INVESTIGATION OF ANTIDIABETIC PROPERTIES, MECHANISMS OF ACTION AND TOXICOLOGY OF *STRYCHNOS HENNINGSII* (GILG) BARK.

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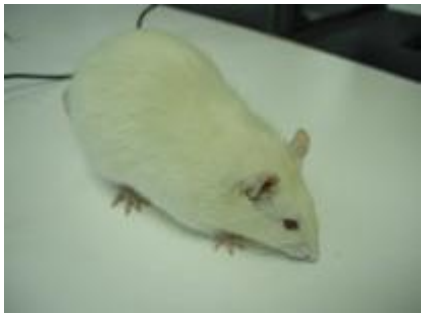

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DEDICATION

TO

GOD ALMIGHTY FOR HIS MERCY OVER MY LIFE

DECLARATION

I, the undersigned, declare that this thesis submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Biochemistry in the Faculty of Science and Agriculture, School of Biological and Environmental sciences and the work contained herein is my original work with exemption to the citations and that this work has not been submitted at any other University in partial or entirety for the award of any degree.

Name:_____

Signature:_____

Date:_____

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LIST OF ABBREVIATION

ATCC	- American Type Cell Culture
BSA	- Bovine Serum Albumin
DM	- Diabetes Mellitus
DMEM	- Dulbecco's Modified Eagles' Medium
DMSO	- Dimethyl sulfoxide
FCS	- Foetal Calf Serum
Fe	- Iron
FRAP	- Ferric Reducing Antioxidant Power
GLUT4	- Glucose Transporter 4
Hb	- Haemoglobin
LUC	- Large Unstained Cell
MCH	- Mean Corpuscular Haemoglobin
MCHC	- Mean Corpuscular Haemoglobin Concentration
MCV	- Mean Corpuscular Volume
MTT	-(3-(4,5-dimethylthiazolyl-2)- 2,5-diphenyltetrazoliumbromide)
Na_2CO_3	- Sodium Carbonate
NAD	- Nicotinamide
NADH	- Nicotinamide Adenine Dehydrogenases
NADPH	- Nicotinamide adenine dinucleotide phosphatease
$NaHCO_3$	- Sodium Hydrogen Carbonate
NIDDM	- Non-Insulin Dependent Diabetes Mellitus
PCV	- Pack Cell Volume

PI 3K	- Phosphatidylinositol 3'Kinase
PPAR	- Peroxisome Proliferator Activator Receptor Gamma
RBC	- Red Blood Cells
RCDW	- Red Cell Distribution Width
RPMI	- Roswell Park Memorial Institute
SH	- *Strychnos henningsii*
STZ	- Streptozotocin
TPRZ	- Tripyridyl-S-Triazine
WBC	- White Blood Cell

GENERAL ABSTRACT

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The apparent reversal of trend from modern drugs to herbal medicine is partly due to the fact that synthetic drugs have always shown adverse reactions and other undesirable side effects. Hence, the use of medicinal plants for the treatment of diseases such as diabetes is very common especially in the rural areas. Majority of these plants are used based on the experience and indigenous knowledge without identification of the therapeutic agents. There is enormous wealth of medicinal plants in the world yet many of them have not been discovered or studied scientifically to substantiate their ethno-medicinal usages.

Ethnobotanical study has been the method often used to search for locally important plant species for the discovery of crude drugs with low side effects. An ethnobotanical survey was conducted on the medicinal plants commonly used for the management of diabetes mellitus in Nkonkobe Municipality, Eastern Cape of South Africa. Information was obtained through structured questionnaire administered to traditional healers and herbalists in the region. The study revealed 15 species of plants belonging to 13 families. *Strychnos henningsii* and *Leonotis leonorus* among others were repeatedly mentioned by the traditional healers as the two mostly used plants for the management of diabetes mellitus. The infusion and decoction of the roots, leaves and barks of these plants are the methods of preparation.

The antioxidant potential of aqueous bark extract of *S. henningsii* was investigated both *in vivo* and *in vitro* using spectroscopic method. The antioxidant activity of the extract against hydrogen peroxide ($H_2O_{2)}$, 2,2′-azinobis[3-

ethylbenzothiazoline-6-sulfonic acid] diammonium salt (ABTS), as well as reducing power was concentration dependent. The extract exhibited lower and average scavenging activities against 1,1- diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) radicals with IC_{50} value of 0.739 and 0.49 mg/ml respectively. The administration of the plant extract at 250, 500 and 1000 mg/kg significantly increased the activities of the antioxidant enzymes in the hepatotoxic rats induced with carbon tetrachloride. On the other hand, the stem bark extract had lower effect on lipid peroxidation level except at the dose of 250 mg/kg.

The effect of oral administration of *S. henningsii* extract was evaluated in normal Wistar rats for 28 days. The observed result indicated non- toxic effect of sub-acute administration of plant extract to the animals except at certain doses. This is because, there was no apparent damage to some haematological and biochemical parameters used in assessing organ specific toxicity. However, the alterations observed on platelet, white blood cells and its differentials imply parameter and dose selective toxicity when repeatedly consumed on daily basis at the doses investigated.

This study also investigated the antidiabetic activities of the extract at the doses of 125, 250 and 500 mg/kg body weight in diabetic rats induced with streptozotocin - nicotinamide for 15 days. The extract appreciably ($P < 0.05$) reduced the blood glucose level, feed and water intake while the best result was obtained at 250 mg/kg. Similarly, the level of triacylglycerol at the three doses investigated was significantly decreased. In addition, the glucose tolerance was reduced to near normal level after 90 min at certain doses. The clinical significance of the extract on some biochemical and haematological parameters lessen both hepatic and renal damages. Anaemic condition in diabetic animals

was also improved after plant extract administration. However, no significant effect was observed in white blood cells and some of its differentials.

The extract demonstrated strong glucose utilization in 3T3-L1 cells with a response of 278.63% of the control at 12.5µg/ml while that of Chang liver cells was 103.54%. The cytotoxicity result revealed non toxic effects of the extract to both cell lines. Treatment of 3T3 L1 cells with the extract did not reduce lipid accumulation. The extract inhibited the activity of α- glucosidase and α- amylase in a concentration dependent manner with IC_{50} values of 38 µg/ml and 60.9 µg/ml respectively. The percentage protein antiglycation of *S. henningsii* was 18.4, 38.2 and 61.2% for 0.25, 0.5 and 1 mg/ml respectively while aminoguanidine a known inhibitor of protein glycation was 87.2% at 1 mg/ml. The FRAP assay values of the extract was 357.05 µmol Fe (II)/g. The findings from this study support the folkloric usage of this plant for the management of diabetes mellitus in the region.

Keywords: *S. henningsii*; ethnobotanical; antioxidant; toxicity; diabetes; glucose metabolism; protein glycation;

INTELLECTUAL PROPERTY AND AGREEMENT STATEMENT

All the role players including the elderly and the traditional healers who volunteered one information or the other, during the preliminary investigation on the folkloric use of *Strychnos henningsii* were adequately financially rewarded with further verbal agreement that this research shall not be for commercial purposes but will serve as an enlightenment information to the community and the entire Eastern Cape, Province on the efficacy, safety and toxicity of this plant.

ETHICAL COMMITTEE APPROVAL

The study involving the use of animals in this project was carried out following the approval of the Ethical Committee on Animal Use and Care of the University of Fort Hare.

COMPLIANCE STATEMENT

No part of this study in any form has been commercialized. The thesis is meant to be used for information dissemination on the medicinal potentials of *Strychnos henningsii* to the immediate community and the entire Eastern Cape Province of South Africa.

_____ _____

Supervisor signature Student signature

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

General introduction..... ..…….1

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GENERAL INTRODUCTION

1.1 Historical background of diabetes mellitus

Diabetes mellitus (DM) has affected human beings as early as prehistoric times. It was first identified by Hesy Ra an Egyptian physician about 3,500 years ago (CDA, 1999). The term siphon was first used to describe the disease as "diabetes" which means excessive loss of body fluid through excess urination from diabetic patients. The ethiopathogenesis of the disease was discovered in the 20^{th} century by ancient Chinese, who observed the attraction of ants to the urine of people suffering from diabetes. In 1672, Thomas Willis added the term "mellitus" to distinguish diabetes from other causes of excessive urination. This disease was further divided into "diabetes gras" (fat) or "diabetes maigre" (thin) by Bouchardat and Lancereaux (1880). They are now recognized as obese and non obese forms of diabetes mellitus. Before the discovery of insulin there was an invariable death sentence due to lack of clinical diagnosis of the disease. In 1922, Banting and Best discovered the use of insulin extracted from the islet tissues of animals for the treatment of diabetes mellitus. This discovery confirmed that some diabetic individuals are not insulin dependent (Berson and Yalow, 1954). Presently, the term diabetes mellitus is now defined as chronic metabolic disorder caused by insulin deficiency or inability of the body to use insulin properly or both (Lanza et al., 1999). It is characterized with hyperglycaemia that plays a key role in the progression of the disease and its complications (Rang et al., 1991).

1.2 Rationale and justification for this study

The current prevalence of DM worldwide constitutes a global public health burden (Boyle et al., 2001). Despite the development of new drugs to prevent the incidence of diabetes, it was still predicted to hit 220 million by 2010 (Erasmus et al., 1999). The prevalence of DM has impeded many developing nations through increased morbidity and mortality (Rang et al., 1991). This is because, there is no satisfactory effective therapy such as insulin or oral hypoglycaemic drugs that could normalize blood glucose homeostasis (Sumana and Suryawanshi, 2001). Moreover, they are not devoid of significant adverse side effects such as hypoglycemia, weight gain, edema, abnormal liver function and diarrhea (ADA, 2006). In addition, they are very expensive and as a result beyond the reach of diabetic patients especially those living in the rural areas. Owing to this fact, diabetic individuals have resorted to the use of herbal medicines with antidiabetic potential and probable fewer side effects (Addy and Nyarko, 1988; Nyarko et al., 1999). Majority of these herbal antidiabetic drugs have long been published but a large number of them remain unexplored. Further studies on pharmacognostic, mechanisms of actions and toxicological properties of these plants are necessitated to justify their folkloric usage (Hansotia and Drucker, 2005).

1.3 The choice of *Strychnos henningsii* for this study

The use of *S. henningsii* (Loganiaceae) by traditional healers for the management of diabetes was discovered in Nkonkobe Municipality, Eastern Cape, South Africa during our ethnobotanical survey (Oyedemi et al., 2009). The plant was frequently mentioned by the traditional healers and herbalists for the treatment of DM. It is a small evergreen tree with leathery leaves. The bark is crown compact with dark green, glossy foliage and the fruit is

oblong which turns brown when ripe. The leaves of the plant have a characteristic aromatic-pungent odour with rough texture (Leeuwenberg, 1969). It is commonly used in south and east Africa traditional medicine for the treatment of rheumatism, gynaecological complaints, abdominal pain, snake bite, gastrointestinal pain, DM as well as malaria (Hutchings, 1989; Bisset, 1970). Despite the acclaimed folkloric use of *Strychnos henningsii* as an antidiabetic agent, there is dearth of scientific evidence to substantiate the claim. Therefore, this study aimed at providing information on the antidiabetic, probable mechanisms of action and toxicity effect of this plant with a view to validating its acclaimed use by the traditional medicine practitioners of Eastern Cape. At the beginning of this study, there was no information in scientific literature on the antidiabetic properties of *S. henningsii*.

Figure 1: *Strychnos henningsii* Gilg (SH). A: SH in its natural habitat in Pirie mission garden (King William's Town). B: Bark of SH.

1.4 The aims and objectives of this study

The primary aim of this study was to validate the folkloric uses of aqueous stem bark extract of *Strychnos henningsii* for the management of DM.

Specific objectives

1.4.1 Ethnobotanical survey of plants

Ethnobotanical study is a process used to search for locally important plant species with low side effects especially for drug development which entails ethnomedical and ethnobotanical field work. The search for anti-diabetic plants has been focused on plants because of leads provided by natural products with better treatment than currently used drugs. Previous studies have revealed antidiabetic plants used for the management of DM in the Eastern Cape Province of South Africa. However, the medicinal plants used traditionally for the management of DM in some areas of the Province had not been explored or documented. In traditional medicine, the number of plants used in the treatment of diseases associated with physiological disorder such as diabetes is limited. Therefore, there is need to document plants and their indigenous knowledge considering the rate at which vegetation is getting depleted in this part of the world.

1.4.2 *In vivo* and *in vitro* antioxidant activities of plant extract

Oxidative stress, the consequence of the imbalance between prooxidants and antioxidants in an organism, is considered to play a very important role in the pathogenesis of several degenerative diseases. These include diabetes, aging, cancer, cardiovascular diseases,

metabolic syndrome, and atherosclerosis. High levels of oxidative stress with excessive generation of free radicals and depleted levels of antioxidant defense system have been demonstrated in animal models and human diabetic subjects (Turk et al., 2002). Free radicals have been reported to induce β – cell dysfunction in type 1 DM by auto-immune reactions and inflammatory cytokines (Cnop et al., 2005). Similarly, these radicals have also been indicated in type 2 diabetes to activate β –apoptotic pathways, impair insulin synthesis and thus contribute to insulin resistance (Evans et al., 2003; Ravi et al., 2004). Recently, an array of medicinal plants has been investigated as new potential sources of antioxidant with lesser or no toxicity effect. The presence of phytochemicals with strong antioxidant activity in these plants may enhance their ability to protect cells against the oxidative damage caused by these radicals. Research into antioxidant properties and phytochemicals of herbs may provide information on their mechanisms of action to support their ethnotherapeutic usage. A vast majority of these plants are used in traditional medicine in South Africa but have not been currently evaluated for their antioxidant potential. One of such plants is *Strychnos henningsii*.

1.4.3 Toxicological effects of *S. henningsii* in rats.

Plants consist of biologically active compounds such as cyanogenic glycosides, glycoalkaloid, salts of oxalic acid and alkaloids. These compounds have been reported to be toxic in animals when ingested at certain concentrations (Joseph and Eloy, 1982). Several reports in both developed and developing countries have indicated adverse side effects allegedly arising from the use of medicinal plants. (Elvin -Lewis, 2001). These effects could be attributed to the presence of phytotoxic compounds in the plant extracts and lack of knowledge of actual dosage necessary for the treatment of diseases (Azaizeh et al., 2003). Assessment of haematological parameters could be used to determine the extent of

deleterious effect of plant extract on the blood constituents of animals. These adverse effects may manifest significant alterations in the levels of biomolecules such as enzymes and metabolic products, normal functioning and histomorphology of the organs. The alterations may be used to predict toxicity in humans when the data are translated from animal studies (Olson et al., 2000). Although, the folkloric usage of these plants was mentioned by the traditional healers, there was no scientific attempt to evaluate their toxicity effects. Therefore, an attempt was made in this present study for scientific investigation on the toxicological profiles of *S. henningsii* extract using different doses in rats.

1.4.4 Antidiabetic and clinical significance of *S. henningsii* extract

The intra-peritoneal induction of DM by streptozotocin has been reported by several authors to damage some vital tissues in diabetic animals model (Kirsch and Groot, 2001; Muhammad et al., 2007). Some of these damages manifested by increased level of alanine (ALT) and aspartate transferases (AST), urea, albumin and creatinine in the liver and kidney of experimental diabetic animals (Sokeng et al., 2005). The findings of Pushparaj et al. (2000) also confirmed coronary heart disease in diabetic animals owing to the elevated level of cholesterol and triacylglycerol. The decreased level of red blood cells and its related indices was also evidenced in diabetic subject that leads to hypochromic microcytic anaemia (Baskar et al., 2006). Therefore, the present study was undertaken to provide scientific credence on the antidiabetic and beneficial effect of *S. henningsii* extract on some clinical parameters in diabetic rats.

1.4.5 In vitro antidiabetic properties of *Strychnos henningsii* stem bark extract used in South African herbal medicine

The impairment of insulin in glucose utilization is known as the main disturbance of nutrient metabolism (Bailey and Day, 2004). This occurs mostly in target tissues such as skeletal muscle, adipose tissue and liver cells of type 2 diabetic patients (Baynes and Dominiczak, 2004; Guyton and Hall, 2005). Similarly, the ability of *S. henningsii* extracts to inhibit α - glucosidase and amylase were explored to establish its usefulness as herbal medicine. An inhibitor of these enzymes is useful to prevent diabetes and obesity by retarding carbohydrate digestion and absorption. It has been recognized that oxidative stress is another factor apart from hyperglycemia that enhances protein glycation that further resulted to diabetic complications (Miyata et al., 1999). To delay or prevent diabetic complications botanical consumption that can inhibit protein glycation as well as lipid accumulation during diabetes is highly needed (Edwin et al., 2008; Hu et al., 2003). The present study was an attempt to understand the mechanisms of action underlying the antidiabetic activities observed in animal model. The inhibitory effect of *S. henningsii* extract against protein glycation, lipid accumulation as well as glucose utilization in 3T3-L1 and Chang liver cell lines including cell viability were tested.

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CHAPTER 2

LITERATURE REVIEW

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CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes Mellitus

Diabetes Mellitus is a group of chronic metabolic disorders characterized by chronic hyperglycaemia as a result of relative or absolute lack of insulin or the actions of insulin (Kumar and Clark, 2002). Chronic hyperglycaemia during diabetes increase non enzymatic glycation of proteins that later leads to secondary complication. These complications include hypoglycaemia, diabetic ketoacidosis, non-ketotic syndrome, thirst, polyuria, visual blurriness, weight loss, hypertension, neuropathy, nephropathy and retinopathy. Several hypoglycaemic drugs, insulin injections and life style changes, such as exercise, weight control and diets have been employed for the treatment of diabetes (Knentz and Nattras, 1991; Kumar and Clark, 2002). However, the management of this disease is still a challenge to the medical system (Kameswararao et al., 2003). This is because none of these hypoglycaemic drugs were able to cure diabetes without causing any adverse side effects. These effects have led to an increase in the prevalence of DM worldwide. Recently, much attention has been focused on antidiabetic plants used in traditional medicine mostly because of better treatment than the modern western medicine.

2.1.1 Types of diabetes mellitus

There are three main types of diabetes mellitus namely: type 1 diabetes (insulin-dependent diabetes mellitus), type 2 diabetes (non insulin- dependent diabetes mellitus) and

gestational diabetes. The symptoms of these forms of diabetes are similar but vary in their intensity. Type 1 DM is caused by immunological destruction of pancreatic β cells leading to absolute insulin deficiency (Notkins, 2002). It is a juvenile disease with 10 % prevalence among diabetic patients worldwide (Ranjan et al., 2002). Patients affected by this form of diabetes are completely dependent on exogenous insulin to maintain a normal life style. Type 2 is another form of DM characterized with insulin resistance and is the most common type of diabetes afflicting 85-95 % of all diabetic individuals over 40 years of age (Lanza et al., 1999). Gestational diabetes is a common disorder that occurs mostly during pregnancy due to hormonal changes or insulin deficiency. It affects approximately 7 % of pregnancies each year (ADA, 2000). This disorder can increase the risk of developing type 2 DM for both mother and the baby if not properly managed.

2.1.2 Cause and prevalence of diabetes mellitus

The causes of diabetes are not clearly known. However it is associated with risk factors that are considered to be the pathogenesis of this disease. These factors include consumption of calorie- rich diet, hereditary, obesity, sedentary life style, smoking, virus infection, age, socio-economic status as well as increasing number of ageing population. The number of people suffering from the disease worldwide is increasing at an alarming rate. According to Wild et al. (2004) about 366 million people were projected to be diabetic by the year 2030 against 191 million estimated in 2000. All over the world, India is ranked as the country with the highest diabetic patients followed by China and United State of America. Unfortunately, Africa and Asia were recently identified as regions where the number of diabetics could rise above the predicted level (ADA, 2006). In South Africa, the prevalence of people suffering from diabetes has been rising steadily over the past two decades. The

prevalence is predicted to hit 3.9 % of the South African population between the age of 20 and 79 by the year 2025 (www.eatlas.idf.org). Insufficient promising therapy to cure diabetes could be responsible for the predicted figure in this part of the world.

2.2 Insulin

Insulin is a peptide hormone secreted by pancreatic β-cells in response to increased blood glucose levels. It was first discovered by Canadian scientists in the year 1921 due to increase death rate of people suffering from type 1 diabetes (Bliss, 1982). Insulin has a molecular weight of 6,000 Daltons with two subunits held together by disulphide bonds. It is secreted in significant amounts as preproinsulin in the ribosome of endoplasmic reticulum. The cleavage of signal peptide of preproinsulin results in the formation of proinsulin and later matures into insulin through the action of endo-peptidases (ADA, 1997).

2.2.1 Mechanism of insulin biosynthesis

The secretion of insulin from pancreatic β cells is a complex process involving a glucose induced biphasic pattern of insulin release. Defects in both phases of insulin secretion may be the earliest detectable stage of type 2 diabetes (Gerich, 2002). Secretion of insulin is based on K_{ATP} channels which are grouped into two principal signaling pathways. These are ATP - dependent and ATP ‗ independent K^+ channels (Chow et al., 1995; Straub and Sharp, 2002). In the K_{ATP} channel pathway, ATP derived from the glycolytic metabolism of glucose closes the K_{ATP} channels and causes β- cell membrane depolarization. The resulting opening of voltage-dependent L-type Ca^{2+} channels increases influx of extracellular Ca^{2+} into the β- cells, leading to rise in $[Ca^{2+}]_i$ which triggers the

release of insulin (Yang and Gillis, 2004). On the other hand, the mechanisms involved in the ATP -independent channel are yet to be defined. Nevertheless, several signaling pathways are employed in this channel for the secretion of insulin. These include mitochondria-generated signaling, Adenylate cyclase and PKA signaling, PLA_2 and arachidonic acid signaling pathways (Chow et al., 1995).

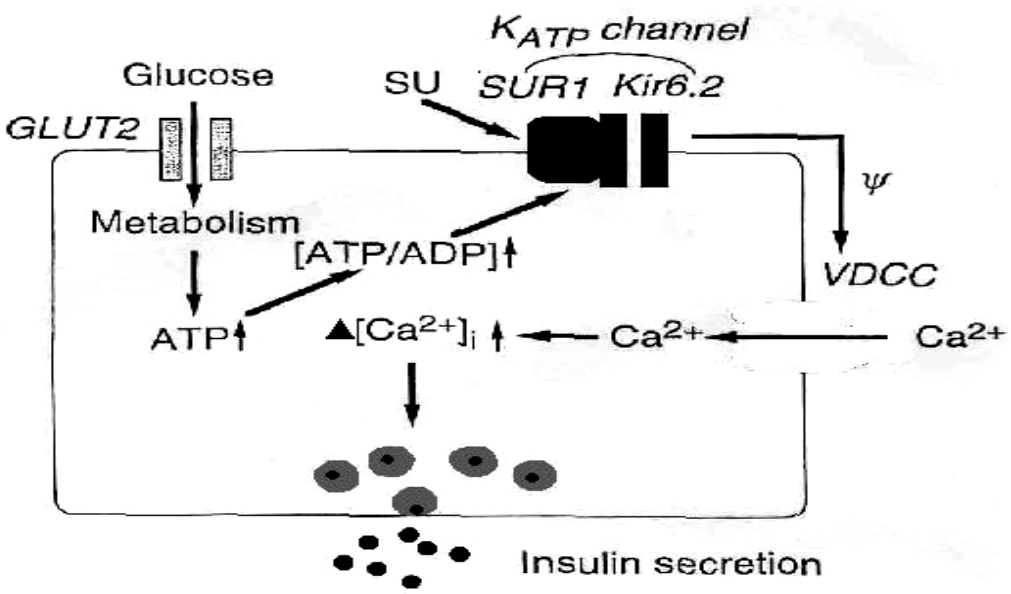

Figure 2: The major pathway of glucose induced and sulfonylurea (SU) -induced insulin secretion. GLUT 2 (glucose transporter 2); VDCC- voltage-dependent calcium channel.

2.3 Functions of insulin

Insulin is a potent anabolic hormone that stimulates the uptake and storage of glucose, fatty acids and amino acids into glycogen, fat and protein respectively. It regulates glucose homeostasis, storage of glycogen and formation of triglycerides, in different target tissues as shown in Figure 2. It is also known to modulate transcriptional processes, replication of cells, DNA synthesis and increase amino acid transport into cells as well as to stimulate lipogenesis (Nakae and Accili, 1999).

