ASSESSING THE ANTI-HYPERGLYCEMIC AND ANTI-HYPERLIPIDEMIC EFFECTS OF AN AQUEOUS EXTRACT OF *LANNEA EDULIS* IN ALLOXAN-INDUCED DIABETIC RATS

BY

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A dissertation submitted to the University of Zambia in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry

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APPROVAL

This dissertation of Michelo Banda has been approved as partial fulfilment of the requirements for the award of the Degree of Master of Science in Biochemistry by the University of Zambia.

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ABSTRACT

Lannea edulis is a perennial dwarf shrub found in Eastern and Southern African countries such as Tanzania, Uganda, Zambia and South Africa, whose leaves are traditionally used for the treatment of sore eyes, boils and abscesses whilst its roots are used to treat diabetes mellitus, diarrhea, gonorrhea and bilharzia. The objectives of this research were to carry out phytochemical screening, and determine the anti-hyperglycemic and anti-hyperlipidemic effect of an aqueous extract of *L. edulis* in alloxan induced diabetic rats. *L. edulis* was collected in December 2016 from different parts of Zambia and an aqueous extract obtained by using the hot infusion and evaporation method. Phytochemical screening tests were carried out and subsequently toxicity studies were performed to establish the Lethal Dose 50 (LD_{50}) in rats. Alloxan monohydrate was used to induce diabetes in rats. The normal and diabetic rats were placed into 6 groups, 6 animals per group. Group 1 (Normal Control) and Group 2 (Diabetic Control) were administered with distilled water. Group 3 was the Positive Control group and was administered with 5 mg/kg glibenclamide, Groups 4, 5 and 6 were administered 100 mg/kg, 300 mg/kg and 500 mg/kg *L. edulis* doses respectively. All doses were given for 14 days. Blood was drawn from the retro-orbital plexus on days 0 and 14 for determination of lipids and on days 0, 1, 3, 5, 7 and 14 from the rat tail vein for blood glucose. Analysis was carried out using one-way ANOVA followed by Dunnett's multiple comparisons test. Phytochemical screening revealed the presence of flavonoids, saponins, tannins, cardiac glycosides, alkaloids and steroids. The Positive Control, 300 mg/kg and 500 mg/kg *L. edulis* treatment groups showed statistically significant difference ($P<0.0001$) in blood glucose levels compared to the diabetic control group by day 3. In addition, when day 0 mean blood glucose levels were compared to day 3 mean blood glucose levels of their respective groups, the positive control group showed a 15.5 percent drop, the 300 mg/kg *L. edulis* group showed a 23.3 percent drop and the 500 mg/kg *L. edulis* group showed a 52.6 percent drop. The 100 mg/kg treatment group showed statistically significant difference ($P<0.0001$) compared to diabetic control group on day 5; when its day 0 mean blood glucose levels were compared to its day 5 mean blood glucose levels, the 100 mg/kg group showed a 25.1 percent drop. In addition, administration of aqueous extract of *L. edulis* to diabetic rats for 14 days significantly decreased ($P<0.0001$) the levels of serum total cholesterol, triglycerides, Low Density Lipoprotein (LDL) and Very Low Density Lipoprotein (VLDL) whilst increasing the levels of High Density Lipoprotein (HDL), when compared to the diabetic control group. Therefore, the aqueous extract of *L. edulis* showed dose dependent reductions in blood glucose and serum lipid levels in Alloxan induced diabetic rats.

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LIST OF ABBREVIATIONS AND ACRONYMS

DM	Diabetes Mellitus
HDL	High Density Lipoprotein
LDL	Low Density Lipoprotein
VLDL	Very Low Density Lipoprotein
TG	Triglycerides
TC	Total Cholesterol
WHO	World Health Organization
L. edulis	*Lannea edulis*
LD	Lethal Dose
USD	United States Dollar

DEFINITIONS

Traditional medicine- Traditional medicine is the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness (World Health Organisation, 2016).

Complementary/alternative medicine (CAM) - The terms "complementary medicine" or "alternative medicine" are used inter-changeably with traditional medicine in some countries. They refer to a broad set of health care practices that are not part of that country's own tradition and are not integrated into the dominant health care system (World Health Organisation, 2016).

Herbal medicines- Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products, that contain as active ingredients parts of plants, or other plant materials, or combinations (World Health Organisation, 2016).

LD_{50}- (median lethal oral dose) is a statistically derived single dose of a substance that can be expected to cause death in 50 per cent of animals when administered by the oral route. The LD_{50} value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg). (Organisation for Economic Co-Operation and Development, 2001)

DEDICATION

This work is dedicated to

my Mother (Mary Haakola Banda) and my Father (Andrew Banda)

For their unwavering support and helping my ideas become a reality,

My family

For their gentle encouragement

My friends

For being supportive throughout this process

Chapter 1 - **INTRODUCTION**

1.1 Background

According to the International Diabetes Federation - Africa, there are over 14 million people in Africa with Diabetes Mellitus (DM), and this number is predicted to double by the year 2040 (International Diabetes Federation - Africa, 2015). In Zambia alone, 218,200 cases of DM were reported in 2015 (International Diabetes Federation - Africa, 2015). It was also recorded that 8,282 deaths of adults aged 20-79 were attributed to DM (International Diabetes Federation - Africa, 2015).

Diabetes Mellitus is a group of metabolic disorders characterized by hyperglycemia (Dipiro J.T., 2008). It develops as a result of defects in insulin secretion, insulin action or both (Rang H.P., 2007). There are two main types of DM:

i. Type 1 which is characterized by an absolute deficiency of insulin as a result of autoimmune destruction of pancreatic β cells. Type 1 DM accounts for 5 to 10% of all cases of DM and is likely initiated by the exposure of a genetically susceptible individual to an environmental agent (Dipiro J.T., 2008). Without insulin treatment, such patients will ultimately die from diabetic ketoacidosis. Type 1 diabetic patients are usually young (children or adolescents) and not obese when they first develop symptoms (Rang H.P., 2007).

ii. Type 2 is accompanied both by insulin resistance (which precedes overt disease) and by impaired insulin secretion, each of which are important in its pathogenesis. Type 2 DM accounts for as much as 90% of all cases of DM. Such patients are often obese and usually the condition presents in adult life, the incidence rising progressively with age as β-cell function declines. Treatment is initially dietary, although oral hypoglycemic drugs such as glibenclamide usually become necessary, and about one-third of patients ultimately require insulin (Rang H.P., 2007).

For the purposes of this research, diabetes will solely mean hyperglycemia that occurs as a result of destruction of the insulin producing pancreatic beta cells, whilst an anti-diabetic effect will solely mean an anti-hyperglycemic effect.

The chronic increase in blood glucose levels results in damage of many of the body's systems, causing microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular (heart attack, stroke and peripheral vascular disease) complications, whose damage is the leading cause of morbidity and mortality associated with DM (Johansen et al., 2005).

Hyperlipidemia is also a known complication of DM that is characterized by elevated levels of cholesterol, triglycerides and changes in lipoproteins (Rajaei et al., 2015). Diabetes-induced hyperlipidemia is attributable to excess mobilization of fat from the adipose tissue due to the underutilization of glucose (Akpan et al., 2012). Despite this, the existing anti-hyperglycemic agents only allow a sharp control of blood glucose levels but insufficient correction of lipid abnormality, especially in hypertriglyceridemia (Maniyar and Bhixavatimath, 2012). This means that many lipoprotein abnormalities are seen in the diabetic patient despite receiving anti-hyperglycemic treatment.

The last few years have observed a rapid growth in the field of traditional medicine, as it has gained popularity in both developing and developed countries due to their possession of fewer side effects and their natural origin (Modak et al., 2007). Plants have always been a very good source of medicinal properties and many of the currently available drugs have been derived directly or indirectly from them. The ethno-botanical information suggests that about 800 plants may possess anti-diabetic activity (Ponnusamy et al., 2011). They play a particularly important role in diabetic therapy in the developing countries where most individuals have limited resources and access to modern therapy (Ali et al., 2006). Several such herbs have shown anti-hyperglycemic and anti-hyperlipidemic activity when evaluated using different types of experimental techniques. Wide arrays of plant derived active principles have been found to possess anti-hyperglycemic and anti-hyperlipidemic activity among which include polyphenols such as flavonoids and tannins, alkaloids, glycosides, saponins, and terpenoids (Grover et al., 2002, Mannan et al., 2014, Ojiako et al., 2013).

Control of diabetes is particularly important in the early stages of disease as changes are reversible (Savickiene et al., 2002). In order to decrease the number of diabetes complications and to postpone their development, the use of biologically active components and plants is recommended. The most important biologically active substances for this purpose include phytochemicals such as saponins and flavonoids (Savickiene et al., 2002).

1.2 Statement of the Problem

The International Diabetes Federation- Africa (2015) reported that there were 8,232 diabetes-related deaths in Zambia. This could be attributed to many factors: Zambia is a developing country that faces challenges such as limited access to health care for its citizens especially in remote areas; as well as erratic supply or non-availability of anti-hyperglycemic drugs. This has potentially fatal consequences for its resource constrained citizens, as government health facilities could be their only source of anti-hyperglycemic drugs. In addition, anti-hyperglycemic medicine such as insulin is quite expensive for an average Zambian citizen. So, when a diabetic patient gets intermittent treatment of diabetes due to any of the factors listed above, they are more prone to chronic hyperglycemia and its related complications such as hyperlipidemia, oxidative damage to body cells, stroke and neuropathies that eventually lead to death.

