$y = 1.11x + 18.7$	$y = 0.98x + 10.7$
R ²	0.63	0.74	0.88
IC ₅₀ (µg/ml)	28.0	28.2	40.1*

FRTV represents fresh methanolic extract of rhizomes of *T. violacea*. DRTV represents dried methanolic extract of rhizomes of *T. violacea*. (*p < 0.05) * indicates significant difference compared to A.A at p < 0.05.

4.5.3.2 Nitric oxide Scavenging Activity of the Extract of *T. violacea*

Nitric oxide has been implicated in inflammation and pathogenesis of various human diseases such as cancer and cardiovascular diseases (Li and Forstemann, 2000). Nitric oxide scavenging capacity of extracts may help to stop the chain of reactions initiated by excess generation of nitric oxide (NO) and therefore play a role in preventing these diseases (Duduku *et al.*, 2011). In this study, we demonstrate that the oil extract significantly inhibited NO production from sodium nitroprusside, The extracts inhibit nitrite formation by directly competing with oxygen during its reaction with nitric oxide and other nitrogen oxides such as NO₃, and N₂O₃ (Osamuyimen *et al.*, 2011). The extract showed strong concentration dependent inhibitory activity with highest percentage inhibition of nitric oxide at 0.5 mg/ml

(Figure 10). Though, the oil extract demonstrated significant inhibitory activity against nitric oxide radical, its 50 % effective inhibition concentration ($EC_{50} = 245 \mu\text{g/ml}$) was comparably higher than what was obtained for garlic oil ($IC_{50} = 50 \mu\text{g/ml}$) (Reena and Kapil, 2011) and the reference compounds BHT ($EC_{50} = 225 \mu\text{g/ml}$) but lower than ascorbic acid ($EC_{50} = 255 \mu\text{g/ml}$) (Table 10). The nitric oxide inhibiting ability of the oil extract could therefore support the use of the plant in the treatment of oxidative stress induced ailments such as cardiovascular disease.

Table 10: Effective inhibitory concentration for DPPH, hydrogen peroxide radical scavenging activity and lipid peroxidation

Extract	Nitric oxide inhibition EC_{50} ($\mu\text{g/ml}$)	LP inhibition EC_{50} ($\mu\text{g/ml}$)
OTV	$Y = 0.120x + 20.51$, $EC_{50} = 245.7$	$Y = 0.04x + 0.20$, $EC_{50} = 1245.3$
BHT	$Y = 0.128x + 21.2$, $EC_{50} = 225.8$	$Y = 0.04x + 0.02$, $EC_{50} = 1245.6$
AA	$Y = 0.122x + 17.3$, $EC_{50} = 255.6$	$Y = 0.04x + 0.16$, $EC_{50} = 1246.5$

OTV represent oil extract of *Tulbaghia violacea*.

BHT represents- Butylated hydroxytoluene

AA represents ascorbic acid.

LP inhibition EC_{50} represents: 50 % effective inhibition concentration of lipid peroxidation.

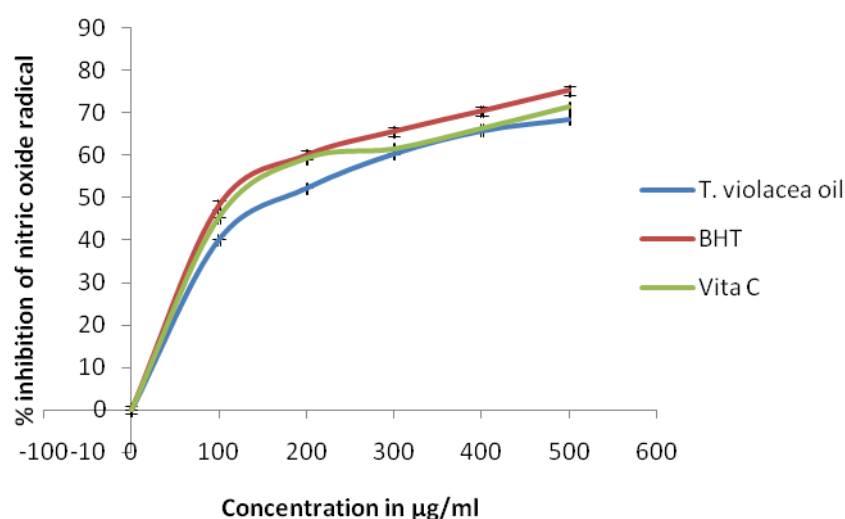


Figure 10: Scavenging effects of oil extracts from rhizomes of *T violacea*, vitamin C and BHT on nitric oxide.

4.5.3.3 Lipid Peroxidation Activity

Reactive oxygen species are known to cross react with lipid constituents of the cell membranes causing changes in fluidity and permeability (Nigam and Schewe, 2000), DNA mutations (Khansari *et al.*, 2009) and lipid peroxidation. The effect of these delirious reactions is the cause of most human diseases.

Lipid peroxidation mediated by free radicals is considered to be the major mechanism of cell membrane destruction and cell damage (Surapaneni and Vishnu, 2009). This cell damage has been implicated in the pathophysiology of various human diseases, such as atherosclerosis, diabetes and cancer. The initiation of peroxidation sequence in membrane or polyunsaturated fatty acids is due to the abstraction of a hydrogen atom from the double bond in the fatty acids (Wagner *et al.*, 1994). Malondialdehyde (MDA) is the major product of lipid peroxidation and is used to study the lipid peroxidation process. Incubation of egg yolk

homogenates in the presence of FeSO₄ causes a significant increase in lipid peroxidation, with subsequent formation of malonodialdehyde (MDA) and other aldehydes that form pink chromogen (Kosugi et al., 1987). In the present study, the ability of the methanolic extract of rhizome of *T. violacea* to inhibit the process of lipid peroxidation was tested using the method of Ruberto *et al.* (2000). Table 9 above shows that the both the fresh and dried extracts of TVR exhibited high lipid peroxidation scavenging activity with 50% inhibition concentration of 28.0 and 40.1 µg/ml respectively. The fresh extract scavenging capacity compared favourably well with the standard (ascorbic acid) and is high than that of dried extract. This result suggested that the extract of rhizome of *T. violacea* possessed high anti-lipid peroxidative agents. This finding is similar to the report that garlic (*A. sativum*) extract significantly reduced lipid peroxidation (Sundaresan and Subramanian, 2005).

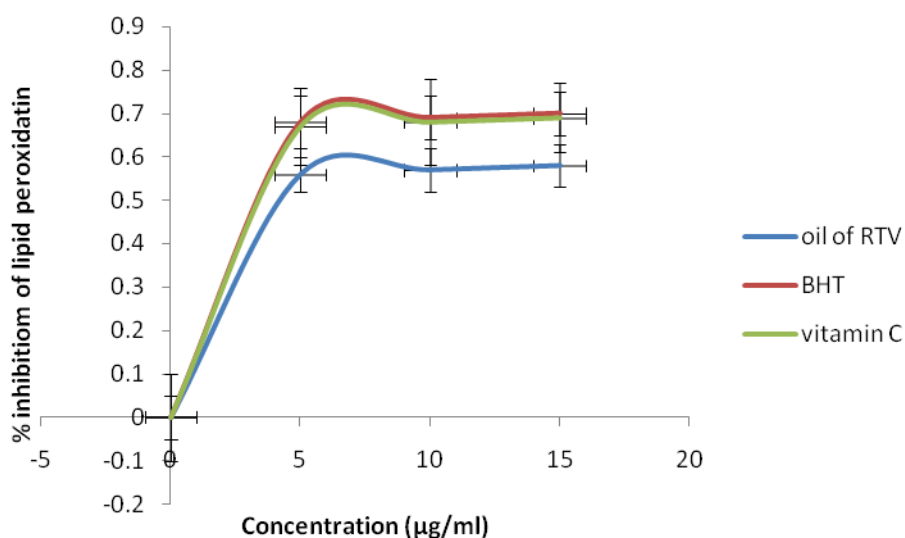


Figure 11: Scavenging ability of methanolic extracts of fresh and dried rhizomes of *T. violacea* (RTV) on lipid peroxidation.

The observed anti-lipid peroxidation activity of the extracts might be due to the high concentration of phenolic compounds in the extract (Maisuthisakul *et al.*, 2007).

Table 10 also showed that the lipid peroxidation scavenging activity of the oil extract of TVR is high and it compared favourably with butylated hydroxytoluene (BHT) and ascorbic acids.

The lipid peroxidation scavenging activity of the oil is concentration dependent (Figure 9). The results of the investigations revealed that oil extract of rhizome of *T. violacea* had potent lipid peroxidation inhibition activity. Although, phytochemical evaluation was not performed in this study, it was reported that for other members of *Alliaceae* species (onion and garlic), the observed inhibition of lipid peroxidation and radical scavenging activities might be due to the phenolic contents (Nuutila *et al.*, 2003).

4.5.3.4 Reducing Power of Extract

The reducing ability of plant extracts is a strong indication of its antioxidant activity (Jayanthi and Lalitha, 2011). Compounds with reducing power presumed to be electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, thereby, acting as primary and secondary antioxidants (Chanda and Dave, 2009). The presence of reductants in *T. violacea* rhizome causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. Since, Fe^{3+} has been implicated in the generation of hydroxyl radical ions, it is logical to assume that the plant extracts are capable of inhibiting hydroxyl radical Fe^{3+} /induced generation and might serves as potential antioxidants. The reducing ability of methanolic extracts of dry and fresh rhizome of *T. violacea* was comparable to the rutin standard and is concentration dependence (Figure 12).

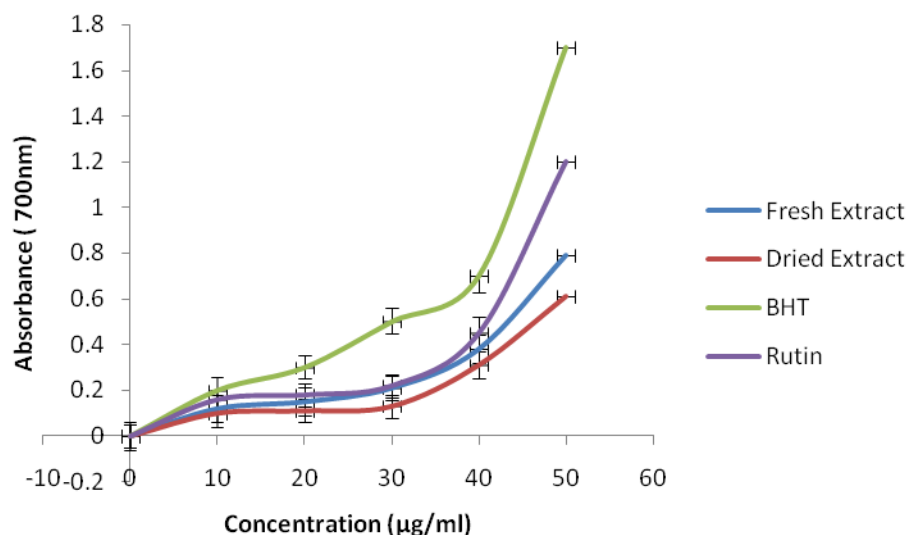


Figure 12: Reducing power of methanolic extract of fresh and dried rhizome of *T. violacea*.

This observation was in agreement with what was reported for other varieties of *A. sativum* bulb (Narendhirakannan and Rajeswari, 2010). Although, the reducing power of the fresh extract was higher than the dry, both were lower than BHT. The antioxidant potential of the oil extract was also estimated from its ability to reduce Fe^{3+} to Fe^{2+} . The reducing power of the oil of *T. violacea* and the reference compounds increased with increasing concentration (Figure 12). In addition, the reducing value of the oil extract was lower than that of BHT and ascorbic acids, used as reference compounds in this study (Figure 13). The reducing activity of the oil extract increased with the concentration of the oil extract and this observation follows similar reports for garlic oil extracts (Reena and Kapil, 2011). The reducing properties of the oil might be due to the presence of reductones (Saha *et al.*, 2008).

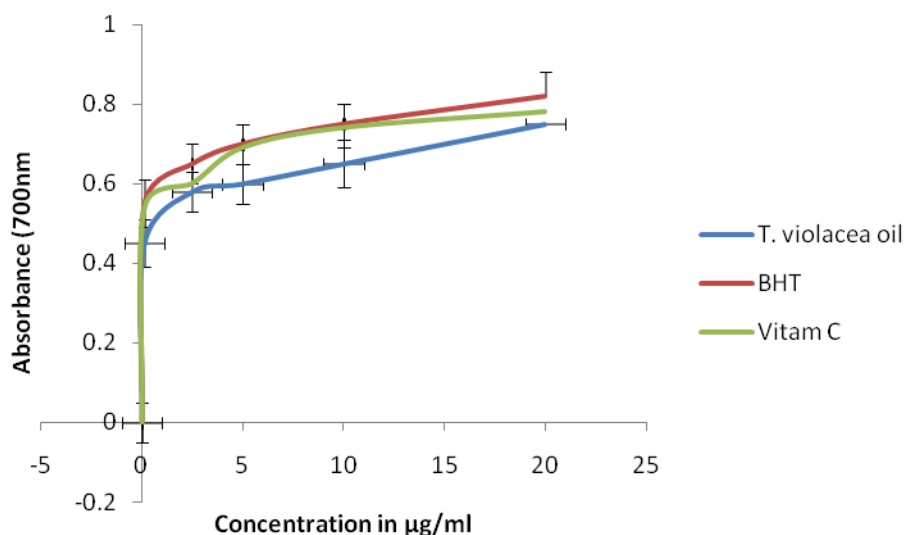


Figure 13: Ferric reducing power of oil extracts from rhizomes of *T. violacea*, vitamin C and BHT.

The present study indicates that the essential oil of rhizome of *T. violacea* exhibit significant antioxidant properties and it further supports its use as a natural antioxidant for the treatment of oxidative stress induced diseases. In view of the reductant activity of the extract, further studies need to be carried out to identify the individual compound(s) that aids in the reducing power.

4.5.3.5 Hydrogen Peroxide Radical Scavenging Activity

Hydrogen peroxide (H_2O_2) is one of the major by-products of incomplete oxygen metabolism (Ilias and Carlos, 2001). It is not a free radical by definition because it lacks free electrons. Nevertheless, its role in reactive oxygen species (ROS) mediated damage is important because of its chemical versatility and diffusibility (Ilias and Carlos, 2001). Hydrogen peroxide is produced from the scavenging activity of superoxide dismutase on superoxide radical (Christophe *et al.*, 2007). In the presence of glutathione peroxidase and reduced glutathione (GSH), hydrogen peroxide is converted to water, thus completely detoxifying ROS (Łukaszewicz-Hussain and Moniuszko- Jakoniuk, 2004; Kryston *et al.*,

2011). However, in the presence of reduced transition metals, such as iron, H_2O_2 can produce the highly reactive $OH^\bullet$, which can cause extensive damage to DNA, proteins and lipids. Therefore, removal of hydrogen peroxide is very important for antioxidant defence in cell systems (Kumar *et al.*, 2011).

The methanolic extracts from fresh and dry rhizomes of *T. violacea* demonstrated a significant hydrogen peroxide scavenging ability (Figure 15). The dry methanolic extract exhibited lower H_2O_2 scavenging activity with a 50 % inhibitory concentration of 5.72 ± 0.16 $\mu\text{g/ml}$ when compared with the fresh extract (3.6 ± 0.11 $\mu\text{g/ml}$) and ascorbic acid standard (3.2 ± 0.14 $\mu\text{g/ml}$) (Table 9). Similar results were reported for three varieties of *A. sativum* L. extracts (Narendhirakannan and Rajeswari, 2010). However, the percentage of H_2O_2 activity of *T. violacea* was slightly higher than what was reported for three varieties of *A. sativum* L. extract by Narendhirakannan and Rajeswari (2010). The H_2O_2 scavenging activity of the extract may be attributed to the presence of phenols, which could donate electrons and transform H_2O_2 into water (Nagavani *et al.*, 2010).

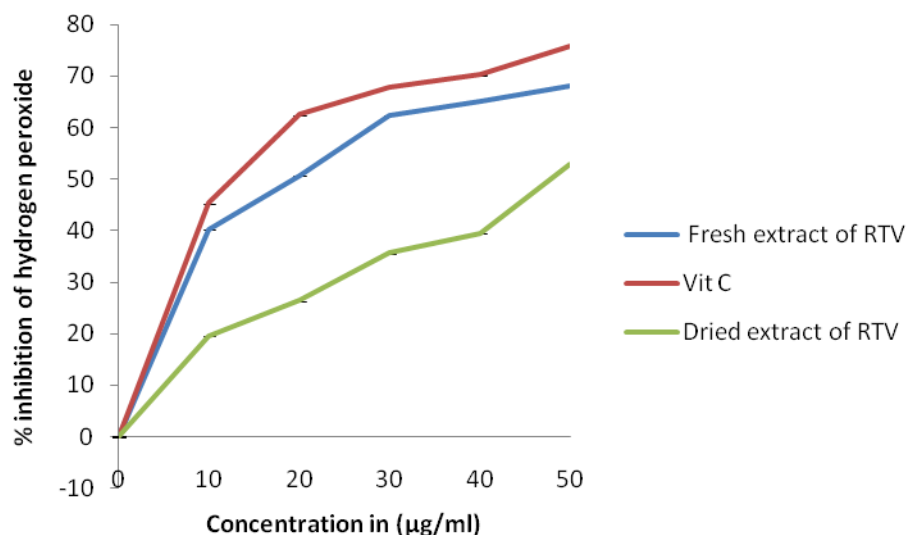


Figure 14: Scavenging ability of methanolic extracts of fresh and dried rhizomes of *T. violacea* (RTV) on hydrogen peroxide.

4.5.4 *In vivo* antioxidant activity

4.5.4.1 Effect of the Extract on Lipid Peroxidation in Normal Rats

The *in vivo* antioxidant potential of extract of *T. violacea* rhizomes was investigated in normal rats. Diminished antioxidant system occasioned by increased free radical generation during normal metabolic functions such as respiration, digestion, immune response and growth introduced from the environment (Nagavani *et al.*, 2010) have been reported to play an important role in the induction of oxidative stress (Prasanna and Purnima, 2011) and development of various human diseases such as ischemia, anaemia, asthma, arthritis, inflammation, heart diseases, Parkinson's diseases, mongolism, ageing process and perhaps dementia (Prasanna and Purnima, 2011). Hence, the use of antioxidant as supplement was recommended as a possible remedy in the control of the aforementioned diseases (Ayoola *et al.*, 2011). In the present study, the effect of extract of *T. violacea*

rhizome on lipid peroxidation in normal rats was analysed and the results revealed that it reduces TBARS generation. Daily administration of *T. violacea* rhizome extract significantly ($p < 0.05$) reduced plasma MDA generation in dose dependant manner in treated. Figure 15 revealed that higher doses of the extract (250 and 500 mg/kg) produced a significant decrease in MDA when compared with the control. There was no significant alteration in the concentration of MDA in the group administered 125 mg/kg body weight of the extract. The results of our experiment showed that the RTV extract significantly protects against lipid peroxidation, a strong marker of oxidative stress, in a dose dependent manner (250 and 500 mg/kg) in normal rats (Figure 15).

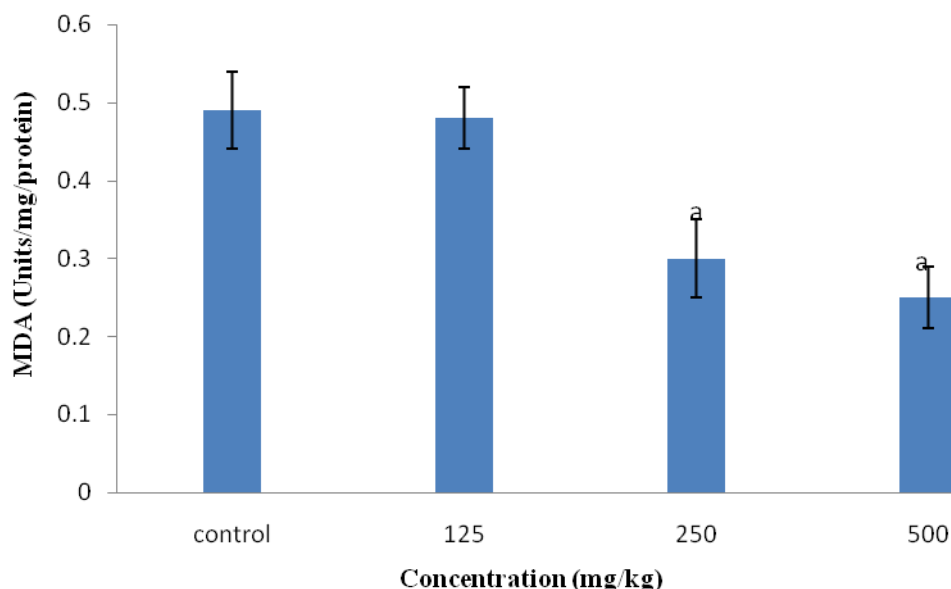


Figure 15: Malondialdehyde (MDA) levels in plasma of rats 28 days after administration of methanolic extract of rhizomes of *T. violacea*. ^a $p < 0.05$ ^a represent significant different from the control.

4.5.4.2 Effect of the Extract on Antioxidant Enzymes in Rats Fed on Normal Diets

The effect of the plant extract on antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in normal rats is shown in figure 16 to 18. The results revealed significantly lower SOD, CAT and GPx activities in saline treated group (control) when compared to the extract treated groups. Higher doses of the extract (250 and 500 mg/kg) produced a significant increase when compared with the control. There was no significant alteration in the concentration of superoxide dismutase in the group administered 125 mg/kg body weight of the extract. Super oxide dismutase (SOD) and Catalase (CAT) are the major enzymes involved with detoxification of reactive oxygen species in most cells (Sivaraj *et al.*, 2011). SOD catalyses the conversion of superoxide anions into hydrogen peroxide while catalase and glutathione peroxidase (GPx) detoxify H_2O_2 and lipid peroxide to non-toxic alcohol. The lower levels of antioxidant activities in untreated rats may be due, in part, to oxidative modification of the enzymatic proteins by excessive ROS generation or may stem from a decrease in their rate of synthesis due to chronic exposure to reactive oxygen insults (Sivaraj *et al.*, 2011). The observed dose dependent increase in the antioxidant enzymes status in the extract treated groups may be due to enhancement of antioxidant enzyme synthesis by the extract acting on the antioxidant response elements in the enhancer region at the promoter site of the gene that codes for the enzymes (Ayoola *et al.*, 2011).

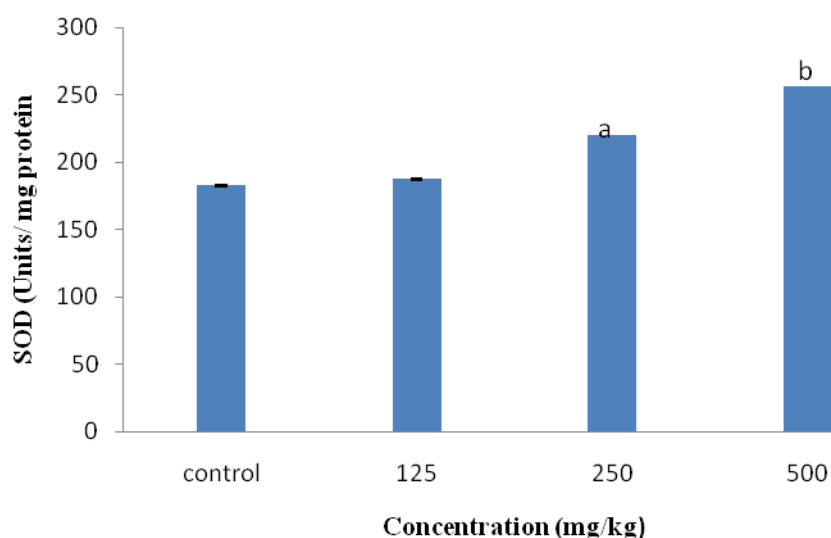


Figure 16: Superoxide dismutase (SOD) levels in the plasma of rats 28 days after administration of methanolic extract of rhizomes of *T. violacea*. * $p < 0.05$ * represent significant different from the control

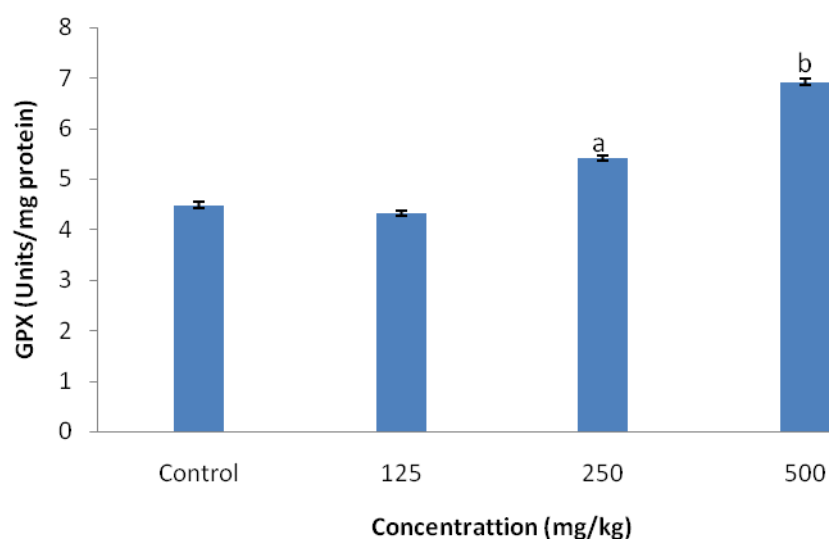


Figure 17: Glutathione peroxidase (GPX) levels in the plasma of rats 28 days after administration of methanolic extract of rhizomes of *T. violacea*. * $p < 0.05$ * represent significant difference from the control.