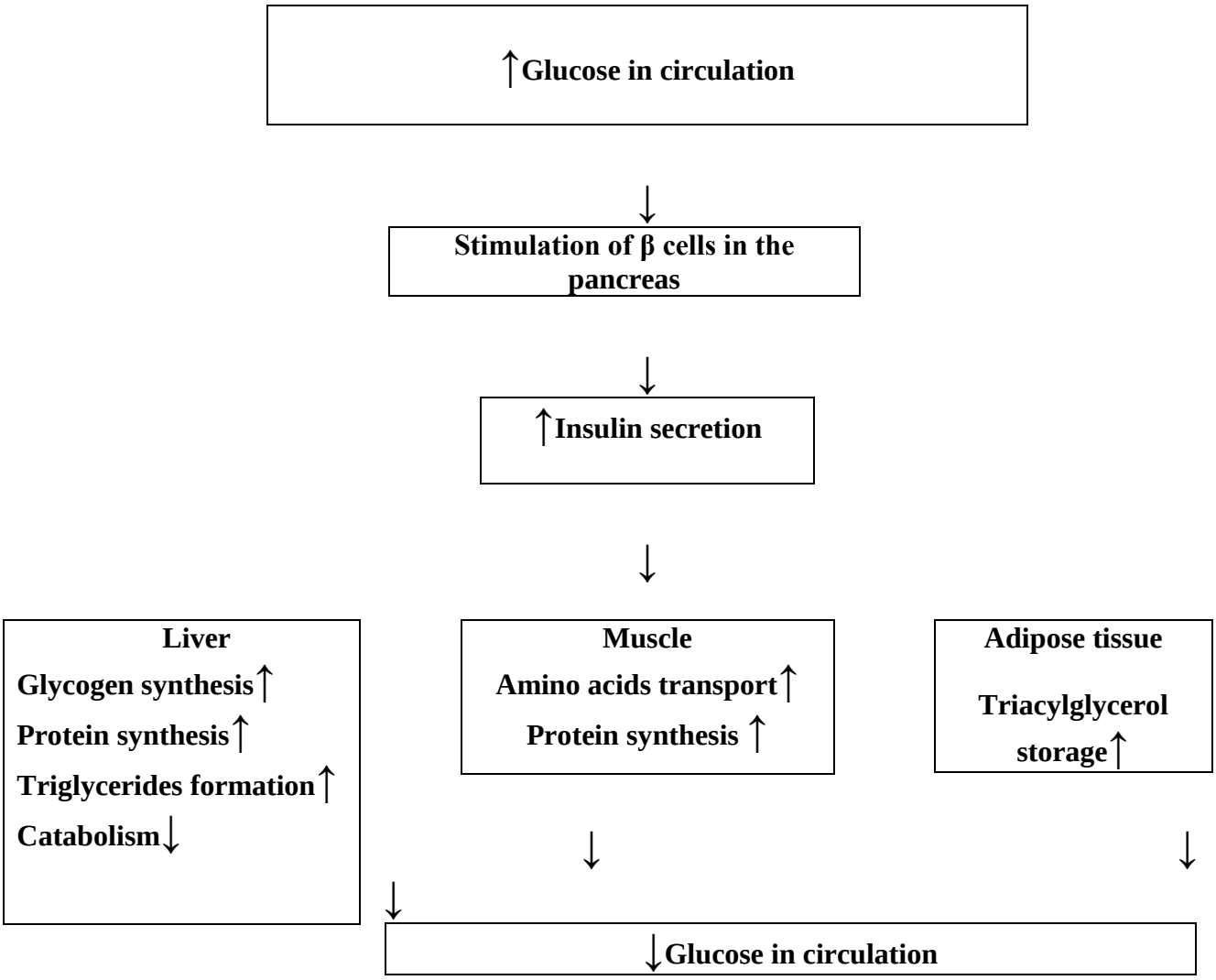

Figure 3: Schematic diagram of function of insulin

2.3.1 Glucose metabolism

Glucose is the prime fuel for the generation of energy with the formula $C_6H_{12}O_6$. During fasting, glucagon is secreted which break down liver glycogen and thus leads to the synthesis of glucose through gluconeogenesis pathway after depletion of glycogen (Voet et al., 2002). Activation of glucagon raises the glucose level in the blood by binding to the tissue receptors to activate adenylyl cyclase which is linked to the cAMP (cyclic adenosine monophosphate) mediated cascade (Figure 3).

2.3.2 Glucose transport

The action of insulin in glucose uptake, involves translocation of glucose transporter proteins (GLUTs) from intracellular compartments to the cellular membrane where glucose uptake is being facilitated (Cushman and Wardzala, 1980). Transport of glucose is initiated upon binding of insulin to the insulin receptor (Schaffer et al., 2003). This binding results into a conformational change and auto-phosphorylations of the receptors as shown in Figure 4. Among several insulin receptors substrates (IRS), IRS1 is recruited and binds to a phosphotyrosine residue (Chen et al., 1996). The increase in enzyme activity of phosphoinositide-3-kinase enhances the recruitment of Protien Kinase B to bind with the plasma membrane (Eldar et al., 1997; Lodish et al., 2004). After localizing on the membrane, protein kinase B is phosphorylated by two membrane bound kinases and then released into the cytosol where glucose uptake and glycogen synthesis are stimulated (Foulds et al., 2004; Lodish et al., 2004). It has been reported that glucose homeostasis is regulated by insulin via uptake of glucose into skeletal muscle, and to a lesser extent, into the liver and adipose tissue (Fielding and Frayn, 1998).

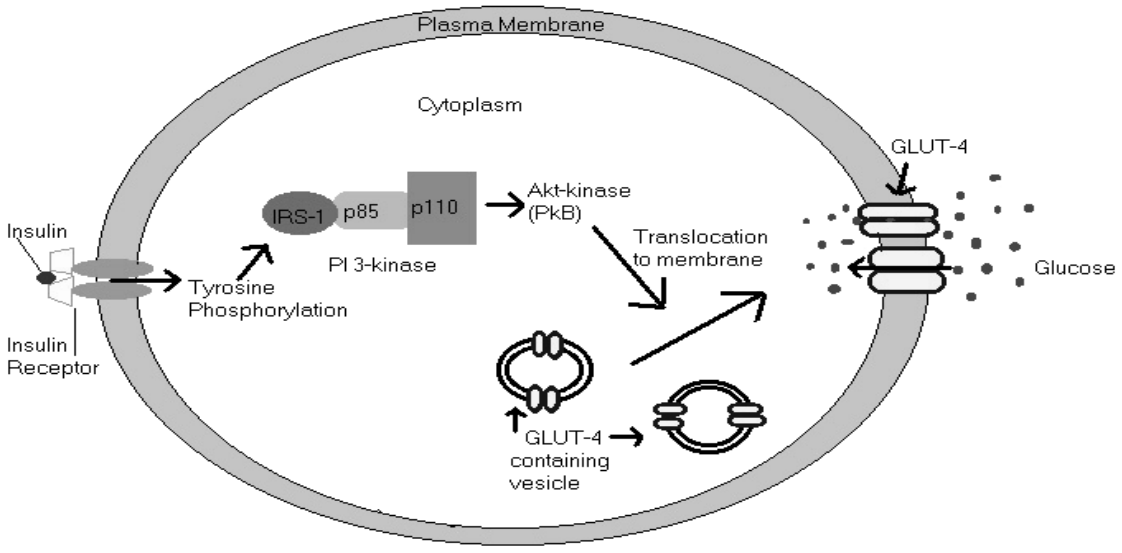

Figure 4: Insulin Signaling Pathway for Glucose Transport Chain (Chen et al., 1996).

2.3.3 Insulin facilitates glucose uptake in the target tissues

Skeletal muscle is one of the key tissues involved in the maintenance of whole body glucose homeostasis. It takes up glucose from the circulation in response to insulin, and is largely responsible for clearing post-prandial hyperglycemia (Moore et al., 2003). The increased glucose uptake by skeletal muscle is mediated by type 4 glucose transporter (GLUT4), which is stored in intracellular vesicles in the absence of insulin (Mueckler, 1994). The adipose tissue is another major site for the uptake of dietary fatty acids. It releases energy in the form of fatty acids (Fielding and Frayn, 1998). In the presence of insulin (Figure 4), adipocytes take up glucose and lipids from the blood and store them as triacylglycerol. After the meal, the adipose tissue functions to supply energy for other tissues by releasing free fatty acid (FFAs) into circulation in the form of chylomicrons for energy production (Fielding and Frayn, 1998). Hepatocytes take up glucose through glucose transporter 2 (GLUT 2) localised at the plasma membrane (Bary and Horuk, 1984). When glucose enters the hepatocytes through facilitated diffusion mechanism, it is retained through conversion by hepatic glucokinase to glucose-6-phosphate in the glycolytic pathways (Pilkis and Granner, 1992; Zhang et al., 2002).

2.3.4 Lipids metabolism

In humans, another major source of fuel or energy apart from carbohydrate is lipids. Lipids are water insoluble biomolecules which includes phospholipids, triglycerides, glycolipids and sterols. Triglycerides among other lipids represent the storage form mainly in the adipocytes and are composed of glycerol and three fatty acid molecules. It is hydrolyzed into free fatty acids and mono-acylglycerols by a process called lipolysis. This process involves multiple lipases that are produced by gastric mucosa and pancreatic cells (Park and Hellerstein, 2000). Free fatty acids and mono-acylglycerols are re-esterified into triglycerides

after uptake by intestinal cells into chylomicrons. Chylomicrons are particles containing a hydrophobic core of triglycerides and cholesteryl esters surrounded by a monolayer of phospholipids and cholesterol. The lipids in these particles both triglycerides and cholesterol are taken up by hepatic and peripheral tissues, mainly muscle and adipose tissue by the action of lipases. In addition, triglycerides are also uptake by the liver from very- low density lipoprotein (VLDL) by which the secretion is regulated by insulin (Park et al., 1999).

2.4 Western medicine for diabetes treatment

Several hypoglycaemic drugs have been employed for the treatment of diabetes with different mechanism of actions. These include sulphonylureas, metformin, acarbose, thiazolidinedione, glibenclamide and insulin. Metformin is a first-line drug of choice for the treatment of type 2 diabetes especially in overweight and obese people (ADA, 2000). It increases insulin sensitivity by suppressing hepatic glucose production as well as increasing muscle glucose uptake. The molecular targets of this drug are currently unknown but activation of the adenosine mono-phosphate (AMP) activated protein kinase has been reported to play an important role (Bailey and Dominiczak, 2004). Sulphonylureas are another first line treatment of type 2 diabetes that acts directly on the pancreatic β cells to potentiate insulin secretion. Acarbose is a new therapeutic drug used basically to inhibit α - glucosidase without directly interfering with glucose uptake (Wehmeier and Piepersberg, 2004). It is often used in combinational therapy with other drugs or insulin to lower the blood glucose level. Rosiglitazone and pioglitazone are thiazolidinedione currently used to improve the body's response to insulin by lowering insulin resistance in the cells. The molecular target for thiazolidinedione is the peroxisome proliferator-activated receptor γ (PPAR), where it acts as an agonist (Suzuki et al., 2010). Lastly, insulin treatment is used in a later stage of type 2

DM progression, when pancreatic β cells can no longer secrete a sufficient amount of insulin to maintain an acceptable glucose concentration in the circulation. It is a life dependent drug for type 1 diabetic patients.

2.5 Limitation to western medicine

The limitation encountered from the use of modern antidiabetic drugs could be linked to the undirectional therapeutic approach, high cost and the presence of adverse side effects (Tiwari and Rao, 2002). For example, the use of sulphonylureas has been reported to worsen heart disease, increase body weight and induce hypoglycaemia (Dey et al., 2003). Liver toxicity was observed in patients treated with thiazolidinedione while diarrhea, abdominal discomfort, flatulence and pain were shown in patients taking glucosidase inhibitors (De Fronzo, 1999). Hypoglycaemia and other symptoms including nausea, hunger, tiredness, palpitation and headache have also been reported as major side effects of insulin therapy (www.medicinenet.com/insulin). Another important factor of western medicine limitation could be attributed to the belief that herbs do provide some benefits over and above allopathic medicine and allow the users to feel that they have some control in their choice of medication (Joshi and Kaul, 2001). These drugs are very expensive and as a result beyond the reach of diabetic patients especially those living in the rural areas. Therefore, there is need to search for natural products of plant origin as a source of novel molecules that can complement the drugs currently used to negate the adverse side effects of western medicine (Marles and Farnsworth, 1995).

2.6 Alternative medicine

Insulin was first introduced in 1922 for the treatment of diabetes, before then diabetes treatment relied heavily on dietary measures which included the use of medicinal plant therapies. Medicinal plants have been used in traditional medicines, since time immemorial to maintain health or cure ailments from the dawn of civilization (Kalemba and Kunicka, 2003). It has been a rich source of new drug discovery. For example, metformin the most commonly used antidiabetic drug was discovered from *Galega officinalis* since the Middle Ages because of its richness in guanidine (Bailey and Day, 2004). In the last few years there has been an exponential growth in the field of herbal medicine and these plants are gaining popularity in some countries for primary health care because of their wide biological and medicinal activities, lower side effects and lesser costs (Farnsworth, 1994). Herbal medicines continue to play an important role in diabetic therapy, particularly in the developing countries where most people have limited resources and do not have access to modern treatment (Ali et al., 2006). The use of herbal medicine for the treatment of diabetes has been authenticated (WHO, 1980) due to the presence of phytochemicals with antidiabetic properties. Some of these compounds include fibres, vitamins, minerals, secondary metabolites and xanthones (Day, 1998). The increase in demand for the use of plant based medicines to treat diabetes may be due to the side effects associated with the use of orthodox drugs such as insulin and oral hypoglycemic agents (Marles and Farnsworth, 1995). Another important factor that strengthens the use of plant materials as antidiabetic could be attributed to the belief that herbs do provide some benefits over and above allopathic medicine and allow the users to feel that they have some control in their choice of medication (Joshi and Kaul, 2001). However, few of these plants have received scientific or medical scrutiny as recommended by the World Health Organization.

2.6.1 Mechanisms of actions of antidiabetic plants

Several antidiabetic plants have been reported in different parts of the world for the treatment of diabetes with different therapeutic targets (Edwin et al., 2008). Some were investigated in streptozotocin (STZ) and alloxan induced diabetic rats at different dosages to evaluate their antidiabetic potentials. Majority of these plants displayed antihyperglycaemic properties while few have shown hypoglycaemic effect. Some of the mechanisms of action reported are related to inhibition of mitochondrial function, stimulation of glycolysis, activation of AMPK (adenosine mono-phosphate kinase) pathway, suppression of adipogenesis, uptake of glucose and induction of low density lipoprotein (Jun et al., 2009; Matsui et al., 2006). In addition, some plants with antidiabetic properties have also been reported to inhibit carbohydrate digestive enzymes such as α- glucosidase and α- amylase (Shibib et al., 1993). Antioxidant properties and modification of insulin structure or insulin receptor sensitivity as well as up-regulation of glucose transporter of some plants have been reported in several studies (Day, 1990; Cummings et al., 2002; Hardman and Limberd, 2001). Although, the metabolic activities of these plants are well established, the molecular mechanism underlying their biological activities remains unknown. Many South African medicinal plants have been investigated which include *Leonotis leonurus*, *Sutherlandia frutescens*, *Cissampelo capensis*, *Schotia latifolia*, *Vernonia amygdalina* and *Momordica foetis.* These plants among others have been reported to possess anti-hyperglycemia, hypoglycaemic, hypolipidaemic and anti-obesity properties due to their action on insulin sensitization, β- cell preservation and antioxidant activities (Jeevathayaparan et al., 1995). Many other antidiabetic plants have been reported as scavenger of free radicals generated at the onset and during diabetic state in the experimental animals (Rice-Evans et al., 1996).

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CHAPTER 3

ETHNOBOTANICAL STUDY OF PLANTS USED FOR THE MANAGEMENT OF DIABETES MELLITUS IN THE NKONKOBE MUNICIPALITY OF SOUTH AFRICA

CHAPTER 3

Ethnobotanical study of plants used for the management of diabetes mellitus in the Nkonkobe Municipality of South Africa.

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Ethnobotanical survey of medicinal plants used for the management of diabetes mellitus in the Nkonkobe Municipality of South Africa

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Abstract

An ethnobotanical survey was conducted on the medicinal plants frequently used for the management of diabetes mellitus in Nkonkobe Municipality, Eastern Cape of South Africa. Information was obtained through structured questionnaire administered to traditional healers and herbalists in the region. The study revealed 15 species of plants belonging to 13 families namely; *Hyacinthaceae, Asphodelaceae, Caryophyllaceae, Aloaceae, Loganiaceae, Portulaceae, Solanaceae, Asteraceae, Hypoxideae, Menispermaceae, Alliaceae, Celastraceae* and *Lamiaceae*. The infusion of the roots, leaves and barks of these plants are the most commonly used while the extracts are taken orally for long period of time usually between six and 12 months, depending on the severity of the ailments. *Strychnos henningsii* and *Leonotis leonorus* of the families *Loganiaceae* and *Lamiaceae* respectively, were repeatedly mentioned by the traditional healers as the two mostly used for the management of diabetes mellitus in the study area.

Key words: Medicinal plants, diabetes mellitus, herbal medicine.

INTRODUCTION

Diabetes mellitus is a chronic disorder that affects the metabolism of carbohydrates, fats, proteins and electrolytes in the body as a result of defect in insulin secretion, insensitivity of target organs to insulin or both. It is characterized by chronic high blood glucose that causes glycation of body protein which could lead to severe complications (Rang et al., 1991). These complications are classified into acute, sub-acute and chronic. Acute complications include hypoglycemia, diabetic ketoacidosis, hyperosmolar and hyperglycaemic non-ketotic syndrome (Knentz and Nattras, 1991) while sub acute complications are thirst, polyuria, lack of energy, visual blurriness and weight loss (Kumar and Clark, 2002). The chronic complications of diabetes mellitus include hypertension, neuropathy, nephropathy, retinopathy and diabetic foot ulcers which could result to amputation.

Diabetes insipidus is another disorder that is associated with lack of vasopressin and characterized by an abnormal increase in urine output, fluid intake, frequent urination, and excessive thirst (Perkin et al., 2003). Vasopressin is an antidiuretic hormone formed in the hypothalamus and secreted by the pituitary gland. The hormone is used primarily to control water retention through the reduction in urine output. Although, both diabetes mellitus and diabetes insipidus share some common symptoms such as frequent urination and excessive thirst, however, the diagnosis and treatments are not the same (Perkin et al., 2003). Therefore, diabetes insipidus should not be confused with diabetes mellitus, which results from insulin deficiency or resistance leading to high blood glucose.

On the basis of aetiology and clinical presentation, diabetes mellitus can be grouped into type 1 known as insulin- dependent diabetes mellitus (IDDM) and type 2 diabetes mellitus also known as non insulin- dependent diabetes mellitus (NIDDM).

Type 1 diabetes mellitus is prevalent in 10 % of diabetic patients and caused by insulin deficiency due to the lack of functional β cells (Notkins, 2002), whereas type 2 diabetes is characterized with insulin dysfunction. Type 2 diabetes includes all cases of diabetes except those that are insulin dependent and afflicting 85-95% of all diabetic individuals. It is a prevalent form of the disease and common in individuals over 40 years of age.

In South Africa, the number of people suffering from diabetes has been rising steadily over the past two decades. Several reports have stressed the high mortality from diabetes especially among the black populations. The prevalence is predicted to hit 3.9 % of South Africans population between the age of 20 and 79 by the year 2025 (www.eatlas.idf.org). This is because; there is no promising therapy, neither insulin injection nor numerous oral hypoglycemic drugs that could cure diabetes mellitus (Sumana and Suryawanshi, 2001). In addition, these drugs are characterized by appreciable level of toxicity, high cost and unavailability to the people in the rural area. Because of this, many South African patients seek relief from traditional healers who administer plant preparations for the treatment of the disease (Morris, 2002; Farnsworth et al., 1985).

The use of plant derived products containing high concentration of dietary fibre and complex polysaccharide for the management of diabetes have been proposed (Jenkins et al., 1976). Natural products especially of plant origin have been found to be potential sources of novel molecules for the treatment of diabetes (Farnsworth, 1994; Marles and Farnsworth, 1995). Considering the rate at which the vegetation is getting depleted in this part of the world, there is need to document the precious knowledge of these plants and to search for more plants with antidiabetic potential.

The search for anti-diabetic agents has been focused on plants because of their ready availability, effectiveness, affordability and low side effects.

Ethnobotanical study has been the method often used to search for locally important plant species with low side effects especially for the discovery of crude drugs (Farnsworth, 1994). The previous ethnobotanical survey conducted by Erasto et al. (2005) revealed 14 plant species used for the management of diabetes in the Eastern Cape Province of South Africa. However, before the commencement of this study, the medicinal plants for the treatment of diabetes in Nkonkobe Municipality area of the Province had not been explored or documented. The present study therefore is a documentation of plants and plant parts used exclusively for the management of diabetes mellitus by traditional healers of the region.

STUDY AREA

Nkonkobe Municipality is an area in South Africa that is situated between 32^{o} 47' S and 26^{o} 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). The major ethnic group is Xhosa speaking people with farming as their main occupation. The people of the region use herbal medications either alone or in combination with orthodox medicines for the treatment of several diseases. The majority of the people are rural dwellers; hence the use of plants for the treatment of common diseases, such as diabetes is very common.

MATERIALS AND METHODS

This study was carried out from May to June 2008 using a well structured questionnaire. The set questions contained the diagnosis of diabetes mellitus, the names of plants, methods of preparation, duration of treatments, adverse effects and mode of administration of the plant materials. Traditional healers and herbalists were interviewed consisting of women and men between 40 and 60 years of age. They have low educational qualifications and were all married. Vouchers of the reported antidiabetic plants were collected, and identified by Prof. DS, Grierson at the Botany Department, University of Fort Hare and deposited at the Giffen Herbarium.

RESULTS AND DISCUSSION

The study revealed 15 species of plants belonging to 11 families that are commonly used by the herbalists, traditional healers and people of Nkonkobe Municipality of South Africa for the management of diabetes mellitus (Table 2). Out of these, 13 species (86.67 %) have not been reported before in the study area for the treatment of diabetes mellitus. Members of *Asphodelaceae* and *Alliaceae* were the most commonly used while other families had one species each. The root of the plants was mostly used (33.3%) followed by the whole plants (20%), corms (20%), bark (13.3%) and leaves (13.3%). Crushing and boiling of plant materials remains the commonly used method for herbal preparation. These are taken orally for long period of time usually between six and 12 months depending on the severity of the ailment. The traditional healers and herbalists consulted in this study claimed to diagnose diabetes mellitus in their patients by observing symptoms such as loss of weight, fatigue, excessive urination and presence of sugar in urine. Two of the plant species,

Leonotis leonorus and *Strychnos henningsii* were frequently mentioned by the traditional healers and herbalists for the treatment of this disease. Information from the literature revealed that both plants are used for the treatment of many other diseases besides diabetes mellitus. For example, *Leonotis leonurus* is used orally for the treatment of cough, cold, influenza, chest infections, diabetes mellitus, eczema, epilepsy, delayed menstruation; intestinal worms, constipation, scorpion stings, spider and snake bite in South Africa (Jager et al., 1996; Van Wyk et al., 2000; Ososki et al., 2002). The plant has also been used topically for the management of haemorrhoids, eczema, skin rashes and boils (Bienvenu et al., 2002). In other parts of Africa, the infusion of the leaves and stem are used as purgative and for the treatment of influenza, tuberculosis, jaundice, hypertension and muscular cramps (Noumi et al., 1999). Whereas, *Strychnos henningsii* is used for various indications in traditional medicine including rheumatism, gynaecological complaints, abdominal pain, snake bite, gastrointestinal pain, malaria and diabetes (Hutchings, 1989; Bisset, 1970); it also has significant medicinal uses in the healing of wounds and as a mouth antiseptic.

In conclusion, this study has revealed 15 medicinal plants, of which *Leonotis leonurus* and *Strychnos henningsii* were frequently mentioned by the traditional healers and herbalists of the area. This primary information may possibly offer effective and affordable management of diabetes mellitus. Further experimental investigations are in progress to validate the pharmacological uses of the plants.

Table 1: Diagnosing methods of diabetes mellitus by the herbalists using herbal drug.

Diagnosis	Respondent (%)
Loss of body weight	100
Body weakness	100
Excessive urination	100
Presence of sugar in urine	100
Excessive thirsty	53
Duration of treatment	
Short duration	40
Long duration	60
Efficacy of plant treatment on patients	
Disappearance of sugar in urine	100
Reduction in body weakness	100
Normal body weight	100
Reduction in frequency of urination	100
Traditional healers claim of no adverse effect after treatment	
Yes	100
No	0
Traditional healers claim of total cure after treatment	
Yes	80
No	20

% of respondent = Number of respondent/Total number of respondent × 100/1

Table 2: Plants used for the management of diabetes mellitus in Nkonkobe Municipality, South Africa

Scientific names	Local names (Xhosa)	Family name	Parts used	Preparation and mode of administration (3 times a day after meal)
Albuca setosa	Ingwe beba	Hyacinthaceae	Corms	Fresh corms are crushed, boiled and ½ of a cup taken orally.
Bulbine abyssinica	Uyakayakana	Asphodelaceae	Whole plants	A fresh plant is crushed, boiled and 2 teaspoonfuls of the infusion is taken orally.
Allium sativum	Ikoronofile	Alliaceae	Whole plants	A fresh plant is crushed, boiled and ½ of a cup of infusion taken orally.
Bulbine natalensis	Ibhucu	Asphodelaceae	Roots	The infusion is made from boiled fresh roots and 2 spoonfuls taken orally.
Aloe ferox Mill	Ikhala- lasekoloni	Aloaceae	Leaves	The liquid from the leaves is boiled to powder, soaked in water and ½ of a cup taken orally.
Strychnos henningsii	Umnonono	Loganiaceae	Barks	The barks are crushed to powder and the infusion is ½ of a cup taken orally.
Cissampelo capensis	Umayisake	Menispermaceae	Roots	Fresh corms are crushed boiled and 2 spoonfuls taken orally.
Anacampseros ustulata	Igwele	Portulaceae	Corms	Fresh corms are crushed, boiled and ½ of a cup taken orally.
Solanum aculeastrum	Umtuma	Solanaceae	Roots	The fresh crushed roots are boiled and 2 teaspoonfuls taken orally

INTELLECTUAL PROPERTY AGREEMENT STATEMENT

All the elderly and the traditional healers who contributed one information or the other during our ethnobotanical survey of medicinal plants used for the management of diabetes mellitus in the Nkonkobe Municipality were adequately financially rewarded with further verbal agreement that this research shall not be for commercial purposes but to serve as an enlightenment information to the community and the entire Eastern Cape, Province on the plants used for the management of diabetes mellitus.

COMPLIANCE STATEMENT

No part of this study in any form has been commercialized, instead is meant to be used as a tool for information dissemination on the medicinal plants used for the management of diabetes mellitus in Nkonkobe Municipality and the entire Eastern Cape Province of South Africa.