Worldwide, the prevalence of DM has increased to 415 million recorded in 2015 alone (International Diabetes Federation - Africa, 2015). As a result, the demand for herbal medicine and its services in several countries has increased (World Health Organisation, 2014). Many countries have recognized the need to develop a cohesive and integrative approach to health care that allows governments, health care practitioners and, most importantly, those who use health care services, to access herbal medicine in a safe, respectful, cost-efficient and effective manner (World Health Organisation, 2014). So most governments have responded by implementing policies and action plans that support the use of traditional medicines either as the main stay of therapy or as complementary medicine (World Health Organisation, 2014). China, India, Nigeria, and the United States of America have all made substantial research investments in traditional herbal medicines (World Health

Organisation, 2014). In support, WHO has updated the WHO Traditional Medicine Strategy from 2002-2005 to 2014-2023, one of whose key goals is to support member states in harnessing the potential contribution of Traditional Medicines to health, wellness and people- centered health care.

Unfortunately, Zambia, despite its many limitations in providing treatment to diabetic patients, has not been as proactive as other countries in their quest to discover traditional medicines that are active against diabetes, neither has it implemented policies that support the use of traditional medicines. Such a project has many benefits such as increasing the treatment options a patient has and prevent diabetes related deaths especially for the resource constrained and those without access to hospitals.

1.3 Justification of the Study

For the year of 2015, the estimated cost of treatment of diabetes per person was calculated to be USD 186.60 (International Diabetes Federation - Africa, 2015). Also, the Central Statistics Office in Zambia reported that the proportion of the population living below the poverty line was 54.4 percent and that poverty still remains predominantly a rural phenomenon with poverty levels at 76.6 percent compared to 23.4 percent in urban areas (Central Statistics Office., 2015). What this means is that most citizens especially in the rural or remote areas are unable to afford anti-diabetes medication in the absence of their provision by government health facilities. The discovery and use of traditional medicine that can be collected and used throughout the year would be very beneficial to these individuals and would reduce the cases of diabetes related deaths.

Lannea edulis (Local names; *Muumbu* (Tonga), *Bukukute* (Kaonde), *Mlamba* (Nyanja)) has been used traditionally for the treatment of DM in South Africa (Deutschländer et al., 2009). However, no laboratory studies have been performed in order to validate its use. The purpose of this study is to determine if *L. edulis* possesses anti-hyperglycemic and anti-hyperlipidemic properties, and will investigate chemical components of *L. edulis* that may cause these effects in an effort

to validate its use. In addition, it will promote further discovery into the field of traditional medicine and hopefully promote inclusion of traditional therapy in Zambian policies either as mainstay therapy or complementary therapy.

1.3.1 Research Question

Is there a difference in mean blood glucose levels and mean serum lipid levels in albino rats that receive an aqueous extract of *L. edulis* and those that do not?

1.3.2 Main objective

The main objective of the research work was to demonstrate experimentally, the effect of administration of an aqueous leaf extract of *L. edulis* on blood glucose levels and serum lipid levels in alloxan-induced diabetic rats.

1.3.3 Specific Objectives

(i) To determine the phytochemical composition of the leaves of *L. edulis*

(ii) To determine the anti-hyperglycemic effect of an aqueous leaf extract of *L. edulis* in alloxan-induced diabetic rats

(iii) To determine the anti-hyperlipidemic effect of an aqueous leaf extract of *L. edulis* in alloxan induced diabetic rats

Chapter 2 - **LITERATURE REVIEW**

2.1 *Lannea edulis* Description, Geographical Distribution and Traditional Uses

L. edulis is a deciduous perennial plant to dwarf shrub that grows from 3 – 60 cm tall with most of the mass of being buried in the soil. The subterranean trunks, that can be 13 cm in diameter, creep along just beneath the surface. The branchlets bearing the leaves, flowers, and fruits stick up only slightly above the ground. It is only found in Africa, particularly in Zambia, Zimbabwe, South Africa, Ethiopia, Democratic Republic of Congo, Uganda, Kenya, Rwanda, Burundi, Tanzania, Mozambique, Malawi, Angola, and Botswana (Fern, 2014).

In Zimbabwe, *L. edulis* root extract is taken as a treatment for bilharzia (schistosomiasis), diarrhea (Chigora P, 2007) and gonorrhea (Maroyi, 2011). The fruit is supposed to be a cure for black water fever (Fern, 2014). Its leaf poultices or leaf infusions are sometimes applied externally to treat sore eyes, boils, and abscesses (Van Wyk B-E, 2005). Herbalists and traditional healers in Venda of Limpopo province in South Africa use *L. edulis* traditionally to treat diabetes (Deutschländer et al., 2009). A thorough search of literature has revealed that no laboratory studies have been performed to validate its use traditionally for the treatment of diabetes.

2.2 Medicinal Plants as a Source of Anti-Hyperglycemic and Anti-Hyperlipidemic Molecules

Medicinal plants are the main source of medicinally important compounds such as polyphenols, saponins, flavonoids, tannins, alkaloids, and steroids. These compounds represent a source for the discovery and development of new types of anti-hyperglycemic molecules (Firdous, 2014). Many compounds isolated from plant sources have been reported to show anti-hyperglycemic and anti-hyperlipidemic activity:

i. *Ocimum gratissimum* leaves are popularly used to treat diabetes mellitus and the phenolic substance Chicoric acid has been identified as the hypoglycemic agent (Casanova et al., 2014).
ii. Cinnamaldehyde, a phytoconstituent extract, has been reported to exhibit significant anti-hyperglycemic effect resulting in the lowering of both total cholesterol and triglyceride levels and, at the same time, increasing HDL cholesterol in Streptozocin-induced diabetic rats (Mannan et al., 2014).
iii. Saponins, tannins and alkaloids in *Melanthera scandens* have been implicated in the demonstration of significant anti-diabetic and hypolipidemic activities in alloxan-induced diabetic rats. They decreased serum total cholesterol, triglycerides, LDL and VLDL with elevations in HDL levels (Akpan et al., 2012).
iv. Literature reports reveal that flavonoids and tannins present in *Eriobotrya Japonica* possess anti-hyperglycemic and anti-hyperlipidemic activity. Ethanolic extract of *E. Japonica* reduced total cholesterol, serum triglycerides, LDL-cholesterol and elevated serum HDL-cholesterol (Shafi and Tabassum, 2013).
v. The anti-hyperlipidemic effect of Total Saponin extract of Kuding Tea was investigated in high-fat diet-induced hyperlipidemic mice. Fifty two saponins were identified or characterized in Total Saponin extract and results revealed that the increased levels of mice serum Total Cholesterol, LDL-Cholesterol, HDL-Cholesterol, and atherogenic index (AI) were significantly reduced after the treatment of Total Saponin extract (Song et al., 2016).

2.3 Phytochemicals in *L. edulis*

In South Africa, *L. edulis'* flavonoids and tannins have been reported to have antimicrobial activity (Van Wyk B-E, 2009). The bark of *L. edulis* has been found to be rich in phenolic compounds and tannins (Van Wyk B-E, 2005). Antidiabetic activity of the Anarcardiaceae family to which *L. edulis* belongs has also been documented (Trease and Evans, 2002).

Activity-guided isolation of radical-scavenging compounds from the dichloromethane extract of the root bark of *L. edulis* led to isolation of two known bioactive alkylphenols, 3-[14'-nonadecenyl]-phenol (cardonol 7) and 3-[16′-heptadecenyl]phenol (cardonol 13), and three new dihydroalkylhexenones were also isolated (Queiroz F.E., 2003). The role of antioxidants or radical scavengers in plant extracts in providing a protective mechanism against reactive oxygen species (ROS) associated with chronic hyperglycemia and diabetic complications has been extensively reviewed by many (Joseph et al., 2013, Tripathi and Chandra, 2010). The presence of phenolic antioxidants in the leaf extract of *L. edulis* could explain its use in the treatment of diabetes.

2.4 Alloxan monohydrate and diabetes

One of the most potent methods to induce experimental diabetes mellitus is chemical induction by Alloxan monohydrate (Rohilla A., 2012). Alloxan monohydrate (2,4,5,6-tetraoxypyrimidine;2,4,5,6-pyrimidinetetrone) induces Type 1 diabetes in experimental animals by destroying the insulin-producing beta cells of the pancreas (Lenzen and Panten, 1988). In vitro studies have shown that alloxan is selectively toxic to pancreatic beta cells, leading to induction of cell necrosis (Jorns et al., 1997). The use of lower dose alloxan (120 mg/kg b.w.) produces partial destruction of pancreatic beta cells even though the animals became permanently diabetic (Stanely et al., 2000).

Chapter 3 - **MATERIALS AND METHODOLOGY**

3.1 Study Design

The study was an experimental randomized controlled study. The aqueous leaf extract of *L. edulis* was administered to albino rats for 14 days and the effect was monitored.

3.2 Study Setting and Population

Healthy male albino rats weighing between 140-200 g body weight, with no previous drug treatment, were acquired from the Animal House, Department of Biological Sciences in the School of Natural Sciences at the University of Zambia. The rats were taken to the Animal House at the School of Veterinary Medicine, University of Zambia and allowed to acclimatize for a period of 7 days. They were maintained under standard animal house conditions (temperature: 26–30 °C, photoperiod: approximately 12 h natural light per day and relative humidity: 55–60 %) with continuous access to pelleted food and water. Research was conducted in accordance with the internationally accepted principles for care and use of laboratory animals (National Research Council (United States) Committee., 2011).

3.3 Sample Size Calculation

The sample size was calculated using G*Power version 3.1.9.2. Under test family, "F test" was selected, and the statistical test selected was "ANOVA: Fixed effects, omnibus, one-way". Sample size was then computed as a function of power level ($1 - \beta$), a pre-specified significance level (α) and the population effect size to be detected with probability. The power level ($1 - \beta$) was set at 0.95, significance level α at 0.05 and pre-detected effect size at 0.717, and 3 groups. Calculation using G*Power gave a sample size of 36. So a total of 36 experimental animals were selected and put into 6 treatment groups with 6 animals each.