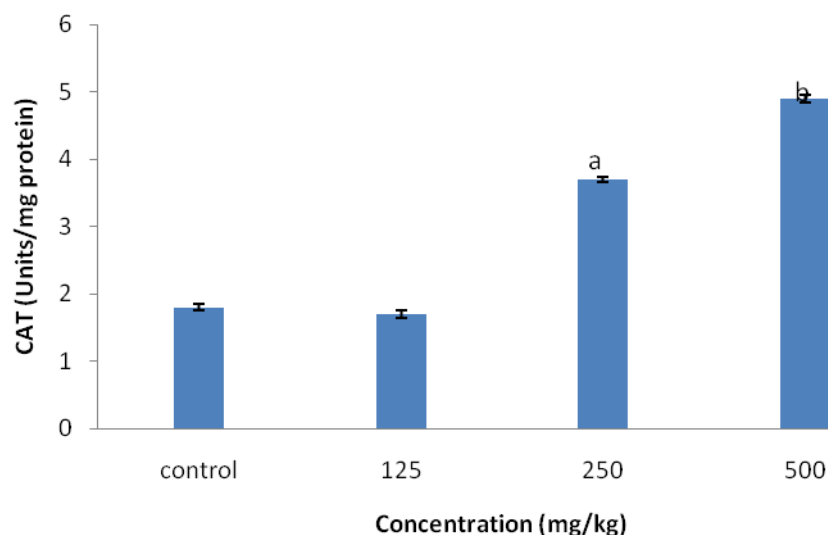


Figure 18: Catalase (CAT) levels in the plasma of rats 28 days after administration of methanolic extract of rhizomes of *T. violacea*. * $p < 0.05$ * represent significant difference from the control.

4.5.4.3 Effect of the extract on Lipid peroxidation and antioxidant enzymes in Diet Induced Hypercholesterolemia

The measurement of thiobarbituric acid (TBARS) is commonly used to monitor lipid peroxidation and indirectly oxidative stress *in vitro* and *in vivo* (Beltowski *et al.*, 2000). Lipid peroxidation is initiated by free radical attack on membrane polyunsaturated fatty acids leading to their transformation and fragmentation to alkanes and reactive aldehyde compounds. Evaluation of the effect of high cholesterol diet (HCD) in experimental rats showed significant ($p < 0.05$) increase in levels of TBARS in the liver, serum, heart, and aorta homogenates of HCD fed rats compared to the normal group (Table 11). The observed increase in lipid peroxidation (TBARS) in the animals fed with high cholesterol is consistent with several clinical and experimental studies (Kallol and Biswadev, 2009). Co-treatment of HCD fed rats with extracts of rhizome of *T. violacea* at a dose of 250 and 500 mg/kg

significantly ($p < 0.05$) reduced the TBARS concentration in serum, aorta, heart and hepatic tissue compared to the untreated HCD fed rats (Table 11). The percentage reduction in the level of TBAR in all the tissues investigated [hepatic (16.34 %, 35.20 %), serum (22.0 %, 28.4 %) heart (31.0 %, 36.8 %) and aorta (18.9 %, 24.4 %)] was dose dependent and it increased with concentration (Table 11). Also the percentage reduction caused by 500 mg/kg TVR extract was comparable to that of gemfibrozil [hepatic tissue (36.56 %), serum (29.9%), (heart 37.2 %) and aorta (27.8 %)]. The ability of the methanolic extract to inhibit the process of lipid peroxidation *in vivo* may be due to the free radical scavenging activities of its phytochemical components, as earlier reported by Olorunnisola *et al.* (2011b). In addition, anti-lipid peroxidative activity of the extracts may be due to the presence of anti-lipidemic agents since activities were similar to those of the standard anti-lipidemic drug used in this research work (Table 11). The results of the present study also showed that the level of glutathione (GSH) was significantly decreased ($p < 0.05$) in the liver, serum, heart, and aorta homogenates of the animals fed with high-cholesterol diet compared to the control diet group. This observation is consistent with previous reports where high cholesterol was administered to experimental rats (BoKang *et al.*, 2011). It can be assumed that the reduction in the tissue glutathione level was as a result of increased oxidative stress and lipid peroxidation occasioned by high cholesterol diet (Novelli *et al.*, 2007; Diniz *et al.*, 2008). Glutathione is a small tripeptide protein synthesized in the liver (Kaplowitz *et al.*, 1985). It is a potent antioxidant with high redox potential and it also serves as co-factor for several detoxifying enzymes (glutathione peroxidase and glutathione transferase) of oxidative stress (Parris, 1997; Valko *et al.*, 2007;). Glutathione has been implicated in the regeneration of some important antioxidants vitamin such as C and E (Parris 1997).

Table 11: Effects of crude extract of rhizomes of *T. violacea* on thiobarbituric acids (TBARS) and reduced glutathione (GSH) in tissues of rats kept on high-cholesterol diet.

Group	NC	HCD	HCD+250 mg/kg of RTV	HCD + 500 mg/kg of RTV	HCD+ Gemfibrozil
Liver					
MDA	2.75 ± 0.12	4.65 ± 0.19 ^a	3.89 ± 0.14 ^b	3.01 ± 0.16 ^b	2.95 ± 0.11 ^b
GSH	5.32 ± 0.17	3.12 ± 0.13 ^a	4.22 ± 0.10 ^b	4.89 ± 0.14 ^b	5.01 ± 0.18 ^b
Serum					
MDA	11.2 ± 0.13	17.45 ± 1.14 ^a	13.65 ± 0.11 ^b	12.50 ± 0.15 ^b	12.41 ± 0.18 ^b
GSH	1.23 ± 0.11	0.19 ± 0.12 ^a	0.89 ± 0.17 ^b	1.02 ± 0.13 ^b	1.01 ± 0.31 ^b
Heart					
MDA	8.52 ± 1.12	14.25 ± 0.17 ^a	9.85 ± 0.21 ^b	9.01 ± 1.10 ^b	8.95 ± 0.12 ^b
GSH	4.85 ± 0.20	2.15 ± 0.28 ^a	3.19 ± 0.15 ^b	3.39 ± 0.24 ^b	3.97 ± 0.13 ^b
Aorta					
MDA	10.5 ± 1.32	15.23 ± 0.52 ^a	12.35 ± 0.15 ^b	11.51 ± 0.16 ^b	10.99 ± 0.20 ^b
GSH	8.25 ± 1.70	4.82 ± 0.19 ^a	6.98 ± 0.17 ^b	7.21 ± 0.13 ^b	7.37 ± 0.10 ^b

Values are mean ± standard error of mean of six Wistar rats. Significant changes are shown by a and b = (p < 0.05). NC= normal control, HCD = high cholesterol diet, RTV= rhizome of *Tulbaghia violacea*, MDA = Malondialdehyde and GSH = glutathione (n =3).

Co-administration of HCD fed rats with methanolic extracts of rhizome of *T. violacea* at a dose of 250 and 500 mg/kg significantly restored the level of GSH to near normal when compared to rats fed with HCD only and the normal control. The protective effect of the extracts is comparable with gemfibrozil (Table 11) and garlic which belong to the same family (Wang *et al.*, 2007; Kim *et al.*, 2011) as RTV. It may be suggested that the activity of the plant is due to its free radical scavenging activities and the rich antioxidant phytochemicals (phenolics and flavonoids) constituents (Olorunnisola *et al.*, 2011). The ability of the extract to protect the heart and aorta against hypercholesterolemia induced glutathione depletion and oxidative stress may be an added advantage in cardioprotective potential of the plant.

Our results also revealed that, hepatic, aorta and heart antioxidant enzyme (SOD, CAT, GPx and GST) activities significantly ($p < 0.05$) decreased in rats fed a cholesterol-rich diet compared to those fed with normal diet (Figure 19, 20 and 21).

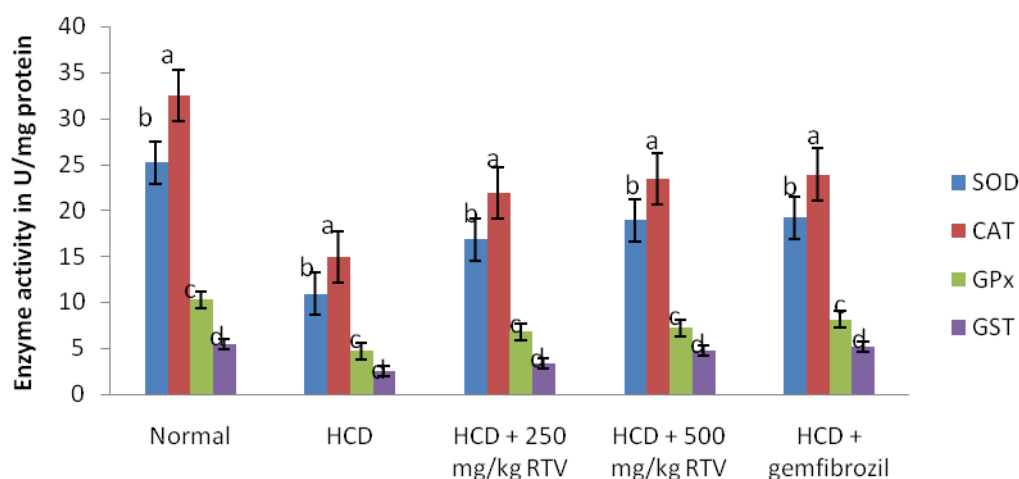


Figure 19: Effects of methanolic extract of rhizomes of *T. violacea* on antioxidant enzymes from aorta homogenate of rats fed a high cholesterol diet.

The decrease in the activities of these enzymes could be attributed to the excessive utilization of these enzymes in inactivating the free radicals generated due to high cholesterol diet (Ma *et al.*, 2011) or insufficient availability of GSH. This observation is further substantiated by the elevated malondialdehyde (Table 11). Our results are in agreement with those of others, who studied the effect of high fat diet on liver (BoKang *et al.*, 2011), aorta (Shela *et al.*, 2011) and heart (Jiangwei *et al.*, 2011) antioxidant enzyme systems.

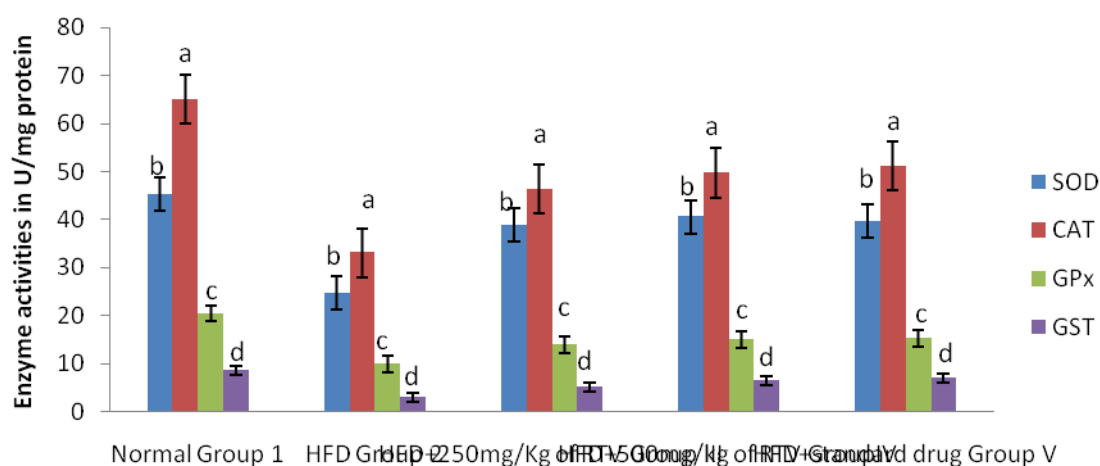


Figure 20: Effects of methanolic extract of rhizomes of *T. violacea* on antioxidant enzymes from liver homogenate of rats fed a high cholesterol diet.

Co-administration of HCD fed rats with extracts of rhizome of *T. violacea* at a dose of 250 and 500 mg/kg significantly prevented the reduction in the level of SOD, CAT, GPx and GST in liver, aorta and heart of rats in dose dependent manner. The protective effect of the extracts is comparable to gemfibrozil (Table 11). Our results indicate that *T. violacea* had a free radical scavenging activity which probably provides organs protection from hypercholesterolemia. The reduction of serum and liver TBARS, and increased cardiac and aorta antioxidant enzymes activities in rats treated with *T. violacea* may be related to lipid-lowering ability because its activity compared favourably with gemfibrozil a lipid lowering drug (Ozansoy *et al.*, 2001) or presence of phenolic compounds. It can also be hypothesized that the anti-oxidant enzyme in aorta, liver or heart may be up-regulated by administration of *T. violacea* in response to hypercholesterolemia enhanced free radical production. Similar observation has been reported for cloves *Syzygium aromaticum* (Gaertn) Linn., a family of Myrtaceae (Shyamala *et al.*, 2003), garlic and onion extracts from the *Alliaceae* family (Shyamala *et al.*, 2003). The extract also compared favourably with garlic oil which was reported to increase hepatic glutathione S-transferase, glutathione reductase and superoxide

dismutase (Shyamala *et al.*, 2003). However, contrary to garlic oil, extract of *T.violacea* increased the activity of glutathione peroxidase.

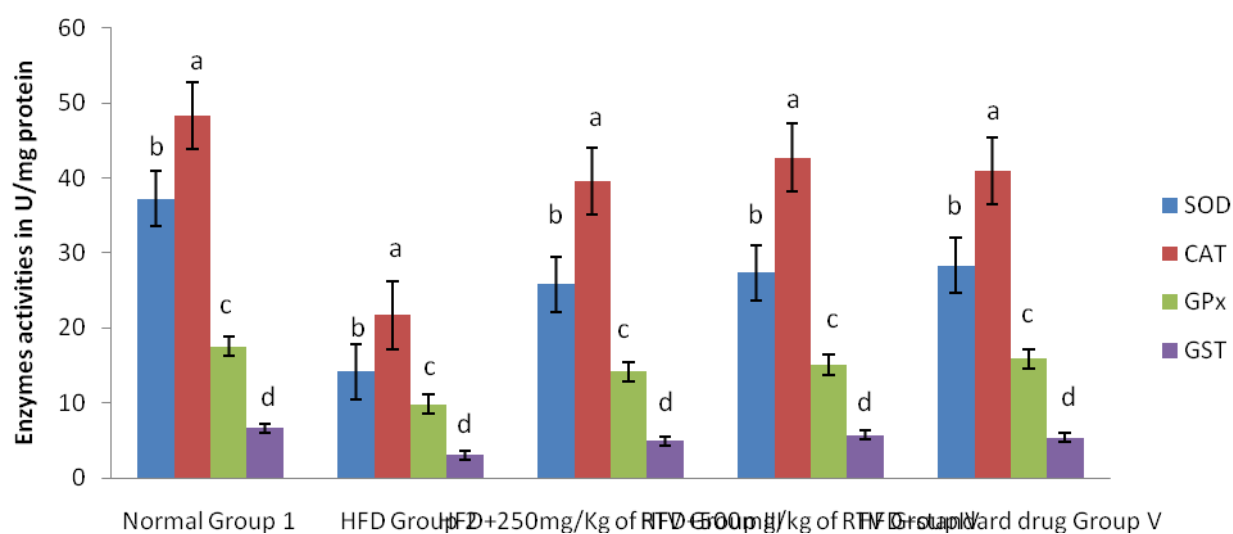


Figure 21: Effects of methanolic extract of rhizomes of *T. violacea* on antioxidant enzymes from heart homogenate of rats feed a high cholesterol diet. Mean $\pm$ SD (n = 6).

4.6 Conclusion

The present study clearly shows that the rhizome of *T. violacea* may be of therapeutic importance, not only as an antioxidant agent but also as a cyto-protective agent to protect the liver, aorta and cardiac injury from hypercholesterolemia.

CHAPTER 5

ANTI-HYPERLIPIDEMIA ACTIVITY OF *T.* *VIOLACEA* RHIZOME EXTRACT

Paper of this chapter is published in Africa Journal of Biotechnology Vol. 11(70), pp.

13498-13505

5.0 INTRODUCTION

Cardiovascular disease (CVD) is one of the major causes of mortality and morbidity in Europe, United States, most parts of Asia (Sans *et al.*, 1997; Ashraf *et al.*, 2005) and recently in developing countries (Karen and Ana, 2009). It encompasses conditions that range from hypertension, acute coronary syndrome, stroke and chronic heart failure (Karen and Ana, 2009). The high burden of CVD in the developing countries is attributable to the increasing incidence of atherosclerotic diseases, urbanization and higher risk factor levels such as obesity, diabetes, dyslipidemia and hypertension (Salim *et al.*, 2009). A large body of studies in experimental animals has clearly demonstrated that the common risk factor for cardiovascular disease is hypercholesterolemia (Shaten *et al.*, 1991; Castelli *et al.*, 1992; Law, 1999). Therefore, treatment of hypercholesterolemia may reduce the risk or development of cardiovascular disease (Doha *et al.*, 2010). Presently, the existing drugs such as statins used in the management of cardiovascular disease are associated with side effects such as abnormal liver disease, diarrhoea, gastric irritation and nausea (Doha *et al.*, 2010). Several herbs have been reported to reduce high blood cholesterol without negative side effects in addition to being affordable (William and John, 2010). These herbs include members of the *Allium sp.* (Garlic, onions and chives) and of the *Labiatae* (mint) family.

One of the frequently used plants in the Nkonkobe Municipality, Eastern Cape, South Africa, in the management of heart diseases, is *Tulbaghia violaceae* (Joanna and Thompson, 2003). *Tulbaghia violacea* belongs to the family of *Alliaceae* and is believed to possess biological activities similar to garlic (*Allium sativum*) (Olorunnisola *et al.*, 2011b). Various scientific publication (Joanna and Thompson, 2003; Bungu *et al.*, 2006) have reported that the leaves, root, stem and rhizomes of *T. violacea* possess pharmacological properties. Bungu *et al.* (2006) reported that the plant is used in the treatment of fever and colds, asthma, tuberculosis, stomach problems and oesophageal cancer. Recently, Olorunnisola *et al.*

(2011a) reported that methanolic extracts of rhizomes of *T. violacea* (RTV) exhibited potent antioxidant activities in a concentration dependent manner. However, despite the reported use of the plant in the management of cardiovascular disease and as an anti-hypercholesterolemia agent, to the best of our knowledge there is no scientific information on its anti-lipidemic activities in the literature. The present study was designed to investigate antilipidemia and biochemical effects of methanolic extracts of *T. violacea* rhizome in high fat diet fed rats.

5.1 Materials and Methods

5.1.1 Collection of Plant Materials was as described in section 3.2

5.1.2 Preparation and Extraction of Plant Materials was as described in section 3.3

5.2 Animals

Healthy female Wister albino rats (130-160 g) were randomly assigned to control and treated groups (6 animals per group/cage). They were maintained in standard environmental conditions ($22 \pm 2^{\circ}\text{C}$, 12:12 h dark/light cycle, humidity: 45-50%) frequent air change and free access to water and food. All animals were obtained from the animal house of the laboratory of the School of Biological Sciences, University of Fort Hare. All procedures used in the present study followed the “Principles of Laboratory Animal Care” from NIH Publication No.85-23 and were approved by the Animal Ethics Committee of UFH.

5.3 Cholesterol Supplemented Diet

Hypercholesterolemia was induced using earlier modified method of Onody et al. (2003). Briefly, cholesterol (2% w/w) powder was thoroughly mixed with crushed pellet diet and reconstituted with water and allowed to dry properly to prevent microbial contamination.

5.4 Experimental Design

Experimental animals were divided into the following groups after 2 weeks of acclimatization. Each group comprised of 6 animals.

Group 1: Control rats fed orally with normal pellet diet.

Group 2: Rats fed orally with cholesterol mixed pellet diet.

Group 3: Rats fed orally with cholesterol (2 % w/w) mixed pellet diet plus (250 mg/kg b.wt./day) RTV by oral gavage

Group 4: Rats fed orally with cholesterol mixed pellet diet plus (500 mg/kg b.wt/ day) RTV by oral gavage

Group 5: Rats fed orally with cholesterol (2 % w/w) mixed pellet diet plus with atorvastatin (30 mg/kg b.wt/day) by oral gavage.

5.5 Determinations of biochemical parameters

5.5.1 Assessment of Lipid Profile and Biochemical Parameters

Blood samples were collected from overnight fasted rats under light anesthesia by cardiac puncture with the aid of a sterilized needle syringe. These animals were thereafter made to bleed through their cut jugular vein and their blood collected in lithium heparinized tubes. Total cholesterol, triglycerides, low density lipoprotein (LDL), very low density lipoprotein, high-density lipoprotein-cholesterol (HDL-C) levels, aspartate amino transferase, alanine amino transferase, alkaline phosphatase, total bilirubin, lactate dehydrogenase, gamma glutamyl transferase (GGT) and glucose were determined in the blood using a piccolo automated chemistry analyser (Abaxis USA).

5.6 Statistical Analysis

Values are given as arithmetic means $\pm$ standard deviation of the mean $\pm$ (SD). Data was statistically analysed by using One-way analysis of variance (ANOVA).

5.7 Results and Discussion

5.7.1 Effect of the *T. violacea* on Lipid profile in Normal Rats

Defects in metabolism and oxidation of lipid molecules have been implicated in the etiopathogenesis and progression of human diseases (Juan *et al.*, 2007). Raised serum lipid levels, particularly of cholesterol along with generation of reactive oxygen species (ROS) play a key role in the development of coronary artery disease and atherosclerosis (Ratheesh *et al.*, 2011). Yakubu and Afolayan (2009) reported that elevated levels of all lipids except the HDL-C are associated with increased risk of atherosclerosis. Ginter and Simko (2010) reported that oxidation of LDL promotes vascular dysfunction, enhances the production of inflammatory mediators and contributes to initiation and progression of heart disease. There is now overwhelming evidence that herbal drugs may be helpful in the treatment and control of hyperlipidemia and this may translate directly or indirectly to the management of cardiovascular diseases. In the present study, oral administration of the methanol extract of rhizomes of *T. violacea* (125, 250 and 500 mg/kg body weight) showed dose dependent hypolipidemic activity. It reduced plasma cholesterol, triglyceride, LDL-c, very low density lipoprotein (VLDL) and atherogenic index in rats fed normal diet (Table 12). This observation is consistent with hypolipidemic activities of other member of *Alliaceae* family such as *Allium sativum* and *Allium tuberosum* (Raghuveer, 2008). The mechanism(s) of hypolipidemic activity of *T. violacea* is unknown but may be due to a direct activating effect on lipoprotein lipase, a vital enzyme in the metabolism of triglyceride, or prevention of

production of cholesterol in the liver by blocking HMG-CoA reductase or increase level of HDL.

Table 12: Effect of methanolic extracts of *T. violacea* on lipid profile in rats fed with normal diet.

Plasma Parameters (mg/dl)	Treatment (mg/kg)			
	Control	125	250	500
TC	110.1 ± 0.25	108.1 ± 0.42	100.4 ± 0.33	95.9 ± 0.13*
TG	98.21 ± 0.81	97.65 ± 0.12	95.82 ± 0.49	84.14 ± 0.91*
LDL-c	23.21 ± 0.62	22.51 ± 0.52	19.78 ± 0.66*	15.05 ± 0.17*
VLDL	20.51 ± 0.32	19.55 ± 0.51	18.93 ± 0.49*	17.89 ± 0.75*
HDL-c	47.56 ± 0.23	52.76 ± 0.10*	58.18 ± 0.97*	63.51 ± 0.71*
TG/HDL	1.6	1.5	1.5	1.5

TC, TG, LDL-c, HDL-c, VLDL and TG/HDL- Represents total cholesterol, total triglyceride, low density lipoprotein, high density lipoprotein, very low density lipoprotein, and ratio of total glyceride and high density lipoprotein respectively. Results are mean ± SD (n = 6)

*Represents (p<0.05) significantly difference from control.

It could also be due to presence of phytochemicals such as organosulphur compounds (Kubec *et al.*, 1999; Olorunnisola *et al.*, 2011) which have been reported to possess anti-atherogenic effects (Matthew *et al.*, 2004). In addition, to hypolipidemic activity, *T. violacea* also caused a significant (p < 0.05) dose dependent increase in the levels of high density lipoproteins (HDL). High density lipoprotein (HDL) commonly referred to as “good cholesterol possesses the ability to reverse cholesterol transport and also protect LDL from oxidation, thereby minimizing the deleterious consequences of LDL oxidation (Bonnetfont-Rousselot *et al.*, 1999). These results imply that *T. violacea* extract may possess beneficial effect (s) by reducing plasma lipid profiles.