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CHAPTER 4

IN VITRO AND *IN VIVO* ANTIOXIDANT ACTIVITIES OF STEM BARK EXTRACT OF STRYCHNOS HENNINGSII GILG

CHAPTER 4

In vitro and *in vivo* antioxidant activities of aqueous stem bark extract of *Strychnos henningsii* Gilg.

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***In vitro* and *in vivo* antioxidant activities of aqueous bark extract of *Strychnos henningsii* Gilg**

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Abstract

The present study assessed the antioxidant activity of aqueous stem bark extract of *Strychnos henningsii* on the occurrence of oxidative stress which plays an important role in chronic complications of diabetes mellitus. The antioxidant and free radical scavenging activity of aqueous extract of this plant was investigated using a spectroscopic method against 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anions, hydrogen peroxide (H_2O_2), nitric oxide (NO), 2, 2'- azinobis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt (ABTS) and the ferric reducing agent. Total phenols, flavonoids, tannins, flavonols, proanthocyanidins and percentage content of alkaloids, saponins and were also determined to assess their effects on the antioxidant activity of the extract. Free radical scavenging activity of the plant extract against H_2O_2, ABTS and NO was concentration dependent with IC_{50} value of 0.023, 0.089 and 0.49 mg/ml respectively. However, *S. henningsii* exhibited lower inhibitory activity against DPPH with IC_{50} value of 0.739 mg/ml. The reducing power of the extract was found to be concentration dependent. The administration of the aqueous extract at 250, 500 and 1000 mg/kg body weight to Wistar rats significantly increased the percentage inhibition of reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) while lipid peroxidation level decreased in hepatotoxic rats induced with CCl_4 treated at the dose of 500 and 1000 mg/kg body weight at the end of 7 days. The extract yielded high phenol content (48 mg/g tannic acid equivalent) followed by proanthocyanidins (8.7 mg/g

catechin equivalent), flavonols (5.5 mg/g quercetin equivalent), tannin (4.2 mg/g tannic acid equivalent) and flavonoids (4.8 mg/g quercetin equivalent) respectively. Percentage contents of alkaloids, saponins and tannins were 1.5 and 6 % respectively of plant sample. A positive linear correlation was observed between these polyphenols and the free radical scavenging activities.

Keywords: *Strychnos henningsii*; enzymes; free radicals; CCl_4; antioxidant activity; phenolics compounds.

INTRODUCTION

Many human diseases are caused by oxidative stress that results from imbalance between the formation and neutralization of pro-oxidants (Hazra et al., 2008). Oxidative stress initiated by free radicals, such as superoxide anions, hydrogen peroxide, hydroxyl, nitric oxide and peroxynitrite, play a vital role in damaging various cellular macromolecules such as DNA, proteins and lipids. These cellular oxidative damages may result into many diseases, including diabetes mellitus, atherosclerosis, myocardial infarction, arthritis, anemia, asthma, inflammation, neurodegenerative diseases and carcinogenesis (Polterat, 1997). However, human cells have an array of protecting mechanisms to prevent the production of free radicals and oxidative damage (Chandra et al., 1994). These mechanisms include enzymic and non- enzymic antioxidants such as superoxide dismutase, catalase, glutathione reductase, ascorbic acid and tocopherol (Niki et al., 1994). The protective roles of these enzymes may be disrupted as a result of various pathological processes and thereby causes damage to the cells. Antioxidant supplement has been reported to reconcile the increase of these radicals by directly reacting and quenching their catalytic metal ions (Robak and Marcinkiewicz, 1995). Several synthetic antioxidant agents such as butylated hydroxylanisole (BHA) and butylated hydroxyltoluene (BHT) are commercially available; however, they are reported to be toxic to animals including human beings (Madhavi and Salunkhe, 1995). Natural products of plant origin have been proposed as a potential source of natural antioxidants with strong activity. This activity is mainly due to the presence of phenolic compounds such as flavonoids, phenols, flavonols and proanthocyanidins (Rice-Evans et al., 1995). A vast majority of plants used in traditional medicine in South Africa have currently not been evaluated for their antioxidant potential. One of such plants is *Strychnos henningsii*.

Strychnos henningsii Gilg. (Loganiaceae) is a small evergreen tree or shrub with leathery leaves. The bark is crown compact with dark green, glossy foliage and the fruit is

oblong which turns brown when ripe. The leaves of the plant have a characteristic aromatic-pungent odour with rough texture. It is one of the most widely distributed species of strychnos in east and southern Africa (Leeuwenberg, 1969).

The bark of *S. henningsii* has been recommended for the treatment of various diseases by the traditional health practitioners in southern Africa including rheumatism, gynaecological complaints, abdominal pain, snake bite, gastrointestinal pain, malaria and diabetes mellitus (Hutchings, 1989; Bisset, 1970). In east Africa, the bark has been documented to have significant medicinal uses in the healing of wounds and as a mouth antiseptic. About five compounds, including indolinic alkaloids, strychnine, brucine, curarine and bitter glycoside have been isolated from this plant (Tits, 1982).

Before the commencement of this work, there was no information in scientific literature on the free radical scavenging and antioxidant activity of the aqueous extract of *Strychnos henningsii* bark both *in vivo* and *in vitro*. Therefore, this study was aimed at providing information on the phytochemicals and antioxidant activities of this plant.

MATERIALS AND METHODS

Plant material

The bark of *S. henningsii* was collected in February, 2009 from a thick forest in Amathole District (Eastern Cape, South Africa). The plant was identified by its vernacular name and later authenticated by Prof. DS. Grierson of the Department of Botany, University of Fort Hare. Voucher specimen (Sun MED 2009) was deposited at the Giffen herbarium of the University.

Preparation of extract

The bark material was air-dried at room temperature in the laboratory. The dried material was then pulverized using an electric blender (Waring Products Division, Torrington, USA). About 60 g of the powdered plant material was extracted in 1 L of distilled water (4 ^{o}C) maintained on a mechanical shaker (Stuart Scientific Orbital Shaker, UK) for 48 h. The extract was filtered using a Buchner funnel and Whatman No.1 filter paper. The filtrate was quickly frozen at -40 ^{o}C and dessicated for 48 h using a freeze dryer (Savant Refrigerated vapor Trap, RV T41404, USA) to give a yield of 8.4 g of dry extract. The resulting extract was reconstituted in distilled water to give desired doses (250, 500 and 1000 mg/ml) used in this study.

Animals

Male Wistar rats (*Rattus norvegicus*) with a mean weight of 175g ± 5.2 were obtained from the animal house of the Agricultural and Rural Development Research Institute, University of Fort Hare. They were kept in clean metabolic cages placed in a well ventilated house conditions (temperature 23 ± 1 ^{o}C: photoperiod: 12 h light and 12 h dark cycle throughout the experimental period; humidity: 45 – 50%). The rats were allowed free access to food (Balanced Trusty Chunks, Pioneer Foods (Pty) Ltd, and Huguenot, South Africa) and water. The experiment was carried out after its approval by the Animal Ethics Committee of the University of Fort Hare in accordance with the recommendations for the proper care and use of laboratory animals.

Animal grouping and extract administration

Twenty five male rats were randomized into five groups consisting of five rats each. Group 1 served as control and was given distilled water alone (0.5 ml) per day for seven days with the aid of oropharyngeal cannula. Group 2 animals served as hepatotoxic control; treated with 50 % CCl_4 prepared in olive oil and were orally administered in a single dose of 0.5 ml for seven days. Animals in group 3-5 were treated like the control except that they received 0.5 ml of the extract corresponding to 250, 500 and 1000 mg/kg body weight respectively. Groups 3-5 were also given 0.5 ml of CCl_4 on the seventh day, 6 h after the administration of plant extract. All the animals from each group were sacrificed by ether anesthesia 24 h after their last dose of the extract and distilled water. The liver from each animal was excised, rinsed in ice cold 0.25 M sucrose solution and 10 %w/v homogenate was prepared in 0.05 M phosphate buffer (pH 7) and centrifuged at 12,000 × g for 60 min at 4 ^{o}C. The supernatant obtained was used for the estimation of catalase, superoxide dismutase, lipid peroxidation (TBARS) and reduced glutathione.

Total phenolics

The total phenolics content in the aqueous bark extract of *S. henningsii* was determined spectrophotometrically with Folin Ciocalteau reagent using the modified method of Wolfe et al. (2003). An aliquot of the extract (0.5 ml) was mixed with 2.5 ml of 10 % Folin-Ciocalteu reagent and 2 ml of Na_2CO_3 (75 % w/v). The resulting mixture was vortexed for 15 s and incubated at 40 ^{o}C for 30 min for colour development. The absorbance of the samples was measured spectrophotometrically at 765 nm using Hewlett Packard, UV/visible light spectrophotometer. Total phenolic content was expressed as mg/g tannic acid equivalent from a calibration curve using the equation: $Y = 0.1216x$, $R^2 = 0.936512$, where x was the

absorbance and Y was the tannic acid equivalent (mg/g). The experiment was conducted in triplicate and the results are reported as mean ± SD values.

Total flavonoids

The method of Ordonez et al (2006) was used to estimate total flavonoid contents of the extract solution based on the formation of a complex flavonoid-aluminium. A volume of 0.5 ml of 2 % $AlCl_3$ ethanol solution was added to 0.5 ml of extract solution. After one hour of incubation at the room temperature, the absorbance was measured at 420 nm using UV-VIS spectrophotometer. A yellow colour indicated the presence of flavonoids. All determinations were done in triplicate and values were calculated from calibration curve obtained from quercetin using the equations: $Y= 0.0255x$, $R^2 = 0.9812$, where x was the absorbance and Y the quercetin equivalent (mg/g).

Total flavonols

Total flavonols content was determined by adopting the procedure described by Kumaran and Karunakaran (2007). The reacting mixture consisted of 2.0 ml of the sample, 2.0 ml of $AlCl_3$ prepared in ethanol and 3.0 ml of (50 g/L) sodium acetate solution. The absorption at 440 nm was read after 2.5 h at 20 ^{0}C. Total flavonols content was calculated as quercetin (mg/g) equivalent from the calibration curve using the equation: $Y= 0.027x$, $R^2 = 0.9912$, where x was the absorbance and Y the quercetin equivalent (mg/g).

Total proanthocyanidins

Total proanthocyanidins was determined based on the procedure of Sun et al. (1998). To 0.5 ml of 1 mg/ml extract solution was added to 3 ml of vanillin-methanol (4 % v/v), and

1.5 ml of hydrochloric acid and then vortexed. The resulting mixture was allowed to stand for 15 min at room temperature followed by the measurement of the absorbance at 500 nm. Total proanthocyanidins content was expressed as catechin equivalents (mg/g) using the following equation from the calibration curve: $Y = 0.5825x$, $R^2 = 0.9277$, where x was the absorbance and Y is the catechin equivalent (mg/g).

Tannins content

Tannins content was determined according to the method of Swain (1979). About 20 ml of 50% methanol prepared in distilled water was added to 0.2 g of plant sample and covered. The mixture was shaking vigorously on the shaker (Stuart Scientific Orbital Shaker, UK) placed in a water bath at 77^{o}C for 1 h to ensure the mixture is mixed uniformly. Extract was filtered using a Buchner funnel and Whatman no 1 filter paper into 100 ml volumetric flask. Twenty milliliter (20 ml) of distilled water containing 2.5 ml of folin- Denis reagent and 10 ml of 17% Na_2Co_3 were added to the filtrate and properly mixed together. The bluish-green colour developed at the end of the reaction (0.02 - 0.1 mg/ml). The absorbance of the tannic acid standard solution was measured after colour development at 760 nm using UV-VIS spectrophotometer (AJ-C03). Total tannins content was expressed as tannin standard equivalents (mg/g) using the following equation from the calibration curve: $Y = 0.087x - 0.06$, $R^2 = 0.9277$, where x was the absorbance and Y is the catechin equivalent (mg/g).

Total alkaloids

Alkaloids contents in plant sample were quantitatively determined following the method described by Harborne (2005). Two hundred milliliter of 10 % acetic acid prepared in ethanol was added to 5 g of powdered plant sample, covered and allowed to stand for 4 h.

The filtrate was collected and concentrated on a water bath to 1/4th of its original volume. Concentrated ammonium hydroxide was added in drop-wise to the mixture until the precipitation was complete. The precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue was dried, weighed to determine the amount of alkaloids in the plant sample. The percentage alkaloids content was calculated using this equation:

$$\% \text{ Alkaloids} = \frac{\text{Weight of residue}}{\text{Weight of sample}} \times 100\%$$

Total saponins

The method described by Obadoni and Ochuko (2001) was used for saponins content determination. Twenty gram of the plant sample was added to 100 ml of 20 % ethanol prepared in distilled water and kept in a shaker for 30 min. The plant sample was heated over a water bath at 55 ^{o}C for 4 h. The resulting mixture was filtered and the residue was re-extracted again with 200 ml of 20 % aqueous ethanol. The mixture was reduced to 40 ml over water bath at 90 ^{o}C. The concentrate was transferred into 250 ml separatory funnel, extracted twice with 20 ml diethyl ether. Ether layer was discarded while aqueous layer was retained followed by adding 60 ml of n-butanol. The butanol extract was washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated over a water bath and evaporated to dryness to a constant at 40 ^{o}C. The saponins content was calculated using the following the equation:

$$\% \text{ Saponins contents} = \frac{\text{Weight of residue}}{\text{Weight of sample}} \times 100\%$$

In vitro antioxidant activity

Determination of reducing power

The reducing power of the extract was evaluated according to the method of Yen and Chen (1986). A volume of 1.0 ml of the extract prepared in distilled water or BHT, Vitamin C or Vitamin E (0 - 5.0 mg/ml) were mixed individually to the mixture containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide ($K_3Fe(CN)_6$) (1%w/v). The resulting mixture was incubated at 50 ^oC for 20 min, followed by the addition of 2.5 ml of trichloroacetic acid (10 % w/v), and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of ferrous chloride (0.1 %, w/v). The absorbance was measured at700 nm against a blank sample containing the mixture without the extract. Increased absorbance of the reaction mixture indicated higher reducing power of the plant extract.

DPPH radical scavenging activity

The method of Liyana-Pathiranan and Shahidi (2005) was used for the determination of scavenging activity of DPPH free radical in the extract solution. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract prepared in methanol containing 0.025 – 0.5 mg of the plant extracts or BHT or rutin. Both BHT and rutin are used as the standard drugs. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The ability of the plant extract to scavenge DPPH radical was calculated by the equation: DPPH radical scavenging activity = $\{(Abs_{control} - Abs_{sample})\}/ (Abs_{control}\} \times 100$ where $Abs_{control}$ is the absorbance of DPPH radical + methanol; Abs_{sample} is the absorbance of DPPH radical + sample extract or standard.

ABTS radical scavenging activity

The method of Re et al. (1999) was adopted for the determination of ABTS activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7 mM ABTS and 2.4 mM potassium persulphate in equal amounts and allowed to react for 12 h at room temperature in the dark. The resulting solution was further diluted by mixing 1ml of freshly prepared ABTS solution to obtain an absorbance of 0.706 ± 0.001 units at 734 nm after 7 min of incubation using spectrophotometer. The percentage inhibition of $ABTS^{\cdot+}$ by the extract was calculated and compared with that of BHT and rutin using the following equation: $ABTS^{\cdot+}$ scavenging activity = {(Abs $_{control}$ − Abs $_{sample}$)}/ (Abs $_{control}$} × 100 where Abs $_{control}$ is the absorbance of ABTS radical + methanol; Abs $_{sample}$ is the absorbance of ABTS radical + sample extract or standard.

Scavenging activity of nitric oxide

The method of Garrat (1964) was used to determine the nitric oxide radical scavenging activity of aqueous extract of *S. henningsii*. A volume of 2 ml of 10 mM sodium nitroprusside prepared in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract or BHT or rutin at various concentrations (0.025-0.5 mg/ml). The mixture was incubated at 25 ^{o}C. After 150 min, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent [1.0 ml sulfanilic acid reagent (0.33 % prepared in 20 % glacial acetic acid at room temperature for 5 min with 1 ml of naphthylethylenediamine dichloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min. The absorbance was then measured at 540 nm. The amount of nitric oxide radical inhibited by the extract was calculated using the following equation: NO radical scavenging activity = {(Abs $_{control}$ − Abs

$_{sample}$)}/ (Abs $_{control}$ } × 100 where Abs $_{control}$ is the absorbance of NO radical + methanol; Abs $_{sample}$ is the absorbance of NO radical + sample extract or standard (BHT and rutin).

Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by the plant extract was estimated using the method of Ruch et al (1989). Plant extract (4 ml) prepared in distilled water at various concentration (0.025-0.5 mg/ml) was mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M pH 7.4) and incubated for 10 min. The absorbance of the solution was measured at 230 nm against blank solution containing the plant extract without H_2O_2. The amount of hydrogen peroxide radical inhibited by the extract was calculated using the following equation H_2O_2 radical scavenging activity = {(Abs $_{control}$ – Abs $_{sample}$)}/ (Abs $_{control}$} × 100 where Abs $_{control}$ is the absorbance of H_2O_2 radical + methanol; Abs $_{sample}$ is the absorbance of H_2O_2 radical + sample extract or standard (BHT or rutin).

Determination of catalase activity

Catalase activity was assayed according to the method of Pari and Latha (2004). The percentage inhibition was done spectrophotometrically following decrease in absorbance at 620 nm. The liver 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 % $K_2Cr_2O_7$ prepared in glacial acetic acid). The changes in the absorbance was measured at 620 nm over 3 min at 1 min interval and recorded. Percentage inhibition was calculated using the equation:

% catalase inhibition = 100 - {Increase in absorbance of the sample × 100}/ Increase in absorbance of the blank.

Determination of superoxide dismutase activity

Superoxide dismutase was assayed following the method of Misra and Fridovich (1972). The assay mixture contained 0.5 ml of hepatic PMS (Protein Microsomal), 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:

$$\% \text{ Superoxide dismutase inhibition} = 100 - \frac{\text{Increase in absorbance of the sample}}{\text{Increase in absorbance of the blank.}} \times 100$$

Determination of reduced glutathione activity

Reduced glutathione was determined using the modified method of Ellman (1951). An aliquot of 1.0 ml of supernatant of liver homogenate was treated with 0.5 ml of Ellman's 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 measured at 412 nm. The percentage inhibition of GSH was calculated using the following equation: % reduced glutathione inhibition = 100 - {Increase in absorbance of the sample × 100}/ Increase in absorbance of the blank.

Estimation of lipid peroxidation

Lipid peroxidation in the liver was estimated colorimetrically by thiobarbituric acid reactive substances (TBARS) using the modification method of Niehius and Samuelsson (1968). In brief, 0.1 ml of liver homogenate (10 %w/v) was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 15 % trichloroacetic acid and 0.25 N HCl). All the tubes were placed in a boiling water bath for 30 min, and cooled. The amount of malondialdehyde formed in each of the samples was assessed by measuring the absorbance of clear supernatant at 535 nm against reference blank. Percentage inhibition was calculated using the equation: % lipids Inhibition = $\{A_o - A_1\}/A_o \times 100$, where A_o is the absorbance of the control and A_1 is the absorbance of the sample extract.

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates.

RESULTS

The total phenolics contents in the aqueous extract of *S. henningsii* are shown in Figure 1. The plant extract possessed high phenol contents (48 mg/g tannic acid equivalent) followed by proanthocyandins (8.7 mg/g catechin equivalent), flavonols (5.5 mg/g quercetin equivalent), tannin (4.2 mg/g tannic acid equivalent) and flavonoids (4.8 mg/g quercetin equivalent). The percentage contents of alkaloids and saponins were 1.5 and 6% respectively. Phenolics compounds, especially flavonoids and phenolics compounds have been shown to possess significant antioxidant activity.

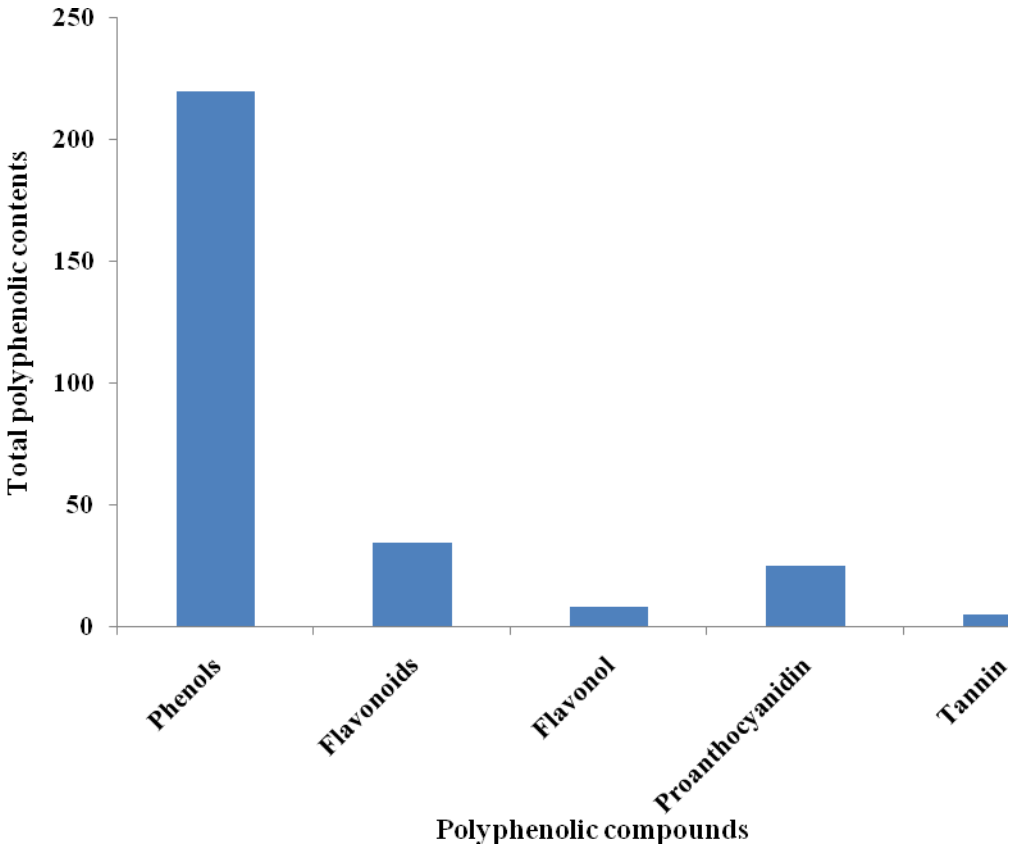

Figure 1: Polyphenolics contents of aqueous stem bark extract of *S. henningsii*.

The antioxidant potentials of the plant extract was estimated from their ability to reduce Fe^{3+} to Fe^{2+}. This was observed from yellow colour of the test solution that changed to various shades of green and blue depending on the concentration of the plant extract. The reducing value of the extract was significantly lower than that of BHT, Vitamin C and Vitamin E used as reference compounds in this study (Figure 2). Even at 0.5 mg/ml, the absorbance of plant extract was still low compared to the reference drugs.

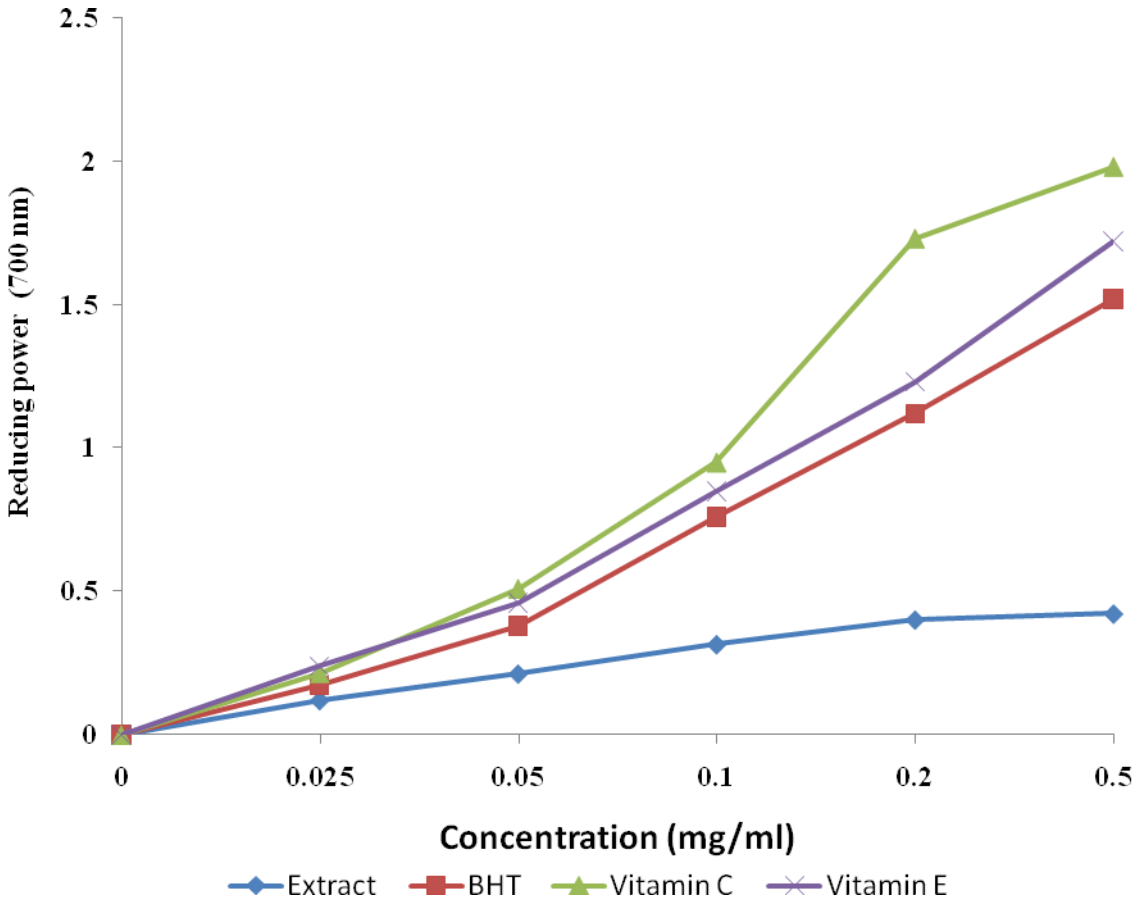

Figure 2: Total ferric reducing potential of aqueous bark extract of *S. henningsii*.