3.4 Data Collection Techniques and Tools

3.4.1 Materials

The materials that were used in this research included: Mission® Cholesterol Kit (Acon Laboratories, Inc. San Diego, USA), Accu-Check glucometer kit (Roche Diagnostics, Germany), examination gloves, ethanol, Alloxan monohydrate (Sigma

Aldrich, Germany), weighing scale, measuring cylinders, electric heaters, Phillips Blender, MN615 150 mm filter paper, permanent marker, and feeding tubes. Albino rats were obtained from the School of Natural Sciences, Biological Sciences Department Animal House at the University of Zambia. The leaves of *L. edulis* were collected from three provinces of Zambia; Lusaka Province, Central Province and Copperbelt Province, in December 2016.

3.4.2 Quality Control

To ensure reliability of results, quality control was performed on the Mission Cholesterol Meter and Accu-Check glucometer according to instructions before each test analysis.

3.4.3 Identification of *L. edulis* and Preparation of its Extract

For the purpose of the taxonomical identification and authentication, a whole plant of *L. edulis* was collected from Ndola, Copperbelt Province, Zambia. The plant had fruits and was identified by Mrs Nyirenda, a botanist from University of Zambia.

The leaves of *L. edulis* were washed completely with water to remove surface contaminants and air dried in the shade for 3 days. They were blended using a Phillips Cucina blender to homogeineity. 150 g of the blended leaves were then placed in 500 ml of hot water and allowed to sit for 30 minutes. Two more extractions were carried out with 300 ml of hot water per extraction. The water was then filtered using MN615 150 mm filter paper. The filtrate was evaporated to dryness on a hot plate and a sticky brown residue was obtained. The residue was weighed, stored in air and water proof containers, and then kept at 4°C. From this stock, a fresh preparation was made whenever required.

The percentage yield was calculated using this formula:

$$\frac{W_2 - W_1}{W_0} \times 100$$

Where: W_2 is the weight of the extract and the container,

W_1 is the weight of the container alone and

W_0 is the weight of the initial dried sample.

3.4.4 Phytochemical screening

In order to determine the presence of saponins, tannins, flavonoids and other phytochemicals, a preliminary phytochemical study (color reactions) with various plant extracts was performed using the procedures outlined in Trease and Evans (Trease and Evans, 2002) and Sofowora (Sofowora A., 1993).

Test for Tannins

Two ml of aqueous extract was shaken vigorously with 2 ml distilled water and few drops of ferric chloride ($FeCl_3$) solution were added. The formation of a green precipitate was an indication of the presence of tannins (Trease and Evans, 2002).

Test for Saponins

Five ml of aqueous extract was shaken vigorously with 5 ml of distilled water in a test tube and warmed. The formation of a stable foam was taken as an indication for the presence of saponins (Sofowora A., 1993).

Test for flavonoids (Shinoda reagent test)

One to two fragments of metallic Magnesium were mixed with 3-4 ml extract, then 0.5 ml concentrated hydrochloric acid was added; after 5 minutes a red color appeared- for the flavonols, orange- for the flavons, red-violaceous-characteristic to flavanones or green- in the case of flavanols.

Borntrager's Test for antraquinones

About 0.2 g of the extract was shaken with 10 ml of benzene and then filtered. 5 ml of 10% ammonia solution was then added to the filtrate and thereafter shaken. Appearance of a pink, red or violet color in the ammoniacal (lower) phase was taken as an indication for the presence of free anthraquinones (Sofowora A., 1993).

Liebermann-Burchard test for steroids

Approximately 4 ml of hydro-alcoholic extract was evaporated in a porcelain capsule, the residue was dissolved in 0.5 ml chloroform then 0.5 ml of acetic anhydride was added. The resulting solution was transferred to a dry test tube,

followed by the addition of 1-2 ml concentrated Sulphuric acid to the test tube bottom; when sterols or triterpenoids are present in solution, a red-brown or violet ring appears after 5-10 minutes at the contact zone between the two liquids as well as a green-bluish or violet upper layer (Sofowora A., 1993).

Test for alkaloids

Three ml of aqueous extract was stirred with 3 ml of 1% HCl on a steam bath. Mayers reagent was then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids. (Sofowora A., 1993)

Test for cardiac glycosides (Keller-killani test)

Two ml of the extract was dissolved in 2 ml of glacial acetic acid containing one drop of $FeCl_3$ solution. The mixture was poured into a test tube containing 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxy sugar characteristic of cardenolides.

3.4.5 Karber method for determination of LD_{50}

In this method (Muhammad A., 2015), seven groups of 5 albino rats each weighing 140-200 g was treated orally once, with different doses of *L. edulis* crude aqueous extract (10, 100, 300, 2000, 5000 and 6000 mg/kg). A hamster dose that was found to be safe was used as a reference (Ndamba et al., 1994)

Table 3-1: Doses used in determination of LD50

Group	Dose	Ratio
1	10 mg/kg	10 times less the safe hamster dose
2	100 mg/kg	Safe hamster dose
3	300 mg/kg	3 times the safe hamster dose
4	2000 mg/kg	20 times the safe hamster dose
5	5000 mg/kg	50 times the safe hamster dose
6	6000 mg/kg	60 times the safe hamster dose
7	Distilled water	Control group

All animals were weighed before *L. edulis* administration and kept under observation (once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours) after the oral administration of the *L. edulis* dose for any change in behavior, weight or physical activities and for symptoms of toxicity and rate of mortality in each group. For signs of toxicity, the Guidance Document On The Recognition, Assessment, And Use Of Clinical Signs As Humane Endpoints For Experimental Animals Used In Safety Evaluation (Organisation for Economic Co-Operation and Development., 2000), was followed to determine toxicity, with the help of the Hodge and Sterner Scale.

At the end of the study period, dead animals were to be counted for the calculation of LD_{50}. The arithmetic method of Karber was used for the determination of LD_{50} (Muhammad A., 2015)

$$LD_{50} = LD_{100} - \sum (a \times b)/n$$

n = total number of animals in a group

a = the difference between two successive doses of administered extract

b= the average number of dead animals in two successive groups

LD_{100} = lethal dose causing the death of 100 % of all test animals

After 24 hours, all surviving mice were euthanized using Diethyl ether for ethical reasons (American Veterinary Medical Association, 2013).

3.4.6 Anti-diabetic activity in alloxan induced diabetes model

Alloxan monohydrate was used to cause pharmacological diabetes to confirm the effectiveness of active anti-hyperglycemic extract in experimental diabetic conditions. Diabetes was induced in 80 male albino rats by injecting 120 mg/kg of Alloxan monohydrate intra-peritoneally dissolved in 0.9 percent w/v cold Normal Saline to overnight-fasted rats (12 hours). The rats were afterwards kept for the next 24 hours on 10 percent glucose solution bottles, in their cages, to prevent hypoglycemia. After 72 hours of injection, fasting blood glucose level was measured. The animals that did not develop more than 200 mg/dl glucose levels were omitted from the study. Thirty six (36) selected diabetic and non-diabetic animals were divided into six groups (n = 6). Group 1 served as normal control and was treated

with 1 ml distilled water orally fed using a feeding tube. Group 2 had alloxan induced diabetes and received 1 ml of distilled water every morning; Group 3 had alloxan induced diabetes and was treated with glibenclamide (5 mg/kg/day) as the reference drug every morning. Groups 4, 5 and 6 had alloxan induced diabetes and were treated with 3 non-toxic doses of *L. edulis* crude aqueous extract lower than the LD_{50}. The animals were given the extracts once every morning by compulsory oral intubations before meals. The treatment was continued for 14 consecutive days.

The scheme below shows the arrangement of the rats in groups:

Table 3-2: Treatment groups and doses administered

Group	Treatment group	Dosage
1	Normal control (Non-diabetic)	Distilled water
2	Alloxan-induced diabetic rats (Diabetic control)	Distilled water
3	Alloxan-induced diabetic rats (Positive control)	5 mg/kg glibenclamide
4	Alloxan-induced diabetic rats	Dose 1
5	Alloxan-induced diabetic rats	Dose 2
6	Alloxan-induced diabetic rats	Dose 3

Key: Dose 1- 100mg/kg *L. edulis*, Dose 2- 300mg/kg *L. edulis*, Dose 3- 500mg/kg *L. edulis*.

3.4.7 Collection of Blood Samples for Glucose Analysis

Blood samples for glucose analysis were collected from the tail vein of the rats after 3 days of administration of Alloxan (day 0) and subsequently on days 1, 3, 5, 7 and 14. The animals were held by their heads and their tails dipped in warm water. The tail tips were then cut with sharp scissors. The first blood drop was wiped away but the second was dropped onto a glucose strip in the Accu-Chek glucometer to get a glucose reading. The tails were then rubbed with ethanol to prevent infection.

3.4.8 Biochemical Parameters

On days 0 and 14, blood samples for serum lipid levels were collected from overnight fasted rats under diethyl ether anesthesia by retro orbital plexus puncture method and were kept aside for 30 minutes for clotting. The serum was separated by centrifuging the sample at 2500 rounds per minute, for 10 minutes at 25°C. It was subsequently analyzed for TG, HDL and TC using the Mission Cholesterol Meter.

Very low density lipoprotein (VLDL) was calculated as Triglycerides (TG); TG/5, and LDL was estimated using the Friedewald formula (Friedewald W. T. et al., 1972) as follows:

$$LDL (mg/dl) = TC - (HDL + VLDL)$$

3.4.9 Data Processing and Analysis

All the values were expressed as mean ± Standard Error of the Mean (SEM). The results were analyzed for statistical significance using one-way ANOVA followed by Dunnett's multiple comparisons test. $P<0.05$ was considered significant.