5.7.2 Effect of RTV on Serum Lipid Profile in Diet Induced Hypercholesterolemia

Derangements in cholesterol metabolism have been associated with the etiology of most human diseases. It is widely reported that hypercholesterolemia occasioned by a defect in cholesterol transportation, biosynthesis or catabolism is a risk factor in coronary heart disease (CAD) and atherosclerosis (Yogendrasinh *et al.*, 2010; Mohammed *et al.*, 2011). Hence, prevention of hypercholesterolemia will make positive contribution to the management and treatment of cardiovascular diseases. The results of the present investigation showed that rats fed with cholesterol rich diet developed hypercholesterolemia with a significant ($p < 0.05$) increase in total cholesterol (TC), low density lipoprotein (LDL), very low density lipoprotein (VLDL), triglyceride levels (TG), and a significant ($p < 0.05$) decrease in HDL-C levels as compared with control rats (Table 13). Increased fat deposition was also observed in the liver of rats fed with high cholesterol diet (Fig 22 b) compared with rats on a standard diet. These results were in agreement with earlier reports on dietary hyperlipidemia (Qadir, 2005; Majeed, 2006 and Abdulazeez, 2011). However, co-administration with RTV (250 and 500 mg/kg bwt) and standard drug Atorvastatin to high cholesterol fed rats significantly ($p < 0.05$) decrease the levels of TC, TG, LDL, VLDL and caused a significant increase in the level of HDL-C when compared with the hypercholesterolemic group (Table 13). The extract and the standard drug also prevented against fat deposition in the liver (Fig 22 c and d). The activity of the extract is dose dependent. At a concentration of 500 mg/kg.bwt *T. violacea* demonstrated a higher percentage reduction in serum triglyceride (79.3 % /60.6 %) and cholesterol (39.8 % /34.2 %) when compared with the standard drug.

Table 13: Effect of extracts *T. violacea* rhizomes on serum lipid profile and glucose in diet induced hypercholesterolemic rats.

Plasma parameters (mg/dl)	Normal	HCD only	HCD +30 mg/kg Atorvastatin	HDC + Dosage of <i>T. violacea</i> in mg/kg	
				250	500
TC	76.21 ± 0.14 ^a	164.55 ± 0.15 ^b	108.21 ± 0.11 ^c	116 ± 0.19 ^c	98.98 ± 0.17 ^d
HDL-C	31.23 ± 0.16 ^a	15.17 ± 0.12 ^b	60.01 ± 0.19 ^c	53.20 ± 0.15 ^c	56.02 ± 0.15 ^c
LDL-C	15.02 ± 0.18 ^a	35.21 ± 0.14 ^b	21.05 ± 0.18 ^c	28.12 ± 0.13 ^c	23.18 ± 0.17 ^c
VLDL-C	14.32 ± 0.16 ^a	29.01 ± 0.11 ^b	12.14 ± 1.18 ^c	11.45 ± 0.14 ^c	12.01 ± 0.15 ^c
TG	58.13 ± 0.17 ^a	261.2 ± 0.14 ^b	103.20 ± 0.19 ^c	110.01 ± 0.12 ^c	54.18 ± 0.16 ^d
GLU.	73.53 ± 0.19 ^a	89.10 ± 0.11 ^b	100.01 ± 0.15	85.02 ± 0.15	90.03 ± 0.19

TC: total cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; VLDL-C: very low density lipoprotein cholesterol; TG: triglycerides; GLU: glucose. Values are mean ± SD triplicate determinations (n = 8). Values in same row with different superscripts are significantly different (p < 0.05).

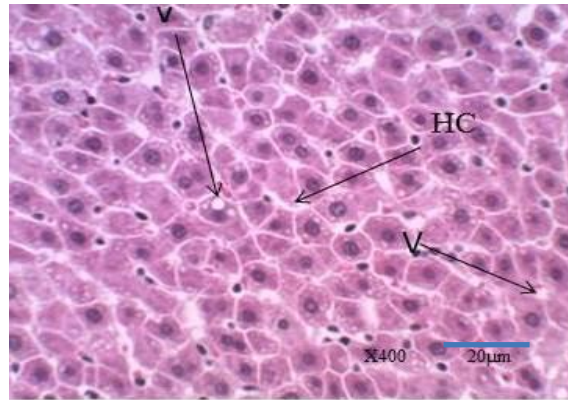


Figure 22a: Photomicrograph of liver section from a rat treated with high cholesterol diet (H&E, X400).

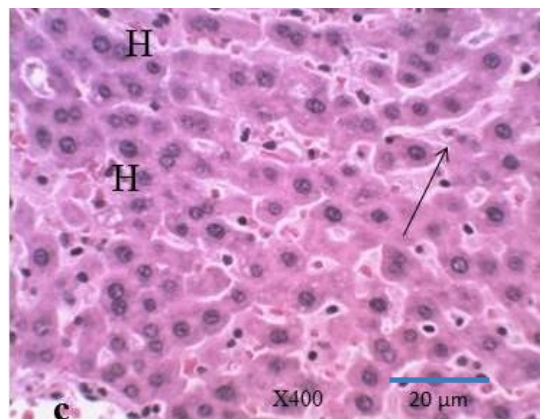


Figure 22b: Photomicrograph of liver section from a normal rat (H&E, X400).

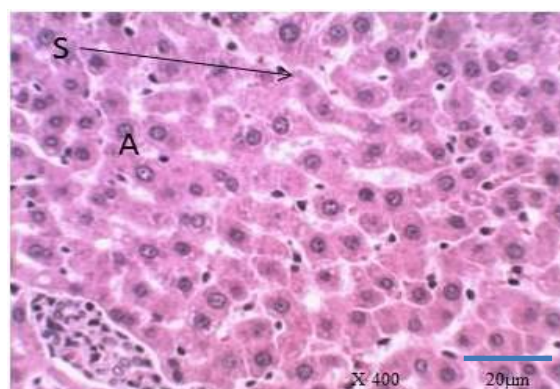


Fig. 22c: Photomicrograph of liver section of rat fed with high cholesterol diet and RTV (500 mg/kg) (H&E, X400).

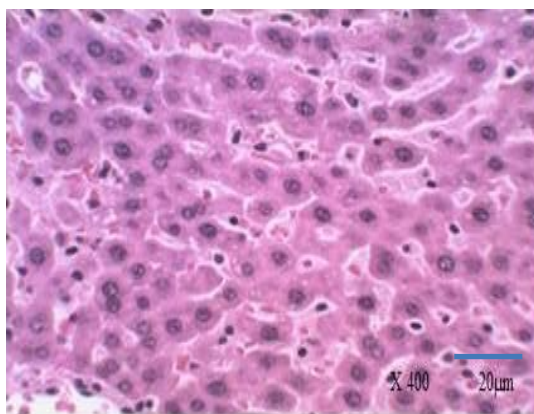


Figure 22d: Photomicrograph of liver section of rat fed with high cholesterol diet and Atorvastatin (30 mg/kg) (H&E, X400).

Atorvastatin is usually employed in the treatment of elevated total cholesterol, LDL and triglycerides. It also increased HDL cholesterol level in the liver, possibly by blocking HMG-CoA reductase (Ginter and Simko, 2010). The mechanism(s) of anti-hyperlipidemia activities of our plant are still unknown. The strong anti-hypertriglyceridemia property of the extract may be employed in the management of hypertriglyceridemia induced atherogenesis, ischemic heart disease, obesity and cholesterol deposition in body tissue.

Also the observed low level of HDL in the hypercholesterolemic rats is consistent with earlier studies (Mohammed *et al.*, 2011). In the current investigation RTV treatment decreased the levels of total cholesterol and triglycerides but increased the levels of HDL-C thereby suggesting a cardioprotective and lipid lowering potential. The lipid lowering potential of the plant may be due to polysulfides (Sanjay *et al.*, 2002), flavonoids and/or saponins (Hostettman, 1995; Ramachandran *et al.*, 2003) which we found to be present in fairly high level in RTV (Table 13 and 14). This lipid lowering ability of the plant is consistent with other members of the family *Alliaceae* such as garlic. Several studies have

shown that garlic possesses anti-hypercholesterolemic activity (Yu-Yan and Lijuan, 2001; Toyohiko *et al.*, 2002).

5.7.3 Effect of Extract of RTV on Body Weight Gain.

A significant ($p < 0.05$) increase was observed in the weight gain of the animals fed with HCD diet compared to normal controls (figure 23). Ramachandran *et al.* (2003) showed that there was no significant weight gain in rats fed with HCD while Harnafi *et al.* (2009) reported a linear weight increase between the control and hypercholesterolemic animals. However, Matos *et al.* (2005) and Otunola *et al.* (2010) reported a significant reduction in weight gain in animals on high a cholesterol diet. In the present study, a consistent weight gain was observed in animals on standard diet throughout the period while those on the HCD only gained weight up till the 2nd week; thereafter a steady decline in weight gain was observed. The resultant weight loss could be due to reduction in nutrient intake caused by high cholesterol content of the diet which might have impaired the absorption of protein and other nutrients (Matos *et al.*, 2005; Woo and Henry, 1996).

Co-treatment with RTV (250 and 500 mg/kg. bwt) or Atorvastatin (30 mg/kg. bwt) to rats on high cholesterol diet significantly ($p < 0.05$) restored the weight gain pattern to near normal (Fig 23). The extract activity is dose dependant and compared favorably with the Atorvastatin. This result agrees with other investigators who noticed an increase in body weight gain upon the improvement of hyperlipidemia status (Lamiaa, 2011). The mechanism of action is unknown but it may be due to improvement in nutritional status of the animals (Prasad, 2010) or improved absorption/digestion of protein.

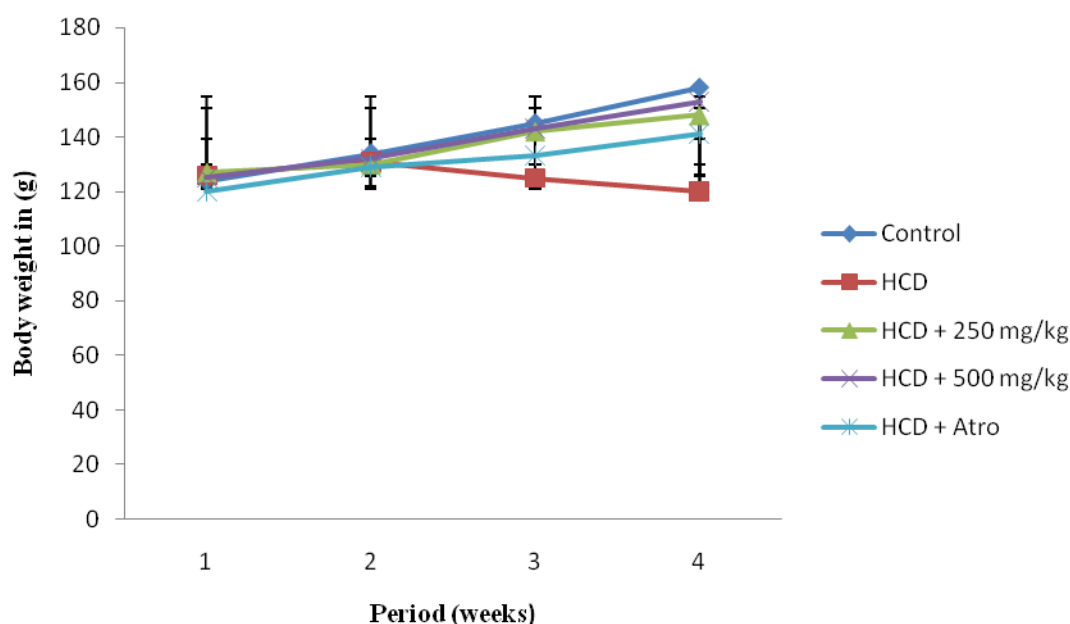


Figure 23: The growth response of rats fed with standard diet, high cholesterol diet (HCD) and plus RTV extract. Data are represented as mean $\pm$ SD (n = 6)

5.7.4 Effect of the Extracts on Biochemical Parameters

There have been conflicting reports on the effect of high cholesterol diet on serum biochemical parameters related to hepatic function of alkaline phosphatase (ALP), Aspartate amino transferase (AST), alanine transferase (ALT) and gamma-glutamyl transpeptidase (GGT). Prasad, (2010) reported that the effects of hypercholesterolemia on serum levels of those enzymes are variable. Lu *et al.*, (2007) and Saki *et al.*, (2011) showed that a high cholesterol diet moderately elevated serum levels of ALT, AST, and ALP in rats and Molgaard *et al.*, (1989) reported that there is no change in the serum level of AST, ALT, and ALP while Mabuchi *et al.* (2007) and Arafa, (2005) reported no changes in serum level of AST and ALP. In addition, Assy *et al.* (2000) observed that there was no change in the serum

level of ALP in hypercholesterolemic rats. The discrepancy in the serum levels of the enzyme could be attributed to the level and duration of hypercholesterolemia (Lu *et al.*, 2007).

The results (Table 14) of the present study indicate that animals on high cholesterol diet have a 3.2 (TB), 1.33 (ALT), 1.17 (AST), 2.61 (ALP), 1.15 (LDH) and 1.80 (γ -GT) fold increase, in their respective enzymatic activities compared with control rats. The observed increase in the enzyme activities is in agreement with Saki *et al.* (2011) and Lu *et al.*, (2007) report. Co-administration of the methanolic extract of *T. violacea* rhizomes to rats leads to a reduction ($p < 0.05$) in elevated serum level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and gamma-glutamyl transpeptidase (GGT). The effect of the extract is dose dependent and it compared favorably with the activity of the standard drug (Atorvastatin). All these enzymes play critical roles in ischemic liver injury, myocardial or pulmonary infarction, hepatobiliary dysfunction and alcohol abuse, cardiovascular and atherosclerotic diseases and therefore any changes in their serum level will affect these conditional disease states.

Table 14: Effect of extracts of *T. violacea* rhizomes on serum levels of biochemical parameters in diet induced hypercholesterolemic rats

Parameters	Control	HCD	HCD + 30 mg/kg ⁻¹ Atro	Extract of TVR	
				HCD +	HCD +
				250 mg/kg	500 mg/kg
TP	7.19 $\pm$ 0.20 ^a	6.10 $\pm$ 0.17 ^b	6.83 $\pm$ 0.22 ^b	6.67 $\pm$ 0.15 ^b	6.79 $\pm$ 0.11 ^b
AL	3.42 $\pm$ 0.51 ^a	2.10 $\pm$ 0.11 ^b	3.35 $\pm$ 0.15 ^a	3.25 $\pm$ 0.13 ^a	3.30 $\pm$ 0.14 ^a
Tb(μ mol/L)	10.21 $\pm$ 1.21 ^a	20.78. 1.00 ^b	8.89 $\pm$ 1.10 ^c	9.34 $\pm$ 1.11 ^c	8.01 $\pm$ 1.01 ^c
ALT (U/L)	38.5 $\pm$ 1.22 ^a	51.3 $\pm$ 1.05 ^b	42.31 $\pm$ 0.23 ^c	45.11 $\pm$ 1.21 ^c	40.12 $\pm$ 1.23 ^c
AST (U/L)	65.2 $\pm$ 2.11 ^a	76.4 $\pm$ 1.12 ^b	68.21 $\pm$ 0.40 ^a	70.10 $\pm$ 0.14 ^b	68.21 $\pm$ 0.31 ^a
ALP (U/L)	11.22 $\pm$ 1.29 ^a	29.2 $\pm$ 1.21 ^b	14.2 $\pm$ 1.42 ^a	17.12 $\pm$ 1.21 ^c	16.4 $\pm$ 0.17 ^c
LDH	128.3 $\pm$ 0.10 ^a	147.5 $\pm$ 0.11 ^b	130.4 $\pm$ 0.20 ^c	133.3 $\pm$ 0.15 ^c	131.1 $\pm$ 0.32 ^c
GGT (μ /L)	31.30 $\pm$ 1.46 ^a	56.34 $\pm$ 1.23 ^b	32.20 $\pm$ 1.62 ^c	39.10 $\pm$ 0.25 ^c	34.23 $\pm$ 0.13 ^c

Total protein (TP) Total bilirubin (Tb); High cholesterol diet (HCD); Aspartate amino transferase (AST), Alanine amino transferase (ALT), Alkaline phosphatase (ALP); Total bilirubin (Tb), Lactate dehydrogenase (LDH), gamma glutamyl transferase (GGT), Total protein, *Tulbaghia violacea* rhizome (TVR). Mean $\pm$ (SD) (n = 6). Values in same row with different superscripts are significantly different ($p < 0.05$).

The enzymes ALT, AST and LDH are notable markers of hepatocyte injury; however; LDH is less specific (Saki *et al.*, 2011). LDH activity has also been reported to increase in ischemic liver injury (Wafeka, 2010), myocardial or pulmonary infarction, kidney, heart, liver, lungs and skeletal muscle damage (Saki *et al.*, 2011). Hence, the reduction of ALT, AST and LDH activities in the RTV extract treated group suggests that the extracts may protect against ischemic liver or hepatocyte injury. Also the reduction in serum γ -GT activity, an enzyme which is an independent risk factor in cardiovascular mortality (Sakuta *et al.*, 2007) or prognostic index in chronic forms of coronary heart disease, congestive heart failure, and ischemic or hemorrhagic stroke (Saki *et al.*, 2011) suggests that the extract may protect against cardiovascular diseases. The exact mechanism of action of the RTV extract is unknown but it may be related to the presence of phytochemicals such as flavonoids and organosulphur compounds which are known to possess anti-cholesterolemic properties (Myron, 2004).

The results revealed an insignificant reduction in the serum concentration of total protein, a significant ($p < 0.05$) reduction in serum albumin and a significant increase ($p < 0.05$) in serum total bilirubin in HCD fed rats compared to animals on standard diet (Table 14). Co-treatment of rats with RTV extract significantly prevented a reduction in total protein, albumin and significantly reduced elevated bilirubin at both doses used. The activity of the extract is similar to the standard drug (Atorvastatin). The reduction in serum albumin concentration and elevated bilirubin may be due to hypercholesterolemia induced liver

damage (Wafeka, 2010) as evident in the increase in activities of marker enzymes of liver damage (Table 14). Co-administration of the extracts or drug might have prevented liver damage resulting in the improvement of the status of albumin and bilirubin.

5.8 Conclusion

The extract of *T. violacea* rhizomes has demonstrated strong anti-lipidemic, hypolipidemia and hepatoprotective properties. This result is in support of its use in folk medicine in the treatment of lipid related diseases.

CHAPTER 6

IN VIVO ANTI-ATHEROGENIC PROPERTY OF RHIZOMES OF *TULBAGHIA VIOLACEA* IN DIET INDUCED ATHEROGENIC RATS.

Published in International Journal of Molecular Sciences, 2012, 13, 12747-12760

6.0 INTRODUCTION

Atherosclerosis is a multifactorial disease of the large and medium-sized muscular arteries and the leading cause of morbidity and mortality in industrialized countries (Braunwald, 1997). It is characterized by endothelial dysfunction, vascular inflammation, and build-up of lipids, cholesterol (Ming-Shi *et al.*, 2008), calcium and cellular debris within the intima of the vessel wall (Anuradha and Raji, 2009). These build-up results in plaque formation, vascular remodelling, acute and chronic luminal obstruction, abnormalities of blood flow and diminished oxygen supply to target organs (Bibave *et al.*, 2011). Depending on the location of the blocked arteries, atherosclerosis may lead to complications generally referred to as cardiovascular diseases (coronary artery disease, carotid artery disease, isperipheral artery disease, aneurysms, heart attack and stroke). Lewis (2009) reported that successful treatment minimizes lifetime chances of cardiovascular events, morbidity, and mortality. It was suggested that risk factors for atherosclerosis should be monitored, beginning in childhood, even in asymptomatic patients. Modifiable factors (e.g., blood pressure, smoking, and serum lipids) and non-modifiable factors (e.g., age and family history) should be assessed (Sandra, 2009). Improved lifestyle such as dietary choices, increased exercise, and smoking cessation combined with lipid-lowering pharmacotherapy and antihypertensive medication may also be employed in the management and prevention of atherosclerosis (Sandra, 2009).

However, the treatment and management of this disease is still a challenge to the medical system (Jain *et al.*, 1986). Though, a large number of hypolipidemic drugs are currently available in the market, they lack desired properties such as efficacy and safety of long-term use, cost and simplicity of administration (Davidson and Tooth, 2004). These deficiencies have led to an increase in the demand for an inexpensive, affordable medication without any adverse side effects. Recently, attention has been focused on a number of

medicinal plants used in the treatment of cardiovascular disease because of their reported lipid lowering, anti-anginal, antioxidant and cardioprotective effect. One of the commonly used plant remedies in the Eastern Cape of South Africa for the treatment of heart disease the rhizomes of *Tulbaghia violacea* Harv (Olorunnisola *et al.*, 2011).

Tulbaghia violacea is a fast-growing, bulbous plant that reaches a height of 0.5 m. It was reported to possess many biological activities such as *in vitro* antithrombotic activity (Bungu *et al.*, 2008), *in vitro* antioxidant activity (Olorunnisola *et al.*, 2011), Antibacterial activities against *Staphylococcus aureus* and *Bacillus subtilis* (Gaidamashivili and Staden (2001), *in vitro* anticancer (Bungu *et al.*, 2006) and anthelmintic activity (McGaw *et al.*, 2000). It is believed that *T. violacea* may possess biological activities similar to garlic (*Allium sativum*) since both belong to the same Alliaceae family (Wyk *et al.*, 1997). However, most of the biological activities demonstrated by garlic remain to be investigated in *T. violacea*.

In the present study, we have for the first time investigated the effect of the extract of *T. violacea* rhizomes on markers of endothelial dysfunction, lipid profile and tissue antioxidant status in diet induced atherosclerogenic rats.

6.1 Materials and Methods

6.2 Collection of Plant Materials was as described in section 3.2

6.2.1 Preparation and Extraction of Plant Materials was as described in section 3.3

6.3 Animals

Healthy, female, Wister albino rats (137-165 g) were randomly assigned to control and treated groups (6 animals per group/cage). They were maintained in standard environmental conditions ($22 \pm 2^{\circ}\text{C}$, 12:12 h dark/light cycle, humidity: 45-50%). The animals were allowed

to acclimatize to the environment for 7 days and supplied with a standard pellet diet (Hindustan Lever Ltd., Bangalore) and water *ad libitum*. Before induction of atherosclerosis, the weight of the individual animals and their plasma cholesterol levels were determined. All animals were obtained from the animal house of the laboratory of School of Biological Sciences, University of Fort Hare Alice, and South Africa. All procedures used in the present study followed the “Principles of Laboratory Animal Care” from NIH Publication No.85-23 and were approved by the Animal Ethics Committee of our university.

6.4 Experiment Design

The experimental animals were divided into five groups and atherogenic diet was prepared according to the method described by Sunder *et al.* (2010). Briefly, Normal rat chow plus 4% cholesterol, 1% cholic acid and 0.5% thiouracil.

Group 1: Experimental animals fed with a normal diet and orally administered 1ml distilled water served as control.

Group II: Negative control rats were fed with an atherogenic diet.

Group III and IV Experimental animals were fed orally with an atherogenic diet supplemented orally with extract of *T. violacea* (0.25 and 0.50g/kg body weight, respectively) once daily for 2 weeks

Group V rats were fed with an atherogenic diet (normal rat chow plus atherogenic die) supplemented with standard atorvastatin orally (30 mg/kg body weight) suspended in distilled water once daily for 2 weeks.

6.5 Sample Collection and Preparation

At the end of the 2 weeks, overnight fasted rats were sacrificed under ether anesthesia. Blood samples were collected with vacuum tubes with/ without EDTA from each rat for determination of hematological parameters and biochemical analysis, respectively. The blood sample collected in a plain tube was centrifuged at 3000 g for 10 minutes to obtain serum for biochemical analysis. Immediately after collecting blood, the liver and aorta from each rat were removed, and placed into clean and dry tubes. Liver and aorta homogenate were prepared as described by Noori *et al.* (2012). Briefly, livers were perfused with saline and homogenized in chilled KCl (1.17%) using a homogenizer. The homogenates were then centrifuged at 800 g for 5 minutes at 4° C to get post mitochondrial supernatant. Whole aorta tissue was homogenized in KH_2PO_4 buffer (100 mM) containing EDTA (1 mM) (pH 7.4) (1:10 w/v) and centrifuged at 12000 g, for 30 min, at 4°C. The supernatant was used for biochemical evaluation.

6.6 Hematological Analysis

Hematological analysis was carried out using using veterinary blood counter (Medonic CA, 620 Sweden). Hematological parameters assayed included fibrinogen, white blood cell (WBC), red blood cell (RBC) and differential leukocyte counts, packed cell per volume (PCV) and platelet counts (PT).