Figure 3 shows the dose-response curve of DPPH radical scavenging activity of *S. henningsii* extract compared with rutin and BHT. It was observed that the extract had DPPH scavenging activity with IC_{50} value of 0.739 mg/ml. The scavenging activity of this plant against DPPH was observed as the weakest among other reactive oxygen species evaluated.

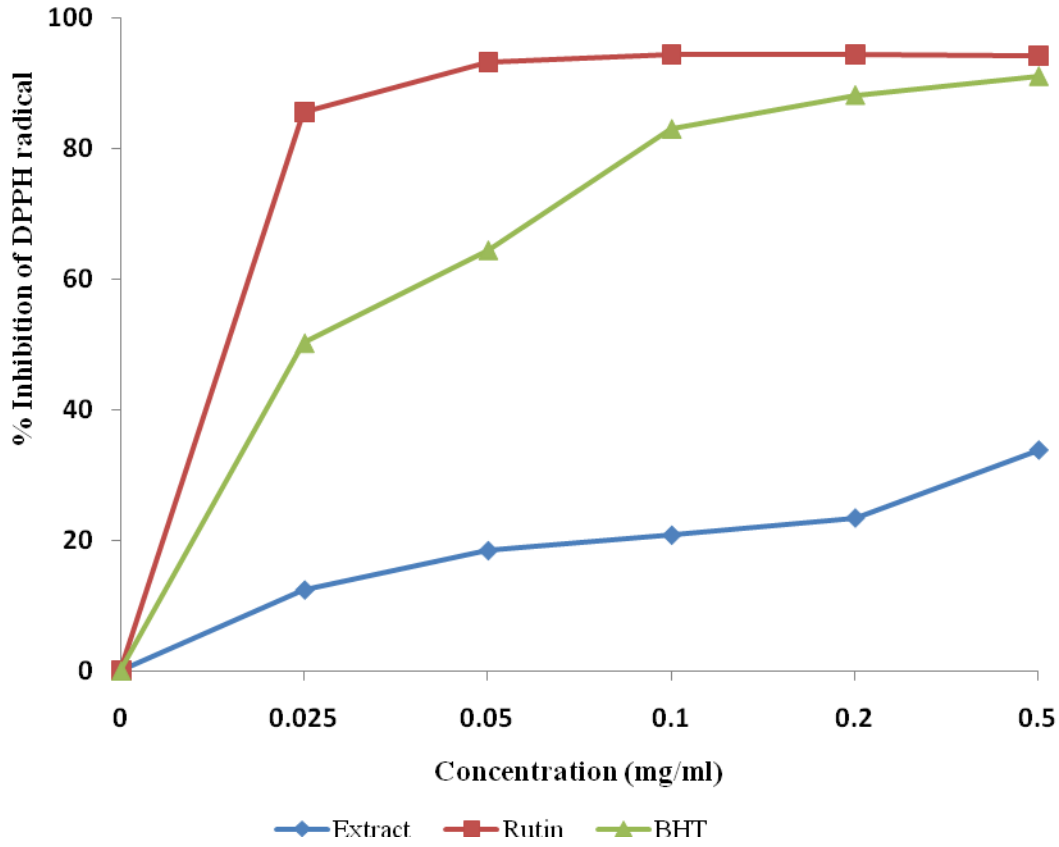

Figure 3: DPPH radical scavenging activity of the aqueous bark extract of *S. henningsii*.

S. henningsii extract was fast and effective scavenger of the ABTS radicals as shown in Figure 4. The scavenging activity of this plant was observed with that of BHT and rutin. The IC_{50} values of the extract, rutin and BHT were 0.089, 0.016 and 0.015 respectively. At 0.5 mg/ml, the plant extract showed strong inhibitory activity in removing ABTS radicals from the reaction system.

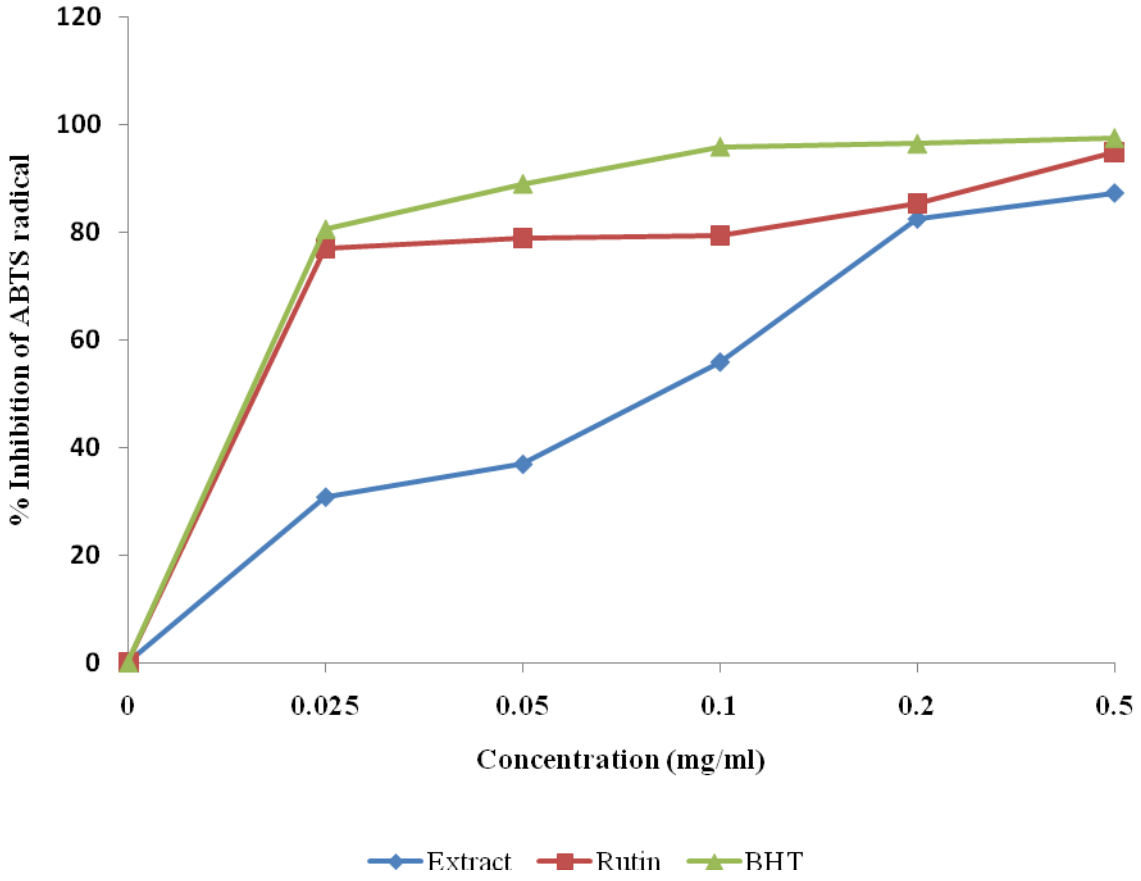

Figure 4: ABTS radical scavenging activity of the aqueous bark extract of *S. henningsii*.

The scavenging activity of aqueous extract of *S. henningsii* compared to BHT and Vitamin C for hydrogen peroxide is shown in Table 1. The results indicated a concentration dependent activity against H_2O_2 with IC_{50} values of 0.023, 0.018 and 0.02 mg/ml for plant extract, BHT and Vitamin C respectively. The percentage inhibition values at 0.5 mg/ml were 92.51, 98.46 and 99.82 % for plant extract, BHT and Vitamin C respectively.

Table 1: Scavenging activity of aqueous extract of *S. henningsii* bark against hydrogen peroxide radical.

Sample	Concentration (mg/ml)	% inhibition	IC_{50}	r^2
Aqueous extract	0.025	54.82		
	0.050	77.80		
	0.100	85.38		
	0.200	87.52	0.023	0.9962
	0.500	92.51		
Vitamin C			0.020	0.9861
BHT			0.016	0.9952

In each group n = 5 rats, Values are expressed as mean ± SD

r^2 – regression co-efficient.

The plant extract caused a moderate dose-dependent inhibition of nitric oxide with an IC_{50} of 0.49 mg/ml as shown in Table 2. The scavenging activity of BHT and Vitamin C showed IC_{50} values of 0.032 and 0.026 mg/ml and percentage inhibition of 85.96 and 94.22% respectively while the percentage inhibition of *S. henningsii* was 50.31 %.

Table 2: Nitric oxide radical scavenging activity of aqueous bark extract of *S. henningsii*

Sample	Concentration (mg/ml)	% inhibition	IC_{50}	r^2
Aqueous extract	0.025	15.80		
	0.050	16.30		
	0.100	28.74		
	0.200	35.56	0.490	0.9982
	0.500	50.31		
Vitamin C			0.026	0.9961
BHT			0.032	0.9946

In each group n = 5 rats. Values are expressed as mean ± SD

r^2 – regression co-efficient.

Table 3 showed the effect of plant extract on the activities of antioxidant enzymes in the liver of control and experimental rats. There was a marked decreased in the percentage inhibition of superoxide dismutase, catalase and the level of GSH in carbon tetrachloride treated rats when compared with normal control group. However, the percentage inhibition of SOD, CAT and the level of GSH were significantly increased followed the oral administration of plant extract at 250, 500 and 1000 mg/kg in a dose dependent manner.

Table 3: Effect of aqueous extract of *S. henningsii* bark on lipid peroxidation, antioxidant enzymes and GSH in carbon tetrachloride induced hepatotoxic rats.

Group	CAT	SOD	GSH	TBARS
Control	89.27 ± 0.24	80.22 ± 0.21	83.55 ± 0.33	45.00 ± 0.35
CCl_4	31.00 ± 0.22	42.60 ± 0.21	31.77 ± 0.31	91.67 ± 0.32
CCl_4 + *S. henningsii* (D1)	37.46 ± 0.34	59.33 ± 0.24	43.33 ± 0.39	47.00 ± 0.43
CCl_4 + *S. Henningsi* (D2)	47.82 ± 0.32	62.11 ± 0.29	48.44 ± 0.36	52.50 ± 0.32
CCl_4 + *S. Henningsii* (D3)	64.00 ± 0.28	67.77 ± 0.26	54.00 ± 0.36	58.33 ± 0.30

D1 = 250 mg/kg; D2 = 500 mg/kg and D3 = 1000 mg/kg. Results are expressed as percentage inhibition of the control. Each value is mean ± S.D (n = 5 rats).

The *in vivo* lipid peroxidation study revealed that rats treated with carbon tetrachloride showed a significant increase ($P < 0.05$) in TBARS when compared with normal control group. Treatment with aqueous extract of *S. henningsii* for 8 days was able to retard the increase in TBARS levels in a dose dependent manner (Table 3).

DISCUSSION

Polyphenols are the major plant compounds with high levels of antioxidant activity. This activity could be due to their ability to adsorb, neutralize and quench free radicals (Duh et al., 1999). Their ability as free radical scavenger could also be attributed to their redox properties, presence of conjugated ring structures and carboxylic group which have been reported to inhibit lipid peroxidation (Rice-Evans et al., 1995).

In the present study, it was found that the aqueous extract of *S. henningsii* contains high levels of phenols that might account for the strong activity observed against ABTS and H_2O_2 radicals. This scavenging activity may be due to the presence of hydroxyl groups attached to the aromatic ring structures and thus help to quench the radicals (Vinson et al., 1998). On the other hand, the weak activity depicted in DPPH and NO radicals scavenging activities may be as a result of lower content of flavonoids which have been reported to possess high antioxidant activity.

The reducing power of aqueous extract of *S. henningsii* obtained in this study was determined by measuring the transformation of Fe^{+3} to Fe^{+2}. The result obtained showed that the extract possessed antioxidant activity in a concentration dependent manner. The reducing power of the extract could be related to electron transfer ability and this may serve as a significant indicator of antioxidant activity observed in this study (Meir et al., 1995). This effect suggests that *S. henningsii* may possess the ability to minimize oxidative damage to some vital tissues in the body (Kojic et al., 1998; Weighand et al., 1999).

The relatively low level of flavonoids might account for the weak activity observed in the DPPH radical scavenging assay as shown in Figure 3. The scavenging of $ABTS^+$ by the plant extract was found to be higher than that of DPPH radical and this could be due to different mechanisms involved in the radical-antioxidant reactions. Factors such as the solubility of the extracts in different testing system, substrate used and quantization method may affect the ability of the plant extract to quench different radicals (Yu et al., 2002). This result corroborate with the report of Wang et al. (1998) found that some compounds with $ABTS^+$ scavenging activity may not exhibit DPPH scavenging activity. As a result, it may be difficult to compare antioxidant activity based on antioxidant assay because of the different test systems used and the substrate to be protected (Frankel and Meyer, 2000).

Hydrogen peroxide is a highly important reactive oxygen species because of its ability to penetrate biological membranes. However, it may be toxic if converted to hydroxyl radicals in the cell (Gulcin et al., 2003). The scavenging activities of the plant extract were nearly the same as the reference compounds. The result obtained could be due to the presence of phenolics compounds that donate electrons to H_2O_2 and thus neutralize it to water (Mathew and Abraham, 2006).

Nitric oxide (NO) is a reactive free radical generated from sodium nitroprusside in aqueous solution at physiological pH and reacts with oxygen to form nitrite. The plant extract inhibits nitrite formation by directly competing with oxygen, nitric oxide and other nitrogen oxides such as NO_3, N_2O_4 and N_2O_3 in the reaction (Marcocci et al., 1994). The percentage inhibition of NO showed that the extract has a moderate activity as compared with the standard drugs (Table 2).

Carbon tetrachloride is one of the most commonly used hepatotoxins in the experimental study of liver damage (Lee et al., 2001). The hepatotoxic effects are largely based on membrane lipid peroxidation and induction of trichloromethyl radical that results in severe cell damage (Johnson and Kroening, 1998). In our study, the rats treated with a single dose of CCl_4 developed a drastic hepatic damage and oxidative stress, which was observed by a substantial increase in the lipid peroxidation. The inability of an antioxidant defense mechanism to prevent formation of excessive free radicals may be responsible for this observation. Treatment with aqueous extract of *S. henningsii* was able to reduce the level of lipid peroxides in a dose dependent manner as compared with hepatotoxic group.

Superoxide dismutase has been reported as one of the most important enzymes in the enzymatic antioxidant defense system (Curtis and Mortiz, 1972). It removes superoxide anion by converting it to hydrogen peroxide, and thus diminishing the toxic effect caused by this radical. The decrease in percentage inhibition of superoxide dismutase as observed in this

study indicated hepatocellular damage by CCl_4. However, an increase in the percentage inhibition of superoxide by SOD after administration of aqueous extract of *S. henningsii* at the doses investigated suggests that the plant has an efficient protective mechanism in response to reactive oxygen species that are generated in hepatotoxic rats.

Catalase is an antioxidant enzyme widely distributed in the animal tissues. It decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals (Chance and Greenstein, 1992). Inhibition of this enzyme may enhance sensitivity to free radical-induced cellular damage. Therefore reduction in the activity of CAT may leads to deleterious effects as a result of superoxide and hydrogen peroxide assimilation. In the present study, the percentage inhibition of catalase was shown to increase after the administration of the aqueous extract at 250, 500 and 1000mg/kg body weight. This indicates the hepatoprotective ability of this plant from liver damage.

Reduced glutathione (GSH) is a tripeptide, non enzymatic biological antioxidant present in the liver. It protects cellular proteins against oxidation in glutathione redox cycle and directly detoxifies reactive oxygen species generated from exposure to carbon tetrachloride (Arivazhagan et al., 2000). Decreased level of GSH is associated with an increased level of lipid peroxidation in CCl_4 treated rats; thus cause liver disorder and injury. Administration of *S. henningsii* extract increased the activity of GSH by the reactivation of hepatic glutathione reductase and thus reduced the level of lipid peroxidation. The reduced glutathione levels of plant extract treated groups are in accordance with the report of Bhandarkar and Khan (2004).

In conclusion, present results demonstrate that aqueous bark extract of *S. henningsii* has both *in vivo* and *in vitro* antioxidant activities due to the presence of phenolic compounds. This result also showed that the plant extract has the ability to prevent the process of initiation and progression of hepatocellular diseases.

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CHAPTER 5

TOXICOLOGICAL EFFECTS OF ORAL ADMINISTRATION OF AQUEOUS STEM BARK EXTRACT OF *STRYCHNOS HENNINGSII* GILG IN WISTAR RATS

CHAPTER 5

Toxicological effects of oral administration of aqueous extract of *Strychnos henningsii* Gilg bark in male Wistar rats.

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Toxicological effects of oral administration of aqueous bark extract of *Strychnos henningsii* Gilg bark in male Wistar rats

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Abstract

Strychnos henningsii Gilg is widely used in southern African traditional medicine for the treatment of various ailments. However, no safety studies have been conducted on its toxicological profiles. The effect of oral administration of aqueous bark extract of the plant at 250, 500 and 1000 mg/kg body weight was investigated on haematological and biochemical parameters in Wistar rats for 28 days. Treatment with the plant extract did not significantly ($P>0.05$) alter the levels of haemoglobin, red blood cell, haematocrit and mean corpuscular haemoglobin concentration. Large unstained cell index and organs body weight ratio of the kidneys, lungs and hearts were also unaffected. The levels of eosinophils, total bilirubin, albumin, urea, creatinine, sodium as well as calcium were also not significantly ($P >0.05$) different from the control. However, the concentration of platelets, monocytes, basophils and white blood cells significantly decreased while those of mean corpuscular haemoglobin, alanine and aspartate aminotransferases were increased. The levels of conjugated bilirubin, mean corpuscular volume and liver body weight ratio were significantly increased at specific doses while lymphocyte was decreased at the dose of 250 mg/kg. The levels of cholesterol and triacylglycerol as well as chlorine were decreased at the higher dose (1000 mg/kg). The results obtained from this study suggest that sub-acute administration of *S. henningsii* bark

extract may not be completely safe as an oral remedy due to the impairment observed on the normal functioning of white blood cells and platelets.

Keywords: *Strychnos henningsii*; biochemical; haematological; serum lipids; toxicity.

INTRODUCTION

The use of medicinal plants for healing purposes is very common in developing countries especially in the rural areas. It has been estimated that 80 % of the population of developing countries relied heavily on the herbal medicine for their primary health care (WHO, 1991). This is probably due to the perceived beneficial and lower adverse side effect of natural products that are extracted from the plants (Leonardo et al., 2000). However, most medicinal plants are used indiscriminately without knowing their possible adverse effect. Over the past decades, several reports in both developed and developing countries indicated adverse effects allegedly arising from the use of medicinal plants (Elvin-Lewis, 2001). Some of these effects include abortion of pregnancy, dizziness, vomiting, diarrhea, abdominal pain, fast heart beat, death, ulcer and loss of appetite (Gessler et al., 1995). These effects could be attributed to the presence of phytotoxic compounds in the plant extracts and lack of actual dosage necessary for the treatment of diseases (Azaizeh et al., 2003). In order to minimize this, there is need for a thorough scientific investigation on the toxicological effect of these plants at different doses. In addition, the World Health Organization has recommended that traditional plants used for the treatment of diseases warrant further evaluation for their toxicological properties (WHO, 1980).

Strychnos henningsii Gilg (Loganiaceae) is a small evergreen tree or shrub with leathery leaves, mostly distributed in east and southern African (Leeuwenberg, 1969). The decoction or infusions of the plant are widely used in southern African traditional medicine for the treatment of various ailments. These include gynaecological complaints, abdominal pain, snake bite, gastrointestinal pain, rheumatism, malaria and diabetes mellitus (Bisset, 1970; Hutchings, 1989). To the best of our understanding, there was no or little scientific report on the biological and pharmacological activities of *S. henningsii* to substantiate its traditional usage. However, previous phytochemical screenings conducted on *S. henningsii*

bark extract have demonstrated the presence of tannins, saponins, alkaloids, phenolics and flavonoids with strong antioxidant activities (Chapter 4). Plants containing tannins and alkaloids have been reported to have toxic side effects in man (Lacomblez, 1989). For instance, strychnine and retuline alkaloid among other compounds isolated by Sandberg and Kristianson (1970) have shown to be toxic and exert some adverse effects on mice.

Despite the traditionally usage of this plant in folk medicine, there was no information in the scientific literature to validate its acclaimed pharmacological properties and the possible toxicological profiles. Therefore the present study was undertaken to assess the safety of aqueous stem bark extract of *S. henningsii* at certain doses using haematology, serum chemistry, liver and kidney functional indices in animal model after acute and sub-acute administration.

MATERIALS AND METHODS

Plant Material

The bark of *S. henningsii* was collected in February, 2009 from a thick forest in Amathole District (Eastern Cape, South Africa). The plant was identified by its vernacular name and later authenticated by Prof. D.S. Grierson of Botany Department, University of Fort Hare. Voucher specimen (Sun MED 2009) was deposited at the Giffen herbarium of the University.

Chemicals and assay kits

The reagents used were of analytical grade and were supplied by Merck Chemicals (Pty) Ltd., Bellville, South Africa. All other assay kits used for the analysis of creatinine,

urea, calcium, sodium, chloride, albumin, cholesterol, triglycerides, alanine and aspartate aminotransferases were obtained from Roche Diagnostic GmbH, Mannheim, Germany.

Preparation of plant extract

The bark material was air-dried at room temperature in the laboratory. The dried bark was thereafter grounded into powdery form using an electric blender (Waring Products Division, Torrington, USA). About 80 g of the powdered plant material was extracted in 1000 ml of cold distilled water maintained on a mechanical shaker (Stuart Scientific Orbital Shaker, UK) for 48 h. The extract was filtered using a Buchner funnel and Whatman No.1 filter paper. The filtrate was quickly frozen at -40 ^{o}C and dried for 48 h using a freeze dryer (Savant Refrigerated vapor Trap, RV T41404, USA) to give a yield of 10.6 g of dry extract. The resulting extract was reconstituted in distilled water to give the doses of 250, 500 and 1000 mg/kg body weight used in this study.

Acute toxicity

Male rats (*Rattus novergicus*) of Wistar strain weighing 159.00 ± 7.20g were obtained from the animal house of the Agricultural and Rural Development Research Institute (ARDRI), University of Fort Hare. They were kept in aluminum cages placed in well ventilated house conditions (temperature 23 ± 1 ^{o}C; photoperiod: 12 h light and 12 h dark cycle; humidity: 40-45%). The animals were allowed free access to rat pellets (Balanced Trusty Chunks, Huguenot, South Africa) and distilled water. The acute toxicity of the plant extract was evaluated in rats (6 rats per group) by preparing five different doses (100, 500, 1000, 2000 and 4000 mg/kg), and administered orally using gavages. Animals were kept without food for 18 h prior to dosing and were monitored continuously on a daily basis for 3

days after dosing for any sign of toxicity. Animals showing any symptom of toxicity were immediately sacrificed. The experiment was carried out after the approval from the Ethics Committee on the Use and Care of Experimental Animals of the University of Fort Hare. LD_{50} value of the extract was calculated arithmetically using the method described by Hamilton et al. (1977).

Sub -acute toxicity

Twenty four rats were randomized into four groups, consisting of six animals in each group. Group 1: control rats administered with drinking water (0.5 ml) on daily basis for 28 days. Groups 2, 3 and 4 received 0.5 ml of the extract corresponding to 250, 500 and 1000 mg/kg body weight respectively. The use of male rats in this study was basically due to animal availability. The toxic manifestation such as body weight was measured every fifth day of the experiment. All the animals from each group were sacrificed at 28 days after their last daily dose of the extract or distilled water. The rats were thereafter quickly dissected to remove the liver, heart, lung and kidney and then transferred into ice-cold 0.25 M sucrose solution. The organs were freed of fat, blotted with clean tissue paper and then weighed. The dosages of the extract were chosen from the result obtained from the acute toxicity study as well as translation of traditional dosage using the equation:

$$\text{Dosage (mg/kg)} = \frac{\text{Volume of extract (ml)} \times \text{Concentration of extract (mg/ml)}}{\text{Weight of animal (kg)}}$$

Preparation of serum

The procedure described by Yakubu et al. (2005) was adopted for the preparation of serum. An aliquot (2 ml) of the blood was collected from the tail vein of the animals into sample bottles containing EDTA (BD Diagnostics, Preanalytical Systems, Midrand, USA) for

the haematological analysis while another 5 ml was allowed to clot at room temperature for 10 min. This was centrifuged at 1282 g x 5 min using Hermle Bench Top Centrifuge (Model Hermle, Z300 Hamburg, Germany). The sera were later aspirated with Pasteur pipettes into sample bottles and used within 12 h of preparation for the assay of biochemical parameters.

Haematological analyses

The full blood count (FBC) including red blood cells (RBC), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), large unstained cell (LUC), red cell distribution width (RCDW), haematocrit, white blood cell (WBC), basophils, neutrophils, eosinophils platelet and monocytes were determined using a semi-automated hematology analyzer (Horiba ABX 80 Diagnostics, ABX Pentra Montpellier, France).