3.5 Ethical Considerations

Ethical approval was sought from the University of Zambia Biomedical Research Ethics Committee. The experimental protocol was followed according to Guidelines for Care and Use of Laboratory Animals in Biomedical Research (National Research Council (United States) Committee., 2011), some of which include;

i. All animals were observed for signs of illness, injury, or abnormal behavior by a person trained to recognize such signs, especially during the determination of LD_{50}.
ii. The researcher and technical assistants conducting surgical procedures were appropriately trained to ensure that good surgical techniques were practiced, that is, asepsis, appropriate use of instruments, obtaining blood from the retro-orbital plexus, and effective hemostasis.
iii. Aseptic technique was used to reduce microbial contamination to the lowest possible practical level, hair removal and disinfection of the operative site, provision of appropriate surgical attire, sterile surgical gloves, sterilization of instruments and supplies, and the use of operative technique to reduce the likelihood of infection.
iv. After recovery from anesthesia, monitoring included attention to basic biologic functions of intake and elimination and to behavioral signs of postoperative pain, monitoring for postsurgical infections, monitoring of the surgical incision site for dehiscence and bandaging as appropriate.

In addition, each animal was used only once and all surviving rats were euthanized with Diethyl ether at the end of the study according to the American Veterinary Medicine Association guidelines (American Veterinary Medical Association, 2013).

Chapter 4 - **RESULTS**

4.1 Plant Identification

L. edulis identification and authentication was done by Mrs Nyirenda, a botanist from the University of Zambia, School of Natural Sciences, Department of Biological Sciences, and deposited in the herbarium with accession number 22,064.

4.2 Percentage Yield of *L. edulis*

The percentage yield of *L. edulis* was calculated and found to be 17.2 %

4.3 Qualitative Phytochemical Screening

The leaves of *L. edulis* were phytochemically screened for tannins, flavonoids, saponins, anthraquinones, steroids, alkaloids and cardiac glycosides and the leaves were positive for these phytochemicals except anthraquinones (Table 4-1, Figure 4-1).

Table 4-1: Results of Phytochemical Screening

Phytochemicals	*L. edulis*
Tannins	+
Flavonoids	+
Saponins	+
Anthraquinones	–
Steroids	+
Alkaloids	+
Cardiac glycosides	+

Key: Present phytochemicals are denoted by (+) sign, absent phytochemicals are denoted by (-) sign.

The presence or absence of phytochemicals was determined by color reactions from procedures outlined in Trease and Evans (Trease and Evans, 2002) and Sofowora (Sofowora A., 1993). The solution in test tube 1 remained colorless indicating the

absence of anthraquinones (Figure 4-1). Steroids were present in test tube 2 as the solution turned red-brown (Figure 4-1). The presence of cardiac glycosides in test tube 3 was indicated by the appearance of a brown ring at the interface of the two liquids (Figure 4-1). The turbidity of the solution in test tube 4 indicated the presence of alkaloids whilst the appearance of the red color in test tube 5 indicated the presence of flavonoids (Figure 4-1). The stable foam in test tube 6 indicated the presence of saponins and the dark green precipitate in test tube 7 indicated the presence of tannins (Figure 4-1).

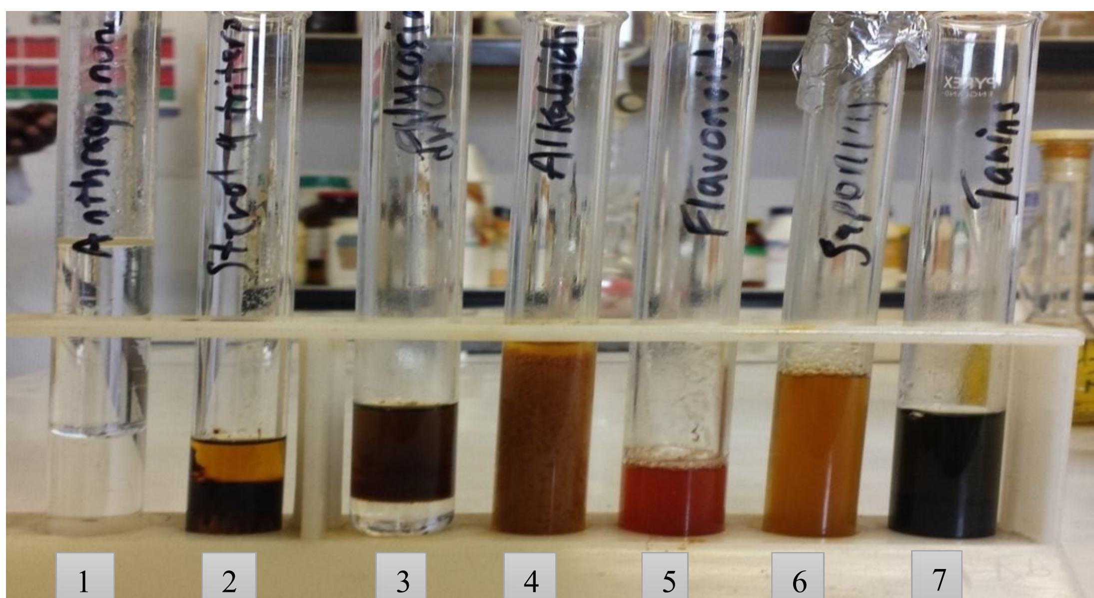

Figure 4-1: Results of Phytochemical Screening of *L. edulis* crude aqueous extract. 1- Test for anthraquinones, 2- Test for steroids, 3- Test for cardiac glycosides, 4- Test for alkaloids. 5- Test for flavonoids, 6- Test for saponins, 7- Test for tannins.

4.4 LD_{50} Results

The LD_{50} of *L. edulis* was tested up to a dose of 6000 mg/kg and none of the doses caused death or caused rats to exhibit symptoms of toxicity (Table 4-2). The LD_{50} value of *L. edulis* was therefore found to be greater than 6000 mg/kg and according to the Hodge and Sterner scale (Table A-1) falls in the practically nontoxic range.

Table 4-2: *L. edulis* LD_{50} results

Group	Dose/day	Mortality (x/N)	Symptoms
1	10 mg/kg	0/5	Nil
2	100 mg/kg	0/5	Nil
3	300 mg/kg	0/5	Nil
4	2000 mg/kg	0/5	Nil
5	5000 mg/kg	0/5	Nil
6	6000 mg/kg	0/5	Nil
7	Normal Saline	0/5	Nil

4.5 Antidiabetic Effect in Alloxan Induced Models

The LD_{50} of *L. edulis* was found to be greater than 6000 mg/kg so dose selection was done below it. Generally, the effective dose 50 (ED_{50}) is considered 10 times less than the LD_{50} (Belhekar et al., 2013), and since one of the tested doses was 5000 mg/kg, a tenth of that dose (500 mg/kg) was chosen as the highest dose. Other doses included 100 mg/kg and 300 mg/kg of *L. edulis*.

All treatment groups had normal blood glucose levels before the induction of diabetes and there were no statistically significant differences between experimental groups and the diabetic control group (Figure 4-2). Mean blood glucose levels after induction of diabetes (day 0 blood glucose levels) of the positive control, diabetic control, and *L. edulis* treatment groups rose to above 200 mg/dl (Figure 4-2). There were still no statistically significant differences between the positive control, *L. edulis* treatment groups compared to the diabetic control group on day 0 (Figure 4-2). The daily dose of *L. edulis* on blood glucose levels of alloxan induced diabetic rats resulted in significant reductions in blood glucose levels (Figure 4-2). One-way ANOVA showed that there was a statistically significant difference ($P<0.0001$) between groups on day 3 (Table 4-3). Further analysis using Dunnetts multiple comparisons test showed that the Positive Control, 300 mg/kg and 500 mg/kg *L. edulis* treatment groups had statistically significant differences ($P<0.0001$) in mean blood glucose levels when compared to the diabetic control group on day 3 (Figure

4-2 and Table 4-4). One-way ANOVA conducted on day 5 also showed statistically significant difference between groups ($P<0.0001$) (Table 4-5). Further analysis using Dunnetts multiple comparisons test also had the 100 mg/kg *L. edulis* treatment group have statistically significant difference ($P<0.0001$) compared to diabetic control group on day 5 (Table 4-6). Administration of the 300 mg/kg and 500 mg/kg *L. edulis* doses resulted in the attainment of normal blood glucose levels by day 5 whilst the 100 mg/kg *L. edulis* dose only achieved this by day 14 (Figure 4-2). Administration of glibenclamide 5 mg/kg (positive control group) also resulted in attainment of normal blood glucose levels by day 5. After induction of diabetes, the diabetic control group had mean blood glucose levels that continued to rise throughout the treatment period and went above 500 mg/dl by day 14 whilst the normal control group had blood glucose levels that remained in the normal range throughout the treatment period.