6.7 Biochemical Assay

Biochemical parameters, serum alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB), Serum triglyceride (TG), total serum cholesterol (TC), high density lipoprotein cholesterol (HDL-c), low density lipoprotein LDL-c and very low density lipoprotein (VLDL-c) were measured using a Piccolo automated

chemistry analyser (Abaxis, Inc. Union City. CA, USA). NO production was determined by measuring the accumulation of nitrite in the aorta supernatant. Nitrite in aorta supernatants was measured, as described by Green *et al.* (1982), by adding 100 µl of Griess reagent [1 % sulfonamide and 0.1% N-(1-Naphthyl) ethylenediamine in 5 % phosphoric acid] to 100 µl supernatant from samples and incubating the mixture for 10 min at room temperature. The OD at 550 nm was measured using a Spectra max micro-plate reader (Molecular Devices, Bio Tek, and USA). The nitrite concentration in µM was calculated from a sodium nitrite standard curve. Tissue antioxidant enzyme activities were also monitored in the liver and aorta supernatant.

6.7.1 Determination of catalase activity (CAT)

Catalase activity was measured, as described by Pari and Latha (2004). Briefly, the tissue was homogenized in 0.01 M phosphate buffer (pH 7.0) and centrifuged at 5000 rpm. The reaction mixture consisted of 0.4 ml of hydrogen peroxide (0.2 M), 1 ml of 0.01 M phosphate buffer (pH 7.0) and 0.1 ml of liver homogenate (10 % w/v). The reaction of the mixture was stopped by adding 2 ml of dichromate-acetic acid reagent (5 % K₂Cr₂O₇ prepared in glacial acetic acid). The change in the absorbance was measured at 620 nm and recorded. Percentage inhibition was calculated using the equation:

$$\% \text{ catalase inhibition} = [(\text{normal activity} - \text{inhibited activity}) / (\text{normal activity})] \times 100\%.$$

6.7.2 Determination of superoxide dismutase activity:

Activity of superoxide dismutase was determined as described by Misra and Fridovich (1972). The assay mixture contained 0.5 ml of hepatic PMS, 1 ml of 50 mM sodium carbonate, 0.4 ml of 25 µM nitroblue tetrazolium and 0.2 ml of freshly prepared 0.1 mM hydroxylamine hydrochloride. The reaction mixture was mixed quickly by inversion

followed by the addition of clear supernatant of 0.1 ml of liver homogenate (10% w/v). The change in absorbance was recorded at 560 nm. Percentage inhibition was calculated using this equation:

$$\% \text{ superoxide dismutase inhibition} = \frac{[(\text{normal activity} - \text{inhibited activity}) / (\text{normal activity})]}{\times 100\%}$$

6.7.3 Malondialdehyde (MDA)

The method described by Ohkawa *et al*, (1979) was employed to determine the level lipid peroxidation in the animal tissues. Briefly, the reaction mixture of 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid was added to 0.2 ml of 10% (w/v) of the homogenate. The mixture was made up to 4.0 ml with distilled water and heated at 95°C for 60 minutes. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol & pyridine (15:1 v/v) was added and the mixture centrifuged at 2,000 g for 10 min. The organic layer was removed and its absorbance measured at 532 nm and compared with those obtained from MDA standards.

6.8 Histopathological Analysis of the Liver and Aorta

The liver and aorta of animals from each group were fixed in 10% formaldehyde, dehydrated and paraffin blocks prepared for histopathological examination. The blocks were sectioned at 5-7 μ and stained with heamatoxylin.

6.9 Statistical Analysis

All data were expressed as mean $\pm$ SD of six replicates and were subjected to one way analysis of variance (ANOVA) followed by Duncan multiple range tests to determine

significant differences in all the parameters. Values were considered statistically significant at $p < 0.05$.

6.10 Results and Discussion

Protection against arterial endothelial injuries such as the development of fatty streak plaques and in-vessel-wall cholesterol accumulation may prevent atherosclerosis. Hyperlipidemia and oxidative stress have been reported to play a critical role in endothelial dysfunction and most chronic diseases such as atherosclerosis (Leopld and Loscalzo, 2008). In the present study, the effect of methanolic extract of *Tulbaghia violacea* on weight in normal and rats fed with atherogenic diet was investigated. Food intake, body weight, and water consumption were similar for all the groups during the two weeks study (Table 15). The results revealed a significant ($p < 0.05$) increased in weight of animal fed with atherogenic diet compared with the animals on normal diet (Table 15). High cholesterol content (cholesterol 1%) in atherogenic diets has been reported to lead to increased body weight in experimental rats (Kanthlal *et al.*, 2012). The present study are consistent with the above observation. Co-administration of the extracts (250 mg/kg and 500 mg/kg) and the standard drug (atorvastatin: 30 mg/kg) significantly reduced ($p < 0.05$) the increase in body weight of animals on atherogenic diet. The results suggest that the extract may be employed in weight control.

Table 15: Food intake and percentage change in weight of experimental animals.

Treatment	AIW (g)	AFW	% weight gain	FI (g/2 weeks)
Control	151.0 ± 0.11	208.0 ± 0.14	38.0	341.11
Athero-diet	152.1 ± 0.12	237.1 ± 0.12 *	55.9	353.62
Athero-diet + TVR (250 mg/kg)	150.2 ± 0.10	215.2 ± 0.11	36.7	346.24
Athero-diet + TVR (500 mg/kg)	153.0 ± 0.13	210.3 ± 0.13	34.6	343.40
Athero-diet + TVR (30 mg ator)	152.4 ± 0.12	209.1 ± 0.11	37.2	342.21

Ath-diet represents an atherogenic diet, TVR represents *Tulbaghia violacea* and Ator represent atorvastatin, AIW represents the average initial weight, FW represents the average final weight and FI represents food intake. * represents a significant difference ($p < 0.05$) from the control.

The results (Table 16) in the present study, revealed that rats on atherogenic diet showed significant ($p < 0.05$) increase in serum TC, TG, LDL-c, VLDL-c and a significant decrease in HDL-c compared with normal rats. This observation is consistent with the reports of Azonov *et al.* (2008) and Sunder *et al.* (2010). Elevated serum cholesterol, TG, and VLDL-c, LDL-c and decreased HDL-c has been implicated in the etiology of cardiovascular diseases (CHD) (Shankar *et al.*, 2008). High serum lipids contribute to the development of cardiovascular diseases in various ways. According to Gokkusu and Mostafazadeh (2003) and Maxfield and Tabas (2005), hypercholesterolemia increases aortic thiobarbituric reacting substance (TBARS) or malondialdehyde and oxygen radicals, resulting in endothelial cell injury, modulation of cell adhesion molecules and eventually the development of atherosclerosis.

Co-treatment of methanolic extracts of TVR along with an atherogenic diet significantly ($p < 0.05$) reduced serum total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) in a dose dependent manner. The

anti-hyperlipidemia activity of the extract was highest at 500 mg/kg.bwt. The extract significantly increased ($p < 0.05$) high density lipoprotein (HDL) when compared to the atherogenic control group (Table 16). Similar observations have been reported for two species of garlic (*Allium sativum* and *Allium tuberosum* (Raghuveer, 2008). This indicates that TVR may help to increase the transport of peripheral tissue cholesterol to the liver and thereby decrease blood cholesterol when concomitantly fed with an atherogenic diet. The atherogenic index ratio ($TC - HDL-C / HDL-C$) is a powerful indicator of the risk of cardiovascular disease (Brehm *et al.*, 2004; Botham *et al.*, 2006). The higher the value (≥ 4.5), the higher the risk of developing cardiovascular disease and vice versa (Saikia and Lama, 2011). In this study, we observed that the TVR significantly ($p < 0.001$) reduced atherogenic index ratio ($TC - HDL-C / HDL-C$) in a dose dependant manner (Table 16). A lower atherogenic index ratio (≤ 3) indicates protection against coronary heart disease (Law, 1999). The antilipidemia activity of TVR extract may be attributed to the inhibitory effect of its organo-sulfur constituents (Olorunnisola *et al.*, 2011). The anti-lipidemia activity of the extracts of compared favorably with the standard drug (Atorvastatin) especially at the highest concentration of (500 mg/kg). The triacylglycerol lowering effect of TVR extract is an added advantage as elevated triglyceride has been identified as an independent risk factor for cardiovascular disease (Bainton *et al.*, 1992). Hypertriglyceridaemia is frequently caused by elevated VLDL levels. The anti-triglyceridemia effect may be due to the ability extract/atorvastatin to inhibit fatty acid synthesis and some metabolic enzymes such as fatty acid synthetase and glucose-6-phosphate dehydrogenase (Yeh and Liu, 2001).

Table 16: Effect of methanolic extract of TVR on serum lipid profile in diet induced atherogenic rats.

Treatment (mg/kg)	TG (mg/dl)	TC (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	HDL (mg/dl)
Control	63.32 ± 0.25 ^a	75.11 ± 0.21 ^a	7.45 ± 0.23 ^a	15.94 ± 0.50 ^a	39.10 ± 0.41 ^a
Ath diet	99.3 ± 1.23 ^b	148.3 ± 0.23 ^b	25.24 ± 0.17 ^b	38.12 ± 0.19 ^b	11.59 ± 0.35 ^b
TVR (250)	79.35 ± 0.43 ^c	95.21 ± 0.22 ^c	19.18 ± 0.15 ^c	25.43 ± 0.23 ^c	21.61 ± 0.27 ^c
TVR (500)	71.78 ± 0.21 ^d	83.11 ± 0.25 ^d	10.44 ± 0.21 ^d	19.25 ± 0.14 ^d	29.41 ± 0.19 ^d
Ator- (30)	66.16 ± 0.24 ^a	80.34 ± 0.31 ^d	8.54 ± 0.32 ^d	16.19 ± 0.11 ^d	31.29 ± 0.16 ^d

Values are Mean ± SD; n = 6, values with different superscripts (a, b, c, d) in the same column are significantly different from the normal control (P < 0.05) or atherogenic control group (Ath diet). TVR represents *Tulbaghia violacea rhizome*, Ath diet represents atherogenic diet and Ator-represents atorvastatin.

The ability of the TVR to protect against aortic, liver antioxidant enzyme depletion and endothelium dysfunction was also investigated. The results (fig 25 and 26) revealed a significant (p<0.05) increase in the activity of superoxide dismutase (SOD), catalase (CAT) and TBARS levels in both aorta and liver of diet induced atherosclerogenic control group compared with the animals on standard diet. The increase in TBARS formation is consistent with observations reported by (Yanling *et al.* (2009). Yanling *et al.* (2009) reported an increase in lipid peroxidation and decrease in the tissue concentrations scavenging enzymes (super oxide dismutase and catalase), in atherogenic diet-fed rats. The elevated antioxidant enzyme activities in both hepatic and aortic antioxidant enzyme activities (SOD and CAT) in this study contradict their reports (fig 25 and 26). The increase antioxidant enzyme activities may be as a result of early response or resistance to oxidative insults and lipid peroxidation occasioned by the atherogenic diet.

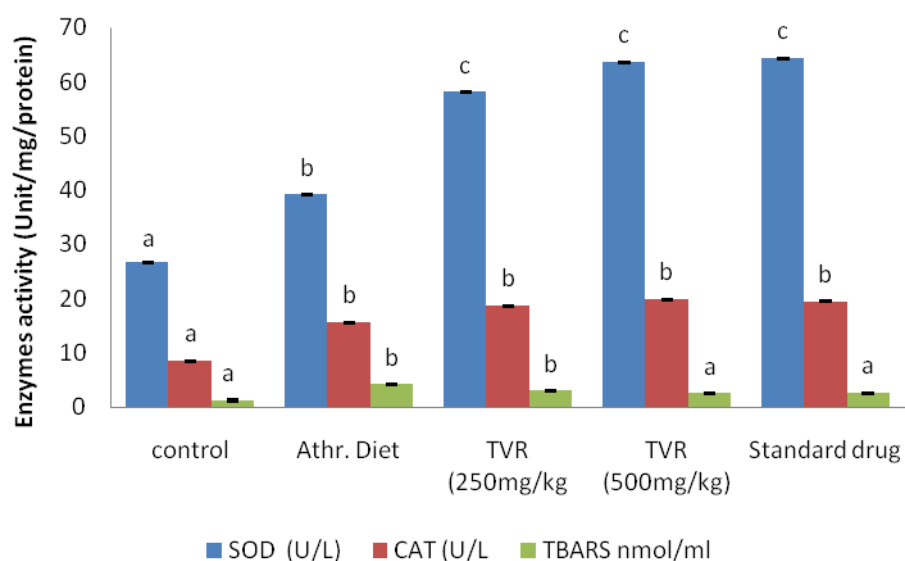


Figure 24: Effect of various doses of RTV on activity of tissue antioxidant enzymes in aorta of diet induced atherogenic rats. Mean values with the same letter subscripts within the different group are not significantly different ($p < 0.05$)

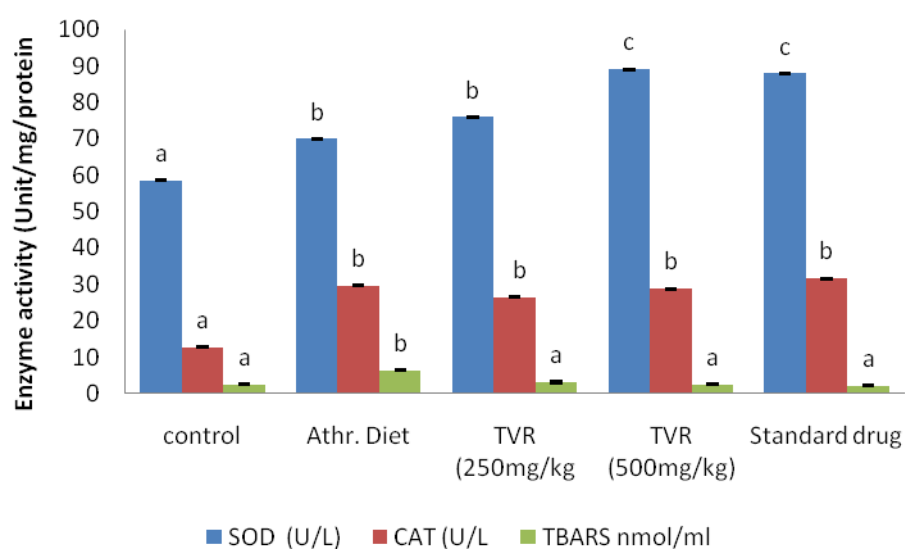


Figure 25: Effect of various doses of RTV on activity of tissue antioxidant enzymes in liver of diet induced atherogenic rats. Mean values with the same letter subscripts within the different group are not significantly different ($p < 0.05$).

Co-administration of the extract (250 and 500 mg/kg. bwt) and atorvastatin (30 mg/kg. bwt) along with the atherogenic diet significantly ($P<0.05$) increased the enzyme activities (SOD and CAT) in both hepatic and aortic tissue but caused a significant reduction ($p<0.05$) in the level of TBARS compared with an atherogenic control group (fig 24 and 25).

The increase in antioxidant enzyme activities in the extract treated group may be due to the ability of the extract to activate enzyme synthesis. Our results also demonstrated that methanolic extracts of TVR may protect the liver and aorta against atherogenic oxidative stress. The protective potential of the extract was further supported by the significant ($P<0.05$) reduction in the levels of platelets and fibrinogen, and a significant increase ($p<0.05$) in the level of nitric oxide (NO) in the extracts treated group compared with atherogenic control (Table 17). The activity of the extract was comparable to the ameliorating effect of the standard drug (atorvastatin) on endothelium dysfunction.

Increased serum platelet, fibrinogen and decreased endothelial nitric oxide have been reported in endothelial dysfunction and the development of atherosclerosis (Sharma *et al.*, 2010). Platelets play major role in the vascular wall homeostasis and etiology of atherosclerosis (Ratanachamnong *et al.*, 2012). They may adhere to exposed sub-endothelium after endothelial injury and release vasoactive substances that induce smooth muscle cell migration and proliferation (Hoak, 1988), induce development of fatty streaks by serving as a lipid source (Chandler and Hand, 1961) or promote foam cell formation (Mendelsohn and Loscalzo, 1988). Platelets may release many substances that can induce further platelet accumulation and activation, vasoconstriction, thrombosis, and mitogenesis, including ADP, serotonin, platelet-derived growth factor, fibroblast growth factor, ADP, serotonin, platelet factor 4, and β -thromboglobulin (Stein *et al.*, 1989; Ware and Collier, 1994) and superoxide anion (Ryszawa *et al.*, 2006), all of which play a significant role in the development and progression of atherosclerosis. In this experiment, increased platelet and fibrinogen

concentration was observed while; Nitric oxide concentration was significantly ($p<0.05$) lower in atherogenic diet-fed rats. The extract at 250 and 500 mg/kg caused a significant ($p<0.05$) dose dependent reduction in platelet and fibrinogen concentrations in atherogenic treated rats. It also restores the level of Nitric oxide to near normal (Table 17). The ability of the extract to restore the level of platelet and fibrinogen to near normal in the atherogenic treated group, showed that the extract can protect against atherogenic diet induced endothelial damage. The anti-platelet properties of the extracts may be due to its polyphenol content (Ryszawa *et al.*, 2006). Pignatelli *et al.* (2006) reported that polyphenols inhibit platelet NADPH oxidase in a protein kinase C (PKC) dependent manner in addition to their free radical scavenging properties.

Table 17: Effect of TVR extract on markers of endothelium dysfunction in diet induced atherosclerogenic rats.

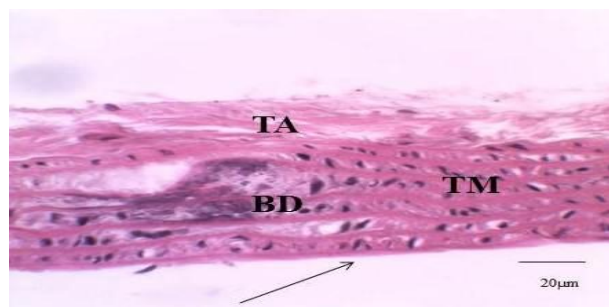
Treatment	PLT (g/l)	Fibrinogen (g/l)	NO ($\mu\text{mol/L}$)
Normal	405.2 $\pm$ 0.25 ^a	1.95 $\pm$ 0.43 ^a	57.33 $\pm$ 0.11 ^a
Ath diet	847. $\pm$ 0.21 ^b	3.21 $\pm$ 0.28 ^b	46.38 $\pm$ 0.18 ^b
TVR (250 mg/kg)	490.6 $\pm$ 0.23 ^c	2.63 $\pm$ 0.25 ^c	51.36 $\pm$ 0.25 ^a
TVR (500 mg/kg)	420.4 $\pm$ 0.16 ^d	2.01 $\pm$ 0.39 ^d	53.28 $\pm$ 0.35 ^a
Ato- (30mg/kg)	415.89 $\pm$ 0.51 ^d	1.99 $\pm$ 0.16 ^d	55.19 $\pm$ 0.22 ^a

Values are Mean $\pm$ SD; n = 6, values with different superscripts (a, b, c, d) in the same column are significantly different from the normal control ($P < 0.05$) and atherogenic control group ($p<0.05$). TVR represents *Tulbaghia violacea rhizome*, Ath diet represents atherosclerotic diet while Ato- represents atorvastatin.

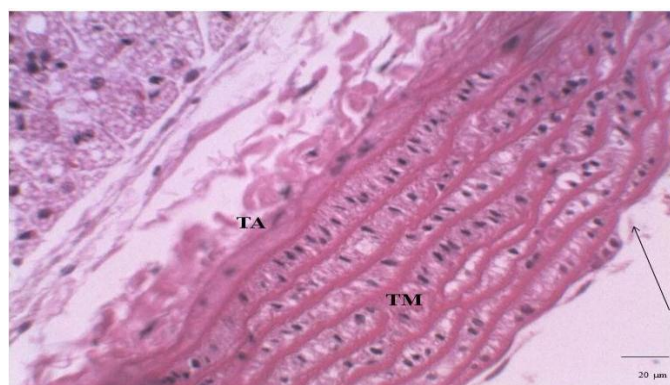
Nitric oxide is a potent vasodilator, an inhibitor of platelet aggregation, smooth muscle cell proliferation and adhesion of monocytes to endothelial cells (Ada and Alfredo, 2004). Endothelial damage occasioned by atherogenic diet induced oxidative insults might result in the destabilization of endothelial enzymes generating NO (endothelial nitric oxide synthase (eNOS)) leading to reduced NO production and endothelium dysfunction (Lokhande *et al.*,

2006). The ability of the extract to restore nitric oxide concentration to near normal in extract treated groups compared with the atherogenic control may be connected to the antioxidant, antilipidemia and antiplatelet properties of the TVR which protect the endothelial against atherogenic diet induced damage. In addition, the extract may share a similar mechanism of action with atorvastatin drug which has been reported to prevent hypoxia-induced down regulation of eNOS in endothelial cell by stabilizing eNOS mRNA leading to increase NO production (Lauf *et al.*, 1997). Histopathology evidence (figure 26) revealed that the extracts protect against swelling of the fibres in the wall or foam cell formation in the aorta by inhibiting mononuclear cell adhesion, their emigration into the intima and an accumulation of lipids in the vessel wall.

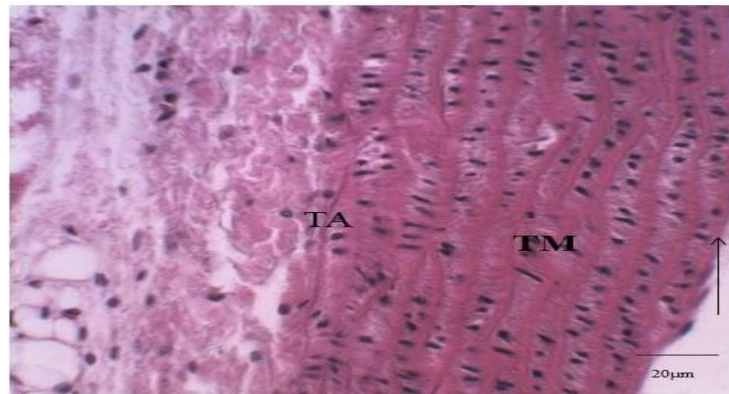
A



B



C



D

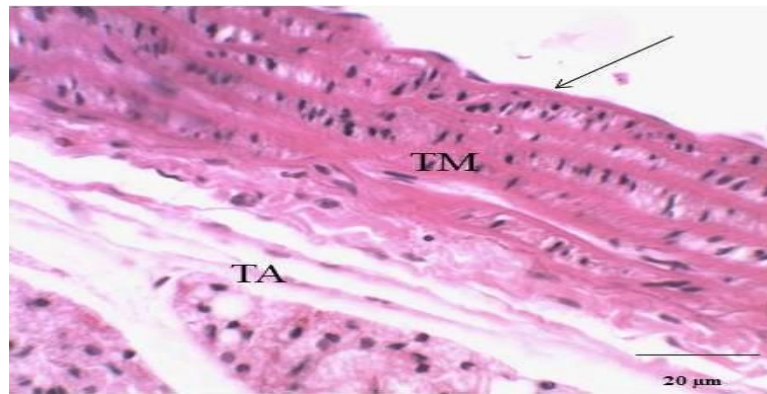


Figure 26: Photomicrograph of aorta of rat. (A) the aorta of atherogenic rat; (B) control aorta of rat; (C) the aorta of (atherogenic diet +500 mg/kg of RTV) rat; (D) the aorta of (atherogenic diet + 30mg/kg of atorvastatin) rat. scale bar: 20 μm, H & E stain, ×400 magnification).

The protective potential of extracts of TVR against atherogenic diet induced liver damage was also assessed in the present study. Table 18 revealed that all serum markers of liver or kidney damage were elevated in rats fed with an atherogenic diet alone compared with animals on a standard rat diet.

Table 18: Effect of methanolic extract of TVR on serum markers of liver and kidney damage in diet induced atherogenic rats.

Group	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	LDH (IU/L)	T-Bilirubin (mg/dl)	Creatinine (μmol/l)
Normal	23.12 ± 0.15 ^a	26.03±0.13 ^a	42.33±0.17 ^a	69.18±0.11 ^a	0.21± 0.21 ^a	61.12±0.50 ^a
Ath diet	47.32 ± 0.21 ^b	51.21±0.18 ^b	92.38±0.14 ^b	138.3±0.23 ^b	5.43±0.25 ^b	67.11±0.32 ^b
TVR (250 mg/kg)	38.61±0.33 ^c	41.13±0.15 ^c	58.22±0.45 ^c	98.12±0.20 ^c	3.98±0.19 ^c	65.01±0.21 ^c
TVR (500 mg/kg)	31.55±0.16 ^c	35.43±0.19 ^d	51.14±0.31 ^c	79.33±0.35 ^d	2.14±0.25 ^d	62.23±0.38 ^a
Ato- (30mg/kg)	28.89±0.51 ^a	34.63±0.16 ^d	50.69±0.42 ^c	73.41±0.31 ^d	2.11±0.24 ^d	61.99±0.26 ^a

Values are Mean ± SD; n = 6, values with different superscripts (a, b, c, d) in the same column are significantly different from the control (P < 0.05). Ath diet represents an atherosclerogenic diet, TVR represent *Tulbaghia violacea* rhizomes while T-Bilirubin represents total bilirubin.