Biochemical parameters

Alanine transaminases (ALT) determination

A volume of 23 µl of the serum sample was added to 250 µl of reagent A (16 mmol/L α- ketoglutarate, 0.18 mmol/LNADH, 500 mmol/L alanine, LDH > 2300 IU/L) with 8 µl of reagent B (Tris buffer 97 mmol/L) and allowed to react for 2 min at room temperature. The activity of the enzyme was determined by measuring changes in absorbance at 340 nm. The ALT reagents were used to measure the enzyme activity by a kinetic rate method (Henry, 1991). The enzyme catalyzes the reversible transamination of L-alanine and alpha ketoglutarate to pyruvate and L-glutamate. The resulting pyruvate is then reduced to lactate in

the presence of Lactate dehydrogenases (LDH) with concurrent oxidation of reduced β - NADH to NAD.

Aspartate transaminases (AST) determination

The reagents of AST were used to measure the activity of the AST by enzymatic rate method (Tietz et al., 1994). In the assay reaction, the AST catalyzed the reversible transamination of L-aspartate and alpha ketoglutarate to oxaloacetate and L- glutamate. The oxaloacetate is then reduced to malate in the presence of malate dehydrogenase (MDH) with concurrent oxidation of β-nicotinamide adenine dinucleotide (NADH) to β- NAD. To 23 µl of serum sample was added 242 µl of reagent A (16 mmol/L α- ketoglutarate, 218 mmol/L L-aspartate, 0.18 mmol/L NADH) and 8 µl of reagent B (Malate > 600 IU/L).

Gamma glutamyl transferases (GGT) determination

A precise volume of 13 µl serum sample was added to 237 µl of reagent A (4.4 mM γ-glutamyl-p- nitroaniline) and 23 µl of reagent B (150 mM glycylglycine) after reaction the change in absorbance was measured at 410 nm to determine enzyme activity. In the reaction, γ-GGT catalyzes the transfer of GGT from the colourless substrate (γ- glutamyl-p-nitroaniline) to the receptor glycylglycine to produce p- nitroaniline.

Alkaline phosphatases (ALP) determination

The ALP reagents were used to measure ALP activity by a kinetic rate method using a 2-amino-2- methyl-1-propanol (AMP) buffer (Tietz et al., 1987). 5 µl of serum sample was mixed with 228 µl of AMP (pH 10.3) and 22 µl of p-nitrophenyl phosphate, allowed to react together for 2 min at room temperature. The enzyme (ALP) catalyzes the hydrolysis of p-

nitrophenyl phosphate (colourless) to p-nitrophenol (yellow). The calculation was performed by the system to produce the final result.

Total bilirubin determination

Serum sample (10 µl) was mixed together with 310 µl of reagent A (442745) and 10 µl reagent B (476861) was allowed to react for 48 s at room temperature as described by the manufacturer. The absorbance was measured at 560 nm.

Estimation of Urea contents

Serum sample (3 µl) was added to 285 µl of reagent A containing 2.9 mmol/L, 0.35 mmol/L NADH, and 1.3 KIU/L of glutamate dehydrogenase with reagent 15 µl B (24 KIU/L of urease) to determine urea concentration in the sample by an enzymatic rate method (Tietz, 1995). The system monitors the changes in absorbance at 340 nm. This change in absorbance was directly proportional to the concentration of urea in the sample. Urea in the reaction was hydrolyzed by urease to CO_2 and ammonia. Glutamate dehydrogenase catalyzes the condensation of ammonia and alpha ketoglutarate to glutamate with the concomitant oxidation of NADH to NAD.

Estimation of Creatinine content

A precise volume (20 µl) of serum sample was reacted with 175 µl of picric acid (8.1 mmol/L) and 44 µl of buffered pH 13.3. In the reaction, creatinine combined with picrate in an alkaline solution to form a creatinine picrate- complex. The complex formation was measured by changes in absorbance at 520 nm to determine the concentration of creatinine in the sample using the method described by Tietz (1995).

Estimation of Cholesterol content

The reagents were used to measure cholesterol in the sample by time-end point method as described by Henry (1991). Serum sample (5 µl) was mixed with 290 µl of reagent A (211 IU/L cholesterol esterase, 216 IU/L of cholesterol oxidase, 6667 IU/L peroxidase) with 10 µl reagent B (0.28 mmol/L 4- aminoantipyrine, 8.06 mmol/L phenol). They are allowed to mix together at room temperature for 2 min. The absorbance was measured at 510 nm to determine cholesterol level in the sample. In the reaction, cholesterol esterase hydrolyzed cholesterol esters in the sample to free cholesterol and fatty acid. Cholesterol oxidase acted on the free cholesterol to cholestene-3-one and hydrogen peroxidase. The enzyme peroxidase catalyzes the reaction of hydrogen peroxide and 4-aminoantipyrine to quinoneimine which is then measured.

Estimation of HDL and LDL contents

To 3 µl of serum sample was added 210 µl of reagent A (211 IU/L cholesterol esterase, 216 IU/L of cholesterol oxidase, 6667 IU/L peroxidase) with 70 µl of reagent B (0.28 mmol/L 4- aminoantipyrine, 8.06 mmol/L phenol). The absorbance was measured at 560 nm to determine both HDL and LDL by a timed- end point method as described above in cholesterol assay.

Estimation of triglyceride content

Serum sample (3µl) was reacted with 285 µl of reagent A (glycerol kinase, glycerol phosphate oxidase with 15 µl of reagent B (961 IU/L horseradish peroxidase). The method by a timed out end point was used to measure concentration of triglycerides in the sample

(NCCLS, 1998). Triglyceride in the sample was hydrolyzed to glycerol and free fatty acid by the action of lipase.

Total protein and albumin

Serum sample (6 µl) was mixed together with 300 µl of reagent A (0.28 mmol/L Bromocresol purple). The change in absorbance was measured at 560 nm and 600 nm to determine the concentration of total protein and albumin respectively.

Electrolytes

A precise volume of 40 µl of the serum sample mixed together with 3.3 ml of ISE electrolyte buffer (467915) and 1.3 ml of ISE buffer (467935) to determine the concentration of calcium, potassium, chlorine and sodium in the sample using indirect potentiometry (ISE) method provided by the manufacturer (Beckmann Coulter). The system monitors the absorbance at 410, 470, 600 and 700 nm for sodium, potassium, chlorine and calcium with different assay principles.

Statistical analysis

Data were expressed as means ± standard deviation of six replicates and were statistically analyzed using one way analysis of variance (ANOVA). Means were separated by the Duncan multiple test using SAS. Values were considered significant at $P < 0.05$.

RESULTS

Acute toxicity studies

The result of the toxicity test of *S. henningsii* extract for 3 days did not show any clinical adverse side effect of substance-related toxicity on the animals such as restlessness, haematuria, diarrhea and muscle coordinated movement. Similarly, there was no mortality or morbidity observed at any tested doses except at 4000 mg/kg. The LD_{50} value of the extract was found to be 3666.66 mg/kg (Table 1).

Table 1: Determination of LD_{50} value by arithmetic method of Hamilton, 1977.

Group	**Dose (mg/kg) Control**	**No of animals dead**
1	100	0
2	500	0
3	1000	0
4	2000	0
5	4000	2

Group	**Dose difference (a)**	**Mean mortality (b)**	**Probit (a×b)**
5	(4000-2000) = 2000	1	2000

Sum of the product = 2000

LD_{50} = Least lethal dose in a group- $\frac{\sum(a \times b)}{N}$

$LD_{50} = 4000 - \frac{2000}{6}$

$= 4000 - 333.3$

$= 3666.66$

Sub-acute toxicity studies

The body weight of the animals treated with the extract at the doses of 250, 500 and 1000 mg/kg and the control group were appreciably increased throughout the experimental periods (Figure 1). There was no significant changes (P >0.05) observed in the organs body weight ratio of the kidney, lung, liver and heart as compared with the control group (Figure 2). Unfortunately, the extract caused the death of two rats, one on the 21st and the other on the 27th day during the experimental period. The observed symptoms before death were respiratory distress, starry hair coat, motionlessness and slow response to external stimuli.

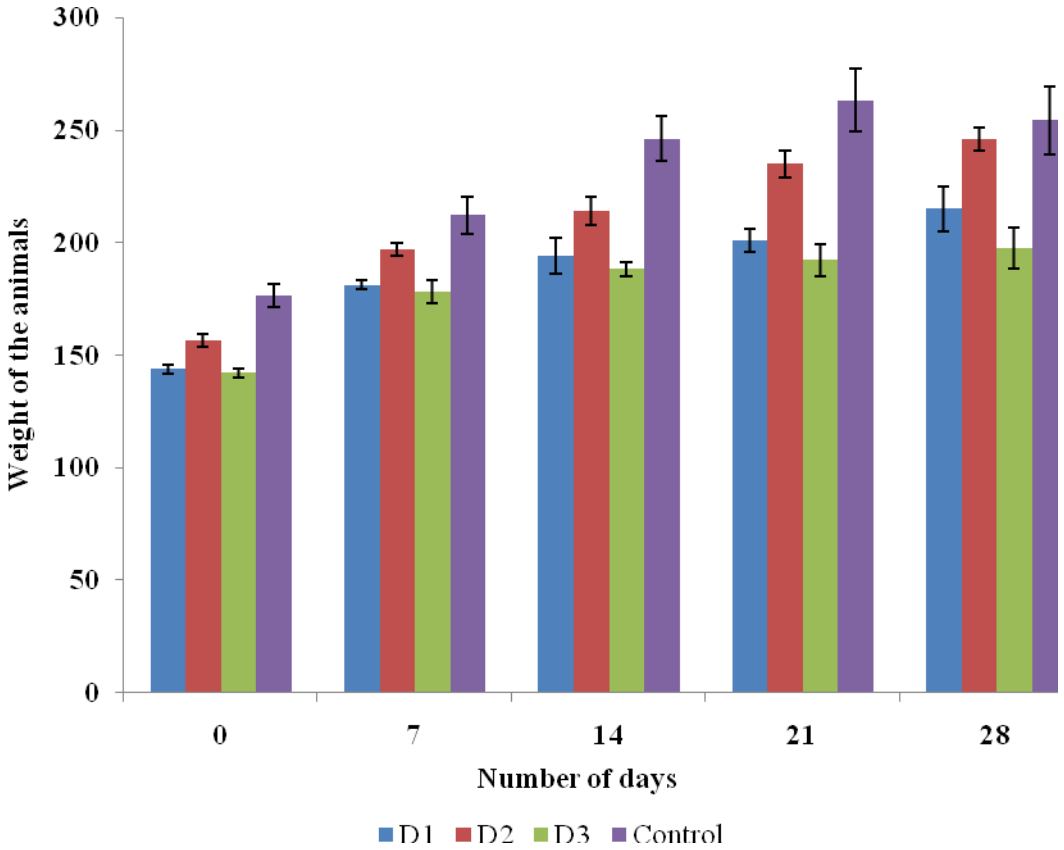

Figure 1: The effect of aqueous extract of *S. henningsii* on the body weight of Wistar rats. Values are mean ± SD of 6 rats in each group. D1 = animals fed with 250 mg/kg body weight of extract, D2 = animals fed with 500 mg/kg body weight of extract, D3 = animals fed with 1000 mg/kg body weight of extract, control = animals receiving only distilled water.

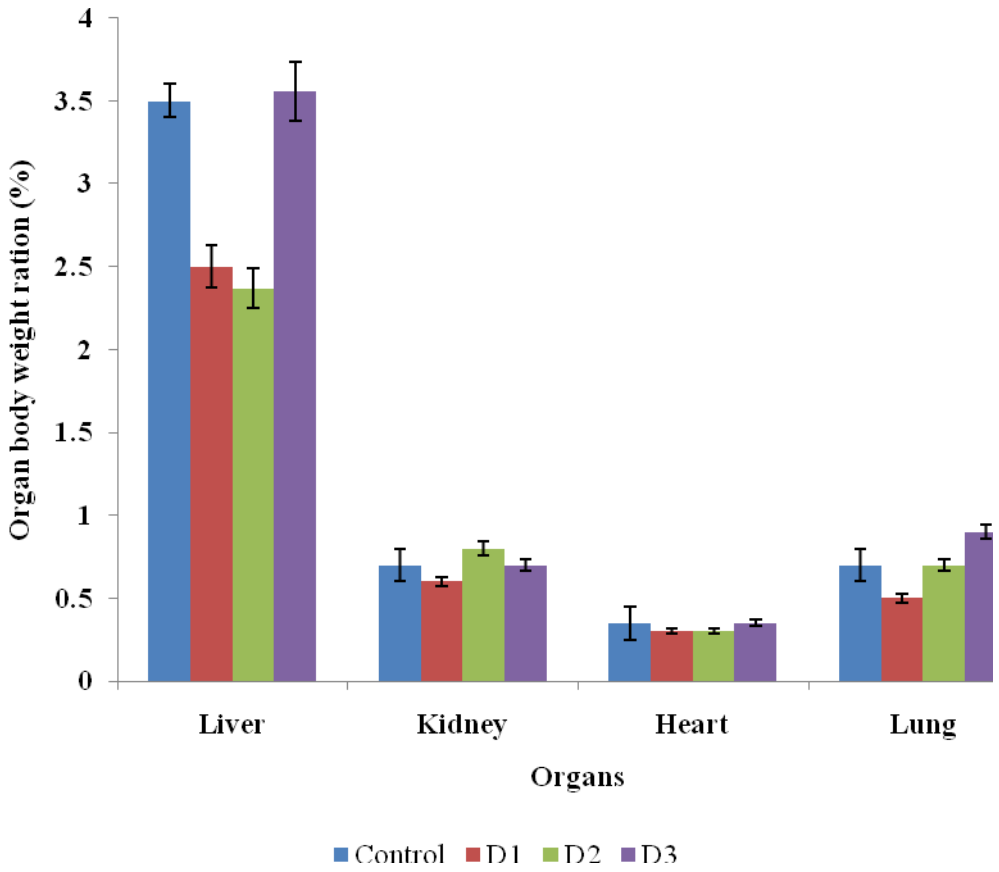

Figure 2: The effect of aqueous extract of *S. henningsii* (SH) on the organ body weight ratio of rats. Values are mean ± SD of 6 rats in each group. D1 = animals fed with 250 mg/kg body weight of extract, D2 = animals fed with 500 mg/kg body weight of extract, D3 = animals fed with 1000 mg/kg body weight of extract, control = animals receiving only distilled water.

Haematological parameters

The effects of oral administration of aqueous bark extract of *S. henningsii* at the doses investigated on red blood cells and its functional indices in male Wistar rats for 28 days are shown in (Table 2). The extract did not significantly alter the levels of Hb, RBC, haematocrit, MCHC and LUC while those of MCV, RCDW and MCH were significantly increased at certain doses.

Biochemical parameters

The plant extract showed varied effect on the kidney and its functional indices (Table 5). The levels of sodium, calcium, urea and creatinine were not significantly affected when compared with the control animals. Contrarily, the level of chlorine ion was decreased drastically at 500 mg/kg body weight while slight increase was apparent in the remaining doses. The extract did not alter the level of total bilirubin and albumin a vital markers of assessing liver damage (Table 6). The concentration of conjugated bilirubin significantly ($P < 0.05$) increased after the treatment with the plant extract at 250 and 500 mg/kg but increased only at the highest dose. The data did not show any notable ($P > 0.05$) changes in serum concentration of AST and ALT.

CHAPTER 6

ANTIDIABETIC ACTIVITY AND CLINICAL SIGNIFICANCE OF AQUEOUS EXTRACT OF *STRYCHNOS HENNINGSII* GILG BARK IN STREPTOZOTOCIN-NICOTINAMIDE INDUCED DIABETIC RATS

CHAPTER 6

Antidiabetic activity and clinical significance of aqueous extract of *Strychnos henningsii* Gilg bark in streptozotocin-nicotinamide induced diabetic rats

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This chapter is accepted in the journal of pharmacognosy

Antidiabetic activities and clinical significance of aqueous extract of *Strychnos henningsii* Gilg bark in streptozotocin-nicotinamide diabetic rats

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Abstract

The aim of this study was to investigate the clinical significance of aqueous bark extract of *Strychnos henningsii* on haematological, hepatic and renal functional indices during diabetes and its resultant effect in blood glucose levels, body weight, feed and water intake. The clinical significance of oral administration of this plant at the doses of 125, 250 and 500 mg/kg body weight was investigated in streptozotocin-nicotinamide induced diabetic rats for 15 days. The administration of the three doses significantly ($P > 0.05$) reduced the level of blood glucose in a dose independently manner while the best result was obtained at 250 mg/kg. The glucose tolerance was reduced significantly to near normal levels after 90 min at certain doses. The weight loss of the diabetic rats after oral administration of plant extracts at doses of 125 and 250 mg/kg was normalized as compared with the normal control rats. Treatment of diabetic rats with the extract did not significantly alter the levels of cholesterol and uric acid. In addition, the extract did not have any significant effect on the levels of basophils, monocytes and eosinophils. Whereas, the level of white blood counts, neutrophils and lymphocyte were remarkably increased at 500 mg/kg. Similarly, the elevated level of triacylglycerol, calcium, urea and liver body weight ratio was drastically reduced at certain doses of the extract. The activities of aspartate aminotransferases and alkaline phosphatase were significantly

lowered in all the three doses. The decreased level of haemoglobin, PCV, MCV, RBC, MCH, total protein, alanine aminotransferases, albumin and globulin in diabetic rats was appreciably increased after oral administration of plant extract at all the three doses. The result showed that *S. henningsii* extract possessed antidiabetic potential and could ameliorate anemic conditions as well as hepatic and renal damage in streptozotocin-nicotinamide induced diabetic rats. It could also prevent various complications in diabetes.

Keywords: *Strychnos henningsii*; diabetes mellitus; streptozotocin; nicotinamide; hypoglycemic.

INTRODUCTION

Streptozotocin- nicotinamide is a method currently used to induce diabetes in animals that resemble non obese type 2 diabetes mellitus in man (Tomonori et al., 2006). The induction of diabetic rats with streptozotocin increases the production of free radicals that damage the pancreatic DNA and thus affect insulin secretion (Oguri et al., 2003). This is achieved by depleting nicotinamide which is a substrate of poly ADP ribose synthetase, an enzyme which involved in DNA repair. The pretreatment of nicotinamide into diabetic rats allows minor damage to pancreatic beta cell by inhibiting poly ADP ribose synthetase activity and prevents NAD depletion (Le Doux et al., 1988). The administration of streptozotocin- nicotinamide induces diabetes with moderate hyperglycaemia associated with loss of early–phase insulin (Atsuo et al., 2008). This form of diabetes is very common in people over 40 years of age (WHO, 1999).

Recently, there is an emergent interest in the use of herbal remedies due to the undesirable effect associated with the use of oral hypoglycaemic drugs. Hence the consumption of herbal extracts has been increasing rapidly, mostly because of less frequent adverse side effect when compared to western medicine (Hu et al., 2003).

Strychnos henningsii Gilg (Loganiaceae) is one of the plants used in southern Africa as a folk remedy for the management of various diseases (Oyedemi et al., 2009). The decoction or infusion of the bark of *S. henningsii* extract is commonly used by traditional health practitioner in the Eastern Cape of South Africa for the management of diabetes mellitus (Oyedemi et al., 2009). Our previous studies on this plant demonstrated strong antioxidant and free radicals scavenging activity of plant extract both *in vitro* and *in vitro* (Oyedemi et al., 2010). The observation which may be due to the high contents of

phenolics, proanthocyanidins and flavonoids which have been reported to enhance insulin secretion coupled with hypoglycemic properties. The results obtained on sub-acute administration of *S. henningsii* bark extract demonstrated that *S. henningsii* extract is not completely safe as an oral remedy due to the impairment observed on the normal functioning of white blood cells and platelets (Chapter 5).

Report regarding the antidiabetic effect and clinical significance of aqueous extract of *Strychnos henningsii* in diabetic rats is scanty in scientific literature. Therefore, present study was undertaken to investigate the scientific basis for the use of this plant for the management of diabetes mellitus in folkloric medicine. We also assessed the effects of the extract on some haematological and biochemical parameters in streptozotocin-nicotinamide induced diabetic rats.

MATERIALS AND METHODS

Plant material

The bark of *S. henningsii* was collected in February, 2009 from a thick forest in Amathole District (Eastern Cape, South Africa). The plant was identified by its vernacular name and later authenticated by Prof. DS. Grierson of Botany Department, University of Fort Hare. Voucher specimen (Sun MED 2009) was deposited at the Giffen Herbarium of the University.

Assay Kits and Reagents

The assay kits for the analyses of triacylglycerol and cholesterol were obtained from Randox Laboratories Limited, Ardmore, Co Antrim, UK. All other reagents used

were of analytical grade and were supplied by Merck Chemicals (Pty) Ltd., Bellville, South Africa.

The animals used

Male Wistar rats (*Rattus novergicus*) weighing between 125 and 255g were obtained from the animal house of the Agricultural and Rural Development Research Institute, University of Fort Hare. The animals were maintained at a controlled temperature of 28 ^{o}C with a 12 h light-dark cycle at room temperature and humidity of 45-50 %. The animals were allowed free access to food and water for 15 days. The experiment was approved by the Animal Ethics Committee of the University of Fort Hare.

Preparation of plant extract

The bark material of *S. henningsii* was air-dried at room temperature in the laboratory and later pulverized after drying using an electric blender (Waring Products Division, Torrington, USA). About 60 g of the powdered plant material was extracted in 1 L of cold distilled water maintained on a mechanical shaker (Stuart Scientific Orbital Shaker, UK) for 48 h. The extract was filtered using a Buchner funnel and Whatman No.1 filter paper. The filtrate was quickly frozen at -40 ^{o}C and dried for 48 h using a freeze dryer (Savant Refrigerated vapor Trap, RV T41404, USA) to give a yield of 8.4 g of dry extract. The resulting extract was reconstituted in distilled water to give desired doses (125, 250 and 500 mg/kg) used in this study.

Induction of diabetes in the rats

The method of Pellegrino et al. (1998) was adopted for the induction of type 2 diabetes mellitus in overnight fasted male rats. The animals were induced by a single intraperitoneal injection (i.p) of freshly prepared solution of streptozotocin (60 mg/kg body weight) in 0.1 M citrate buffer (pH 4.5), 15 min after the i.p administration of nicotinamide (110 mg/kg) prepared in normal saline. Diabetes was confirmed in the animals by the elevated plasma glucose levels after 24 h of injection. The rats with diabetes having glycosuria and hyperglycaemia (blood glucose > 8.1 mmol/L) were used for the experiment.

Animal grouping and extract administration

Thirty six male Wistar rats were randomized into six groups of six animals each (30 diabetic surviving rats, 6 normal rats). Group I: normal control rats administered with drinking water daily for 15 days; Group II: diabetic animals received 0.5 ml of distilled water; Group III-V: diabetic rats treated daily with 0.5 ml of 125, 250 and 500mg/kg body weight of *S. henningsii* extract respectively; Group VI: diabetic animals received 0.5 ml of glibenclamide only. No detectable irritation, restlessness, respiratory distress, catalepsy or abnormal locomotion was observed after extract administration at different doses. Blood samples were drawn every fifth days till the end of experimental period. At the end of 15 days, all the animals from each group were sacrificed by ether anesthesia 24 h after their last daily doses of the extract, glibenclamide and distilled water.

Oral glucose tolerance test (OGTT)

Thirty male rats (normal) were fasted for 12 h and assigned randomly into 5 equal groups (n = 6/group). Group I-III: six normal rats were fed orally with 0.5 ml of aqueous bark extract of *S. henningsii* at the doses of 125, 250 and 500 mg/kg body weight respectively using gavage. Group IV: six normal rats were fed orally with 0.5 ml of glibenclamide (0.6 mg/kg) an antidiabetic standard drug and Group V: consisted of six normal rats receiving 0.5 ml of distilled water. Glucose (2 g/kg) was orally administered 30 min prior to the extract and glibenclamide administration and blood was withdrawn from the tail vein at 30, 60 and 90 min (Latha et al., 2004). The fasting plasma glucose level was measured using glucometer (Bayer Health Care, Japan).

Preparation of serum

The preparation of serum was carried out using the method of Yakubu et al. (2005). The blood samples were collected into clean dry centrifuge tubes. An aliquot (2 ml) of the blood was collected into sample bottles containing EDTA (BD Diagnostics, Pre-analytical Systems, Midrand, USA) for the haematological analysis while another 5 ml was allowed to clot at room temperature for 10 min. This was centrifuged at 1282 g x 5 min using Hermle Bench Top Centrifuge (Model Hermle, Z300, Hamburg, Germany). The sera were later aspirated with Pasteur pipettes into sample bottles and used within 12 h of preparation for the assay of biochemical parameters. The rats were thereafter quickly dissected in the cold; the liver and kidney were excised and transferred into ice-cold 0.25 M sucrose solution. The organs were thereafter freed of fat, blotted with clean tissue paper and then weighed.

Haematological analysis

Haematological parameters includes red blood cells (RBC), haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), large unstained cell (LUC), and red cell distribution width (RCDW) as well as white blood cell (WBC) and other related indices such as basophils, monocytes, lymphocytes, neutrophils and eosinophils were analyzed by using Horiba ABX 80 Diagnostics (ABX Pentra Montpellier, France).

Serum biochemical analysis

The levels of alanine and aspartate aminotransferase, alkaline phosphatase, urea, uric acid, total bilirubin, total protein, calcium, albumin, globulin, triacylglycerol and cholesterol in the serum of the animals were determined as described in chapter 5. They were determined spectrophotometrically using assay kits from Randox Laboratories Limited, Ardmore, Co Antrim, UK.

Effect of extract on the weight, feed and water intake of the animals

Feed and water intake were measured everyday at the same hour during the experimental periods while the body weight of the animals were measured before the start and every fifth day throughout the experimental period (15 days).