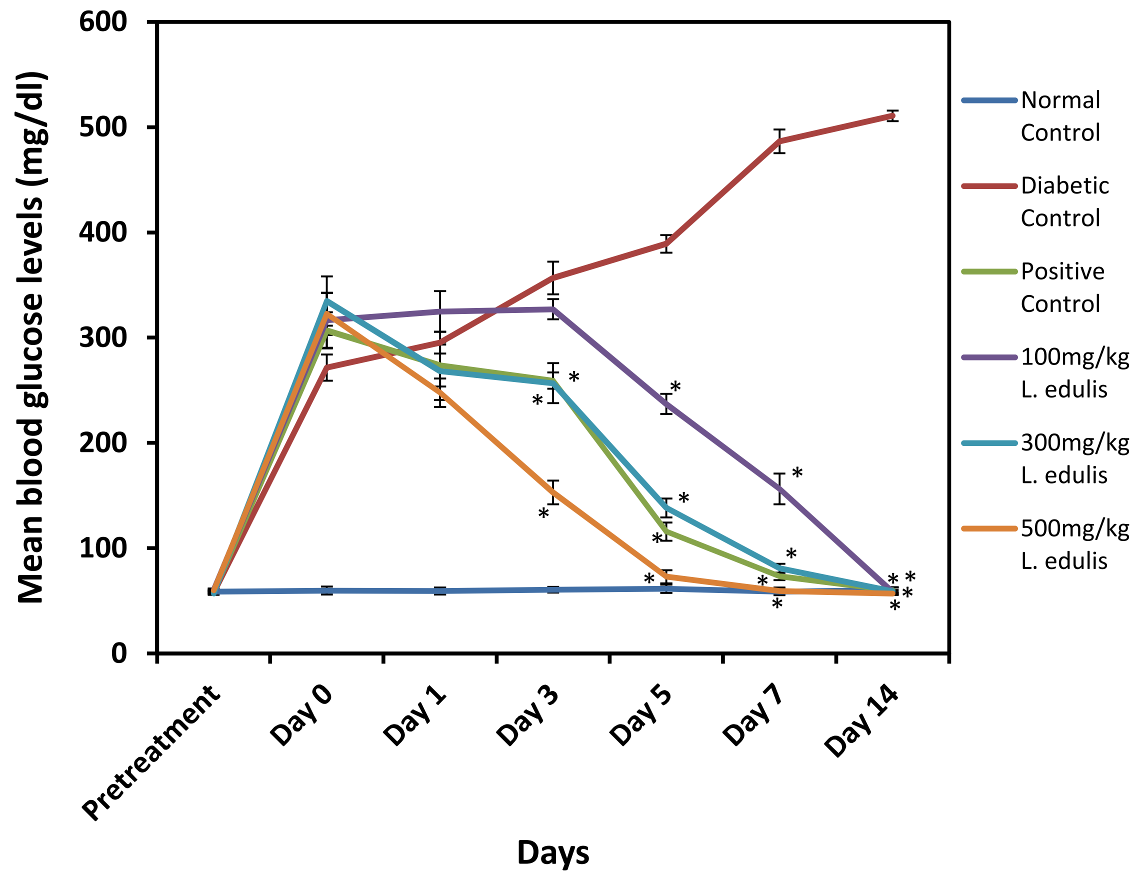

Figure 4-2: Antidiabetic effect of aqueous extract of *L. edulis* on blood glucose levels of alloxan-induced diabetic rats for 14 days treatment. Data is expressed as Means ± SEM.*P<0.0001 vs diabetic control group

Table 4-3: One-way ANOVA of day 3 mean blood glucose levels

ANOVA			
Day 3 mean blood glucose levels			
	Sum of Squares	Mean Square	Sig.
Between Groups	148488.461	37122.115	<0.0001

Table 4-4: Dunnetts multiple comparison test of mean blood glucose levels on day 3

Multiple Comparisons					
Day 3 mean blood glucose levels Dunnett t (<control)					
(I) Treatment Group	(J) Treatment Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Upper Bound
Positive Control	Diabetic Control	-97.500*	18.8046	<0.0001	-54.733
100mg/kg	Diabetic Control	-29.667	18.8046	0.174	13.101
300 mg/kg	Diabetic Control	-99.900*	18.8046	<0.0001	-57.133
500mg/kg	Diabetic Control	-203.700*	18.8046	<0.0001	-160.933
* The mean difference is significant at the .05 level.					

Table 4-5: One-way ANOVA of day 5 mean blood glucose levels

ANOVA			
Day 5 mean blood glucose levels			
	Sum of Squares	Mean Square	Sig.
Between groups	382451.525	95612.881	<0.0001

Table 4-6: Dunnetts multiple comparisons test of mean blood glucose levels on day 5

Multiple Comparisons					
Day 5 mean blood glucose levels Dunnett t (<control)					
(I) Treatment Group	(J) Treatment Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Upper Bound
Positive Control	Diabetic Control	-273.300*	11.9815	<0.0001	-246.050
100mg/kg	Diabetic Control	-152.067*	11.9815	<0.0001	-124.817
300 mg/kg	Diabetic Control	-250.800*	11.9815	<0.0001	-223.550
500mg/kg	Diabetic Control	-316.200*	11.9815	<0.0001	-288.950
* The mean difference is significant at the .05 level.					

When day 3 mean blood glucose levels were compared to their respective day 0 mean blood glucose levels, the positive control group showed a 15.5 percent drop, the 300 mg/kg *L. edulis* group showed a 23.3 percent drop and the 500 mg/kg *L. edulis* group showed a 52.6 percent drop (Table 4-7).

Table 4-7: Percent reductions in mean blood glucose levels

Treatment group	Day 0 mean blood glucose levels (mg/dl)	Day 3 mean blood glucose levels (mg/dl)	% Reduction
Positive Control	306.9 ± 17.2	259.2 ±7.7	15.5
300 mg/kg *L. edulis*	334.8 ± 23.5	256.8 ±19.1	23.3
500 mg/kg *L. edulis*	322.5 ±19.7	153.0 ± 11.2	52.6

When day 5 mean blood glucose levels were compared to day 0 mean blood glucose levels, the 100 mg/kg *L. edulis* group showed a 25.1 percent drop (Table 4-10).

Table 4-8: Percent reduction in mean blood glucose levels

Treatment group	Day 0 mean blood glucose levels (mg/dl)	Day 5 mean blood glucose levels (mg/dl)	% Reduction
100 mg/kg *L. edulis*	316.5 ± 26.2	237.0 ± 9.7	25.1

Administration of *L. edulis* to alloxan induced diabetic rats for 14 days resulted in an increase in HDL levels compared to the diabetic control (Figure 4-3). One way ANOVA showed that there was a significant difference ($P<0.0001$) in HDL levels between groups on day 14 (Figure 4-3 and Table 4-9). Further analysis using Dunnetts multiple comparisons test showed that the positive control, 300 and 500 mg/kg *L. edulis* treatment groups showed statistical significance of $P<0.0001$ compared to the diabetic control group whereas the 100 mg/kg *L. eduis* dose had $P = 0.017$ (Table 4-10). By the 14th day of treatment, only the 500mg/kg *L. edulis* dose had raised the HDL levels to normal range (Figure 4-3). The positive control, 100 mg/kg and 300 mg/kg *L. edulis* treatment groups were not able to achieve normal

range HDL levels by the 14th day of treatment (Figure 4-3). The positive control group however improved the HDL levels better than the 300 mg/ kg and 100 mg/kg doses of *L. edulis*. The 300 mg/kg *L. edulis* dose raised HDL levels better than the 100 mg/kg *L. edulis* dose. Therefore the HDL levels were decreased in a dose dependent manner. The diabetic control group had the lowest HDL levels on the 14th day of treatment.

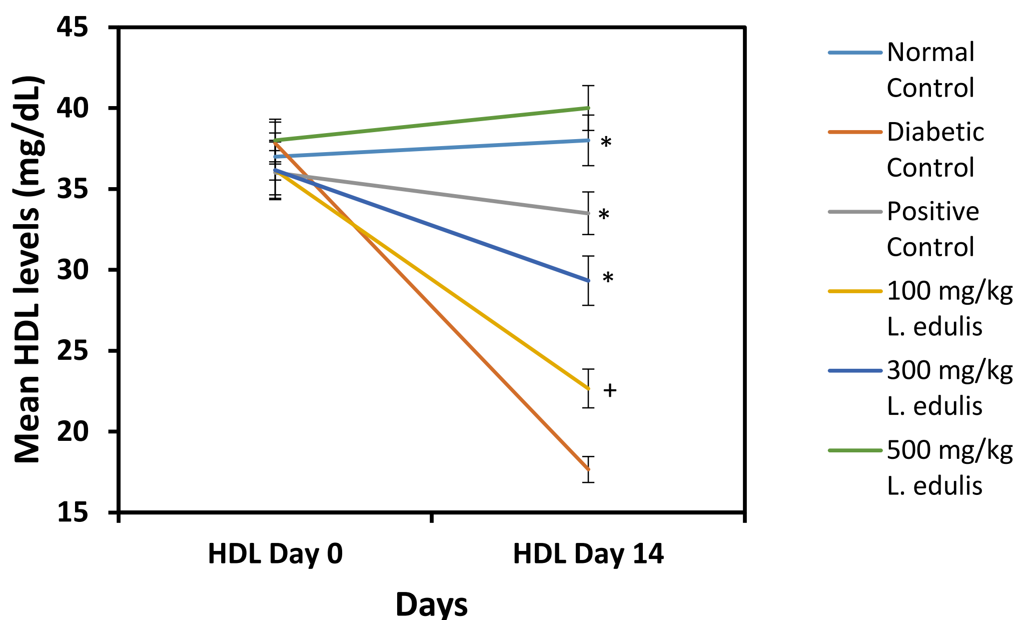

Figure 4-3: Serum HDL levels on day 0 and day 14**.** Data is expressed as Mean ± SEM. HDL-High Density Lipoprotein. *P<0.0001 vs diabetic control $^{+}$P<0.05 vs diabetic control

Table 4-9: One-way ANOVA of mean HDL levels on day 14

ANOVA			
High Density Lipoprotein levels day 14			
	Sum of Squares	Mean Square	Sig.
Between Groups	1855.467	463.867	<0.0001

Table 4-10: Dunnetts multiple comparisons of mean HDL levels on day 14

Multiple Comparisons					
High Density Lipoprotein levels day 14 Dunnett t (>control)					
(I) Treatment Group	(J) Treatment Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound
Positive Control	Diabetic Control	15.833*	1.7944	<0.0001	11.752
100 mg/kg	Diabetic Control	5.000*	1.7944	0.017	.919
300 mg/kg	Diabetic Control	11.667*	1.7944	<0.0001	7.586
500 mg/kg	Diabetic Control	22.333*	1.7944	<0.0001	18.252
*. The mean difference is significant at the .05 level.					