The observed increased in the serum marker enzymes of hepatic tissue of rats fed with atherogenic diet is likely due to cellular damage caused by atherogenic diet induced steatohepatitis with cellular ballooning via cholesterol-induced oxidative stress (Matsuzawa *et al.*, 2007) which leads to lipid peroxidation of biomolecules and leakage of cellular components. The results are in agreement with the report of Sunder *et al.* (2010) in which they reported that atherogenic diet caused a significant increase in the serum markers of liver (LDH, AST, ALT, ALP and bilirubin) and kidney damage (creatinine). Atherogenic diet has been reported to induced glomerulosclerosis / nephropathy and mild tubular and hepatic damage in experimental rats (Sunder *et al.*, 2010). Co-treatment with RTV with an atherogenic diet significantly protected against elevated serum markers of liver and kidney damage in a dose dependant manner. The results of the present, study revealed that the

kidney and hepatoprotective properties of the extract compared favorably with the standard drug used as positive control in this study. The protection against kidney hepatic damage by the extract may be due to its phytochemical components such flavonoids and saponins (Olorunnisola *et al.*, 2011) which have been reported to possess hepatoprotective effects (Panigrahi *et al.*, 2010; Khatri *et al.*, 2010).

6.11 Conclusion

The present study showed that the methanolic extract of TVR is protective against atherogenic diet induced aortic pathology, enzyme depletion and hepatic damage by preventing oxidative stress induced endothelium dysfunction.

CHAPTER 7

GENERAL DISCUSSION

7.0 General Discussion

Atherosclerosis will be one of the leading causes worldwide of mortality and morbidity by the year 2030 (Garcia-Garcia *et al.*, 2010). The expected increase in the burden of atherosclerosis is associated with rapid urbanization and 21st-century lifestyles: tobacco use, unhealthy diet, insufficient physical activity and the harmful use of alcohol (WHO, 2010). Hyperlipidemia due to diet, particularly elevated serum cholesterol and its carrier LDL, are known to be associated with an increased risk of atherosclerosis (Orolin *et al.*, 2007). In humans, a 15% increase in level of LDL-C can increase the risk of coronary heart diseases by about 15- 45% (Radjabian and Huseni, 2007).). It has been well established that oxidative stress occasioned by lipid peroxidation of LDL plays a critical role in etiology of many pathological processes including atherosclerosis (Skottova *et al.*, 2000; Skottova and Krecman, 1998). The side effect of orthodox drugs and high cost of procurement has turned the attention of the world, especially developing countries, to phytotherapy. It has been shown that extracts from plants such as garlic and onion possess a large group of naturally occurring antioxidants that could inhibit lipid peroxidation of LDL and could serves in scavenging free radicals (Rankin and Leake, 1988). Recent evidence revealed that garlic and onion are effective in preventing cardiovascular disease because of their hypocholesterolemic, hypolipidemic and antilipidemia activity (Shela *et al.*, 2011).

In the present study, medicinal plants employed in the management of cardiovascular disease and hypercholesterolemia was identified through structured questionnaires and interviews with traditional healers in Nkonkobe Municipality, Eastern Cape of South Africa. *Tulbaghia violacea* Harv. a member of Liliaceae family, was repeatedly noted in the survey and was therefore selected for further study to evaluate its potential as a treatment for heart diseases. The results of our ethnobotanical study (Olorunnisola *et al.*, 2011a) informed the choice of *Tulbaghia violacea* rhizomes for further study.

7.1 Toxicity Evaluation

In this study, a dose range for lethality of extracts of *Tulbaghia violacea* rhizomes was determined using brine shrimps toxicity test. The results of the *in vitro* toxicity test revealed that the essential oil, methanolic fresh and dried extracts of the plant were toxic. IC₅₀ values of (18.18 and 19.24 µg/ml) fresh and dried methanolic extract respectively, and a considerably lower IC₅₀ (12.59 µg/ml) for the oil were obtained. The high toxicity recorded might be due to the presence of polysulfides which have been implicated as cytotoxic agents with potential anticancer, antimicrobial and antifungal activities (Leonid and Kent, 2000). The observed results also revealed that the oil extract is more toxic when compared with the fresh and dried extracts and that these cytotoxic effects were concentration dependent. However, the results of the *in vivo* acute toxicity study conducted using the OECD guidelines 420 (OECD, 2001) where the limit test dose of 5000 mg/kg of methanolic extract of rhizome of *T. violacea* was used indicated that a single oral administration of 5000 mg/kg/bwt dose does not produce mortality or significant behavioral changes during 14 of days observation. Also the extract administered at a dose of 125, 250 and 500 mg/kg/bwt respectively for a period of 28 days does not cause mortality, morbidity or change in relative weight of organs. However, significant weight gain was observed in both treated and control groups. Indicators of liver damage alanine amino transferase (ALT), aspartate amino transferase (AST) as well as total protein and albumin were not negatively affected although, the extracts caused an insignificant increase in the level of AST and a significant ($p < 0.05$) decreases in Alkaline phosphatase (ALP) and gamma glutamic transferase (gamma-GT) (ALP), suggesting that the plant may possess hepatoprotective properties.

The extract did not show any significant effect on markers of kidney functions such as albumin, creatinine and urea. Oral administration of the plant extracts also had no significant effect on hematological parameters except lymphocytes and red blood cell. The extract

produced a significant ($p < 0.05$) dose dependent increase in lymphocytes population and insignificant decrease in red blood cell. The alterations produced by the extract of *T. violacea* rhizomes on the lymphocytes as well as the insignificant decrease on the red blood cells suggest a dose selective, stimulatory effect on the bone marrow (Andrew, 1965). Histopathology examination of the liver revealed no detectable inflammation. The consumption of extract from RTV in the range of 125 – 500 mg/kg/bwt can therefore be considered relatively safe with no observed effects on normal growth, liver, kidneys or blood enzymes

7.2 Antioxidant Activity

The role of free radicals in the etiopathogenesis of human diseases such as atherosclerosis, and several other diseases has gained a lot of recognition (Flora, 2007). Under physiological conditions reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are products of normal cellular metabolism. They play a dual role, first as deleterious and then as beneficial species in living systems. The use of medicinal plants rich in antioxidants may therefore protect the human body against free radical induced oxidative damage and diseases (Chanda and Nagani, 2010). In the current study, *in vitro* antioxidant and *in vivo* antioxidant activities of the extracts of rhizomes of *Tulbaghia violacea* were assessed. The results of the *in vitro* quantitative analysis revealed that extracts of rhizomes of *T. violacea* are very rich in bioactive compounds such as flavonoids, tannins, phenolics and saponins, flavonol and proanthocyanidin. GC-MS analysis of the essential oil of the plant showed that it contains various concentrations of polysulfide. *In vitro* antioxidant activity evaluation results revealed that the fresh, dried and the oil extracts of *T. violacea* possessed strong DPPH, nitric oxide, lipid peroxidation scavenging activity, reducing power and hydrogen peroxide inhibitory capacity. Furthermore, the extract demonstrated strong antioxidant capacity in normal and hypercholesterolemic induced rats. It increased the

activities of superoxide dismutase, catalase, and glutathione peroxidase and decreased the level of malondialdehyde (MDA) in normal and hypercholesterolemic rats. The observed strong *in vitro* and *in vivo* antioxidant activities of the plant may be due to the presence of the observed bioactive compounds.

7.3 Antilipidemia and Antiatherosclerotic Activities

Derangements in lipid metabolism which result in hyperlipidemia have been reported in the pathology of various human diseases such as diabetes and atherosclerosis (Raghuveer, 2008). It has been well established that reduction of total cholesterol or low density lipoprotein cholesterol (LDLc) could lead to decreased risk of atherosclerosis and coronary heart disease (Brown *et al.*, 1998).

The anti-hyperlipidemic and anti-atherosclerotic effect of methanolic extract of *T. violacea* rhizomes at the doses of 250, and 500 mg/kg body weight were investigated in 2 % cholesterol rich diet-induced hypercholesterolemia rats for 28 days and diet (4 % cholesterol, 1 % cholic acid and 0.5 % thiouracil) induced atherosclerosis for 2 weeks. Co-administration of extracts *T. violacea* along with the high cholesterol diet or atherogenic diet significantly ($p < 0.05$) reduced serum level of total cholesterol (TC), low density lipoprotein (LDL-c), very low density lipoprotein (VLDL), triglycerides and significantly increased serum levels of high density lipoprotein in a dose dependent manner when compared with negative controls. Co-administration of the extract (250 and 500 mg/kg. bwt) and atorvastatin (30 mg/kg. bwt) along with the atherogenic diet significantly ($P < 0.05$) increased the enzyme activities (SOD and CAT) in both hepatic and aortic tissue but caused a significant reduction ($p < 0.05$) in the level of TBARS compared with an atherogenic control group (fig 24 and 25). The extract was able to prevent derangements in lipid profile and also keep the cholesterol level, LDL-c, VLDL-c to near normal. Contrary to garlic extract, *T. violacea* increased the serum

concentration of HDL-c. The extract also reduced ($p < 0.05$) TBARS formation and reversed endothelial dysfunction parameters (fibrinogen and total NO levels) and tissue antioxidant enzyme activities to near normal in atherogenic treated groups compared with atherogenic control. Histopathology revealed that the extracts prevented accumulation of cholesterol a necessary step for fatty streak formation in the aorta. In addition, the extract protected against elevated markers of liver, kidney damage and defect in hematological parameters usually observed in hypercholesterolemia and atherogenic rats.

The extract of RTV reduces the effects of hypercholesterolemia induced cardiovascular diseases and biochemical defect and therefore these studies support the folk usage of this plant the in treatment of hypercholesterolemic induced heart diseases

7.5 Possible Mechanisms of Action

The present study showed that rhizomes of *Tulbaghia violacea* Harva. possess considerable antioxidant, antilipidemia and anti-atherosclerogenic properties. Our results indicated that *T. violacea* contains bioactive compounds such as dimethyl disulfide, dimethyl trisulfide, (methyl-methylthio) methyl-2,4-dithiapentane, ethanol,3-(methylthio)-, propanenitrile, tannins, flavonoids , phenol, proanthocyanidin and flavonol (Olorunnisola *et al.*, 2011) which may be responsible for the observed pharmacological activities.

The organosulphur and other photochemical compounds especially flavonoids detected in this plant have been reported to protect the vascular endothelium from free radical damage by reducing NO oxidation and increasing NO bioavailability, thereby contributing to endothelium membrane stability and protection against atherosclerosis (Grassi *et al.*, 2009). This may be one of the mechanisms by which *T. violacea* protects the aorta against hypercholesterolemia induced atherosclerosis. The *in vivo* superoxide ion scavenging ability of the extract may protect against oxidation of proteins and the lipid moiety of LDL-

Cholesterol (Dillon *et al.*, 2003) which has been identified as a critical step in the etiopathology of atherosclerosis.

Extract of TVR significantly decrease plasma or serum lipid and it possible mechanism of action may be related to inhibition of HMG-CoA reductase a rate limiting enzyme in the conversion of HMG-CoA to mevalonic acid, a cholesterol precursor (Camelia, and Anca, 2001). Sequence of biochemical events that follows this action include decreased hepatic cholesterol synthesis, up regulated LDL receptor synthesis and increased LDL cholesterol clearance from the plasma to the liver cells (Camelia and Anca, 2001). According to Ginsberg *et al.* (1987) atorvastatin inhibit hepatic synthesis of apolipoprotein B-100 with a resultant reduction in synthesis and secretion of triglyceride rich lipoproteins. This may explain another possible mechanism by which *T. violacea* reduced serum triglyceride and lipoproteins.

7.6 CONCLUSION

Our results showed for the first time that the extract of *T. violacea* rhizomes can protect against hypercholesterolemia diet induced atherosclerosis. The likely mechanisms of its anti-atherogenic action may be related to its strong antioxidant, antilipidemia and protection against endothelium dysfunction. Our findings also revealed that the observed pharmacological activities of the extract might be due to its phytochemical constituents such as .polysulfide, flavonoids and phenolics compounds. We also confirmed folk usage of *T. violacea* rhizomes in the treatment of cardiovascular diseases and that its consumptions are relatively safe for humans.

Recommendation

We recommended that further work need to be carried out to isolate and characterised the actual compound (s) responsible for the observed pharmacological activities. The direct or indirect effect of the extract on LDL oxidation, HMG CoA synthase, macrophages, NADPH oxidase or any superoxide generating enzymes which are involved in cholesterol metabolism must be investigated in other to confirmed the actual mechanism(s) of action of *T. violacea*

References

- Abascal, K., Ganora, L., Yarnell, E (2005). The effect of freeze-drying and its implications for botanical medicine: A Review. *Phytotherapy Research* **19**: 655-660.
- Abdulazeez, Mansurah (2011). Effect of *Peristrophe bicalyculata* on lipid profile of P- 407 induced hyperlipidemic Wistar rats. *Journal of Medicinal Plants Research* **5(4)**: 490 - 494.
- Abeer temraz., Walid h el-tantawy (2008). Characterization of antioxidant activity of extract from *artemisia vulgaris*. *Pakistan Journal Pharmaceutical Sciences* **21**: 321-326.
- Abou-Arab, A.A., Abou-Donia, M.A (2000). Heavy metals in Egyptian spices and medicinal plants and the effect of processing on their levels. *Journal of Agricultural Food Chemistry* **48(6)**: 2300 -2304.
- Ada, M.C., Alfredo M. G (2004). Diet and Endothelial Function. *Biological Research* **37**: 225-230.
- Adebayo, A.H., Abolaji, A.O., Opata T.K., Adegbenro K.I (2010). Effects of ethanolic leaf extract of *Chrysophyllum albidum* G. on biochemical and haematological parameters of albino Wistar rats. *African Journal of Biotechnology* **9(14)**: 2145-2150.
- Adebayo, J.O., Adesokan, A.A., Olatunji, L.A., Buoro, D.O., Soladoye, A.O (2005). Effect of Ethanolic extract of *Bougainvillea spectabilis* leaves on haematological and serum lipid variables in rats. *Biokemistry* **17**: 45-50.
- Adel K., Néji G., Mohamed D., Radhouane, G (2011). Chemical composition and in vitro antioxidant properties of essential oil of *Ricinus communis* L. *Journal of Medicinal Plant Research* **5(8)**: 1466-1470 18.

- Admas, R.P (1995). Identification of essential oil components by gas chromatography and mass spectroscopy. Carol Stream, IL: Allured Publication.
- Akanji, M. A., Yakubu, M. T (2000). α - tocopherol protects against metabisulphite – induced tissue damage in rats. Nigerian Journal of Biochemistry. and Molecular Biology **16**:147-148.
- Akiibinu, O.M., Ogunyemi, O.E., Arinola, O.G., Adenaike, A.F., Adegoke, O.D (2008). Assessment of antioxidants and nutritional status of pulmonary tuberculosis patients in Nigeria. European. Journal General. Medicine **5(4)**: 208-211.
- Aleksander, Siger., Malgorzata, Nogala-Kalucka, Eleonora Lampart-Szczapa (2008). The content and antioxidant activity of phenolic compounds in cold-pressed plant oils. Journal of Food Lipids. **15**: 137–149.
- Ali, Ramazani., Sedigheh, Zakeri., Soroush, Sardari., Nastaran, Khodakarim., Navid, Dinparas Djadidt (2010). *In vitro* and *in vivo* anti-malarial activity of *Boerhavia elegans* and *Solanum surattense*. Malaria Journal. **9**:124
- Ali K. Ozturk and Ketan R. Bulsara (2012). The Evolving Paradigmin the Management of Intracranial Atherosclerotic Disease. International Journal of Vascular Medicine. **2012**: 5 Pg doi:10.1155/2012/289852
- Amagase, H (2006). Significance of Garlic and Its Constituents in Cancer and Cardiovascular Disease: Clarifying the Real Bioactive Constituents of Garlic. Journal of Nutrition. **136**: 716S-725S.
- Amal, A Mohamed., Gehan, A., EI- Emary., Hanaa, F.Ali (2010). Influence of some citrus essential oils on cell viability, Glutathione-s-transferase and lipid peroxidation in Ehrlich ascites carcinoma cells. Journal of American Science **6**: 10.

- Amresh, G.R., Singh, P.N., Rao, V.C (2008). Toxicological screening of traditional medicine *Laghupatha (Cissampelos pareira)* in experimental animals. *Journal. Ethnopharmacology*. **116**: 454–460.
- Amy, Berkov., Barbara, Meurer-Grimes., Kenneth, L. Purzycki (2000). Do Lecythidaceae specialists (Coleoptera, Cerambycidae) shun fetid tree species? *Biotropic* **32 (3)**: 440–451.
- Anagnostopoulou, M.A., Kefalas, P., Papageorgiou, V.P., Boskou, D (2006). Radical scavenging activity of various extracts and fractions of sweet orange peel (*Citrus sinensis*). *Food Chemistry* **94**: 19-25.
- Andrew, W (1965). *Comparative hematology* New York: Grune & Strattonp. 144 - 146.
- Anne, W and Allison, G (2006). *Anatomy and Physiology in health and illness*. Elsevier, Spain, 9th edn. 116 - 117.
- Anuradha, S., Raji, Sugumar. V (2009). Impact of coconut oil replacement in diet among obese adolescent girls. *Indian Coconut Journal*, 12-16.
- Arafa, H.M (2005). Curcumin attenuates diet-induced hypercholesterolemia in rats. *Medical Science Monitor* **11**: BR228–BR234.
- Archer, R. K., Jeffcott, L.B., Lehmann, H (1977). *Comparative clinical haematology*, Oxford: Blackwell 611-613.
- Ashafa, A.O.T., Sunmonu, T.O., Afolayan, A.J (2011). Effects of leaf and berry extracts of *Phytolacca dioica* L. on haematological and weight parameters of Wistar rats. *African Journal of Pharmacy and Pharmacology* **5(2)**: 150-154.

- Ashraf, Z.A., Hussain, M.E., Fahim, M (2005). Antiatherosclerotic effects of dietary supplementation of garlic and turmeric: restoration of endothelial function in rats. *Life Science* **77**: 837–857.
- Assy, N., Kaita, K, Mymin, D., Levy, C., Rosser, B., Minuk, G (2000). Fatty infiltration of liver in hyperlipidemic patients. *Digestive Disease of Scienc* **45**:1929–1934.
- Attia, D.M., Ni, Z.N., Boer, P., Attia, M.A., Goldschmeding, R., Koomans, H.A., Vaziri N.D., Joles, J.A (2002). Proteinuria is preceded by decreased nitric oxide synthesis and prevented by a NO donor in cholesterol-fed rats. *Kidney International* **61**: 1776-1787.
- Awais, A., Burkholz, T., Scherer, C., Abbas, M., Lehr, Claus-Michael., Diederich, M., Jacob, C (2008). ‘Naturally occurring reactive sulfur species, their activity against Caco-2 cells and possible mode of biochemical action’, *Journal of Sulfur Chemistry* **29**: 3, 251-268
- Ayoola, A.O., Akinloye, O., Oguntibeju, O.O., Oke, J. M., Odetola, A.A (2011). Antioxidant activities of *Parquetinanigrescens*. *African Journal Biotechnology* **10(24)**: 4920-4925.
- Azonov, J.A.; Khorram Khorshid, H.R.; Novitsky, Y.A.; Farhadi, M.; Ghorbanoghli, Z.; Shahhosseiny, M.H. Protective effects of setarud (IMODTM) on development of diet induced hypercholesterolemia in rabbits. *DARU*. **16 (4)**: 218-222.
- Bainton, D., Miller, N.E., Bolton, C.H., Yarnell, J.W., Sweetnam, P.M., Baker, A.I., Lewis, B., Elwood, P.C (1992). Plasma triglyceride and high density lipoprotein cholesterol as predictors of ischaemic heart disease in British men. *British Heart Journal* **68**: 60-66.

- Balakumar, P.S., Jindal, D.I., Shah, Singh, M (2007). Experimental models for vascular endothelial dysfunction. *Trends Medical Research* **2**: 12-20.
- Banerjee, K .Saurabh., Bonde, C. G (2011). Total phenolic content and antioxidant activity of extracts of *Bridelia Retusa* Spreng Bark: Impact of dielectric constant and geographical location. *Journal Medicinal Plants Research*. **5(5)**: 817-822
- Barry, S.L (1995). Animal Clinical Pathology. In: Micheal J.D, Mannfred A.H, eds. CRC Handbook of Toxicology, CRC Press, Inc. U.S.A. 517-537
- Beata, Drużyńska., Magdalena, Wojda (2007). Antioxidant properties of acetone extracts from three selected fresh and dried spices. *Polish Journal Food Nutrition Science*. **57**: 47-51.
- Beltowski, J., Wójcicka, G., Górny, D., Marciniak, A (2000). The effect of dietary-induced obesity on lipid peroxidation, antioxidant enzymes and total plasma antioxidant capacity. *Journal Physiology Pharmacology* **51(4)**: 883-896.
- Bibave, K.H., Shenoy, P.A., Mahamuni, S.P., Bandawane, D.D., Nipate, S.S., Chaudhari, P.D (2011). Preclinical Evaluation Methods for Screening of Anti-Atherosclerotic Drugs: An Overview. *Asian Journal of Biomedical and Pharmaceutical Sciences*. **1(2)**: 01-14.
- Birgel Junior, E.H., Benesi, F.J., Lenz, F.F., Birgel, E.H (1986). Estudo das variações de parâmetros hematológicos de animais de laboratório I hemograma de ratos (*Rattus norvegicus*). In: Conferência Anual da Sociedade Paulista DE Medicina Veterinária. 41., São Paulo, 1986. *Resumos*. São Paulo: Sociedade Paulista de Medicina Veterinária. 1-3.

- Blum, C.B., Levy, R.I (1987). Role of dietary intervention in the primary prevention of coronary heart disease. Individuals with high normal or elevated serum cholesterol levels should be placed on cholesterol-lowering diets. *Cardiology*. **74**:2-21.
- Bodhankar, S (2006). Text book of Pathophysiology. Nirali prakashan, 4th .edn, 2.22-2.24.
- Leon AS, Sanchez OA. 2001. Response of blood lipids to exercise training alone or combined with dietary intervention. *Medicine and Science in Sports and Exercise* **33**: S502–S515.
- Boguslaw, L (2011). Hydroxyl Radical and Its Scavengers in Health and Disease. *Oxidative Medicine and Cellular Longevity*. 1-9
- BoKang, Cui., Su, Liu., Xiao, Jun., Lin., Jun, Wang., ShuHong, Li., QiBo, Wang ShengPing, Li (2011). Effects of *Lycium Barbarum* Aqueous and Ethanol Extracts on High Fat Diet Induced Oxidative Stress in Rat Liver Tissue. *Molecucles*. **16**: 9116 - 9128.
- Botham, K.M., Mayes, P.A (2006). Harper's Illustrated Biochemistry, 26th ed.; The McGraw-Hill Companies Inc: New York, NY, USA 217–245
- Bonnefont-Rousselot, D., Therond, P., Beaudeux, J.L., Peynet, J., Legrand, A., Dlatte, J (1999). High density lipoproteins (HDL) and the oxidative hypothesis of atherosclerosis. *Clinical Chemistry and Laboratory Medicine* **37**: 939-948.
- Boudjeltia, K.Z., Legssyer, I., Van, Antwerpen P., Kisoka, R.L., Babar, S., Moguelevsky, N., Delree, P., Ducobu, J., Remacle, C., Vanhaeverbeek, M., Brohee, D (2006). Triggering of inflammatory response by myeloperoxidase-oxidized LDL. *Biochemistry and cell biology = Biochimie et biologie cellulaire*. **84 (5)**: 805-812.

- Bourcier, T., Sukhova, G., Libby, P (1997). The nuclear factor kappa-B signalling pathway participates in dysregulation of vascular smooth muscle cells in vitro and in human atherosclerosis. *Journal of Biological Chemistry*. **272**: 15817-15824.
- Boyne, A.F., Ellman, GL (1972). A methodology for analysis of tissue sulfhydryl components. *Analytical Biochemistry*. **46**: 639-653.
- Bradford, R.H., Shear, C.L., Chremos, A.N., Dujovne, C., Downton, M., Franklin, F.A., Gould, A.L., Hesney, M., Higgins, J., Hurley D.P (1991). Expanded Clinical Evaluation of Lovastatin (EXCEL) study results. I. Efficacy in modifying plasma lipoproteins and adverse event profile in 8245 patients with moderate hypercholesterolemia. *Archives of Internal Medicine* **151(1)**: 43-9.
- Braunwald, E (1997). Shattuck lecture - cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. *New England Journal of Medicine* **337**:1360-1369.
- Brehm, A., Pfeiler, G., Pacini, G., Vierhapper, H., Roden, M (2004). Relationship between serum lipoprotein ratios and insulin resistance in obesity. *Clinical Chemistry* **50**: 2316–2322.
- Brown A. S., Bakker, Arkema., R. G. and Yellen L. (1998). Treating patients with documented atherosclerosis to National Cholesterol Education Program recommended low density lipoprotein cholesterol goals with atorvastatin, fluvastatin, lovastatin and simvastatin. *Journal Molecular Cellular Cardiology*. **32**: 665- 692.
- British, Pharmacopoeia II (1980). 1909, H.M, Stationary office London.