Statistical analysis

Data were expressed as mean ± SD (standard deviation) of six replicates and were statistically analyzed using one way analysis of variance (ANOVA). Means were separated by the Duncan multiple test using SAS (SAS, 2002). Values were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The earlier studies of *S. henningsii* extract on *in vitro* and *in vivo* antioxidant activities (Chapter 5) showed the prevention of oxidative stress as a result of strong antioxidants and free radical scavenging activities (Oyedemi et al., 2010). In this present study, the results of glucose tolerance test in diabetes, control and normal rats treated with *S. henningsii* extract and glibenclamide after oral administration of glucose at 30, 60 and 90 min are shown in Figure 1. The increased plasma glucose level observed at 30 min was significantly reduced by plant extract at various doses as compared with diabetic control rats. The dose at 250 and 500 mg/kg showed a similar blood glucose lowering capacity while glibenclamide treated rats was comparable to normal control rats. However, the dose of 125 mg/kg did not show strong effect as compared with the control and glibenclamide treated rats. The significant reduction of peak levels of blood sugar level within 90 min manifests the antidiabetogenic potential of *S. henningsii* extract in rat models. This observation could be attributed to antihyperglycemic property of the plant by restoring the delayed insulin response.

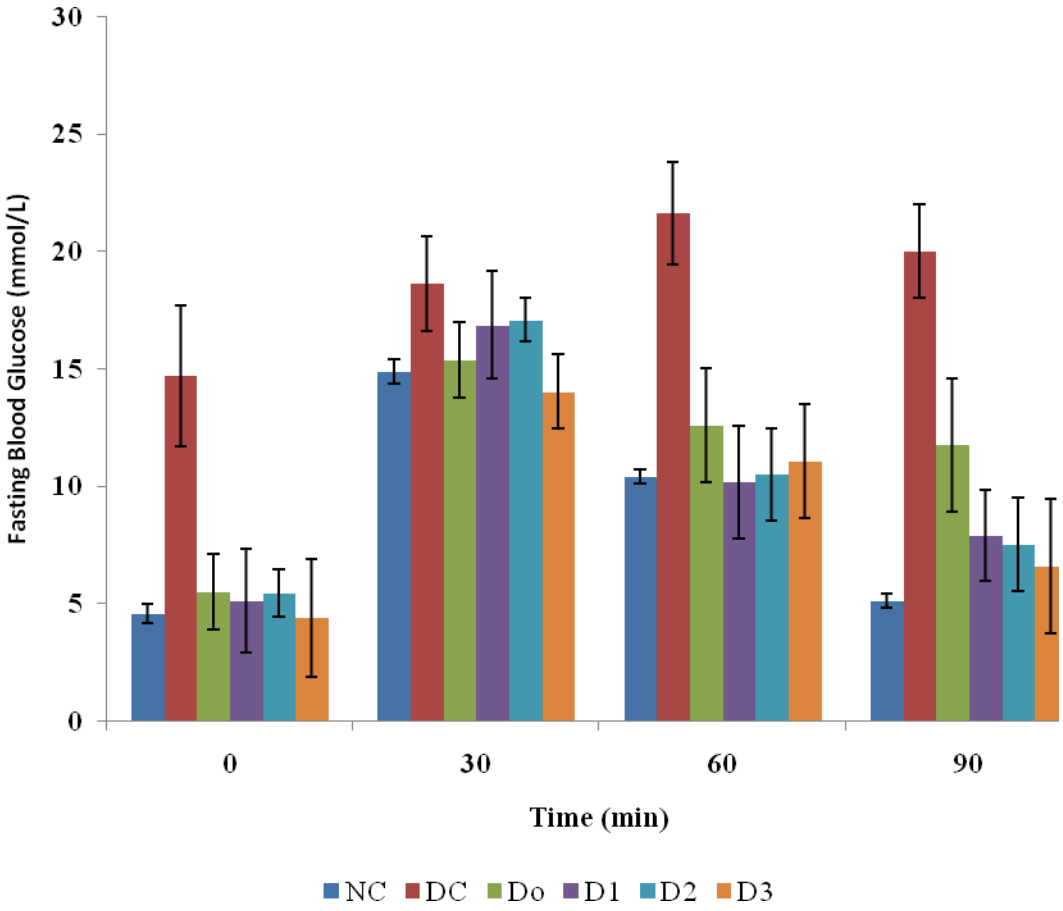

Figure 1: Effect of *S. henningsii* extract on oral glucose tolerance test in rats. The results are expressed as mean ± SD. NC- Normal control; DC- Diabetic control; Do- Normal rats +125 mg/kg SH; D1- Normal rats+250 mg/kg SH; D2- Normal rats + 500 mg/kg SH; D3- Normal rats +glibenclamide (0.6 mg/kg).

A significant decrease in the body weights of diabetic animals was observed 15 days after STZ-NAD treatment when compared with normal control rats. This observation was in agreement with the findings of Sharma et al. (1993). The oral administration of plant extract effectively increased the body weight gain of the animals near that of normal rats but not dose related (Figure 2). The observed result indicated that extract of *S. henningsii* possess the ability to manage glucose properly as well as controlling muscle wasting and induced adipogenesis (Swantson-Flatt et al., 1990).

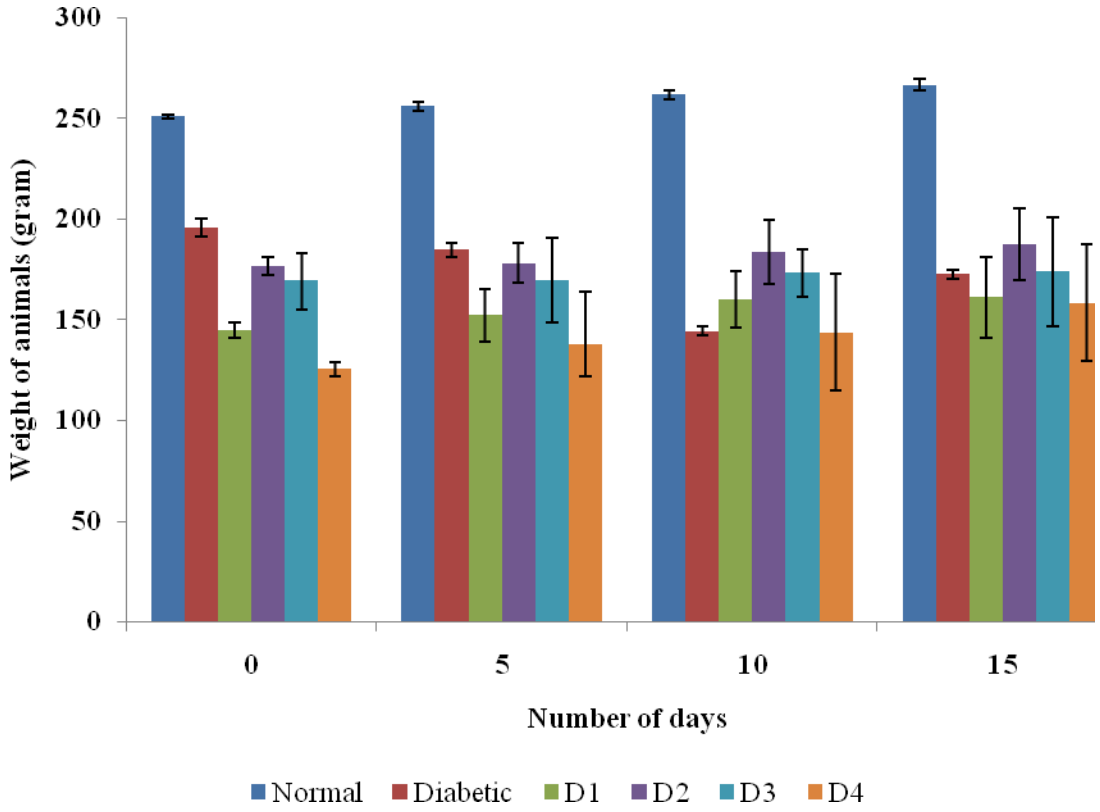

Figure 2: The effect of aqueous extract of *S. henningsii* on the body weight of Wistar rats. Values are mean ± SD of 6 rats in each group. D1 = animals fed with 250 mg/kg body weight of extract, D2 = animals fed with 500 mg/kg body weight of extract, D3 = animals fed with 1000 mg/kg body weight of extract, control = animals receiving only distilled water

Also, the feed and water intake of the diabetic rats were significantly increased in comparison with the normal control rats (Figure 3 and 4 respectively). These symptoms are well known markers of type 2 diabetes in both human and animal models which are direct consequence of insulin deficiency (Shenoy and Ramesh, 2002). The daily administration of plant extract to diabetic rats for 15 days caused a significant decrease in feed and water intake which was an indication of proper glucose utilization in the animals. All three doses of the plant extract improved the feed and water intake of the diabetic rats.

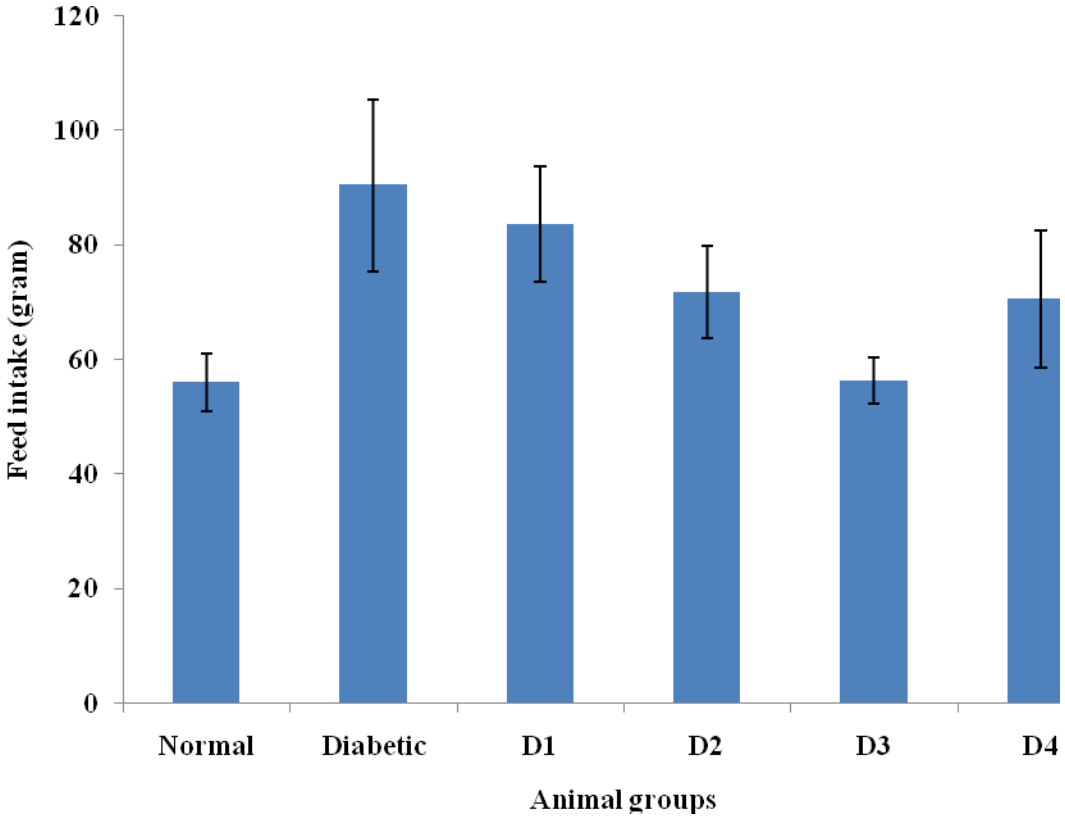

Figure 3: The effect of aqueous extract of *S. henningsii* on the feed intake of diabetic rats. D1 =Diabetic + SH (125 mg/kg), D2 = Diabetic + SH (250 mg/kg), D3 = Diabetic + SH (500 mg/kg) and D4 = Diabetic + glibenclamide (0.6 mg/kg).

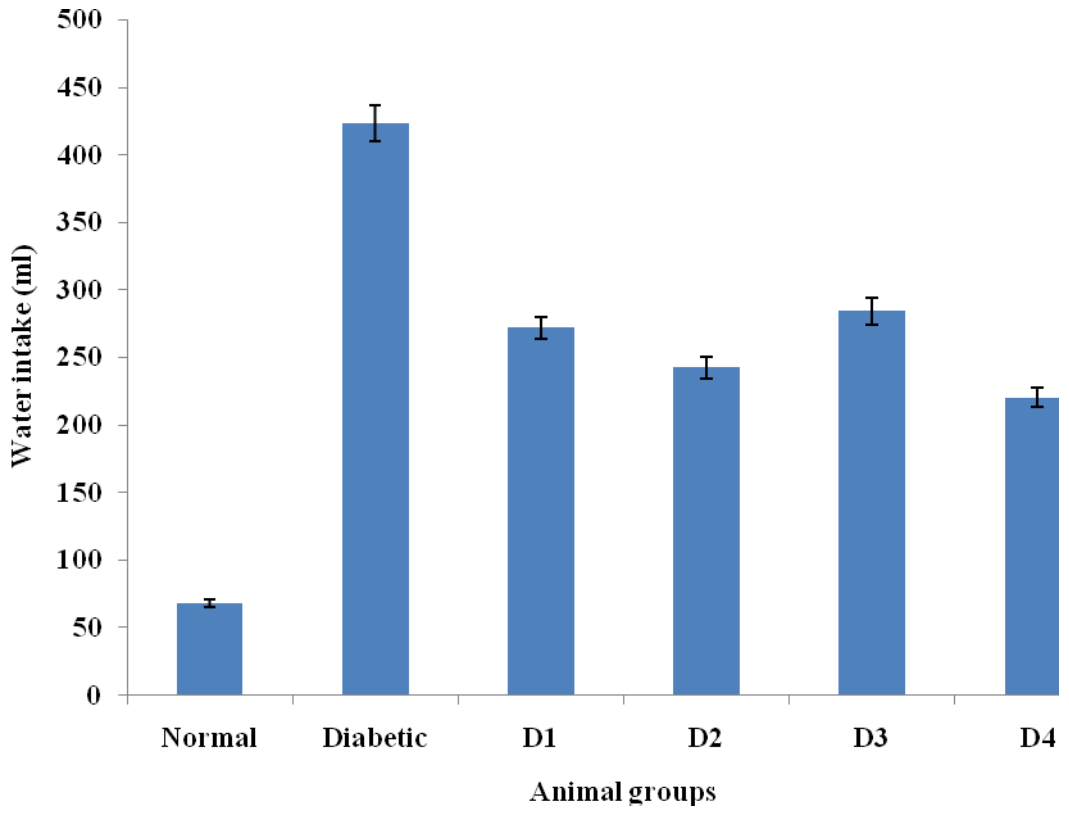

Figure 4: The effect of aqueous extract of *S. henningsii* on the water intake of diabetic rats. D1 = Diabetic + SH (125 mg/kg), D2 = Diabetic + SH (250 mg/kg), D3 = Diabetic + SH (500 mg/kg) and D4 = Diabetic + glibenclamide (0.6 mg/kg).

In this present study, the intraperitoneal injection of streptozotocin-nicotinamide resulted to a significant increase ($P < 0.05$) of plasma glucose level in the diabetic animals than the normal rats (Table 1). The normal rats were euglycemic during the course of the study whereas the diabetic control rats were hyperglycemic throughout the experimental period. The blood glucose in diabetic rats was reduced significantly after oral

administration of aqueous extract of *S. henningsii* in a dose independent manner for 15 days. The reduction of plasma glucose level by the different dosages of the extract was calculated by subtracting the blood glucose level of the final day from the zero day. It was reduced by 6.7, 10.03 and 7.6 mmol/L at the doses of 125, 250 and 500 mg/kg respectively. Meanwhile that of glibenclamide treated group was 8.98 mmol/L. The extract demonstrated hypoglycemic effect throughout the experimental period while the dose at 250 mg/kg showed the best result. The observed result indicated the possibility of increased glucose utilization by the extract owing to the significant reduction of blood glucose (Table 1). This view is supported by the antioxidant activities demonstrated by the plant in our previous study which might increase the resistant of beta cells to the toxic effect of streptozotocin by activating antioxidant mechanism (Yamamoto et al., 1981).

Table 1: Effect of oral administration of aqueous extract of *S. henningsii* on plasma glucose level of STZ induced diabetic rats.

Treatment	Plasma blood glucose (mmol/L)			
	0 (day)	5 (day)	10 (day)	15 (day)
Normal control	5.60 ± 0.40	5.40 ± 0.53	5.6 ± 0.30	5.70 ± 0.32
Diabetic control	18.20 ± 0.30[a]	23.45 ± 0.35[a]	28.30 ± 0.40[a]	30.10 ± 0.40[a]
Diabetic+ SH (125 mg/kg)	19.33 ± 1.20[a]	16.35 ± 1.14[b]	15.53 ± 1.30[b]	12.56 ± 0.90[b]
Diabetic+ SH (250 mg/kg)	24.30 ± 0.09[b]	22.20 ± 0.08	17.57 ± 1.02[c]	14.27 ± 1.20[c]
Diabetic+ SH (500 mg/kg)	25.30 ± 0.01[b]	20.20 ± 0.04[c]	19.28 ± 0.10[d]	17.70 ± 0.30[d]
Diabetic+ glibenclamide	22.50 ± 3.30[c]	19.30 ± 3.20[c]	17.78 ± 2.40[c]	13.52 ± 2.50[c]

Values are mean ± SD of 6 rats in each group.

[a-e] Test values carrying superscripts different from the control for each parameter are significantly different ($P < 0.05$).

In our study the level of red blood cells, Hb, MCV, MCH, MCHC, LUC, RCDW and PCV were measured in diabetic rats induced with STZ-NAD to determine the anaemic conditions of the animals. The diabetic animals were severely anaemic while those treated with the plant extract showed no signs of anaemia. The level of red blood cells, Hb, MCV, MCH, MCHC, RCDW and PCV was significantly decreased in diabetic induced rats when compared with non diabetic groups (Table 2). This observation corroborated with the report of Baskar et al. (2006). However, it contradicts the report of Junod et al. (1969) who observed that diabetogenic action of streptozotocin may be lost

due to prior administration of nicotinamide. This observation support that intraperitoneal injection of nicotinamide into the rats may not completely prevent the effect of streptozotocin on pancreatic beta cells but allow minor damage by repairing poly ADP ribose synthetase (Giroix et al., 2002). The disturbed haematological alteration of red blood cells in diabetic animals was improved upon treatment with the plant extract by preventing haemolysis of erythrocytes caused by lipid peroxidation (Ceriello, 2003).

CHAPTER 7

IN VITRO ANTIDIABETIC PROPERTIES OF SOUTH AFRICAN MEDICINAL PLANT: *STRYCHNOS HENNINGSII* (Gilg)

CHAPTER 7

In vitro antidiabetic properties of South African medicinal plant: *Strychnos henningsii* (Gilg)

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In vitro antidiabetic properties of South Africa medicinal plant: *Strychnos henningsii* (Gilg)

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Abstract

Strychnos henningsii Gilg is a plant commonly used in southern Africa traditional medicine for the management of diabetes. However, the mechanism of action of this plant for the management of diabetes has not been elucidated. The plant extract was tested for the potential to induce differentiation in 3T3-L1 preadipocytes using Oil Red O staining. The *in vitro* antidiabetic effect of the extract was screened against differentiated 3T3-L1 cells and Chang liver cells by measuring glucose uptake. We also assessed the therapeutic approach of *S. henningsii* extract against gastrointestinal carbohydrate digestion through inhibition of α - glucosidase and α – amylase. The antioxidant potential and ability of the plant extract to inhibit protein glycation was also investigated. Plant extract significantly increased glucose uptake in the 3T3 L1 cells with a response of 278.63% above the control at 12.5µg/ml but showed no effect on Chang liver cells. No cytotoxicity was recorded for preadipocyte at the concentrations investigated in this study while Chang liver cells exhibited toxic effect at 250 and 500 µg/ml. The extract inhibited the activity of α- glucosidase and α- amylase in a concentration dependent manner with IC_{50} 0.84 and 2.31 mg/ml respectively. The plant extract exhibited weak inhibition of protein glycation relative to aminoguanidine. The FRAP

assay values of the extract was 357.05 µmol Fe (II)/g while that of BHT, catechin and quercetin were 75, 859.32 and 2907 µmol Fe (II)/g respectively. The finding from this study provides some evidences that *S. henningsii* extract may indeed be useful in the treatment of diabetes mellitus.

Keywords: *Strychnos henningsii*; 3T3-L1; Chang liver cells; α –glucosidase; α –amylase; cytotoxicity; glucose uptake; oil red-O; protein glycation; diabetes mellitus

INTRODUCTION

Diabetes mellitus is a heterogeneous group of metabolic disorder characterized by hyperglycemia and glucose intolerance as a result of inadequate supply of insulin or impaired effectiveness of insulin to stimulate glucose utilization or both. The impairment of insulin secretion from pancreatic beta cells has been reported to affect glucose utilization as a result of defect in insulin signaling cascade (Zick, 2001; Else et al., 2009). This occurs most importantly in target tissues such as skeletal muscle, adipose tissue and liver cells of type 2 diabetic patients (Baynes, 2004; Guyton, 2005).

Persistent hyperglycaemia in diabetes mellitus induces non enzymatic glycation of protein, hyperlipidemia and oxidative stress which are believed to be pathogenetic in the development of diabetic complication such as retinopathy, neuropathy and nephropathy (Jakus et al., 1999). This could be linked to imbalance of antioxidant defense mechanism to neutralize the formation of excessive free radicals generated in the system (Hazra et al., 1999). Despite the recent surge of new antidiabetic drugs such as insulin releasers, insulin sensitizers and alpha glucosidase traditional healers are heavily reliant upon medicinal plants to treat this disease (Ebbeling and Ludwig, 2001). These plants are known to possess antidiabetic properties due to a wide range of phytochemical compositions with cost effectiveness and lesser side effects (Edwin, 2008). Moreover, the majority of plants use in southern Africa traditional medicine need to be thoroughly explored to understand the mechanism of action underlying their activity.

Strychnos henningsii Gilg (Loganiaceae) is one of the plants used in southern and eastern Africa as a folk remedy for the management of various diseases (Oyedemi et al., 2009). The decoction of the plant (branch, stem and root) has been used in traditional Kenyan medicine for the treatment of rheumatism, gynaecological complaints, chest pain, internal injuries and malaria (Bisset, 1970; Hutching, 1989; Kareru et al., 2007). It is also used in

Tanzania for the treatment of snake bite and hookworm infections. The aqueous bark extract of the plant is used in South Africa traditional medicine for the treatment of stomach ache, colic, dizziness, purgative, nausea and anthelminthic. This plant was discovered recently during our ethnobotanical survey in Nkonkobe Municipality, Eastern Cape, South Africa as herbal mixture for the treatment of diabetes mellitus (Oyedemi et al., 2009). The isolated alkaloids such as holstiine, holstilinne, rindline and strychinine from the stem bark of this plant were shown to possess pharmacological and physiological properties (Ohiri et al., 1983; Tits et al., 1991). These include convulsive, anti-cancer, anti-malarial, hypotensive, anti-inflammatory, anti-spasmodic and cardiac depressant activities (Neuwinger, 1999).

Previously, we have demonstrated the effect of aqueous stem bark extract of *S. henningsii* on fasting blood glucose and glucose tolerance in diabetic induced rats (Chapter 6). The present study was therefore designed to further investigate the antidiabetic properties of the plant extract responsible for its antidiabetic activity using various in vitro models designed to simulate specific known antidiabetic target. This study examined the effect of *S. henningsii* extract on *in vitro* glucose utilization in hepatic (Chang liver) and adipocyte (3T3-L1) cell lines; the two cell lines used in this study were chosen because of the importance of the liver and adipose tissue in the regulation of blood glucose homeostasis. Furthermore they have different glucose transporters, which react differently to insulin stimulation. In addition, preadipocyte differentiation cytotoxicity, inhibition of protein glycation and intestinal carbohydrate digestions were also investigated as potential therapeutic targets.

MATERIALS AND METHODS

Plant material

The bark of *S. henningsii* was collected from a thick forest in February, 2009 by a traditional healer in the Amathole District (Eastern Cape, South Africa). The plant was identified by its vernacular name and later authenticated by Prof. D.S. Grierson of Botany Department, University of Fort Hare. Voucher specimen (Sun MED 2009) was deposited at the Giffen herbarium of the University. The herbal drug was prepared by soaking 3 g of the plant sample in 750 ml of water for the treatment of diabetic patient according to traditional healers interviewed.

Preparation of plant extracts

The bark material was air-dried at room temperature in the laboratory. The dried bark was further grounded into powdery form using an electric blender (Waring Products Division, Torrington, USA). About 80 g of the powdered plant material was extracted in 1000 ml of cold distilled water (4 ^{o}C) maintained on a mechanical shaker (Stuart Scientific Orbital Shaker, SOS1, Essex, UK) for 48 h. The extract was filtered using a Buchner funnel and Whatman No.1 filter paper. The filtrate was quickly frozen at -40 ^{o}C and dried for 48 h using a freeze dryer (Savant Refrigerated vapor Trap, RV T41404, USA) to give a yield of 10.6 g of dry extract. The dried extracts were reconstituted in diluted DMSO, vortexed and left for at least 15 min before further dilution with the respective growth medium for acute exposure. The final DMSO concentration did not exceed 0.25%.