Repeated administration of the different *L. edulis* doses resulted in a dose dependent decrease in TC levels of alloxan induced diabetic rats (Figure 4-4, Table 4-11). TC levels of the *L. edulis* and positive control groups were still in the normal range on day 0 with no statistically significant differences compared to the diabetic control. However, one-way ANOVA carried out on day 14 indicated significant difference (P<0.0001) between groups (Table 4-12). Further analysis using Dunnetts multiple comparisons test showed that all the *L. edulis* treatment groups and the positive control group had significant difference (P<0.0001) compared to the diabetic control by day 14 (Figure 4-4, Table 4-13). In addition, all *L. edulis* doses were able to reduce TC levels to within normal ranges by day 14 (Figure 4-4, Table 4-11). The diabetic control group had TC levels rise above 300 mg/dl by day 14 whilst the normal control had TC levels that were still in the normal range (Figure 4-4, Table 4-11).

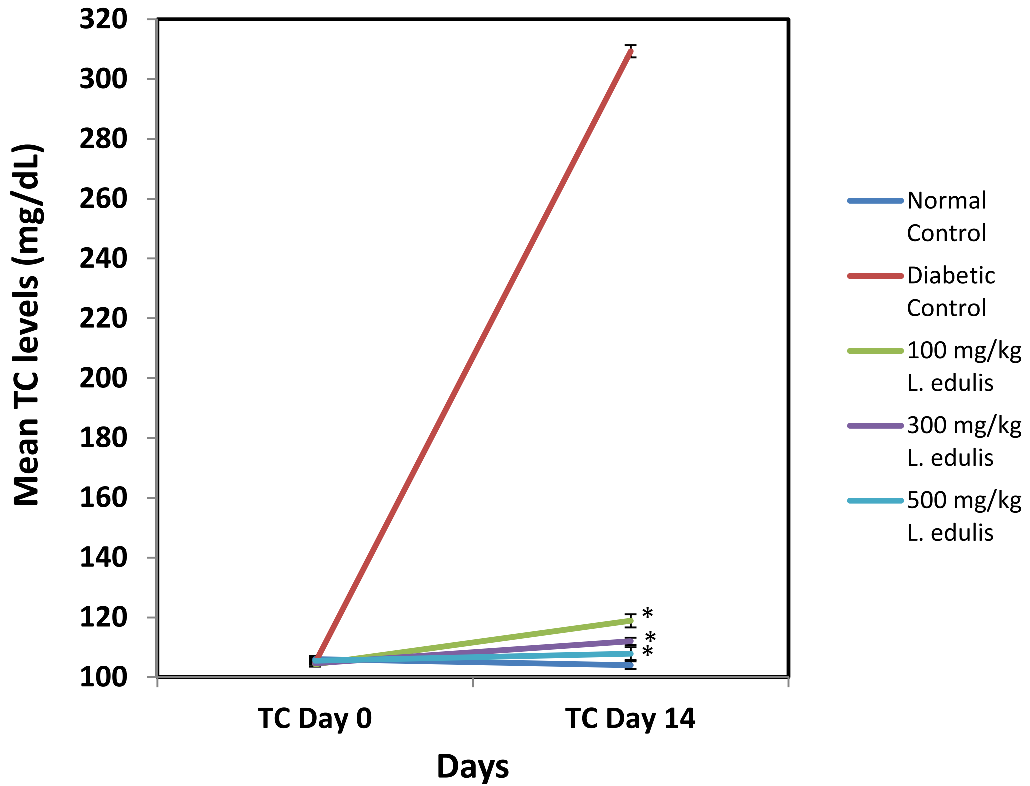

Figure 4-4: Serum Total Cholesterol levels on day 0 and day 14. Data is expressed as Mean ± SEM. TC- Total Cholesterol. *P<0.0001 compared to the diabetic control

Table 4-11: Descriptive Statistics of mean Total Cholesterol levels on day 14

Descriptive Statistics			
Dependent Variable: Total Cholesterol levels day 14			
Treatment Group	Mean	Std. Deviation	N
Normal Control	104	3.0261	6
Diabetic Control	309.333	5.0067	6
Positive Control	108.333	2.5033	6
100 mg/kg	118.833	5.3821	6
300 mg/kg	112.000	2.9665	6
500 mg/kg	107.833	5.2313	6

Table 4-12: One-way ANOVA of mean Total Cholesterol levels on day 14

ANOVA			
Total Cholesterol levels day 14			
	Sum of Squares	Mean Square	Sig.
Between Groups	187851.533	46962.883	<0.0001

Table 4-13: Dunnetts multiple comparisons of mean Total Cholesterol levels on day 14

Multiple Comparisons					
Total Cholesterol levels day 14 Dunnett t (<control)					
(I) Treatment Group	(J) Treatment Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Upper Bound
Positive Control	Diabetic Control	-201.000*	2.5360	<0.0001	-195.232
100 mg/kg	Diabetic Control	-190.500*	2.5360	<0.0001	-184.732
300 mg/kg	Diabetic Control	-197.333*	2.5360	<0.0001	-191.566
500 mg/kg	Diabetic Control	-201.500*	2.5360	<0.0001	-195.732
*. The mean difference is significant at the .05 level.					

The TG levels of alloxan induced diabetic rats were reduced in a dose dependent manner after repeated administration of 100 mg/kg, 300 mg/kg and 500 mg/kg *L. edulis* doses (Figure 4-5, Table 4-14). Analysis of day 14 data using one-way ANOVA showed statistical significance of P<0.0001 (Table 4-15). Further analysis using Dunnetts test showed that the positive control, 100 mg/kg, 300 mg/kg and 500 mg/kg *L. edulis* treatment groups were statistically significant (P<0.0001) from the diabetic control by day 14 (Figure 4-5, Table 4-16). By day 14 of treatment, the positive control, and all *L. edulis* doses reduced TG levels significantly but none were able to reduce them to normal range (Figure 4-5, Table 4-14). The diabetic control had mean TG levels rise over 300 mg/dl by the 14th day of treatment whilst the normal control still had normal TG levels (Table 4-14).

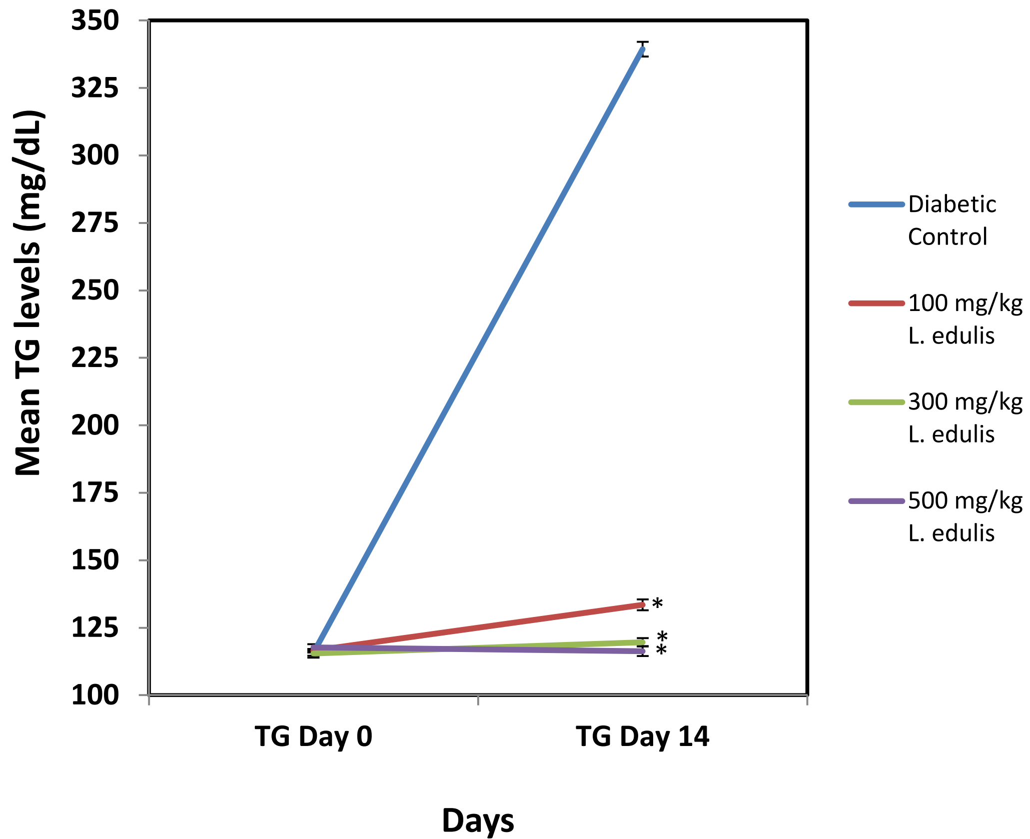

Figure 4-5: Serum Triglyceride levels on day 0 and day 14. Data expressed as Mean +/-SEM. TG- triglyceride. *P<0.0001 vs diabetic control

Table 4-14: Descriptive statistics of mean Triglyceride levels on day 14

Descriptive Statistics			
Dependent Variable: Triglyceride levels day 14			
Treatment Group	Mean	Std. Deviation	N
Normal Control	115.333	2.1945	6
Diabetic Control	339.333	6.7132	6
Positive Control	117.000	3.0332	6
100 mg/kg	133.500	5.0100	6
300 mg/kg	119.667	3.7771	6
500 mg/kg	116.333	4.5019	6

Table 4-15: One-way ANOVA of mean Triglyceride levels on day 14

ANOVA			
Triglyceride levels day 14			
	Sum of Squares	Mean Square	Sig.
Between Groups	228670.667	57167.667	<0.0001

Table 4-16: Dunnetts multiple comparisons of mean Triglyceride levels on day 14

Multiple Comparisons					
Triglyceride levels day 14 Dunnett t (<control)					
(I) Treatment Group	(J) Treatment Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Upper Bound
Positive Control	Diabetic Control	-222.333*	2.7556	<0.0001	-216.066
100mg/kg	Diabetic Control	-205.833*	2.7556	<0.0001	-199.566
300mg/kg	Diabetic Control	-219.667*	2.7556	<0.0001	-213.400
500mg/kg	Diabetic Control	-223.000*	2.7556	<0.0001	-216.733
*. The mean difference is significant at the .05 level.					