- Bungu, L., Carminita, Frost., Clint, Brauns., Maryna, van de Venter (2006). *Tulbaghia violacea* inhibits growth and induces apoptosis in cancer cells *in vitro*. Africa Journal Biotechnology **5 (20)**: 1936 -1943.
- Bungu, Lelethu., Maryna, van de Venter., Carminita, Frost (2009). Evidence for an *in vitro* anticoagulant and antithrombotic activity in *Tulbaghia violacea*. Africa Journal Biotechnology **7 (6)**: 681-688.
- Bungu, L., Maryna, van de Venter., Carminita, Frost (2008). Evidence for *in vitro* anticoagulant and antithrombotic activity in *Tulbaghia violacea*. Africa Journal Biotechnology **7 (6)**: 681- 688.
- Burke, A.P., Kolodgie, F.D., Farb, A., Weber, D.K., Malcom, G.T., Smialek, J., Virmani, R (2001). Healed plaque ruptures and sudden coronary death: evidence that subclinical rupture has a role in plaque progression. Circulation. **103**:934-940
- Buwa, L.V., Afolayan, A.J (2009). Antimicrobial activity of some medicinal plants used for the treatment of tuberculosis in the Eastern Cape Province. South Africa. Africa Journal Biotechnology **8(23)**: 6683-6687.
- Camelia, Stancu., Anca, Sima (2001). Statins: mechanism of action and effects. Journal of Cellular and Molecular Medicine **5(4)**: 378-387.
- Carlberg, I., Mannervik, B (1985). Glutathione reductase. Methods Enzymology **113**:484 490.
- Castelli, W.P., Anderson, K., Wilson, P.W., Levy, D (1992). Lipid risk of coronary heart disease: The Framingham Study. Annal of Epidemiology **2**: 23 - 28.

- Catapano, A.L., Maggi, F.M., Tragni, E (2000). Low density lipoprotein oxidation, antioxidants, and atherosclerosis. *Current. Opinion. Cardiol.* **15 (5)**: 355-63.
- Cenarro, A (2007). Individual variation of scavenger receptor expression in human macrophages with oxidized low-density lipoprotein is associated with a differential inflammatory response. *Journal of Immunology* **179 (5)**: 3242-3248.
- Chanda, S., Daves, R (2009). In vitro models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties: An overview. *African Journal Microbiology Research* **3(13)**: 981-996.
- Chandler, A., Hand, R (1961). Phagocytized platelets: A source of lipids in human thrombi and atherosclerotic plaques. *Science* **134**: 946.
- Chanda, S. V., Nagani, K. V (2010). Antioxidant Capacity of Manilkara zapota L. Leaves Extracts Evaluated by Four in vitro Methods. *Nature and Science* **8(10)**: 260-266
- Chen, D., Misra, A., Garg, A (2002). Lipodystrophy in Human Immunodeficiency Virus Infected Patients. *The Journal of Clinical Endocrinology & Metabolism* **87 (11)**: 4845-4856.
- Cheruvu, P.K., Finn, A.V., Gardner, C., Caplain, J., Goldstein, J., Stone, G.W., Virman, R., Muller, J.E (2007). Frequency and distribution of thin-cap fibroatheroma and ruptured plaques in human coronary arteries: a pathologic study. *Journal of American College Cardiology* **50**: 940 949.
- Choi, D.J., Lee, S.J., Kang, M.J., Cho, H.S., Sung, N.J., Shin, J.H (2008). Physicochemical characteristics of black garlic (*Allium sativum* L.). *Journal of Korean Society of Food Science and Nutrition* **37**: 465–471.

- Christophe, Dunand., Michèle, Crèvecoeur., Claude, Penel (2007). Distribution of superoxide and hydrogen peroxide in Arabidopsis root and their influence on root development: possible interaction with peroxidases. *New Phytologist* **174**: 332–341
- Commerford, P.J (2005). Valvular heart disease in South Africa in 2005. *South Africa Medical Journal* **95**: 568-574.
- Crook, M.A (2006). *Clinical Chemistry and Metabolic Medicine*. 7th edn. Hodder Arnold, London 426.
- Davison, C., Levendal, R. A., Frost C. L (2012). Cardiovascular benefits of an organic extract of *Tulbaghia violacea*: Its anticoagulant and anti-platelet properties. *Journal of Medicinal Plants Research* **6(33)**: 4815-4824
- Davidson, M.H., Tooth, P.P (2004). Comparative effect of lipid lowering therapies. *Prognostic Cardiovascular Disease* **47**: 173-204.
- Decker, E.A (1998). Antioxidant mechanism. In *Food Lipids* (C. C. Akoh, D.B. Min, eds.), 397-421. Marcel Dekker, New York
- Diniz, Y.S., Burneiko, R.M., Seiva, F.R.F., Almeida, F.Q.A., Galhardi, C.M., Novelli Filho, J.L.V.B., Mani, F., Novelli, E.L.B (2008). Diet compounds, glycemic index and obesityrelated cardiac effects. *Internatinal Journal of Cardiology* **124**: 92-99.
- Dillon, S.A., Burmi, R.S., Lowe, G.M., Billigton, D., Rahman, K (2003). Antioxidant properties of aged garlic extract: an in vitro study incorporating human lowdensity lipoprotein. *Life Science* **72**: 1583-1594.

- Diouf, P.N., Stevanovic, T., Cloutier, A (2009). Study on chemical composition and anti-inflammatory activities of hot water extract *Piceamariana* bark and its proanthocyanidin-rich fractions. *Food Chemistry* **113**: 897–902.
- Divya, B.T., Mini, S (2011). *In vitro* radical scavenging activity of different extracts of *butea monosperma* bark. *International Journal of Current Pharmacology Research*. **3(3)**: 114-116.
- Doha, A, Mohamed., Thanaa, E. Hamed., Sahar, Y. Al-Okbi (2010). Reduction in hypercholesterolemia and risk of cardiovascular diseases by mixtures of plant food extracts: a study on plasma lipid profile, oxidative stress and testosterone in rats. *Grasas Y aceites* **61 (4)**: 378-389.
- Drużyńska, Beata., Magdalena, Wojda (2007). Antioxidant Properties of Acetone Extracts From Selected Fresh and Dried Spices. *Polish Journal of Food and Nutrition. Science* **57 (2)**: 47-51
- Duduku, Krishnaiah., Rosalam, Sarbatly., Rajesh, Nithyanandam (2011). A review of the antioxidant potential of medicinal plant species. *Food and Bioproducts Processing*. **89 (3)**: 217–233
- Duke, J.A (1997). *The Green Pharmacy: New Discoveries in Herbal Remedies for Common Diseases and Conditions from the World's Foremost Authorities on Healing Herbs*. Rodale,. Retrieved from: www.greenpharmacy.com
- Duncan, A.C., Jager, A.K., Van Staden, J (1999). Screening of Zulu medical plants for angiotensin converting enzyme (ACE) inhibitors. *Journal Ethnopharmacology* **68**: 63-70.

- Dyson, Alex (1998). Discovering Indigenous Healing plants of the herb and fragrance gardens at Kristenbosch (National Botanical Garden).
- El-Demerdash, F.M., Yousef, M.I., Abou El-Naga, N.I. (2005). Biochemical study on the hypoglycemic effects of onion and garlic in alloxan-induced diabetic rats. Food Chemistry and Toxicology **43**: 57
- Ellison, D Hank (1999). Handbook of chemical and biological warfare agents. CRC Press LLC, 2000 N.W. Corporate Blvd., Boca Raton, Florida 33431: 162.
- Ellman, G. L (1959). Tissue sulfhydryl groups. Archive of Biochemistry and Biophys. **17**: 214-226.
- Emad, A Shalaby., Nasr, N.F., Samia, M.El S (2011). An *in vitro* study of the antimicrobial and antioxidant efficacy of some nature essential oils. Journal of Medicinal Plant Research. **5 (6)**: 922-931.
- Erasto, P., Mbwambo, Z.H (2009). Antioxidant activity and HPTLC profile of *Lagenariasiceraria* fruits. Tanzania Journal Health Research **11(2)**: 79-83.
- Ezeonwumelu, J.O.C., Julius, A.K., Muhoho, C.N., Ajayi, A.M., Oyewale, A.A., Tanayen, J.K., Balogun, S.O., Ibrahim, A., Adzu, B., Adiukwu, C.P., Oloro, J., Kiplagat, D.M., Goji, A.D.T., Okoruwa, A.G., Onchweri, A.N., Reddy, P.M.K (2011). Biochemical and Histological Studies of Aqueous Extract of *Bidens pilosa* Leaves from Ugandan Rift Valley in Rats. British Journal of Pharmacology and Toxicology **2(6)**: 302-309.
- Feldman, B.V., Zinkl, J.G (2000). Schalm's Veterinary Hematology, 5th ed. Philadelphia, Lea Febiger 1210 -1218.

- Flora, S.J (2007). Role of free radicals and antioxidants in health and disease. *Cell and Molecular Biology* **53**: 1-2.
- Folin, O.V Ciocalteu (1927). On tyrosine and tryptophan determination in proteins. *Journal of Biological Chemistry* **73**: 627–650.
- Frederick, J.R (2009). Hypercholesterolaemia in children and young adults—current management. *Journal of Endocrinology, Metabolism and Diabetes of South Africa*. **14 (1)**: 09-12.
- Fridovich, I (1986). Superoxide dismutases. *Advanced Enzymology* **58**: 61–97.
- Furtado, J, Zambrini, H., Neto, D., Scozzafave, G., Brasileiro, R (2007). Ambulatório de Lipodistrofia do Hospital Heliópolis - Uma experiência com as correções cirúrgicas em dois anos de atendimento. *Prática hospitalar*. Vol. 58, 28, (Set-Out IX), **(28-32)**: 1679-5512.
- Garcia-Garcia, H. M., Costa, Marco A., Serruys, P.M (2010). Imaging of Coronary atherosclerosis: intravascular ultrasound. *European Heart Journal* **3**: 2456–2469
- Gaidamashvili, M., Van, Staden J (2001). Interaction of lectin-like proteins of South African medicinal plants with *Staphylococcus aureus* and *Bacillus subtilis*. *Journal of Ethnopharmacology* **80**: 131-135.
- Ganong, W.F. (2001) Review of medical physiology. 20th Edition. Lange Medical Books/McGraw Hill Companies Inc., New York. 500-515.
- Gauthaman, K., Adaikan, P.G., Prasad, R.N.V (2003). Sexual Effects of Puncturevine (*Tribulus terrestris*) Extract. *Journal of Alternative Complementary Medicine* **9**: 257-265

- George, J., Laing, M.D., Drewes, S.E (2001). Phytochemical research in South Africa. South African Journal of Science **97(3)**: 93-105.
- Georgia, Vogiatzi., Dimitris, Tousoulis., Christodoulos, Stefanadis (2009). The Role of Oxidative Stress in Atherosclerosis. Hellenic Journal Cardiology **50**: 402-409.
- Gian, P.F., Carlo, A., Saverio, S., Angelo, A (2007). Endothelial progenitor cells in the natural history of atherosclerosis. Atherosclerosis **194**: 46–54.
- Ginsberg H.N., Le N.A., Short M.P., Ramakrishnan R., Desnick R.J (1987) Suppression of apolipoprotein B production during treatment of cholesteryl ester storage disease with lovastatin: implication for regulation of apolipoprotein B synthesis. Journal Clinical Invest **80**:1692-1697.
- Ginter E, Simko V (2010). Garlic (*Allium sativum* L) and cardiovascular diseases. Bratisl Listy **111 (8)**: 452-456.
- Ginter, E., Simko, V (2010). Garlic (*Allium sativum* L.) and cardiovascular diseases. Bratisl Leky Lister **111 (8)** 452-456.
- Gokkusu, C., Mostafazadeh, T (2003). Changes of oxidative stress in various tissues by long-term administration of vitamin E in hypercholesterolemic rats. Clinica Chimica Acta **328**: 155 – 161.
- Govindarajan, R., Rastogi, S., Vijayakumar, M (2003). Antioxidant activities of *Desmodium gangeticum*. Biological and Pharmaceutical Bulletin **26**: 1427-1427
- Gowri, J., Vijaya, A.A., Achi, R.S., Archunan, G., Kalavathy, S., Sampath, K.S., Vijaya, K.K (2011). Redemptive benefit of atorvastatin in the risk factors of coronary artery disease. Journal of Pharmacy Research **4 (3)**: 627-629.

- Grassi, D., Desideri, G., Tiberti, S., Ferri, C (2009). Oxidative stress, endothelial dysfunction and prevention of cardiovascular diseases. *Agro FOOD Industry Li-tech.* **20**: 76-79
- Green, L.C., Wagner, D.A., Glogowski, J., Skippe, P., Wishnok, J.S., Tannenbaum, S.R (1982). Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. *Analytical. Biochemistry* **126**: 131-138.
- Greenland, P., Smith, S.C., Grundy, S.M. (2001). Improving Coronary Heart Disease Risk Assessment in Asymptomatic People. *Circulation.* **285**: 2486-2497 [online]. Available from: <http://circ.ahajournal.org/cgi/content/fall/101/>
- Gutteridge, J.M.C., Halliwell, B (2000). Free radicals and antioxidants in the year 2000: a historical look to the future *Annals of the New York Aca. Science* **899**: 136-147.
- Guyton, A.C., Hall, J.E (2000). *Textbook of Medical Physiology (Tenth Edition)* Harcourt International Edition, W.B Saunder Company Philadelphia. 279 - 281.
- Guyton, A.C., and Hall, J.E. (2000) *A textbook of medical physiology.* 10th Edition. W.B. Saunders Company, Philadelphia. 382-401.
- Habig, W.H., Pabst M.S., Jekpoly, W.B (1974). Glutathione transferase: a first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry* **249**: 7130-7139.
- Hansotia, T and Drucker, D.J (2005). GIP and GLP-1 as incretin hormones: lessons from single and double incretin receptor knockout mice. *Regulatory Peptides* **15**: 125-134.
- Harborne, J.B (1973). *Phytochemical Methods*, Chapman and Hall, London, pp: 113.
- Harnafi, H., Aziz, M., Amrani, S (2009). Sweet Basil (*Ocimum basilicum* L.) improves lipid metabolism in hypercholesterolemic rats. *E- SPEN, the European e-Journal of Clinical Nutrition and Metabolism* **4**: e181- e186 doi:10.1016/j.eclnm.2009.05.011.

- Hasan, S.M., Hossain, M.M., Faruque, A., Mazumder, M.E.H., Rana, M.S., Actor, R., Alam M.A (2008). Comparison of antioxidant potential of different fractions of *Commelina benghalensis* Linn. Bangladiash Journal of Life Science **20 (2)**: 9-16.
- Hassan, S.W., Ladan, M.J., Dogondaji, R.A., Umar, R.A., Bilbis, L.S., Hassan, L.G., Ebbo, A.A., Matazu, I.K (2007). Phytochemical and toxicological studies of aqueous leaves extracts of *Erythrophleum africanum*. Pakistan Journal of Biological Science **10**: 3815–3821.
- Hennekens, H.C (2000). Clinical and research challenges in risk factors for cardiovascular diseases. European Heart Journal **21**: 1917- 1921.
- Hoak, J.C (1988). Platelets and atherosclerosis. Seminars in Thrombosis Hemostasis. **14**: 202.
- Hostettman, K (1995). Marston, A. Saponins. Cambridge University Press, Cambridge. 232-286.
- Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A.B (1996). Zulu Medicinal Plants: An Inventory. Natal University Press, Kriel, S (2007). The heart and stroke foundation South Africa heart disease in South Africa Media data document.<http://www.heartfoundation.co.za/docs/heartmonth/HeartDiseaseinSA.pdf>.
- Ilhami, G.I., Haci, A.A., Mehmet, A.A (2005). Determination of in Vitro Antioxidant and Radical Scavenging Activities of Propofol. Chemical Pharmaceutical Bulletin **53(3)**: 281-285.
- Ilias, G.K., Carlos, T.M (2001). Reactive oxygen species and mitochondrial diseases. Cell & Developmental Biology **12**: 449-457.

- Imelouane, B., Amhamdi, H., WatheleT, J.P., Ankit, M., Khedid, K., Bachiri, A.EL (2009). Chemical Composition and Antimicrobial Activity of Essential Oil of Thyme (*Thymus vulgaris*) from Eastern Morocco. International Journal of Agriculture & Biology 1560–8530.
- Inhye, Kim., Jin-Young, Kim., Yu-Jin, Hwang., Kyung-A, Hwang., Ae-Son, Om., Jae Hyun, Kim., Kang-Jin, Cho (2011). The beneficial effects of aged black garlic extract on obesity and hyperlipidemia in rats fed a high-fat diet. Journal of Medicinal Plants Research **5(14)**: 3159-3168.
- Insull, W. Jnr. (2009). The Pathology of Atherosclerosis: Plaque Development and Plaque Responses to Medical Treatment. The American Journal of Medicine. **122**: S3–S14.
- Jacques, P (1994). Ascorbic acid and plasma lipids. Epidemiology. **5(1)**: 19-26.
- Jadeja, R., Thounaojam, M., Ansarullah, R.A.V., Devkar, R (2009). Phytochemical constituents and free radical scavenging activity *Clerodendronglandulosum* Coleb methanolic extract. Journal of Complementary Integrated Medicine 1: 19.
- Jadeja, Ravirajsinh., Thounaojam, Menaka., Ansarullah., Ramachandran, A.V., Devkar, Javanmardi J., Stushnoff, C., Locke, E., Vivanco, J.M (2003). Antioxidant activity and total phenolic content of Iranian Ocimum accessions. Food Chemistry. **83(4)**: 547-550.
- Jain, N. C (1993). *Essentials of veterinary hematology*. Philadelphia: Lea & Febiger. 54-71.
- Jain, N.C., Schalm, O.W., Carroll, E.J (1986) (. *Veterinary hematology*. Philadelphia: Lea & Febiger. 235-245.

- Janssen-Heininger, Y.M., Poynter, M.E., Baeuerle, P.A (2000). Recent advances towards understanding redox mechanisms in the activation of nuclear factor kappa B. *Free Radical Biology Medicine* **28**:1317–27.
- Jayanthi, P., Lalitha, P (2011). Reducing power of the solvent extracts of *eichhornia crassipes (mart.) solms.* *International Journal of Pharmacy and Pharmaceutical Science* **3 (3)**: 126128.
- Joanna, S., Thompson, Coony (2003). Herbs for serum cholesterol reduction. *The Journal of family practice* **52** (5).
- Joulain, D., König, W.A (1998). The atlas of spectral Data of Sesquiterpenes, Hydrocarbons E.B- Verlag Hamburg, Germany.
- Joulain, D., König, W.A., Hochmuth, D.H (2001). Terpenoids and related constituents of essential oils. Hamburg, Germany: Library of Mass Finder 2.1
- Juan, Ascaso., Pedro, Gonzalez Santos., Antonio, Hernandez Mijares., Alipio, Mangas Rojas., Luis, Masana., Jesus, Millan., Luis, Felipe Pallardo., Juan, Pedro-Botet., Francisco, Perez Jimenez., Xavier, Pint’o., Ignacio, Plaza., Juan, Rubi’es., Manuel, Z ‘u~ niga (2007). Management of Dyslipidemia in the Metabolic Syndrome. *America Journal of Cardiovascular Drugs* **7 (1)**: 39-58.
- Kakkar, P., Das, B., and Viswanathan, P.N (1984). “A modified spectrophotometric assay of superoxide dismutase”. *Indian Journal of Biochemistry. Biophysic* **21(2)**: 130-132.
- Kallol, Dutta., Biswadev, Bishayi (2009). *Escherichia coli* lipopolysaccharide administration alters antioxidant profile during hypercholesterolemia. *Indian Journal of Clinical. Biochemistry* **24(2)**: 179-183.

- Kanthlal, S.K., Sureesh, V., Arunachalam .G., Royal Frank, P., Kameshwaran, S (2012). Anti obesity and hypolipidemic activity of methanolic extracts of *Tabernaemontana Divaricata* on atherogenic diet induced obesity in rats. *International Research Journal of Pharmacy* **3**: 157–161
- Kaplowitz, N., Aw, T.Y., Ookhtens, M (1985). The regulation of hepatic glutathione. *Annual Review of Pharmacology Toxicology* **25**: 715-744.
- Karen, Sliwa., Ana Olga, Mocumbi (2009). Forgotten cardiovascular diseases in African *Clinical Research of Cardiology* DOI 10.1007/s00392-009-0094-1.
- Kets, V.V., Liebenberg, L., Wainwright, H., Martin, L., Gunston, G., Alexander, R (2011). Atherosclerotic lesions in the thoracic aorta: a South African anatomical and histological mortuary study. *South Africa Medical Journal* **110**: 409-412.
- Khatrri, A., Garg, A., Agrawal, S.S (2010). Evaluation of hepatoprotective activity of aerial part of *Tephrosia purpuria* L. and stem bark of *Tecomella undulata*. *Journal of Ethnopharmacology* **122**: 1-5.
- Kim, I., Kim, J.-Y., Hwang, Y.-J., Hwang, K.-A., Om, A.-S., Kim, J.-H., Cho, K.-J (2011). The beneficial effects of aged black garlic extract on obesity and hyperlipidemia in rats fed a high-fat diet. *Journal Medicinal Plants Research* **5**: 3159–3168
- Kneifel, W., Czech, E., Kopp, B (2002). Microbial contamination of medicinal plants—A Review. *Planta Medica* **68 (1)**: 5-15.
- Koba, k., Poutouli, P.W., Raynaud, C., Jaen-Pierre, C., Komla, S (2009). Chemical composition and antimicrobial properties of different basil essential oils chemotypes from Togo. *Bangladesh Journal of Pharmacology* **4**: 1-8.

- Kolar, Firdose, R., Vaishali, S.k., Ghansham, B.D (2011). Phytochemical constituents and antioxidant potential of some underused fruits. *Africa Journal of Pharmacy Pharmacology* **5(18)**: 2067–2072.
- Kosugi, H., Kato, T., Kikugawa, K (1987). Formation of yellow, orange and red pigments in the reaction of alk-2-enals with 2-thiobarbituric acid. *Analytical. Biochem* **165**: 456–464.
- Kotze, M.J., Langenhoven, E., Theart, L., Loubse, r O., Micklem, A., Oosthuizen, C.J (1995). Recurrent LDL-receptor mutation causes familial hypercholesterolaemia in South African Coloureds and Afrikaners. *South Africa Medical Journal* **85(5)**:357-361.
- Kovats, E (1958). Characteristic of Organic compounds by gas Chromatography. Part 1 Retention indices of aliphatic halides, alcohols, aldehydes and ketones, *Helv, Chim.*
- Kryston, T.B., Georgiev, A.B., Pissis, P., Georgakilas, A.G (2011). Role of oxidative stress and DNA damage in human carcinogenesis. *Mutat Res* 3, 711, (Jun) (193-201), *Acta* **41**: 1915-1932.
- Krisela S (2007). The heart and stroke foundation South Africa heart disease in South Africa Media data document.
<http://www.heartfoundation.co.za/docs/heartmonth/HeartDisease> in SA.pdf
- Kubec, R., Svobodova, M., Velisek, J (1999). Gas- chromatographic determination of S alk(en)lcysteinesulfoxdes. *Journal Chromatograph A* **862**: 85-94.
- Kubec, R., Kim, S., Musah, R.A (2002). S-Substituted cysteine derivatives and thiosulfinate formation in *Petiveria alliacea* – part II. *Phytochemistry* **61**: 675–680.
- Kumar, R.S., Rajikapoor, B., Perumal, P (2011). Antioxidant potential of *indigoferaLinnaei* Ali- An *in vitro* studies. *Pharmacologyonline* **1**: 710-720.

- Laakso, I., Seppane-Laakso, T., Hiltunen, R., Muller, B., Jansen, H., Knobloch, K (1989). Volatile garlic odor components: gas phases and adsorbed exhaled air analyzed by headspace gas chromatography-mass spectrometry. *Planta Medica* **55**: 257-261.
- Lamiaa, A.A. Barakat (2011). Hypolipidemic and Antiatherogenic Effects of Dietary Chitosan and Wheatbran in High Fat- High Cholesterol Fed Rats. *Australian Journal of Basic and Applied Science* **5(10)**: 30-37.
- Lamon, B.D., Hajjar, D.P (2008). Inflammation at the molecular interface of atherogenesis: an anthropological journey. *American Journal Pathology* **173** (5):1253-1264.
- Laporta, O., Perez-Fons, L., Mallavia, R., Caturla, N., Micol, V (2007). Isolation, Characterization and anti-oxidant capacity assessment of the bioactive compounds derived from *Hypoxis rooperii* corm extract (African potato). *Food Chemistry* **101**: 1425-1437.
- Latha, P.B., Reddy, I.R.M., Ismail, S. M., Vijaya, T (2010). Medicinal Plants and Their Derivatives as Potential Source in Treatment of Obesity. *Asian Journal Experimental Biological Science* **1(4)**:719- 727.
- Lau Benjamin, H.S (2001). Suppression of LDL oxidation by garlic. *Journal Nutrition* **131(3s)**: 9585 - 9885.
- Lauf, U., Fata, V. La., Liao, J.K (1997). Inhibition of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase blocks hypoxia-mediated down-regulation of endothelial nitric oxide synthase. *Journal of Biological Chemistry* **272**: 31725-31729.
- Law, MR (1999). Lowering heart disease risk with cholesterol reduction: evidence from observational studies and clinical trials. *European Heart Journal Supplements* **1**:S3 S8.