Cell lines, media and chemicals

Chang liver and 3T3-L1 (Preadipocyte) cell lines were obtained from the American Type Culture Collection (Highveld Biological, Johannesburg, South Africa). Glucose oxidase reagent (SERA-PAK Plus, Bayer), and RPMI 1640 medium was obtained from Sigma Aldrich, S. Africa, whereas fetal calf serum (FCS) was purchased from Highveld Biological, South Africa. The chemicals and other reagents used in this study were of analytical grade and were purchased from Merck Chemicals (Pty) Ltd, S. Africa.

Maintenance of cell cultures

All cell cultures were incubated at 37 ^{o}C in a humidified atmosphere with 5% CO_2. The Chang liver cells were fed fresh with growth medium every 2-3 days, consisting of RPMI 1640 (Highveld Biological, South Africa) medium supplemented with 10 % fetal bovine serum. 3T3-L1 cells were cultured in DMEM (1.5g/l $NaHCO_3$) (Highveld Biological, South Africa) with 10 % fetal bovine serum under rigorous aseptic techniques to avoid cell culture contamination. Both cell lines were sub-cultured after 90 % confluence was reached.

Glucose utilization in 3T3-L1 cell line

The 3T3-L1 cells were cultured in DMEM (1.5g/L NaHCO3) with 10% fcs. For glucose utilization determination, 3T3-L1 preadipocytes were seeded at a density of 3 000 cells per well into a 96-well plate and cultured for five days in a growth medium (Erasto et al., 2009). The method described by van de Venter et al. (2008) was adopted to determine the glucose uptake. The cells were left without feeding after seeding to allow glucose depletion from the medium. On day three after seeding, 1 µl of *S. henningsii* extract (12.5µg/ml) was added to give the final concentration of 2.5µg/well of the extracts. On day five after seeding,

the medium was removed and 50 µl of incubation medium (8mM glucose RPMI 1640 + 0.1% BSA) containing the plant extract (12.5µg/ml) was added to the appropriate wells. The control wells contained only incubation medium while the positive control contained 1 µM of insulin. After 90 min of incubation, 10 µl of the incubated medium was removed and placed into a new 96 well plate into which 200 µl glucose oxidase reagent (SERA-PAK Plus, Bayer) was added. After incubation for 15 min at 37 ^{o}C, the absorbance was measured at 492 nm using a Multiscan MS microtitre plate reader (Labsystems). The amount of glucose utilized was calculated by subtracting the amount of glucose left in the medium from the amount given at time 0 (8mM).

Glucose utilization in Chang liver cells

The Chang-liver cells were cultured and maintained in an antibiotic-free growth medium as described above. The cultured cells were seeded into 96 well plates at a density of 6 000 cells per well in a growth medium (RPMI 1640 medium supplemented with 10 % fetal bovine serum) for three days without feeding, prior to glucose utilization assay. On day three after seeding, 1 µl of *S. henningsii* extract (12.5µg/ml) was added to give the final concentration of 2.5µg/well of the extracts. On day five after seeding, the medium was removed and 50 µl of incubation medium (8mM glucose RPMI 1640 + 0.1% BSA) containing the plant extract (12.5µg/ml) was added to the appropriate wells. 1 µM metformin was used as the positive control while the control contained only incubation medium. After 180 min of incubation, 10 µl was removed and placed into a new 96 well plate into which 200 µl glucose oxidase reagent (SERA-PAK Plus, Bayer) was added. After incubation for 15 min at 37 ^{o}C, the absorbance was measured at 492 nm using a Multiscan MS microtitre plate reader (Labsystems). The amount of glucose utilized was calculated by subtracting the amount of glucose left in the medium from the amount given at time 0 (8mM).

Oil - red - O staining

Treated 3T3-L1 cells of were stained with 100 µl of Oil Red O and incubated for 30 min at37°C. The assay was conducted following the method described by Kasturi and Joshi (1982). Excess dye was removed by washing thrice with water and the plate was later dried in an oven at 37 ^{o}C. The dye was further extracted by adding 100 µl of isopropanol followed by measuring the absorbance at 520 nm.

Total phenolics content

The amount of phenolics compound in the aqueous leaves extract of *L. leonurus* was determined with Folin Ciocalteu reagent using the method of Lister and Wilson (2001). To 0.5 mL of plant extract solution (1 mg/mL) was added 2.5 mL of 10 % Folin-Ciocalteu reagent and 2 mL of Na_2CO_3 (2 % w/v). The resulting mixture was incubated at 45 ^{o}C with shaking for 15 min. The absorbance of the samples was measured at 765 nm using UV/visible light. Total phenolics content was expressed as mg/g tannic acid equivalent using the following equation from the calibration curve: $Y = 0.1216x$, $R^2 = 0.936512$, where x was the absorbance and Y was the tannic acid equivalent (mg/g). The experiment was conducted in triplicate, and the results are reported as mean ± SD values.

Ferric reducing antioxidant potential (FRAP)

The method described by Benzie and Strain (1999) was used to determine total antioxidant activity of *S. henningsii* extract**.** The FRAP reagent was prepared by mixing 20 ml acetate buffer (300mM, pH 3.6), 2 ml TPTZ solution, with 2 ml $FeCl_3$ solution and 2.4 ml distilled water. The solution was straw coloured and kept in a water bath at 37 ^{o}C. A volume of 10 µl of the sample was transferred into a 96 well plate with 250 µl of freshly prepared FRAP reagent. The absorbance at 593 nm after 5 min against blank containing distilled water

was taken and recorded. The antioxidant capacity of this plant based on the ability to reduce ferric ions of the extract was expressed as catechin equivalents (mg/g) using the following equation from the calibration curve: $Y = 0.3329x$, $R^2 = 0.9763$ where x was the absorbance and Y the catechin equivalent (mg/g).

Inhibition of Protein glycation

Albumin glycation was determined using the method of Matsuura et al (2002). Bovine serum albumin (10 mg/ml) was incubated with 250 mM of glucose in 200 mM potassium phosphate buffer (pH 7.4; 0.02% sodium azide) for 72 h. During this time, samples were kept in a 37 ^{o}C under 5% CO_2. Results were read using a microtiter plate reader with an excitation/emission wavelength of 370/440 nm. The assay was routinely conducted using 12.5 µl of plant extract in a 1 ml assay. The inhibition of glycation was assumed when fluorescence was reduced to that of albumin in the absence of glucose. All experiments were performed in triplicate and the extract was tested at least twice at a minimum of three dilutions.

Alpha-glucosidase inhibiting activity

Alpha glucosidase solution was prepared from rat intestinal acetone powder. Approximately 100 mg of acetone powder was suspended in 3 ml of 0.01 M phosphate buffer (pH 7.0) and sonicated for 30 s for 12 times in ice bath followed by centrifugation at 3000 rpm, 4 ^{o}C for 20 min. The supernatant containing the enzyme was obtained and kept on ice for further dilution. The enzyme inhibition activity of α-glucosidase was evaluated according to the method previously reported by Sancheti et al (2010). The reaction mixture consisted of 50 µl of 0.5 mM MPNG (dissolved in 0.1 M phosphate buffer pH 7.0), 10 µl of test sample and 25 µl of a glucosidase solution. The glucosidase solution was prepared using

a stock solution of 1 mg/ml in 0.01M phosphate buffer (pH 7.0), which was diluted to 0.1 U/ml with the same buffer (pH 7.0) just before the assay. This reaction mixture was then incubated at 37 ^{o}C for 30 min. The reaction was then terminated by the addition of 100 µl of a 0.2 M sodium carbonate solution. The enzymatic hydrolysis of the substrate was monitored based on the amount of p-nitrophenol released into the reaction mixture by a microtiter plate reader {Multiscan MS microtitre plate reader (Labsystems)} at 410 nm to measure the absorbance. The % inhibition was calculated as follows: (1 - B/A) × 100 where A is the absorbance of control and B is the absorbance of plant extract. All experiments were carried out in triplicate and the results are expressed as the mean ±SD of three determinations.

Alpha- amylase inhibitory activity

The α-amylase inhibitory activity of *S. henningsii* extract was tested based on the method described by Zhizhuang et al. (2005) using microplate-based starch-iodine assay. The reaction was initiated by adding 40 µl (2 g/L) of starch (Sigma S-2630) solution and 40 µl of enzyme in 0.1 M phosphate buffer at pH 7.0 to microplate wells. After 30 min of incubation at 50 ^{o}C, 20 µl of 1 M HCl was added to stop the enzymatic reaction. 100 µl of iodine reagent (5 mM I_2 and 5 mM KI) was added to the mixture. After colour development, 150 µl of iodine treated sample was transferred to a transparent flat bottomed 96 well microplate to measure the absorbance at 580 nm.

MTT cell viability assays

Cell viability in the individual wells of both cell lines treated with plant extract was evaluated using a modified method of Mosman (1983) to determine cytotoxicity and to normalize glucose utilization results. Toxicity assays of both cell lines were done by adding

100µl of MTT (0.5 mg/ml in RPMI1640: 10% fbs) directly into the last three rows of Chang liver and 3T3-L1 cell lines individually with 10µl of plant extracts (12.5 and 25µg/ml). MTT (3-(4,5-dimethylthiazolyl-2)- 2,5-diphenyltetrazoliumbromide) is a yellow water soluble tetrazolium dye that is reduced by metabolically active cells to a purple formazan product that is insoluble in aqueous solution within 2 hrs of exposure. After 2 hrs of incubation (5% humidified incubator) at 37^{o}C, the MTT was removed and the resulting formazan dissolved in DMSO was read spectrophotometrically (Multiscan MS, Labsystems) by measuring the absorbance at 540 nm.

RESULTS

Glucose uptake in 3T3-L1 cells

The plant extract demonstrated high insulin-sensitive glucose uptake of 178.6% at 12.5µg/ml while the cells treated with 25µg/ml were 20.2% over the control (Figure 1). In the positive control, insulin caused a two-fold increase in glucose utilization for 24 h over the basal. However, in the presence of insulin, the extract further increases glucose utilization to 325 % at 12.5 µg/ml and 290 % at 25µg/ml over the control. This result indicates synergistic or additive interaction of the extract with insulin for the induction of glucose utilization in 3T3-L1 adipocytes.

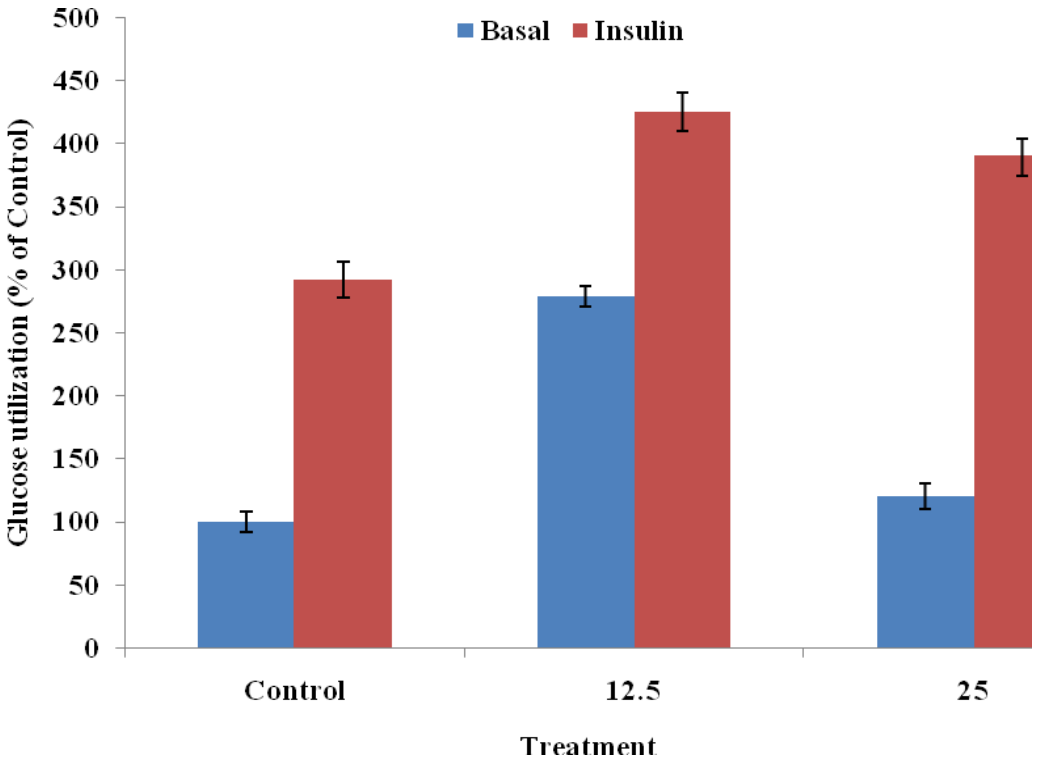

Figure 1: The effect of aqueous extract of *S. henningsii* bark on glucose utilization in the 3T3-L1 cells. Cells were exposed to extract concentration at 12.5, 25µg/ml and insulin (1µM) prior to glucose utilization. Data are represented as % control ± SD of mean (n = 6).

Glucose uptake in Chang liver cells

Figure 2 revealed the effect of *S. henningsii* extract on glucose uptake in Chang liver cells. The extract did not have any significant effect (P>0.05) on glucose utilization in the cells when compared with the control. The percentage glucose uptake by the extract at 12.5µg/ml and metformin at 1uM was 103.5% and 160.24% respectively. Metformin is a popular drug prescribed for the treatment of type 2 diabetes however the precise molecular mechanism remains poorly defined. The glucose lowering effects associated with metformin results mainly from decreased hepatic glucose production via the inhibition of gluconeogenesis, and to a lesser extent due to increased insulin stimulated glucose utilization

in peripheral tissue. It has been shown to inhibit the mitochondrial respiratory chain at complex 1 with concomitant up-regulation of glucose transporters and glycolytic enzymes resulting in increased glycolytic glucose utilization, and hence is used as positive control in the Chang liver assay. Metformin exhibited higher glucose uptake activity with response of 56.74% over the extract. The plant extract appeared to inhibit the action of metformin when incubated together with the Chang liver cells (102.9%).

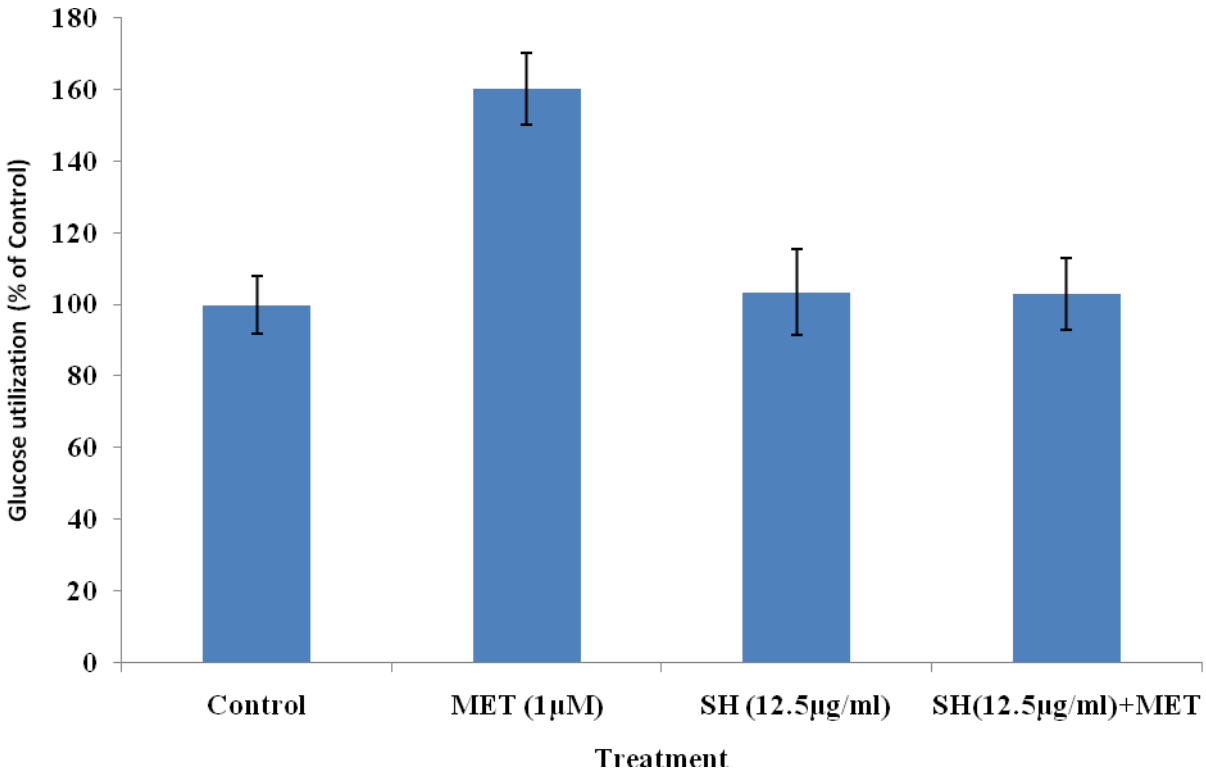

Figure 2: The effect of aqueous bark extract of *S. henningsii* on glucose utilization in the Chang liver cells. Cells were exposed to extract concentration of 12.5µg/ml, metformin (MET) and combination of extract and metformin (SH+MET) prior to glucose utilization measurement. Data are expressed as % control ± SD (n =6).

Cell viability assay

The effect of aqueous stem bark extract of *S. henningsii* on the growth of 3T3-L1 and Chang liver cell lines were examined by MTT assay. The data obtained on Chang liver cells revealed non toxic effect of the extract at both concentrations investigated (Figure 3).

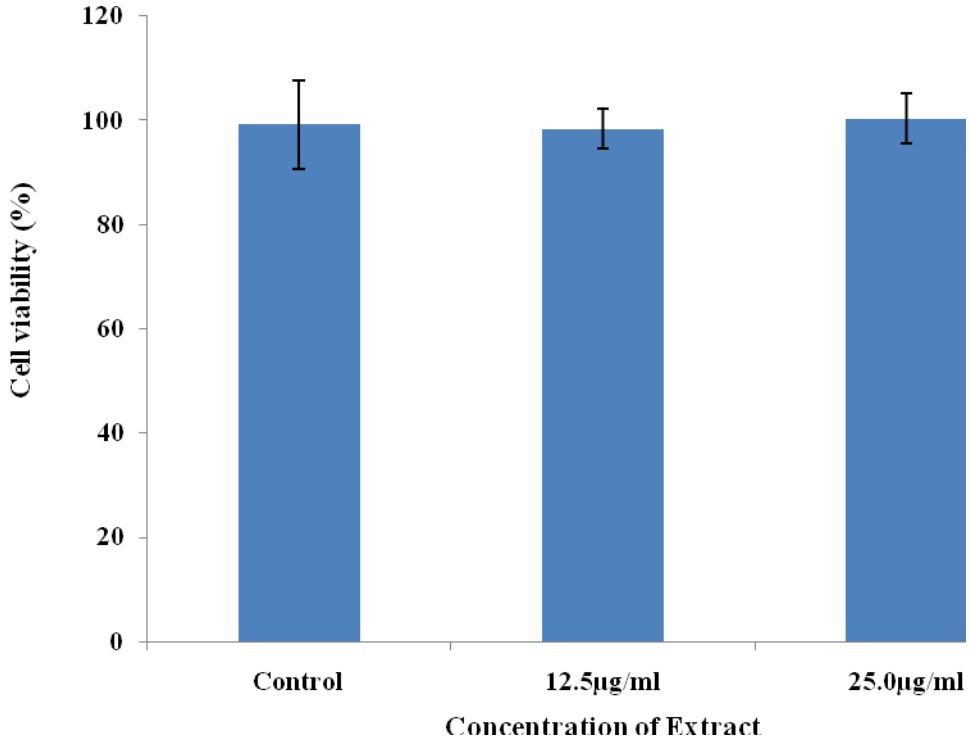

Figure 3: MTT assay of aqueous extract of *S. henningsii* in the Chang liver cells. Data are expressed as % control ± SD (n = 6).

In 3T3-L1 adipocytes, the plant extract reduced the relative cell viability to 90.2 and 85.7% at the dose of 12.5 and 25µg/ml compared with 100 % of the control (Figure 4). These data suggest that *S. henningsii* extract at concentrations investigated have ability to enhance glucose metabolism by the cells with slight toxic effect on 3T3-L1 cell line as shown in this study.

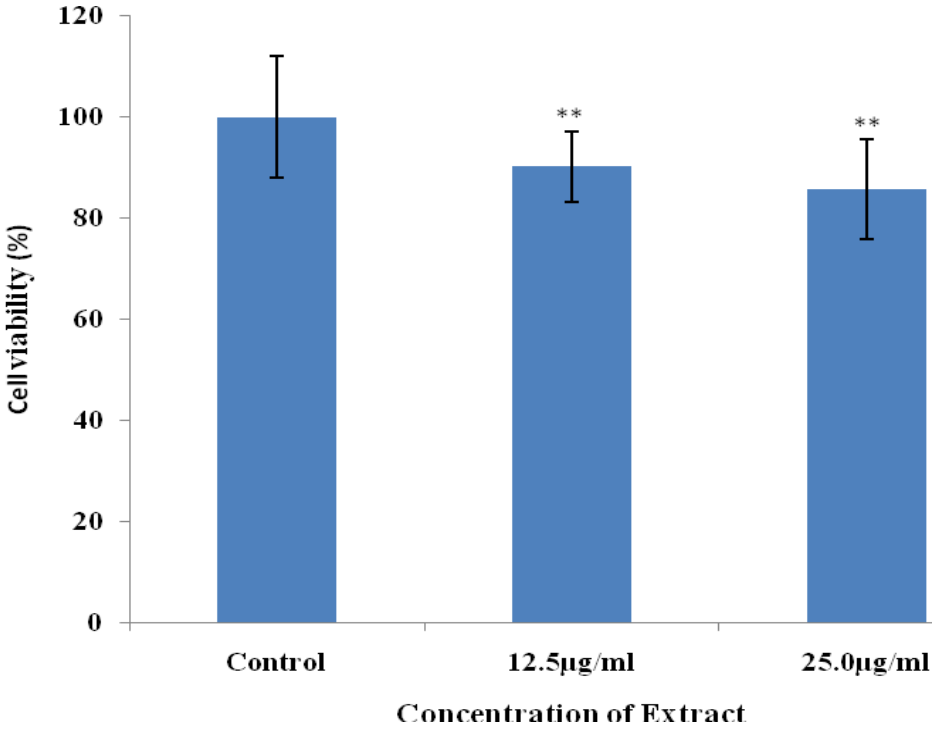

Figure 4: MTT assay of aqueous extract of *S. henningsii* in 3T3-L1 cells exposed to 12.5 and 25µg/ml of the extract. Data are expressed as % control ± SD (n = 6). **P< 0.01 compared to control

Lipid accumulation assay

The effect of plant extract on preadipocyte differentiation was examined using triglyceride accumulation as a marker for adipogenesis. Lipid droplets were quantified by oil-red-O staining (Figure 5). Plant extract did not have any significant effect on the lipid accumulation as compared with insulin treated cells at physiological concentration (12.5µg/ml). The observed results give an indication that the extract might not be a good therapeutic agent in lowering lipid profiles associated with obesity especially in the *in vitro*

studies. Rosiglitazone is a standard antidiabetic drug used as an insulin sensitizer and thus make the cells more responsive to insulin.

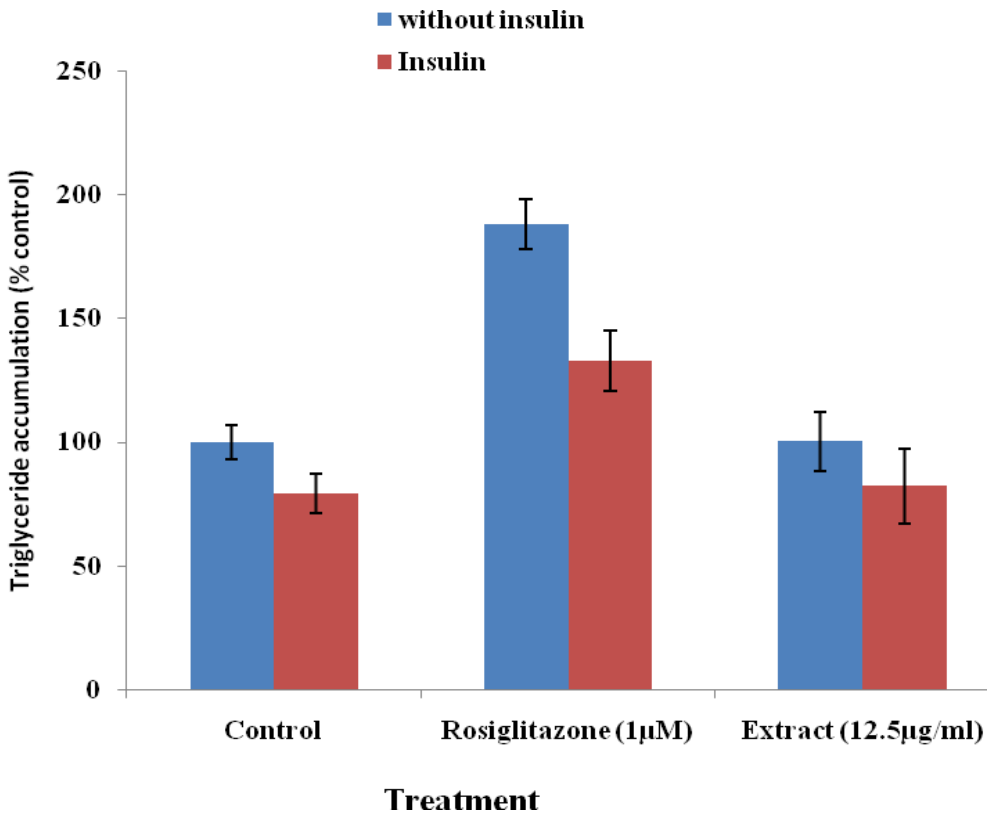

Figure 5: Effect of *S. henningsii* extract on 3T3-L1 preadipocyte triglyceride accumulation.