VLDL levels of all treatment groups were still in the normal range on day 0 with no statistically significant difference between groups (Figure 4-6). By day 14 the diabetic control group had VLDL levels rise above 60 mg/dl with the normal control group still having normal VLDL levels (Figure 4-6, Table 4-17). VLDL levels of alloxan induced diabetic rats were reduced in a dose dependent manner after 14 days of *L. edulis* administration (Figure 4-6, Table 4-17). One-way ANOVA for day 14 results revealed statistical significance (P<0.0001) between groups (Table 4-18). Dunnetts multiple comparison test showed that the positive control and all *L. edulis* doses had significantly reduced VLDL levels (P<0.0001), but none were able to reduce VLDL levels to normal range (Figure 4-6, Table 4-17, Table 4-19). The 500 mg/kg *L. edulis* dose was more effective at reducing VLDL levels than the 300 mg/kg *L. edulis* dose. Of the 3 *L. edulis* doses, the 100 mg/kg dose was the least effective at reducing VLDL levels (Figure 4-6, Table 4-17).

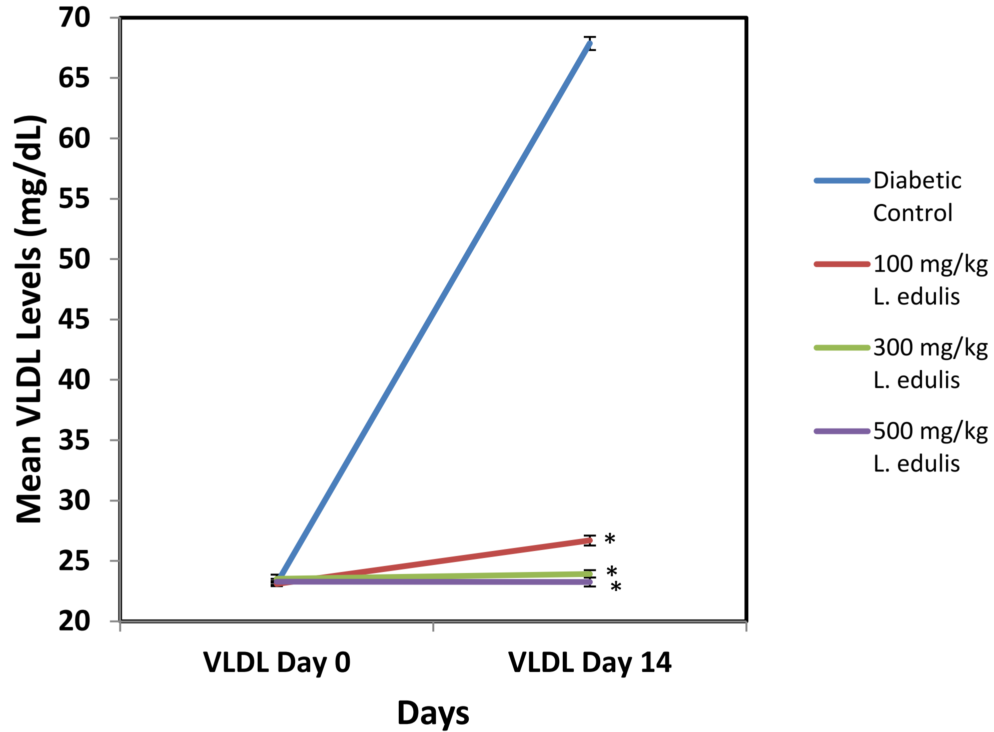

Figure 4-6: Serum VLDL levels on days 0 and 14. Data expressed as Mean +/- SEM. VLDL- Very Low Density Lipoprotein

Table 4-17: Descriptive statistics of mean VLDL levels on day 14

Descriptive Statistics			
Dependent Variable:Very Low Density Lipoproteins day 14			
Treatment Group	Mean	Std. Deviation	N
Normal Control	23.0667	0.65098	6
Diabetic Control	67.8667	1.34263	6
Positive Control	23.4000	.60663	6
100mg/kg	26.7000	1.00200	6
300mg/kg	23.9333	.75542	6
500mg/kg	23.2667	.90037	6

Table 4-18: One-way ANOVA of mean VLDL levels on day 14

ANOVA			
Very Low Density Lipoproteins day 14			
	Sum of Squares	Mean Square	Sig.
Between Groups	9146.827	2286.707	<0.0001

Table 4-19: Dunnetts Multiple comparisons of mean VLDL levels on day 14

Multiple Comparisons					
Very Low Density Lipoproteins day 14 Dunnett t (<control)					
(I) Treatment Group	(J) Treatment Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Upper Bound
Positive Control	Diabetic Control	-44.4667*	.55112	<0.0001	-43.2132
100mg/kg	Diabetic Control	-41.1667*	.55112	<0.0001	-39.9132
300mg/kg	Diabetic Control	-43.9333*	.55112	<0.0001	-42.6799
500mg/kg	Diabetic Control	-44.6000*	.55112	<0.0001	-43.3466
*. The mean difference is significant at the .05 level.					

Administration of *L. edulis* to alloxan induced diabetic rats also resulted in a dose dependent reduction in LDL levels (Figure 4-7, Table 4-20). One-way ANOVA revealed a significant difference (P<0.0001) between groups on day 14 (Table 4-21). Dunnetts multiple comparisons test was then performed and revealed that the positive control, 100 mg/kg, 300 mg/kg and 500 mg/kg *L. edulis* doses had significant difference (P<0.0001) compared to the diabetic control on day 14 (Figure 4-7, Table 4-22). By day 14, the 500 mg/kg *L. edulis* dose appeared to be the most potent in reducing LDL levels of alloxan induced diabetic rats whilst the 100 mg/kg dose appeared to be the least potent (Figure 4-7, Table 4-20). The positive control group appeared more potent than the 300 mg/kg and 100 mg/kg *L. edulis* doses. However all *L. edulis* doses reduced the LDL levels to normal ranges. The diabetic control group had LDL levels rise to above 200 mg/dl by day 14 (Figure 4-7, Table 4-20).

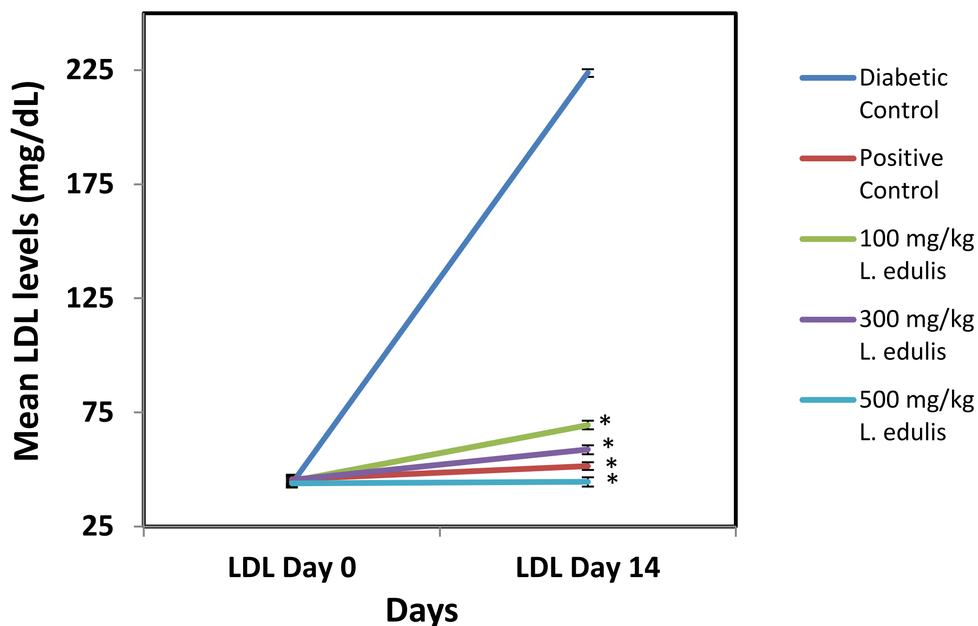

Figure 4-7: Serum LDL levels on days 0 and 14. Data expressed as Mean +/- SEM. LDL-Low Density Lipoprotein

Table 4-20: Descriptive statistics of mean LDL levels on day 14

Descriptive Statistics			
Dependent Variable: Low Density Lipoproteins day 14			
Treatment Group	Mean	Std. Deviation	N
Normal Control	43.7	4.06566	6
Diabetic Control	223.800	3.91101	6
Positive Control	51.4333	4.14423	6
100 mg/kg	69.4667	4.66590	6
300 mg/kg	58.7333	4.41029	6
500 mg/kg	44.5667	4.89803	6

Table 4-21: One-way ANOVA of mean LDL levels on day 14

ANOVA			
Low Density Lipoproteins day 14			
	Sum of Squares	Mean Square	Sig.
Between Groups	137114.627	34278.657	<0.0001

Table 4-22: Dunnetts multiple comparisons of mean LDL levels on day 14

Multiple Comparisons					
Low Density Lipoproteins day 14 Dunnett t (<control)					
(I) Treatment Group	(J) Treatment Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Upper Bound
Positive Control	Diabetic Control	-172.3667*	2.55190	<0.0001	-166.5629
100mg/kg	Diabetic Control	-154.3333*	2.55190	<0.0001	-148.5295
300mg/kg	Diabetic Control	-165.0667*	2.55190	<0.0001	-159.2629
500mg/kg	Diabetic Control	-179.2333*	2.55190	<0.0001	-173.4295
*. The mean difference is significant at the .05 level.					