- Lelethu, Bungu., Maryna, van de Venter., Carminita, Frost (2006). Evidence for an *in vitro* anticoagulant and antithrombotic activity in *Tulbaghia violacea*. African Journal Biotechnology **7 (6)**: 681-688.
- Lelethu, Bungu., Maryna, van de Venter., and Carminita, Frost (2008). Evidence for an *in vitro* anticoagulant and antithrombotic activity in *Tulbaghia violacea*. African Journal Biotechnology **7 (6)**: 681–688, 18.
- Leon, AS., Sanchez, O.A (2001). Response of blood lipids to exercise training alone or combined with dietary intervention. Medicine Science in Sports and Exercise **33**: S502–S515
- Leonid Breydo and Kent S. Gates (2000). Thiol-Dependent DNA Cleavage by 3H-1,2 Benzodithiol-3-one 1,1-dioxide. Bioorganic & Medicinal Chemistry Letters **10**: 885-889
- Leopold, J.A., Loscalzo, J (2008). Oxidative mechanisms and atherothrombotic cardiovascular disease. Drug Discovery Today **5**: 5-13.
- Li, H., Forstermann U (2000). Nitric oxide in the Pathogenesis of vascular disease. Journal of Pathology **190 (3)**: 244-54
- Libby, P., Aikawa, M., Schonbeck, U (2000) .Cholesterol and atherosclerosis. Biochimica et Biophysica Acta : **1529**: 299–309.
- Lipnick, R.L., Cotruvo, J.A., Hill, R.N., Bruce, R.D., Stitzel, K.A., Walker, A.P., Chu, I., Goddard, M., Segal, L., Springer, J.A., Myers, R.C (1993). Comparison of the Up and-Down, Conventional LD₅₀ and Fixed Dose Acute Toxicity procedures. Food. Chemistry Toxicology. **33**: 223-231.

- Liyana-Pathiranan, C.M., Shahidi, F (2005). Antioxidant activity of commercial soft and hard wheat (*Triticum aestivum* L) as affected by gastric pH conditions. *Journal of Agricultural and Food Chemistry* **53**: 2433–2440
- Lokhande, P.D., Dhawaren, B.S., Jagdale, S.C., Chabukswar, A.R., Mukawar, S.A (2006) Cardiac activity of isolated constituents of *Inula racemosa*. *Journal Herbal Pharmacotherapy* **6**: 81-88.
- Long-Sheng, Lu., Chau-Chung, Wu., Li-Man, Hung., Meng-Tsan, Chiang., Ching-Ting, Lin., Chii-Wann, Lin., Ming-Jai, Su (2007). Apocynin alleviated hepatic oxidative burden and reduced liver injury in hypercholesterolaemia. *Liver International* 529 537.
- Lu, L.S., Wu, C.C., Hung, L.M., Chiang, M.T., Lin, C.T., Lin, C.W., Su, M.J (2007). Apocynin alleviated hepatic oxidative burden and reduced liver injury in hypercholesterolaemia. *Liver International* **27**:529–537.
- Łukaszewicz-Hussain, A., Moniuszko-Jakoniuk, J (2004). Liver Catalase, Glutathione Peroxidase and Reductase Activity, Reduced Glutathione and Hydrogen Peroxide Levels in Acute Intoxication with Chlorfenvinphos, an Organophosphate Insecticide. *Polish Journal of Environmental Studies* **13(3)**: 303-309.
- Lusis A.J (2000). Atherosclerosis. *Nature*. **407(6801)**: 233–241.
- Ma, Jiangwei., Qiao, Zengyong., Xiang, Xia (2011). Aqueous extract of *Astragalus mongholicus* ameliorates high cholesterol diet induced oxidative injury in experimental rats models. *Journal of Medicinal Plants Research* **5(5)**: 855-858.
- Mabuchi, H., Nohara, A., Kobayashi, J., Kawashiri, M.A., Katsuda, S., Inazu, A., Koizumi, J Hokuriku (2007). Lipid Research Group. Effects of CoQ10 supplementation on

- plasma lipoprotein lipid, CoQ10 and liver and muscle enzyme levels in hypercholesterolemic patients treated with atorvastatin: a randomized double blind study. *Atherosclerosis* **195**:182–189.
- Mac, D.J.A., Marchand, M.E., Langler, R.F (2004). Improving upon the *in vitro* biological activity of antithrombotic disulfides. *Blood Coagulation Fibrinolysis* **15**: 447-450.
- Malomo, S.O., Adebayo, J.O., Olorunniji, F.J. (2002) Modulatory effect of vitamin E on some haematological parameters in dihydroartemisinin-treated rats. *Tropical Journal of Health Science* **9**:15-20
- McGaw, L.J., Jager, A.K., Van, Staden (2000). Antibacterial, anthelmintic and anti-amoebic activity in South African medicinal plants. *Journal Ethnopharmacology* **72**: 247-263.
- Mackay, JM., George A (2004). The atlas of heart disease and stroke. Geneva: World Health Organization.
- Madamanchi, N.R., Vendrov, A., Runge, M.S (2005). Oxidative stress and vascular disease. *Arteriosclerosis Thrombosis Vascular Biology* **25**: 29-38.
- Magdy, N Ashour., Hoda, A. Megahed., Safenaz, I. Eltoukhy., Eman, R. YounesDawound, F. Habib., Hanaa A. Wafai. (2011) Antioxidant and Radical Scavenging Properties of Garlic oil in streptozotocin induced Diabetic Rats. *Australian Journal of Basic and Applied Science* **5 (10)**: 280-286.
- Maisuthisakul, P., Suttajit, M., Pongsawatmanit, R (2007). Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants. *Food Chemistry* **100**: 1409-1418.

- Majeed, Z.R (2006). Effects of L-carnitine and melatonin on some biochemical and haematological parameters in L-NAME and cholesterol-treated male albino rats. M.sc. thesis; Salahaddin Univ. Coll. Science.
- Mann, A., Ibrahim, K., Oyewale, A.O., Amupitan, J.O., Fatope, M.O., Okogun, J.I (2011). Brine Shrimp Toxicity Evaluation of the Root bark Extract of *Terminalia avicennioides*. African Journal Scientific Research 4(1): 221-229.
- Marinova, D., Ribarova, F., Atanassova, M (2005). Total phenolics and total flavonoids in Bulgarian fruits and vegetables. J. Univer. Chem. Tech. Metallurgy **40(3)**: 255-260.
- Martin-Fuentes, P., Civeira, F., Recalde, D., Garcia-Otin, A.L., Jarauta, E., Marzo, I., Mathers, C.D., Loncar, D (2007). Projections of global mortality and burden of disease from 2002 to 2030. PLOS Medicine **3(11)**: 442.
- Masika, P.J., Afolayan, A.J (2003). An ethenobotanical study of plants used for the treatment of livestock diseases in the Eastern Cape Province, South African Pharmaceutical Biology **41**: 16-21.
- Mathew, B.C., Prasad, N.V., Prabodh, R (2004). Cholesterol-lowering effect of organosulphur compounds from garlic: a possible mechanism of action. Kathmandu University Medical Journal **2(2)**: 100-2
- Mathers, C D and Loncar, D (2006). Updated projections of global mortality and burden of disease, 2002-2003: data sources, methods and results. Evidence and information for policy World Health Organization October 2005.
- Matos, S.L., Paula, H., Pedrosa, M.L., Santos, R.C., Oliveira, E.L., Chianca, Jr D.A, Silva M.E (2005). Dietary models for inducing hypercholesterolemia in rats. Brazilian Archives of Biology Technology **48(2)**: 203-209.

- Matsuzawa, N., Takamura, T., Kurita, S., Misu, H., Ota, T., Ando, H., Yokoyama, M., Honda, M., Zen, Y., Nakanuma, Y., Kaneko, S (2007). Lipid-induced oxidative stress causes steatohepatitis in mice fed an atherogenic diet. *Hepatology* **46**: 1392–1403
- Maxfield, F.R., Tabas, I (2005). Role of cholesterol and lipid organization in disease. *Nature* **438**: 612-621.
- Mayosi, B., Robertson, K., Volmink, J., Adebo, W., Akinyore, K., Amoah, A., Bannerman, C., Biesman-Simons, et al. (2006). The Drakensberg declaration on the control of rheumatic fever and rheumatic heart disease in Africa. *South African Medical Journal* **96(3)**: 246.
- Medscape (2007). "EBCT Coronary Calcium Imaging for the Early Detection of CAD." Lorenz, M., *Circulation* **115**: 459-467.
- Mendelsohn, M.E., Loscalzo, J (1988). Role of platelets in cholesteryl ester formation by U 937 cells. *Journal Clinical Investigation* **81**: 62
- Michael, E. Debakey., Gerald, M. Lawrie., Donald H. Glaeser (1985). Patterns of Atherosclerosis and their Surgical Significance. *Annals of Surgery* **201(2)**: 115 131
- Millogo-Kone, H (2008). Study of the phytochemical parameters and biological activities of *Parkia biglobosa* (Jacq) Benth (Mimosaceae) Ph.D. Thesis, University of Quagadougou.
- Ming-Shi, Shiao., Jing-Jing, Chiu., Bao-Wen, Chang., Jane, Wang., Wei-Ping, Jen., Ye-Jer, Wu., and Yuh-Lien, Chen (2008). In search of antioxidants and anti-atherosclerotic agents from herbal medicines. *BioFactors* **34**: 147–157.

- Misharina, T.A., Terenina, M.B., Krikunova, N. I (2009). Antioxidant Properties of Essential Oils. *Applied Biochemistry and Microbiology* **6**: 642–647.
- Mishra, A.K., Dubey, N.K (1994). Evaluation of some essential oils for their toxicity against fungi causing deterioration of stored food commodities. *Applied Environmental Microbiology* **60**: 1101-1105.<http://aem.asm.org/cgi/reprint/60/4/1101>.
- Misra, HP., Fridovich, I (1972). The role of superoxide anion in the autooxidation of epinephrine and simple assay for superoxide dismutase. *Journal Biological Chemistry* **247**: 3170-3175.
- Misra, M., Mittal, M., Singh, R., Verma, A., Rai, R., Chandra, G., Singh, D., Chousdhary V., Singh, R., Mall, A., Khan, M.J., Khane, S., Yadav, R (2007). Prevalence of rheumatic heart disease in school going children of eastern Uttar Pradesh. *Indian Heart Journal* **59**: 42-43.
- Mohammad, S.F., Woodward, S.C (1986). Characterisation of a potent inhibitor of platelet aggregation and release reaction isolated from *Allium sativum* (Garlic). *Thrombosis Research* **44**:793-806
- Mohammed, AL-Dosari, Saleh, Alqasoumi., Majid, Ahmad., Mohammed, AL-Yahya M., Nazam, Ansari., Syed, Rafatullah (2011). Effect of *beta vulgaris* l. on cholesterol rich diet-induced hypercholesterolemia in rats. *FARMACIA* **59 (5)**: 669-678.
- Mohammed, S. Al-Dosari (2011). Hypolipidemic and antioxidant activities of avocado fruit pulp on high cholesterol fed diet in rats. *Africa Journal of Pharmacy and Pharmacology* **5(12)**: 1475-1483.

- Molgaard, J., von, Schenck H., Olsson, A.G (1989). Comparative effects of simvastatin and cholestyramine in treatment of patients with hypercholesterolaemia. *European Journal Clinical Pharmacology* **36**: 455–460.
- Moreno, P.R., Purushothaman K.R (2006). Neovascularization in human atherosclerosis. *Current Molecular Medicine* **6**:457-477.
- Morshed, M.A., Uddin, M. Azim., Rahman,A.,Tahrim, H., Uddin, Tareq Ahmed Fakhar., Zakaria Mudasser., Haque Anwarul., Parvez, Anowar Khasru (2011). Evaluation of analgesic and anti-inflammatory effect of terminalia arjuna ethanol extract. *International Journal of Bioscience* **2(10)**: 2577-2585
- Moody, J.O., Robert, V.A., Connolly, J.D., Houghton, P.J (2008). Anti-inflammatory activities of the methanol extracts and an isolated furanoditerpene constituent of *Sphenocentrum jollyanum* Pierre (Menispermaceae). *Journal of Ethnopharmacology* **104**: 87-91.
- Motsei, M.L., Lindsey, K.L., Van, Staden. J., Jager, A.K (2003). Screening of traditionally used South African plants for antifungal activity against *Candida albicans*. *Journal of Ethnopharmacology* **86**: 235-241.
- Mohammad Ahmad., Mudassir, Ahmad Khan., Abdus Sattar, Khan (2009). Oxidative stress and level of iron indices in coronary heart disease patients. *Journal Ayub Medical College Abbottabad* **21(2)**: 56-59
- Munchberg, U., Anwar, A., Mecklenburg, S., Jacob, C (2007). Polysulfides as biologically active ingredients of garlic. *Organic and Biomolecular Chemistry* **5**: 1505-1518.

- Mune, M., Meydani, M., Gong, J., Fotouhi, N., Ohtani, H., Smith, D., Blumberg, J.B (1999).
Effect of dietary fish oil, vitamin E, and probucol on renal injury in the rats. *Journal of Nutritional Biochemistry* **10**: 539-546.
- Musk, S.R., Preobrazhenskaya, M.N., Belitsky, G.A., Korolev, A.M., Kytcheva, T.A., Khitr, I.A., Johnson., Nocentini, S., Guggiari, M., Rouillard, D., Surgis, S (2001):
Exacerbating effect of vitamin E supplementation on DNA damage induced in cultured human normal fibroblasts by UVA radiation. *Photochemistry Photobiology*. **73**: 370-377.
- Myron, Gross (2004). Flavonoids and Cardiovascular Disease. *Pharma*. 42, Supplement, pp. 21–35.
- Nader, Saki., Ghasem, Saki., Fakher, Rahim., Abolfazl, Shiravi khoozani., Soheila, Nikakhlagh (2011). Modulating effect of soy protein on serum cardiac enzymes in cholesterol-fed rats. *International Journal of Medicine and Medical Science* **3(14)**: 390-395.
- Nagavani, V., Madhavi, YD., Bhaskar, Rao P., Koteswara, Rao,, Raghava, Rao T (2010).
Free radical scavenging activity and qualitative analysis of polyphenols by RP-HPLC in the flowers of *Couroupita guianensis* abul. *Electronic Journal of EnviromentalAgricultural and food chemistry* **9(9)**: 1471 - 1484.
- Narendhirakannan, R.T., Rajeswari, K (2010).In Vitro Antioxidant Properties of Three Varieties of *Allium Sativum* L. Extracts. *Electronical Journal of Chemistry* **7(1)**: S573-S579.
- Naoto, Matsuzawa., Toshinari, Takamura., Seiichiro, Kurita., Hirofumi, Misu., Tsuguhito, Ota., Hitoshi, Ando., Masayoshi, Yokoyama., Masao, Honda., Yoh, Zen., Yasuni,

- Nakanuma., Ken-ichi, Miyamoto., Shuichi, Kaneko (2007). Lipid-Induced Oxidative Stress Causes Steatohepatitis in Mice Fed an Atherogenic Diet. *Hepatology* **46**:1392-1403
- Nemat, Khansari.,Yadollah, Shakiba., Mahdi, Mahmoudi (2009). Chronic Inflammation and Oxidative Stress as a Major Cause of Age-Related Diseases and Cancer. *Recent Patents on Inflammation & Allergy Drug Discovery* **3**: 73-80
- NCEP, III (2001). Executive summary of the third report of the national cholesterol education program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). *Journal of American Medical Association* **285**: 2486-2497.
- Nigam, S., Schewe, T (2000). Phospholipase A2 and lipid peroxidation. *Biochimica Biophysica Acta* **1488**: 167-181.
- Noureddine, Bankeblia., Said, Dahumouni., Shuichi, Onodera., Norio, Shiomi (2005). Antimicrobial Activity of phenolic compound extracts of various Onions (*Allium cepa* L.) cultivars and garlic (*Allium sativa* L.). *Journal of Food Technology* **3(1)**: 30-34.
- Noori, S. Azmat., Mahboob, M (2012). Study on antioxidant effects of cinnamon and garlic extract in liver, kidney and heart tissue of rat. *Bioscience Research*. **9(1)**: 17-22.
- Norman, R., Bradshaw, D., Schneider M., Pieters, D., Groenewald, P (2006). Revised Burden of Disease Estimates for the Comparative Risk Factor Assessment, South Africa 2000. Methodological Note. Cape Town: MRC.

- Nove lli, E.L.B., Diniz, Y.S., Galhardi, C.M., Ebaid, G.M.X., Rodrigues, H.G., Mani, F., Fernandes, A.A.H., Cicogna, A.C., Novelli Filho, J.L.V.B (2007). Anthropometric parameters and markers of obesity in rats. *Laboratory Animal (NY)* **41**: 111-119.
- Nuutila, A.M., Puupponen-Pimia, R., Aarni, A., Oksman-Caldentry, M (2003). Comparison of antioxidant activities of onion and garlic extracts by inhibition of lipid peroxidation and radical scavenging activity. *Food Chemistry* **81**: 485-493
- Obdoni, B.O., Ochuko, P.O (2001). "Phytochemical studies and comparative efficacy of the crude extracts of some Homostatic plants in Edo and Delta States of Nigeria" *Global Journal of Pure Applied Science* **8**: 203-208.
- OECD (2001). The OECD guideline for testing of chemical. The organization of Economic Co-operation Development, Paris. 1 - 14.
- OECD (2003). Guidelines for the Testing of Chemicals/Section 4: Health Effects Test No. 423: Acute Oral toxicity - Acute Toxic Class Method. Organization for Economic Cooperation and Development, Paris, France.
- OECD (2008). Repeated dose oral toxicity test method. In: OECD Guidelines for testing of chemicals, N° 407. Organization for Economic Cooperation and Development, Paris, France.
- Ogbonnia, S.O., Mbaka, G.O., Anyika, EN., Ladiju, O., Igbokwe, H.N., Emordi, J.E., Nwakakwa, N (2010). Evaluation of Anti-diabetics and Cardiovascular Effects of *Parinari curatellifolia* Seed Extract and *Anthoclista vogelli* Root Extract Individually and Combined on Postprandial and Alloxan-Induced Diabetic Albino Rats. *British Journal of Medicine & Medica Research* **1(3)**: 146-162.

- Ogbonnia S.O., Florence E., Nkemehule, Emmanuel N., Anyika E.N (2009). Evaluation of acute and subchronic toxicity of *Stachytarpheta angustifolia* (Mill) Vahl (Fam. Verbanaceae) extract in animals. *African Journal of Biotechnology* **8(9)**:1793-1799
- Ohkawa, H., Ohishi, N., Yagi, K (1979). Assay for lipid peroxides in animal tissues by Thiobarbituric acid reaction. *Analytical Biochemistry* **95**: 351-358.
- Okrainec, K., Banarjee, D.K., Eisenberg, M. J (2004). Coronary artery disease in the developing world. *American Heart Journal* **148**: 7-15. Available from <http://www.elsevier.com>.
- Okwu, D.E., Emenike, I.N (2006). Evaluation of the Phytonutrients and vitamins content of Citrus Fruits. *International Journal Molecular Medicine and Advanced Science* **2(1)**: 1- 6.
- Olorunnisola, O.S., Amao, S., Ehigie, L.O., Ajayi, A.F (2008). Anti-Hyperglycemic and Hypolipidemic Effect of Ethanolic Extracts of *Chrysophyllum Albidum* Seed Cotyledon in Alloxan Induced-diabetic Rats. *Research Journal of Applied Science*. **3(2)**: 123-127
- Olorunnisola, O.S., Bradley, G., Afolayan, A.F (2011b). Antioxidant properties and cytotoxicity evaluation of methanolic extract of dried and fresh rhizomes of *Tulbaghia violacea*. *Africa Journal of Pharmacy and Pharmacology* **5(22)**: 2490-2497.
- Olorunnisola, O.S., Bradley, G., Afolayan, A.J (2011a). Ethnobotanical information on plants used for the management of cardiovascular diseases in Nkonkobe Municipality, South Africa *Journal Medicinal Plants Research* **5(17)**: 4256-4260

- Olorunnisola, Olubukola S., Graeme Bradley, and Anthony J. Afolayan (2012). Protective Effect of *Tulbaghia violacea* Harv. on Aortic Pathology, Tissue Antioxidant Enzymes and Liver Damage in Diet-Induced Atherosclerotic Rats. *International Journal of Molecular Science* **13**: 12747 -12760
- Olson, H., Betton, G., Robinson, D., Thomas, K., Monro, A., Kolaja, G., Lilly, P., Sanders, J., Spes, G., Bracken, W., Dorato, M., Deun, K.V., Smith, P., Berger, B., Heller, A (2000). Concordance of toxicology of pharmaceuticals in humans and animals. *Regulatory Toxicology and Pharmacology* **32**: 56–67.
- Orolin, J., Vecera, R., Jung, D., Meyer, U.A., Skottová, N., Anzenbacher, P (2007). Hypolipidemic effects of silymarin are not mediated by the peroxisome proliferator activated receptor alpha. *Xenobiotica* **37**: 725-35.
- Osamuyimen, O.I., Isoken, H.I., Vincent, N.C., Olohirere, E.U., Sunday, O.O., Emmanuel, E.O, Anthony, I.O., Etinosa, O.I (2011). Polyphenolic Contents and Antioxidant Potential of Stem Bark Extracts from *Jatropha curcas* (Linn). *International Journal of Molecular Science* **12**: 2958-2971.
- Osawa, T (1999). Protective role of dietary polyphenols in oxidative stress. *Mechanism of Ageing and Development* **111**: 133–139.
- Otunola, A Gloria., Oloyede Oyelola, B., Adenike, T Oladiji., Anthony, A. Afolayan (2010). Effects of diet-induced hypercholesterolemia on the lipid profile and some enzyme activities in female Wistar rats. *Africa Journal of Biochemical Research* **4 (6)**: 149-154.

- Oyaizu, M (1986). Studies of products browning reaction: Antioxidative activity of products of browning reaction prepared from glucosamine. Japan Journal of Nutrition **44**: 307-315.
- Oyedemi, S.O., Bradley, G., Afolayan, A. J (2010). *In -vitro* and *-vivo* antioxidant activities of aqueous extract of *Strychnos henningsii* Gilg. African Journal of Pharmacy and Pharmacology **4(2)**: 070-078.
- Ozansoy, O., Akin, B., Aktan, F., Karasu, C (2001). Short-term gemfibrozil treatment reverses lipid profile and peroxidation but does not alter blood glucose and tissue antioxidant enzymes in chronically diabetic rats. Molecular and Cellular Biochemistry **216**: 59 – 63.
- Ozsoy, N., Can., Yanardag, R., Akev, N (2008). Antioxidant activity of Smilax excels leaf extracts. Food Chemistry **110**: 571–583.
- Panigrahi, B.B., Panda, P.K., Patro, V.J (2010). Comparative hepatoprotective activity of different extracts of spirulina against CCl₄ induced liver damage in rats International Journal of pharmaceutical Sciences Review and Research **4(2)**: 200-202
- Pari, L., Latha, M (2004). Protective role of Scoparia dulcis plant extract on brain antioxidant status and lipid peroxidation in STZ diabetic male Wistar rats. BMC Complementrayand Alternative Medicine **4**: 16; doi: 10.1186/1472-6882-4-16
- Parris, M.K (1997). Glutathione systemic protectant against oxidative and free radical damage. Alternative Medicine Review **2 (3)**: 155-176.
- Pavel Poredos (2011). Markers of Preclinical Atherosclerosis and their Clinical Relevance. The Open Atherosclerosis & Thrombosis Journal **4**: 1-10.

- Pencina, M.J, D, Agostino, R.B., Larson, M.G., Massaro, J.M., Vasan, R.S (2009). Predicting the 30-year risk of cardiovascular disease: the framingham heart study. *Circulation* **119(24)**: 3078-3084.
- Peter Libby., Paul M.Ridker., AttilioMaseri (2002). Inflammation and atherosclerosis. *Circulation* **105 (9)**: 1135 – 1143.
- Pignatelli, P., Di, Santo S., Buchetti, B., Sanguigni, V., Brunelli, A., Violi, F (2006). Polyphenols enhance platelet nitric oxide by inhibiting protein kinase C-dependent NADPH oxidase activation: effect on platelet recruitment. *Federation of American Societies aseb J.* **20**: 1082-1089.
- Pino, J., Rosado, A., Gonzalez, A (1991). Volatile flavor components of garlic essential oil. *Acta Alimentaria* **20 (3-4)**:163-171.
- Pilaipark Chumark., Panya Khunawat., Yupin Sanvarinda., Srichan Phornchirasilp., Noppawan Phumala Morales., Laddawal Phivthong-ngame., Piyanee Ratanachamnong., Supath Srisawat., Klai-upsorn S. Pongrapeeporn (2008). The *in vitro* and *ex vivo* antioxidant properties, hypolipidaemic and antiatherosclerotic activities of water extract of *Moringa oleifera* Lam. Leaves. *Journal of Ethnopharmacology* 8. doi:10.1016/j.jep.2007.12.010.
- Ponmari, G., Sathishkumar, R., Lakshmi, P.T.V (2011). Effect of drying treatment on the contents of antioxidants in *Cardio sperm umhalicacabum* Linn. *International Journal Pharm. Bio. Science* 304-313.
- Prabuseenivasan, S., Jayakumar, M., Ignacimuthu, S (2006). In vitro antibacterial activity of some plant essential oils. *BMC Complementary Alternative Medicine* **6**: 39.