Protein glycation

In this study, we evaluated the inhibitory activity of plant extract on protein glycation which is implicated as a major pathogenesis process to diabetic complications (Figure 6). The formation of protein glycation was assessed by monitoring the production of fluorescent products at excitation and emission maxima of 370 and 440 nm respectively. The fluorescence intensity was highly increased through incubation of BSA with glucose at 37 ^{o}C compared to the control without glucose. The presence of high molecular weight substances

in the plant extract that may interfere with fluorescence measurements were dialyzed and tested. The plant extract suppressed the intensity of fluorescence in a concentration dependent manner. The percentage protein glycation in the medium was reduced to 86.8, 73.7 and 48.8 % at 3, 6 and 12.5µg/ml when compared with the control while that of aminoguanidine a known inhibitor of protein glycation process was 12.5%. The result obtained demonstrated that *S. henningsii* extract possesses antiglycation activity especially at the concentration of 12.5µg/ml.

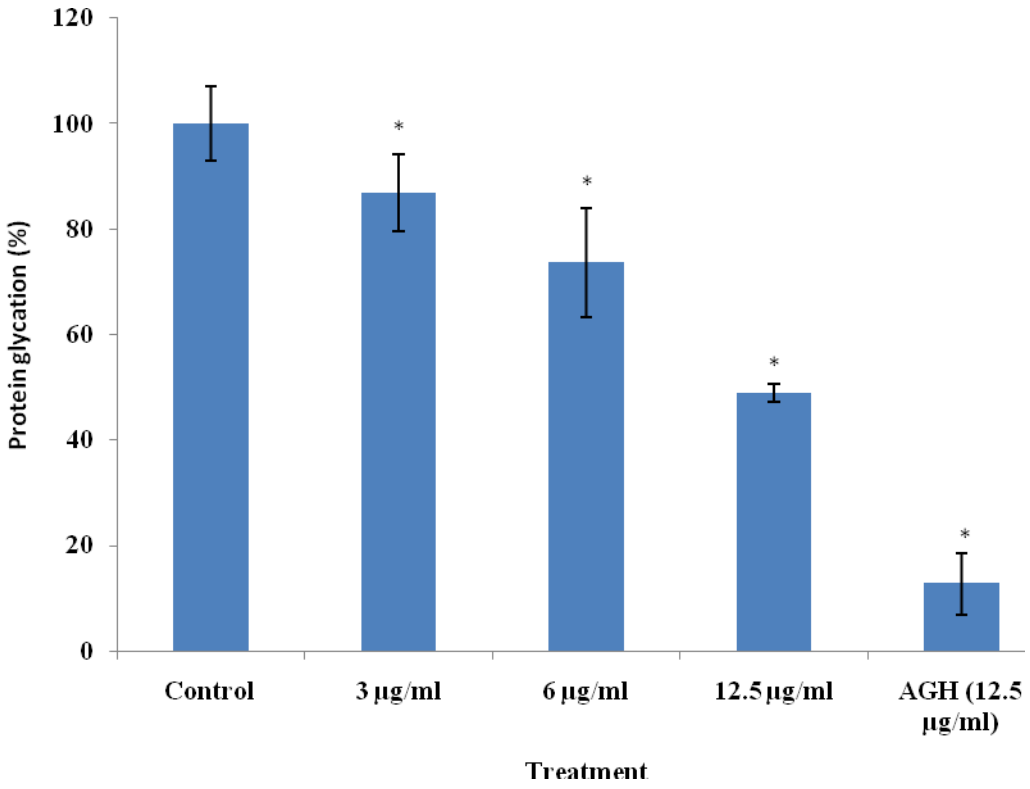

Figure 6: Effect of *S. henningsii* extract and aminoguanidine (AGH) on BSA glycation. Data are expressed as mean ± SD (n = 6). *P<0.01 compared to control

Ferric reducing antioxidant power assay (FRAP)

The quantitative phytochemical analysis in previous studies reported a total phenolics content of 48 mg/g tannic acid equivalent. Phenolics have been documented to possess significant antioxidant activity which may be responsible for the reduction of TPRZ-Fe (III) complex to TPTZ-Fe (II) as shown in this study. The results obtained depicted high antioxidant property of the plant extract (357.05 µmol Fe (II)/g) when compared with BHT (butylated hydroxyltoluene) (75 µmol Fe (II)/g) but significantly lower to that of catechin (859.32µmol Fe (II)/g) and quercetin (2907.25 µmol Fe (II)/g) as depicted in Table 2.

Table 1: Total antioxidant (FRAP) activity of the bark extract of *S. henningsii*

Sample	FRAP
Extract	357.05 ± 9.21[e]
Catechin	859.32 ± 15.67[e]
BHT	75.22 ± 5.16[e]
Quercetin	2907.25 ± 23.67[e]
Total phenolics content (extract)	48 .02 ± 1.52[d]

[e]Expressed in units of µmol Fe (II)/g.

[d]Expressed as Tannic acid equivalent (mg/g)

Alpha glucosidase and α-amylase assay

In vitro α- glucosidase and α- amylase inhibitory activities showed that *S. henningsii* extract had an appreciable inhibitory effect on α – glucosidase but weak inhibitory effect on α -amylase. The percentage inhibitory activity of the extract against α–glucosidase at 0.25, 0.5

and 1 mg/ml was 14.8, 35.27 and 58.04% respectively (Table 1). The value of 0.84 mg/ml was obtained for the extract which is lower than the positive control (Acarbose) which had an IC_{50} of 0.007mg/ml. The extract did not have a significant inhibitory effect on α-amylase (IC_{50} = 2.31 mg/ml) inhibitory activity when compared to that of Acarbose with IC_{50} value of 0.024 mg/ml.

Table 2: The inhibitory effects of aqueous *S. henningsii* extract against α–amylase and α-glucosidase. Data are expressed as the mean (n=3).

Concentration (mg/ml)	% inhibition	
	α–amylase	α-glucosidase
0.25	5.40 ± 0.01	14.80 ± 0.03
0.5	18.70 ± 0.03	35.27 ± 0.02
1.0	31.28 ± 0.12	58.04 ± 0.07
Extract (IC_{50} mg/ml)	2.31 ± 0.02	0.84 ± 0.04
Acarbose(IC_{50} mg/ml)	0. 024 ± 0.31	0.007 ± 0.19

DISCUSSION

Type 2 diabetes is a complex metabolic disorder characterized by abnormal insulin secretion and insulin action caused by impaired cell function and insulin resistance in liver, skeletal and adipose tissues (Pessin et al., 2000). It is also characterized by chronic high blood glucose that causes glycation of body proteins due to defect in postprandial glucose uptake (Rang et al., 1991). The underlying mechanism responsible for this disease and its complication remain unclear though possible events such as activation of series of protein in insulin signaling pathway has been suggested (Giugliano et al., 1996). Other pathway that may be evidenced includes polyol pathway, oxidative stress, non-enzymatic glycation, inhibition of carbohydrate digestive enzymes and glucose uptake (King, 1996). Previously, we have reported the antihyperglycemic and antilipidemic effect of *S. henningsii* extract in streptozotocin-nicotinamide induced diabetic rats (unpublished). The mechanism behind this effect was not fully understood, hence the present study was undertaken to partially assess the possible actions of this plant.

Glucose uptake is a rate limiting step for glucose metabolism which has been shown to regulate increased postprandial blood glucose. Defects in insulin receptors and its signal transduction pathway have been reported in diabetic patients as the underlying cause (Docio et al., 1992). The result obtained in this present study on glucose uptake using differentiated 3T3-L1 cells in the basal state established that *S. henningsii* extract possess the ability to improve glucose uptake in the adipose tissue . The effects of the Plant extract on adipocyte glucose utilization was biphasic with the lower concentration significantly ($P< 0.01$) stimulating glucose uptake in a magnitude comparable to 10^{-6} M of insulin but at the higher concentration basal glucose utilization was strongly inhibited an effect which may at least in part reflect its toxicity towards these cells . The increased basal glucose utilization suggests

the extract may possess insulin mimetic activity; however the precise mechanism needs to be established. In the insulin stimulated state, *S. henningsii* extract exhibited enhanced effect by significantly increased glucose uptake by 325% at 12.5µg/ml and 270% at 25µg/ml over control. The percentage difference of glucose uptake at 12.5µg/ml was 146.4% and that of 25µg/ml was 269.8% which indicate insulin-sensitizing and potentiating activity of the plant extract at higher concentration. The presence of polyphenolics compounds such as phenolics, flavonoids and proanthocyanidins as shown in chapter 4 have been reported to mimic insulin action and thus may be responsible for this observation (Gomes et al., 1995). On this basis, a mechanism of action of *S. henningsii* may be hypothesized which could be linked to activation of series of proteins involved in insulin- mediated glucose transport transduction pathway. This could lead to the translocation of glucose transporter - 4 (GLUT4) to the plasma membrane to facilitate the uptake of glucose from the bloodstream into the cells (Zierath et al., 2002).

Insulin is well known as a regulator of wide variety of metabolic and mitogenic events that involves activation of intracellular signaling pathways (Zierath et al., 2000). The binding of insulin to the insulin receptor provides docking site for several downstream of signaling molecules that transduce to activation of phosphatidylinositol (PI) – 3 kinase (Chen et al., 1996). PI 3-kinase is well recognized as an important step in the insulin signaling to glucose transport through GLUT4 translocation. Increase in enzyme activity of phosphatidylinositide-3-kinase enhances the recruitment of Protein Kinase C (Akt) to bind with the plasma membrane which directly promotes glucose transport and translocation of GLUT 1 and GLUT 4 to the plasma membrane (Eldar et al., 1997; Lodish et al., 2004). In this study, the treatment of 3T3-L1 cells with *S. henningsii* extract at the concentrations investigated enhance glucose uptake and potentiate insulin activity which may be due to the increased expression of PI3K and PKC. Activation of these signaling proteins have been implicated in

several studies to mediate glucose transport due to the presence of polyphenolics compounds (Jorge et al., 2004; Aseervatham et al., 2010; Samane et al., 2006). Thus, the presence of polyphenolics compounds in this plant might therefore account for the up-regulation of these proteins though not investigated in this study (Oyedemi et al., 2010b). Flavonoids and alkaloids have been reported to exert antidiabetic effect through the insulin secretory response and modulation of carbohydrate metabolic enzymes (Hii and Howell et al., 1985; Garcia- Lopez et al., 2004). Owen et al (2008) also demonstrated increase glucose uptake of *Psidium guajava* by activating PPAR in the adipocyte due to the presence of phenolics compounds. It can also be suggested that *S. henningsii* exert indirect benefits on glucose uptake through its strong antioxidant activities (Singh et al., 1993). The results from this study clearly indicate that amelioration of insulin resistance is a potential new field of *S. henningsii* extracts in the treatment of diabetes which warrants further investigation.

The plant extract did not stimulate glucose utilization in Chang liver cells when compared with metformin an antidiabetic standard drug that improves glycemic control primarily by inhibiting hepatic gluconeogenesis and glycogenesis (Gerich, 1989). The extract was also tested with metformin standard drug but did not have synergistic or additive effects. However, reduction of the glucose uptake activity by addition of extract to metformin was observed when compared with metformin alone. The interaction of these agents on the glycaemic control is antagonistic and thus may cause a serious problem for diabetic patients who co-treat diabetes with metformin and *S. henningsii* extract.

The MTT toxicity assay indicated that *S. henningsii* extract was not toxic to the Chang liver and 3T3- L1 cell lines at the concentrations used to determine cellular glucose utilization. Meanwhile, the result exhibited by *Coptis chinensis* (Berberine) on the adipocyte (3T3- L1) as reported by Yin et al. (2008) corroborates with our findings in this study. The cytotoxic effects of this plant extract towards hepatocytes is concerning as this is one of the

main clinical adverse effects reported for use of medicinal plants. Such hepatotoxicity effects are particularly problematic in the treatment of chronic diseases such as diabetes which requires long term medication. Furthermore, the increased prevalence of diseases affecting liver function (hepatitis, obesity and alcohol abuse) and use of hepatotoxic drugs in the treatment of TB and HIV could further increase the potential for toxic side effects. The sub-acute administration of *S. henningsii* barks in our previous studies revealed the relatively non toxic to animals. This is because, there was no apparent damage to some haematological and biochemical parameters used in assessing organ specific toxicity except the white blood count and its relative indices (Oyedemi et al., 2010a). The alterations observed on some parameters suggest parameter and dose selective toxicity. These observations corroborated with the report of Neuwinger (1998) who reported that *S. henningsii* bark produce a marked decrease in toxicity even at high doses in mice due to the opening of lactam and introduction of hydroxyl group at the 17-position of strychnine compound (Neuwinger, 1998).

The adipogenic potential of aqueous stem barks extract of *S. henningsii* on lipid accumulation during differentiation of preadipocytes of 3T3-L1 cells to adipocytes was determined using oil-red-O stained cells. In this present study, the extract did not exhibit any significant effect on the accumulation of lipids in the adipose tissues as compared with the untreated cells but reduced significantly (20 %) when combined with insulin.

Protein glycation is one of the consequences of elevated blood glucose in diabetic patients (Ulrich and Cerami, 2001). This is caused by the non - enzymatic reaction between reducing sugar such as glucose and free amino group of proteins (Singh et al., 2001). This reaction results in the formation of Schiff's base, which can bond covalently to each other to form Amadori products. The rearrangement of this product either by oxidation or reduction further leads to the formation of advanced glycation end-products (AGEs). This product has been reported to cause various complications in patients with diabetes due to loss of collagen

(Kiho et al., 2000). The result obtained in this study demonstrated that *S. henningsii* extract could inhibit protein glycation especially at higher concentration (12.5 µg/ml) although not as efficient as aminoguanidine. The presence of phenolics compounds which have been reported to prevent advance glycation end-product formation could be responsible for this observation (Rice-Evans et al., 1995). The antioxidant potential of this plant as estimated in this study could play a role in inhibiting protein glycation. Several citations of antihyperglycemic and antidiabetic activities of medicinal plants has been done based on their free radical scavenging properties as well as increased glucose metabolism (Zhang and Tan 2000b).

Some antidiabetic drugs act through inhibition of digestion of complex carbohydrates in the gastrointestinal tract (Guyton and Hall, 2000). The plant extract was tested at this level to determine its inhibitory activity against α-amylase and α-glucosidase. These enzymes are well known in the metabolism of carbohydrates. Alpha amylase degrades complex dietary carbohydrates to oligosaccharides and disaccharides, which are ultimately converted into monosaccharide by alpha glucosidase. The inhibition of intestinal alpha glucosidase limits postprandial glucose levels by delaying the process of carbohydrate hydrolysis and absorption, making the inhibitors useful in the management of type 2 diabetes. The ability of *S. henningsii* extract to reduce postprandial glucose level by inhibiting these digestive enzymes was tested to determine its efficacy at this level. The results obtained showed an appreciable inhibitory activity for α-glucosidase but not for α- amylase when compared with Acarbose a standard digestive enzymes inhibitor. Future studies could focus on the isolation of the inhibitory components of this plant and their potential as dietary supplement to control the postprandial glucose level in type 2 diabetes.

It is obvious from the data obtained from this study that the aqueous stem barks extract of *S. henningsii* improves glucose metabolism and insulin sensitivity in the adipocytes. Moreover, the percentage protein glycation and α – glucosidase was moderately improved at the highest concentration (12.5 µg/ml). Therefore, it can be concluded that *S. henningsii* extract exhibits antidiabetic activity through glucose uptake in the adipocytes. Further studies on the insulin signaling pathways are needed to assess the specific mechanism for the observed result.

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CHAPTER 8

GENERAL DISCUSSION

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General Discussion and Conclusion

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GENERAL DISCUSSION

Diabetes mellitus is the commonest endocrine disorder that affects more than 100 million people worldwide (WHO, 1992). It is caused by deficiency in insulin secretion or impaired effectiveness of insulin's action or combination of both. The global prevalence of diabetes has risen drastically over the past two decades. Hence, necessitates constant re-assessment of glycaemic control in people with diabetes and appropriate adjustment of therapeutic regimens. This fact is supported based on the side effects such as gastrointestinal upsets and lactic acid intoxications shown by the synthetic anti-diabetic drugs (Li et al., 2004). Phytotherapy is an alternative medicine widely used as an herbal remedies for the management of diabetes. In developing countries about 80% of the populations are now using traditional medicine as their primary health care (Grover and Yadav, 2004). The people of the Eastern Cape province of South Africa have a long history of traditional medicine usage for the treatment of various ailments including diabetes mellitus (Van Wyk et al., 1997).

The uses of medicinal plants have long played a significant role in the lives of the Xhosa people, the primary inhabitants of the study area. Most rural communities of the region are regularly use wild harvested herbal medicines as an initial response to illness (Dold and Cocks, 2002). Although, there is an increasing interest in the use of medicinal plants for the treatment of diseases still it is necessary to provide scientific evidence to justify their ethnomedicinal usages. This view is supported by WHO (2007) who recommended further evaluation of traditional plants use for the treatment of diabetes mellitus.

Ethnobotanical information

Medicinal plants used for the management of diabetes mellitus were discovered during our ethnobotanical survey conducted at the study area. This study was carried out to documents the indigenous knowledge of experienced traditional herbalists considering the current rate of deforestation and the concurrent loss of biodiversity (Kambizi and Afolayan, 2001). The information on the plants used for the treatment of diabetes were obtained through structured questionnaire and interviewed with the traditional healers of the region. The plants were collected from the thick forest and field accompanied by local traditional healers. *Strychnos henningsii* Gilg (*Loganiaceae*) among other plants was repeatedly mentioned and highly recommended by the traditional healers and rural dwellers (Oyedemi et al., 2009). Thus, the choice of *Strychnos henningsii* for further study was based on the ethnomedical information obtained from indigenous Xhosa people of South Africa who have been using the plant for many years for the management of diabetes mellitus.

Antioxidant activity

The role of oxidative stress in the pathogenesis of diabetes mellitus is well established due to increased production of oxygen free radicals and a sharp reduction of antioxidant defense systems (Fridlyand et al., 2005). Prolonged exposure to oxidative stress could lead to oxidative damage which possibly contributes to pancreatic beta cell dysfunction (Song et al., 2007). Antioxidant agents of natural origin have attracted special interest because they can protect human body from oxidative damages caused by free radicals (John, 1991). Investigation into the antioxidant properties of *S. henningsii* both *in vitro* and *in vivo* was conducted to understand its mechanism of actions. The phytochemical analysis of the plant extract revealed the presence of bioactive compounds such as tannins, flavonoids, phenolics,

saponins, steroids and alkaloids. These compounds are known to be biologically active aiding the activities against diabetes through different mechanisms (Price, 1987; Teotia and Singh, 1997; Marles and Farnworth, 1995). These mechanisms include enzymatic degradation and scavenging of free radicals that are generated during or at the onset of diabetes (Penckofer et al., 2002). Further quantitative analysis of the plant extract showed high levels of phenolics contents followed by proanthocyanidins, flavonols and flavonoids. These compounds may account for the strong antioxidant activities observed both *in vitro* and *in vivo* studies. Thus antihyperglycemic effect observed in this study could be attributed to the strong antioxidant capacity demonstrated by this plant. The decreased level of lipid peroxidation in hepatotoxic treated rats induced with CCl_4 at the dose of 500 and 1000 mg/kg body weight suggest an efficient protective mechanism in response to reactive oxygen species that are generated in hepatotoxic rats.

Toxicological studies

The effect of oral administration of aqueous bark extract of *S. henningsii* at 250, 500 and 1000 mg/kg body weight was investigated on some haematological and biochemical parameters in male Wistar rats for 28 days. In this study, the levels of Hb, RBC, haematocrit or PCV, MCHC and LUC were not significantly altered. However, white blood cells and its differentials were appreciably affected at certain doses. The observed results indicate the influence of the extract on the immune system and phagocytic activity of the animals (Ghasi et al., 2000). The biochemical indices of liver and kidney damage monitored in this study are useful markers for assessing the functional capacities of the organs. A selective effect at specific doses was indicated in liver marker indices, the fact which is strengthened by the organ- body weight ratios of the liver while others did not show any evidence of extract

toxicity either by inflammation or cellular constriction (Idell et al., 1985). The alterations observed at some haematological and biochemical parameters at different dosages imply parameter and dose selective toxicity of the plant. The available evidence from this study implies that the extract may not be completely safe as oral remedy.

Antidiabetic and clinical significance

Diabetes mellitus is a complex syndrome involving decreased sensitivity of target tissues to insulin or deterioration of insulin secretion or both (Alberti and Zimmet, 1998). The antidiabetic and antilipidemic property of *S. henningsii* extract at the doses of 125, 250 and 500 mg/kg body weight were investigated in diabetic rats induced with streptozotocin - nicotinamide for 15 days. These chemicals are well known for the induction of diabetes mellitus in rats that resemble non obese type 2 diabetes (Tomonori et al., 2006). The plasma glucose level was very high with polyuria, weight loss and polyphagia to confirm diabetic state. The plant extract was able to reduce blood glucose levels either by potentiate insulin secretion from the existing beta cells or by its release from the bound form. Similarly, various complications associated with this disease were ameliorated after extract administration as shown in this study. Furthermore, the plant extract exhibited strong antilipidemic effect which might implies the presence of bioactive compounds that inhibit or activate some enzymes involves in lipid metabolism (Babu et al., 2007; Swantson-Flatt et al., 1990). In addition, the result obtained from this study on the clinical parameters provides evidence that administration of *S. henningsii* extract could reverse anemic condition as well as alleviating some disturbed markers of the liver. The elevated kidney markers were drastically reduced at certain doses while there was no significant improvement on the white blood cells and some

of its related indices. The study also revealed the ability of the extract to utilize glucose properly which is reflected on the body weight gain of the animals.

Probable mechanisms of action

The results obtained from this study has established that *S. henningsii* extract possessed strong glucose utilization on the 3T3-L1 adipocytes cell lines with a respond of 278.63% of the control at 12.5µg/ml. Meanwhile the extracts lacked efficacy on Chang liver cell lines with a respond of 103.54%. The extract displayed a promising effect on the liver cell lines when combined with metformin an antidiabetic standard drug. This showed a probable synergistic potential of the extract if combined with other plants of medicinal values. It may also justify the rationale behind the therapeutic benefits attributed to plant mixtures by the herbalists. Moreover, *S. henningsii* is a potent antidiabetic plant, hence the people using its bark for the management of diabetes, have possibly been relieved through the ability to stimulate glucose metabolism in the body. The cytotoxicity result revealed non toxic effects of the extract on both cell lines. The observation which contradicts the report of Deutschlander et al. (2009) who observed increased glucose utilization might be due to toxic effect on fat cells. In addition, the results obtained on α-glucosidase and amylase showed appreciable and weak inhibitory activities respectively. Based on previous phytochemical studies, it can be conclude that S. *henningsii* should be further investigated to identify the compounds responsible for its glucose metabolism especially on the adipocytes tissue

Protein glycation is one of the consequences of elevated blood glucose in diabetic patients (Ulrich and Cerami, 2001). The reaction of glucose and other reducing sugars with protein reversibly produces Amadori products and over a long period produced glycation end products (Singh et al., 2001). These reactions are greatly accelerated and are important in the

pathogenesis of diabetic complications (Kiho et al., 2000). The *in vitro* glycation was studied with bovine serum albumin as the model protein. In this study, the plant extracts significantly suppressed protein glycation at concentration of 1 mg/ml. The presence of high phenolics contents and the antioxidant potential as shown in this study could be potent inhibitors of both glycation reaction and the subsequent end products. The plant extract was effective at physiological concentrations and exhibited dose-response relationships. Oxidative stress is known to increase lipid accumulation which promotes generation of reactive oxygen species (Mika et al., 2008). The study revealed inhibitory activity of *S. henningsii* extract on hydrogen peroxide while there was no significant effect on lipid accumulation on 3T3-L1 cell lines using Oil Red O staining method. The scavenging activity against hydrogen peroxide might be responsible for the protection of lipid peroxidation a contributing factor to protein glycation. These results underline the importance of *S. henningsii* extract in diabetes and justify the possibility of therapeutic use of this plant for the prevention of diabetic complications by the traditional healers.

Conclusion

So far, this study has justified the ethnomedicinal usage of *Strychnos henningsii* for the management of diabetes mellitus. The antidiabetic activity of the aqueous bark extract of *S. henningsii* may be attributed to the presence of antioxidant compounds such as tannins, saponins, flavonoids, flavonols and other phytochemical compounds. These compounds have contributed to the antioxidants and free radical scavenging activity of this plant as a means of delaying, lessening and preventing the pathogenesis or secondary complications of diabetes mellitus. The beneficial effect of this plant on the red blood cells and its differentials together with liver and kidney functional indices of diabetic animals has given credence to the

ethnotherapeutic usage of this plant as an antidiabetic plant. Moreso, the significant *in vitro* glucose utilization on the 3T3-L1 adipocytes cell lines as well as protein glycation inhibitory activity also support the antidiabetic potential of *S. henningsii*. The toxicological evaluation of the plant extract did not have a remarkable adverse side effect on both haematological and biochemical parameters except at certain dosages.

Recommendation

The use of animals as a tool is an effective model that allowed determination of relative metabolic factors that play a significant role in disrupting the glycemic homeostasis of man. However, it will be of great importance if analysis of some enzymes, hormones, insulin signaling pathway and pancreatic beta cell mass and insulin secretion was investigated. Further studies on the exact mechanisms of action for the hypoglycemic effect of this plant need to be evaluated to understand its therapeutic target. Similarly, isolation and characterization of compounds responsible for the antidiabetic potentials of *Strychnos henningsii* extract would be of great importance.

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