Chapter 5 - **DISCUSSION**

The first objective of this study was to determine the phytochemicals present in the leaves of *L. edulis.* Phytochemical screening revealed the presence of flavonoids, saponins, tannins, alkaloids, cardiac glycosides and steroids. Several reports have implicated phytochemicals as being responsible for anti-hyperglycemic and anti-hyperlipidemic activities (Kumar et al., 2011a, Kumar et al., 2011b). Plants which have flavonoids, alkaloids, and glycosides have antioxidant activity and are claimed to possess anti-hyperglycemic effect (Petchi et al., 2013). Flavonoids present in some plants regenerate the damaged beta cells of pancreases, whilst the polyphenolic compounds are reported to inhibit alpha (α) amylase and sucrose (Petchi et al., 2013). It has also been reported that isoflavones, tannins and crude saponins have been found to inhibit glucose transport by inhibiting sodium glucose co-transporter-1 (S-GLUT-1) in intestinal brush border cells (Petchi et al., 2013, Tiwari Ak, 2002). Other studies have demonstrated that saponins isolated from different plants produce significant anti-hyperlipidemic effects mainly by suppression of cholesterol luminal absorption and also by increasing cholesterol secretion through biliary excretion (Francis et al., 2002, Ma et al., 2002). Therefore, the phytochemicals present in *L. edulis* could work in such a manner either alone or in synergism to produce anti-hyperglycemic and anti-hyperlipidemic effects.

The last two specific objectives of this research were to determine the anti-hyperglycemic and anti-hyperlipidemic effect of aqueous extract of *L. edulis* in alloxan induced diabetic rats. All selected doses of *L. edulis* showed statistically significant reductions ($P<0.0001$) in mean blood glucose levels, TC, TG, LDL, VLDL as compared to the diabetic control group during the 14 day treatment period. In addition, mean serum HDL levels were statistically increased ($P<0.0001$) in the 300 mg/kg and 500 mg/kg *L. edulis* treatment groups when compared to the diabetic control with $P=0.017$ for the 100 mg/kg *L. edulis* group. The *L. edulis* doses showed dose dependent reductions in blood glucose levels, with the 500 mg/kg dose being the most potent and the 100 mg/kg dose being the least potent. TC and LDL levels for all *L. edulis* treatment groups were also within the normal range on the 14th day of treatment. The 500 mg/kg *L. edulis* treatment group also raised HDL levels to

normal range by the 14th day of treatment. This is the first study that has demonstrated not only the anti-hyperglycemic but also the anti-hyperlipidemic effects of *L. edulis*.

Alloxan is reported to selectively destroy insulin secretory β-cells to impair insulin secretion and function (Lenzen and Panten, 1988). Alloxan induced diabetic rats were observed to have increased blood glucose and lipid levels. Insulin inhibits hormone sensitive lipase which breaks down triglycerides into fatty acids. In the diabetic state, the activity of hormone sensitive lipase increases, thereby increasing lipolysis and discharge of fatty acids into circulation. This in turn promotes the conversion of excess fatty acids into phospholipids and cholesterol in the liver. These two substances along with excess of TG formed in the liver may be discharged into the blood in the form of lipoproteins (Rajaei et al., 2015). Also, under normal circumstances, insulin activates the enzyme lipoprotein lipase, which hydrolyses triglycerides. However, in the diabetic state, lipoprotein lipase is not activated due to insulin deficiency, resulting in hypertriglyceridemia (Pushparaj et al., 2007). Decreased HDL cholesterol levels are associated with defective lipoprotein lipase catabolism of triglyceride rich lipoproteins, as insulin stimulates this enzyme. Insulin deficiency is also associated with hypercholesterolemia because insulin has an inhibitory action on β-hydroxy-β-methylglutaryl-Coenzyme-A (HMG-CoA) reductase, a key rate-limiting enzyme responsible for the metabolism of cholesterol-rich LDL particles. (Murali et al., 2002). Lowering of serum lipid levels with elevation of HDL through dietary or drug therapy seems to be associated with a decrease in the risk of vascular disease and related complications (Rajaei et al., 2015). Sulphonylureas such as glibenclamide mediate anti-hyperglycaemic activity by stimulating insulin release from pancreatic beta cells, reducing glucagon production, promoting glycogenesis and thereby reducing blood glucose levels (Davis Sn, 2001). Sulfonylureas have also been shown to suppress gluconeogenesis (Maniyar and Bhixavatimath, 2012).

The mechanism of action of *L. edulis* extracts remains unknown, but it may be related to the presence of antioxidants in the plant that have been reported in a previous study (Queiroz F.E., 2003). Antioxidants are protective agents that inactivate the reactive oxygen species (ROS) which cause cell damage (Joseph et al., 2013). Alloxan induces diabetes through reactive oxygen species that lead to a rapid

destruction of pancreatic beta cells (Stanely et al., 2000). Hyperglycemia in turn increases the generation of free radicals by glucose auto-oxidation (Bajaj and Khan, 2012). Joseph et al. (2013) reported that the antioxidant activity of herbal hypoglycemic plant extracts may represent a protective mechanism against reactive oxygen species (ROS) associated with chronic hyperglycemia and diabetic complications such as microvascular and macrovascular conditions. In addition, because the use of lower dose alloxan (120 mg/kg body weight) produces partial destruction of pancreatic beta cells, the albino rats have surviving beta cells and regeneration is possible (Aybar et al., 2001). *L. edulis* extracts may have led to the regeneration of the β-cells of the pancreas and potentiation of insulin secretion from surviving β-cells; the increase in insulin secretion and the consequent decrease in blood glucose levels may have led to inhibition of lipid peroxidation and control of lipolytic hormones. A number of plants have been reported to have anti-hyperglycemic and anti-hyperlipidemic effects in such a manner (Adeneye et al., 2011). This makes the use of *L. edulis* very important as it not only controls blood glucose levels but also controls lipid levels; this is important as many anti-hyperglycemic agents merely correct blood glucose levels without sufficient control of dyslipidemia.

Chapter 6 - **CONCLUSION, LIMITATIONS AND RECOMMENDATIONS**

6.1 Conclusion

On the basis of the results exhibited by this study, it can be concluded that *L. edulis* leaf aqueous extract has significant anti-hyperglycemic activity in alloxan induced diabetic rats. *L. edulis* exhibited dose dependent reductions in both blood glucose and serum lipid levels. By the 3rd day of treatment, the 300 mg/kg *L. edulis* dose reduced blood glucose levels by 23.3 percent whilst the 500 mg/kg *L. edulis* dose reduced blood glucose levels by as much as 52.6 percent. The 100 mg/kg *L. edulis* dose was able to reduce blood glucose levels by 25.5 percent by day 5. In addition, *L. edulis* was found to be effective in managing the diabetes associated complication hyperlipidemia. The 500 mg/kg *L. edulis* dose raised the HDL to normal ranges, whilst all *L. edulis* doses reduced LDL and TC to normal ranges by day 14. Therefore, *L. edulis* can be considered as alternative treatment not only for diabetes but especially that associated with dyslipidemia.

6.2 Limitations

Isolation of the active ingredient(s) could not be performed due to resource and time constraints.

6.3 Recommendations

Extensive research has shown that this is the first study to report not only anti-hyperglycemic but also anti-hyperlipidemic activity of an aqueous extract of the leaves of *L. edulis*. Because many anti-hyperglycemic drugs do not correct dyslipidemia as well, this makes further research into this plant more important. Further investigations are needed to isolate the plant extract(s) responsible for the observed anti-hyperglycemic and anti-hyperlipidemic effect.

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APPENDICES

APPENDIX A - HODGE AND STERNER TOXICITY SCALE

Table A-1 : Hodge and Sterner toxicity scale

Term	LD_{50} (rat/oral)
Extremely Toxic	Less than 1 mg/kg
Highly Toxic	1-50 mg/kg
Moderately Toxic	50-500 mg/kg
Slightly Toxic	500-5000 mg/kg
Practically Non-Toxic	5000-15000 mg/kg

APPENDIX B - REFERENCE RANGES

Table B-1: Glucose reference range and analysis

Variable	Nature	Description	Units	Analysis
Glucose levels	Categorical	<50 (hypoglycemia 50-160 (normoglycemia) >160 (hyperglycemia)	mg/dL	One-Way ANOVA

Table B-2: TC reference range and analysis

Variable	Nature	Description	Units	Analysis
Total Cholesterol	Categorical	<95.24 (hypocholesterolemia) 95.24–129.52(normal cholesterol) >129.52 (hypercholesterolemia)	mg/dL	One-Way ANOVA

Table B-3: TG reference range and analysis

Variable	Nature	Description	Units	Analysis
Triglycerides	Categorical	<47.57 (hypotriglyceridemia) 47.57-114 (normal triglyceride) >114 (hypertriglyceridemia)	mg/dL	One-Way ANOVA

Table B-4: HDL reference range and analysis

Variable	Nature	Description	Units	Analysis
HDL	Categorical	<35 (low HDL) 35.00–66.67 (normal HDL) >66.67 (high HDL)	mg/dL	One-Way ANOVA

Table B-5: LDL reference range and analysis

Variable	Nature	Description	Units	Analysis
LDL	Categorical	<20.28 (low LDL) 20.28–81.55 (normal LDL) >81.55 (high LDL)	mg/dL	One-Way ANOVA

Table B-6: VLDL reference range and analysis

Variable	Nature	Description	Units	Analysis
VLDL	Categorical	<9.51 (low VLDL) 9.51-22.8 (normal VLDL) > 22.8 (high VLDL)	mg/dL	One-Way ANOVA