- Prasad, K (2010). Effects of vitamin E on serum enzymes and electrolytes in hypercholesterolemia. *Molecular Cell Biochemistry* **335**: 67–74.
- Prasanna GS, Purnima A (2011). Protective Effect of Leaf Extract of *Trichiliaconnaroides* on Hypercholesterolemia Induced Oxidative Stress. *International Journal of Pharmacology* **7 (1)**: 106-112.
- Qadir, AB (2005). Effect of Ubiquinone and Omega-3 on some induced hypertensive Parameters in Male Albino Rats. Ph.D. Thesis Salahaddin University Coll. Science.
- Raal, F J (2009). Hypercholesterolaemia in children and young adults – current management. *Journal of Endocrinology, Metabolism and Diabetes of South Africa* **14(1)**: 09-12.
- Radjabian, T., Huseini, F. H (2010). Anti-Hyperlipidemic and Anti-Atherosclerotic Activities of Silymarins from Cultivated and Wild Plants of *Silybum marianum* L. with Different Content of Flavonolignans. *IJTP*. **9(2)**: 63 -67.
- Raghuveer, Choudhary (2008). Beneficial effect of *allium sativum* and *allium tuberosum* on experimental hyperlipidemia and atherosclerosis. *Pakistan Journal of Physiology* **4(2)**: 7-9
- Rahman, M.A.A., Moon, S.S (2007). Antioxidant polyphenol glycosides from the Plant *Draba nemorosa*. *Bulletin of Korean Chemical Society* **28(5)**: 827-831.
- Ramachandran, H.D., Narasimhamurthy, K., Raina, P.L (2003). Modulation of cholesterol induced hypercholesterolemia through dietary factors in Indian desert gerbils (*Meriones hurricinae*). *Nutritional Research*. **23**: 245-256.
- Ramesar, S., Baijnath, H., Govender, T., and Mackraj, I. (2008). Angiotensin I-converting enzyme inhibitor activity of nutritive plants in KwaZulu-Natal. *Journal of Medicinal Food* **11(2)**: 331-336

- Ramesh, B., Satakopan, V.N (2010). In vitro antioxidant activities of *Ocimum* species: *Ocimum basilicum* and *Ocimum sanctum*. Journal of Cell and Tissue Research **10(1)**: 2145-2150.
- Rang, H., Dale M (2007). Pharmacology. Elsevier, India, 5th edn. 321-326.
- Rankin, S.M, Leake, D.S (1988). The modification of low density lipoproteins by macrophages by oxidation or proteolysis. Agents Actions Supplement **26**: 233-39.
- Rainer W. Bussmann ., Ashley Glenn (2011). Fighting pain: Traditional Peruvian remedies for the treatment of Asthma, Rheumatism, Arthritis and sore bones. Indian. Journal Traditional Knowledge **10(3)**: 397-412.
- Rantanachamnong, P., Matsathit, Y., Sanvarinda, Y., Piyachaturawat, P., Phivthong Ngam, L (2012). Effects of *Curcuma comosa* Roxa. On platelet aggregation and atherosclerosis plaque development in hypercholesterolemic Rabbits. International Journal of pharmacology. **8(4)**: 234 - 242.
- Rao, M.V., Alice, K.M (2001). Contraceptive effects of *Phyllanthus amarus* n female mice. Phytotherapy Research. **15**: 265-267.
- Ratheesh, M., Shyni, G.L, Sindhu, G., Helen, A (2011). Inhibitory effect of *Ruta graveolens* L. on oxidative damage, inflammation and aortic pathology in hypercholesteremic rats. Experimental and Toxicologic Pathology **63**: 285–290
- Ravirajsinh, Jadeja., Menaka, Thounaojam., Ramachandran, AV., Ranjitsinh, Devkar (2009). "Phytochemical Constituents and Free Radical Scavenging Activity of *Clerodendron glandulosum* Coleb Methanolic Extract. Journal of Complementary and Integrative Medicine **6**: 1-19.

- Reiko, Inagi (2006). Oxidative Stress in Cardiovascular Disease: A new avenue toward future therapeutic approaches. *Recent Patents on Cardiovascular Drug Discovery* **1**: 151-159.
- Reena, L., Kapil, L (2011). Antioxidant activity of garlic essential oil (*Allium Sativum*) grown in north Indian plains. *Asian Pacific Journal of Tropical Biomedicine* 1-3.
- Rhiouani, H., El-Hilaly, J., Israili, H.Z., Lyoussi, B (2008). Acute and sub-chronic toxicity of an aqueous extract of the leaves of *Herniaria glabra* in rodents. *Journal of Ethnopharmacology* **118**: 378–386.
- Rodrigues, A. G., & Casali, R. A. B. (2002). Plantas medicinais, conhecimento popular e etnociência. In A. G. Rodrigues, F. M. C. Andrade, F. M. G. Coelho, M. F. B. Coelho, R. A. B. Azevedo, & V. W. D. Casali (Eds.), *Plantas medicinais e aromáticas: etnoecologia e etnofarmacologia* (pp. 25–76). Vicosa: UFV, Departamento de Fitotecnia.
- Rolls, B.J (2000). The role of energy density in the over consumption of fat. *Journal of Nutrition* **130**: 268 S-271S.
- Ross, R (1999). Atherosclerosis—an inflammatory disease. *New England Journal of Medicine* **340**:115–126.
- Rotruck, J.T., Pope, A.L., Ganther, H.E., Swason, A.B (1973). Selenium: biochemical role as a component of glutathione peroxidase. *Science* **17**: 588-590.
- Ruberto, G., Baratta, M.T., Deans, S.G., Dorman, H.J (2000). Antioxidant and antimicrobial activity of *Foeniculumvulgare* and *Crithmummaritimum* essential oils. *Plant Medicine* **66 (8)**: 687-693.

- Rui-Li, Yang., Yong-Hui, Shi., Gang, Hao., Wu, Li., Guo-Wei Le (2008). Increasing Oxidative Stress with Progressive Hyperlipidemia in Human: Relation between Malondialdehyde and Atherogenic Index. *Journal of Clinical Biochemistry and Nutrition* **43**:154–158.
- Ryszawa, N. A., Kawczyńska-dród, J., Pryjma, M., Czesnikiewicz-guzik, T., Adamek guzik, M., Naruszewicz, R., Korbut, T., Guzik, J (2006). Effects of novel plant antioxidants on platelet superoxide production and aggregation in atherosclerosis. *Journal of Physiology and Pharmacology*. **57 (4)**: 611-626.
- Saad, B., Azaizeh, H., Abu-Hijleh, G., Said, S (2006). Safety of traditional Arab herbal medicine. *Evidence-Based Complimentary & Alternative Medicine* **3**: 433-439.
- Sacher, R.A (1991). Mepherson, R.A. Widmann's clinical interpretation of laboratory test, U.S.A., Pennsylvania. 3rd edition. 416-443.
- Saha M.R., Hasana S.M.R., Aktera, R., Hossain,a M.M., Alamb, M.S., Alam, M.A., Mazumder, M.E.H (2008). In vitro free radical scavenging activity of methanol extract of the leaves of *mimusops elengi* linn. angl. *Journal of Veterinary Medicine* **6(2)**: 197–202.
- Saikia, H., Lama, A (2011). Effect of *Bougainvillea spectabilis* leaves on serum lipids in albino rats fed with high fat diet. *International Journal Pharmaceuical Science and Drug Research* **3**: 141–145
- Sakuta, H., Suzuki, T., Ito, T (2007). Serum gamma-glutamyl transferase is associated with plasma total homocysteine in Japanese patients with type 2 diabetes. *Acta Diabetology* **44(4)**:177-80.

- Salim, Yusuf., Srinath, Reddy., Stephanie, Ôunpuu., Sonia, Anand (2001). Global Burden of Cardiovascular Diseases Part I: General Considerations, the Epidemiologic Transition, Risk Factors, and Impact of Urbanization. *Circulation* **104**: 2746-2753.
- Sam, T.W., Ng, A.S, Cheah, P.B (1988). Toxicity screening with the brine shrimp (*Anemia salina*) of plant extracts. 50- 57 in Ikram M. Said & Laily Din (Eds .) Proceedings of the UNESCO Sub-regional Seminar/Workshop on the Systematic Identification of Natural Products.
- Sandra, J Lewis (2009). Prevention and Treatment of Atherosclerosis: A Practitioner's Guide for 2008. *The American Journal of Medicine* **122**: S38–S50.
- Sanjay, K Banerjee., Subir, K Maulik (2002). Effect of garlic on cardiovascular disorders: a review. *BMC Nutrition Journal* 1:4
- Sans, H., Kesteloot, H., Kromhout, D (1997). On behalf of the Task Force. The burden of cardiovascular diseases mortality in Europe. *European Heart Journal* **18**: 1231–1248.
- Saritha, V., Anilakumar, K.R., Farhath, Khanum (2010). Antioxidant and antibacterial activity of *Aloe Vera* gel extracts. *International Journal of Pharmaceutical & Biological Archives* **1(4)**: 376-384.
- Saurabh, K., Bonde, C.G (2011). Total phenolic content and antioxidant activity of extracts of *Bridelia Retusa Spreng* Bark: Impact of dielectric constant and geographical location. *Journal of Medicinal Plant Research* **5(5)**: 817-822.
- Shankar, V., Kaur, H., Dahiya, K., Gupta M.S (2008). Comparison of fasting and postprandial lipid profile in patients of coronary heart disease. *Bombay Hospital Journal* **50**: 445–449.

- Sharma, S. Kumar., Wiseman, D.A., Jing Tian, Imran Rehmani., Sanjeev, Datar., Peter, Oishi., Sohrab, Fratz., Richard, C. Venema., Jeffrey, R. Fineman., Stephen, M. Black (2010). Perinatal changes in superoxide generation in the ovine lung: Alterations associated with increased pulmonary blood flow *Vascular Pharmacology* **53**: 38–52
- Shaten, B.J., Kuller, L.H., Neaton, J.D (1991). Association between baseline risk factors, cigarette smoking and CHD mortality after 10.5 years. *Preventive Medicine* **20**: 655-669.
- Shela, Gorinstein., Hanna, Leontowicz., Maria, Leontowicz., Katarzyna, Najman., Wojciech, Bielecki., Kyung-Sik, Ham., Seong-Gook, Kang., Octavio, Paredes-Lopez., AlmaLeticia, Martinez-Ayal., Simon, Trakhtenberg (2011). Aorta and Liver Changes in Rats Fed Cholesterol-Containing and Raw Vegetable-Supplemented Diets: Experiments *in vitro* and *in vivo*. *Agricultural Food Chemistry* **59**: 7441–7451.
- Shyam, A Sunder., Rama, A Narsimha., Reddy, Rajeshwar Y., Kiran, G., Krishna, D., Prasad, Baburao B., Thirumurugu, S., Karthik, A (2010). Protective effect of methanolic extract of *Trianthema portulacastrum* in atherosclerotic diet induced renal and hepatic changes in rats. *Der Pharmacia Lettre* **2 (1)**: 540-545.
- Shyamala, M.P., Venukumar, M.R., Latha, M.S (2003). Antioxidant potential of the *syzygium aromaticum* (gaertn.) linn. (Cloves) in rats fed with high fat diet. *Indian Journal of Pharmacology* **35**: 99-103.
- Sing, C.F, Moll, P.P (1990). Genetic Athrosclerosis *Annual Review of Genetics* **24**: 171 187
- Sinha, K.A (1972). Colorimetric assay of catalase. *Anal Biochem.* 1972, **47**: 389-394.
- Sivaraj, A., Vinothkumar, P., Sathiyaraj, K., Sundaresan, S., Devi, K., Senthilkumar, B (2011). Hepatoprotective potential of *Andrographispaniculata* aqueous leaf extract on

- ethanol induced liver toxicity in *albino* rats. Journal of Applied Pharmaceutical Science **01(06)**: 204-208..
- Skottova, N., Krecman, V., Vana, P., Chmela, Z., Ulrichova, J., Simanek, V (2000). Effect of silymarin and silibinin-phosphatidylcholine complex on plasma and lipoprotein cholesterol, and oxidation of LDL in rats fed on high cholesterol diet supplemented with currant oil. Acta University Palacky Olomuc Faculty of Medicine **144**: 55-8.
- Skottova, N., Krecman, V (1998). Dietary silymarin improves removal of low-density lipoproteins by perfused rat liver. Acta University Palacky Olomuc Faculty of Medicine **141**:39-40.
- Sliwa, K., Damasceno, A., Mayosi, B.M (2005). Epidemiology and etiology of cardiomyopathy in Africa. Circulation **112**: 3577-3583.
- Smith, SC, Jr., Greenland, P., Grundy, S.M (2000). AHA Conference Proceedings. Prevention conference V: Beyond secondary prevention: Identifying the high-risk patient [105] for primary prevention: executive summary. American Heart Association Circulation **101(1)**: 111-116.
- Sary, H.C (2000). Natural history and histological classification of atherosclerotic lesions: an update. Arteriosclerosis Thrombosis and Vascular Biology **20(5)**:1177-1178.
- Sary, H.C (2003). Atlas of Atherosclerosis: Progression and Regression. 2nd ed. New York: Parthenon Publishing Group.
- Sary, H.C., Chandler A.B., Glagov, S., Guyton, J.R., Insull, W., Jr., Rosenfeld, M.E., Schaffer, S.A., Schwartz, C.J, Wagner, W.D., Wissler R.W (1994). A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the

- Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arteriosclerosis Thrombosis* **14(5)**: 840-856.
- Sary, H.C., Blankenhorn, D.H., Chandler, A.B., Glagov, S., Insull, W., Richardson, M., Rosenfeld, M.E., Schaffer, S.A., Schwartz, C.J., Wagner, W.D., et. al. (1992). A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arteriosclerosis Thrombosis* **12(1)**:120-134.
- Sary, H.C., Chandler, A.B., Dinsmore, R.E., Fuster, V., Glagov, S., Insull W, Jr., Rosenfeld M.E., Schwartz, C.J., Wagner, W.D., Wissler, R.W (1995). A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* **92(5)**: 1355-1374.
- Stein, B., Fuster, V., Israel, D.H, et al (1989). Platelet inhibitor agents in cardiovascular disease: An update. *Journal of America College Cardiology* **14**: 813.
- Steinberg, D (2005). Hypercholesterolemia and inflammation in atherogenesis: two sides of the same coin. *Molecular Nutrition and Food Research* **49**: 995-998.
- Steve, O. Ogbonnia., Florence E. Nkemehule., Emmanuel, N., Anyika (2009). Evaluation of acute and subchronic toxicity of *Stachytarpheta angustifolia* (Mill) Vahl (Fam. Verbanaceae) extract in animals. *Africa Journal of Biotechnology* **8 (9)**: 1793-1799.
- Stocker, R., Keaney, J.F., Jr (2004). Role of oxidative modifications in atherosclerosis. *Physiological Review* **84**: 1381-1478.

- Suman, B.S, Reenu, S.T., Rini, A.C., Usha, R.S., Shipra, G., Santosh K.S (2010). Protective effect of *Morus rubra* L. leaf extract on diet –induced atherosclerosis in diabetic rats. *Indian Journal of Biochemistry and Biophysics* **47**: 26-31.
- Sun, J.S., Tsuang, Y.W., Chen, J.J., Huang, W.C., Hang, Y.S., Lu, F.J (1998). An ultra-weak chemiluminescence study on oxidative stress in rabbits following acute thermal injury *Burns* **24**: 225-231.
- Sundaresan, S., Subramanian, P (2005). Preventive effects of garlic extract on lipid peroxidation and antioxidant defence system in nnitrosodiethylamine induced hepato carcinogenesis in rats. *Acta Horticulture* **680**: 171-175.
- Surapaneni, K.M., Vishnu, P.V (2009). Lipid peroxidation, glutathione, ascorbic acid, vitamin E, antioxidant enzyme and serum homocysteine status in patients with polycystic ovary syndrome. *Biology and Medicine* **1(3)**: 44-49.
- Taner, Y., Elif, G.K., Tanju, H.B., Gonca, Y., Bülent, G., Ankara, T (2004). The role of oxidants and antioxidants in otitis media with effusion in children. *Otolaryngology Head and Neck Surgery* Volume 131 Number 6.
- Tewtrakul S, Subhadhirasakul S (2007). Anti-allergic activity of some selected plants in the Zingiberaceae family. *Journal of Ethnopharmacology* **109**: 535-538.
- Thanaboripat, D., Suvathi, Y., Srilohasin, P., Sripakdee, S., Patthanawanitchai, O., Charoensettasilp, S (2007). Inhibitory Effect of Essential Oils on the Growth of *Aspergillus flavus*. *King Mongkut's Institute of Science and Technology Journal* **7**: 1-7.
- Thim, T., Hagensen, M.K., Bentzon, J.F., Falk, E (2008). From vulnerable plaque to atherothrombosis. *Journal of Internal Medicine* **263 (5)**: 506-516.

- Thomas, B. Kryston., Anastassiya, B. Georgiev., Polycarpos, Pissis., Alexandros G. Georgakilas (2011). Role of oxidative stress and DNA damage in human carcinogenesis. *Mutation Research* **711**: 193–201.
- Tietz, N.W., Prude, E.L., Sirgard- Anderson, O (1994). Tietz textbook of Clinical Chemistry. (Burtis, C.A and Ashwood, E.R eds). W.B. Saunders Company: London 1354-1374.
- Toyohiko, Ariga., Hitomi, Kumagai., Masakiyo, Yoshikawa, Hajime Kawakami., Taiichiro, Seki., Hidetoshi, Sakurai., Isao, Hasegawa., Takeomi Etoh., Hiromichi, Sumiyoshi., Tadamitsu, Tsuneyoshi, Shinichiro Sumi., Kazuo Iwai (2002). *Journal of the Japanese Society for Horticultural Science* **71 (3)**: 362-369
- Ute, M., Awaia, A., Susanne, M., Claus, J (2007). Polysulfides as biologically active ingredients of garlic. *Organic Biomolecular. Chemistry* **5**: 1505-1518.
- Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M., Telser, J (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The international Journal of Biochemistry Cell biology* **39 (1)**: 44 -48.
- Van Wyk, B-E., Van Oudtshoorn, B., Gericke, N (1997). *Medicinal Plants of South Africa*. Briza Publications, Pretoria. (304 pages). (Revised edition 2000, reprint 2002, 2005).
- Van Wyk, B-E., & Gericke, N (2000). *People's Plants: a guide to useful plants of southern Africa*. Briza Publications, Pretoria. (351 pages). (Reprinted 2002, 2005).
- Vandewoude, M.F., Vandewoude, M.G (1987). Vitamin E status in normal population: the influence of age. *Journal of America College of Nutrition* **6 (4)**: 307-11.
- Varadarasou, Mouttaya Mounnissamy., Subramanian, Kavimani., Gnanapragasam, Sankari., Sabarimuthu, Darlin Quine.; Kuppuswamy, Subramani (2010). Evaluation of acute

- and sub-acute toxicity of ethanol extracts of *Cansjera rheedii* J. Gmelin (Opiliaceae). Journal of Brewing and Distilling **1(1)**: 011-014.
- Vasthi, K. E., Devarajan N (2011). Evaluation of free radical scavenging activity and biological properties of *spinacia oleracea l.* Inational Journal Enviromental Science and Technology **3 (1)**: 25-30.
- Vepa, S., Scribner, W.M., Parinandi, N.L., et al. (1999). Hydrogen peroxide stimulates tyrosine phosphorylation of focal adhesion kinase in vascular endothelial cells. Americal Journal of Physiology **277**: L150-L158
- Vinay, R Patel., Prakash R Patel., Sushil, S. Kajal (2010). Antioxidant Activity of Some Selected Medicinal Plants in Western Region of India. Advances in Biological Research **4(1)**: 23-26.
- Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. 2000a. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arteriosclerosis Thrombosis Vascular Biology **20(5)**:1262-1275.
- Viuda-Martos, M., Navajas, Y.R., Zapata, E.S., Fernandez-Lopez, J., Perez-Alvarez, J.A (2010). An Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet. Flavour Fragrant Journal **25**:13-19.
- Wafeka, Abdulah., Al, Hamedan (2010). Protective Effect of *Lepidium sativum* L. Seeds Powder and Extract on Hypercholesterolemic Rat. Journal of American Science **6(11)**: 873-879.

- Wagner, B.A., Buettner, G.R., Burns, C.P (1994). Free radical-mediated lipid peroxidation in cells: oxidizability is a function of cell lipid bis-allylic hydrogen content. *Biochemistry* **33**: 4449-4453.
- Wang, Q., Zeng, T., Yu, L., Xie, K (2007). Preventive effect of garlic oil on fatty liver in experimental rats. *Dulixue Zazhi* **21**: 450–453.
- Ware, J.A., Collier, B.S (1994). Platelet morphology, biochemistry and function. In: Williams' Hematology, Beutler, E, Lichtman, MA, Collier, BS, Kipps, TJ (Eds), McGraw Hill, New York. 1161.
- Wickens, A.P (2001). Ageing and the free radical theory. *Respiration Physiology* **128(3)**: 379-391.
- William, R Lagor., John, S. Millar (2010). Overview of the LDL receptor: relevance to cholesterol metabolism and future approaches for the treatment of coronary heart disease. *Journal of Receptor, Ligand and Channe Research* **3**: 1–14.
- Woo, J., Henry, J.B (1996). Metabolic intermediates and inorganic ions in clinical diagnosis and management by laboratory animals. Philadelphia: W.B Saunders Company. 162 193.
- Woodman, D.D (1996). Assessment of hepatotoxicity. In: Evans, G. O. (ed.) *Animal Clinical Chemistry, A Primer for Toxicologist*. Taylor & Francis, London. 71- 86.
- Wolfe, K., Wu, X., Liu, R.H (2003). Antioxidant activity of apple peels. *Journal of Agricultural and Food Chemistry* **51**: 609–614.
- World Health Organization, (2003). WHO guidelines on good agricultural and collection practices (GACP) for medicinal plants. World Health Organization, 2003.Geneva.

World Health Organization (2003). The World Health Report, Shaping the Future. World Health Organization, Geneva.

World Health Organization (2009). World Health Statistics 2008.

World Health Organization (2010). Global status report on non-communicable diseases 2010. World Health Organization 2011 Reprinted 2011.

Yakubu, M.T., Afolayan, A.J (2009). Effect of aqueous extract of *Bulbine natalensis* Baker stem on haematological and serum lipid profile of male Wistar rats. *Indian Journal of Experimental Biology* **47**: 283-288.

Yakubu, M.T., Akanji, M.A., Oladiji, A.T (2005). Aphrodisiac potentials of aqueous extract of *Fadogia agrestis* (Schweinf. Ex Heirn) stem in male albino rats. *Asian Journal Andrology* **7(4)**: 399-404.

Yakubu, M.T., Bilbis, L.S., Lawal, M., Akanji, M.A (2003). Effect of repeated administration of sildenafil citrate on selected enzyme activities of liver and kidney of male albino rat. *Nigerian Journal of Pure and Applied science* **18**:1395-4000.

Yanling, Wua., Junyun, Li., Jusu, Wangb., Qiuju, Si., Jianping, Zhanga., Ye, Jiangd., Li, Chu (2009). Anti-atherogenic effects of centipede acidic protein in rats fed an atherogenic diet. *Journal of Ethnopharmacology*. **122**: 509–516.

Yeh, Y.Y., Liu, L (2001). Cholesterol lowering effect of garlic extracts and organosulphur compounds: Human and animal studies. *Journal of Nutrition* **131(3s)**: 389s–93s.

- Yen, G.C., Chen, H.Y (1995). Antioxidant activity of various tea extracts in relation to their Anti-mutagenicity. *Journal of Agricultural and Food Chemistry* **43(1)**: 27-32. ijpt. 4 (1): 32-35 .
- Yogendrasinh, B., Solanki, Rajendra., V, Bhatt (2010). Effects of antioxidant vitamins along with atorvastatin and atorvastatin–niacin combination on diet-induced hypercholesterolemia in rats. *International of Journal Physiology Pathophysiology and Pharmacology* **2(1)**: 5763-17.
- Yuanxi, Xu., Yidong Wei., Yajun Zhang., Jianyun Gu., Jun Ma., Liyang Zheng., Dayi, Hu (2007). The characteristics of living and behaviour factors in Chinese Patients metabolic syndrome. *Journal of Health Science* **53 (1)**: 84-91.
- Yu-Yan, Yeh., Lijuan, Liu (2001). Cholesterol-Lowering Effect of Garlic Extracts a Organosulfur Compounds: Human and Animal Studies. *SAC. Journal of Nutrition* **131**: 989S-993S.