EFFICACY OF CHIA SEEDS (*Salvia hispanica L*) ON SERUM CARDIOVASCULAR RISK FACTORS IN MALE WISTAR RATS FED A HIGH FAT AND FRUCTOSE DIET

FABIAN DOMINICUS MIHAFU

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Efficacy of Chia Seeds (*Salvia hispanica L*) on Serum Cardiovascular Risk Factors in Male Wistar Rats Fed a High Fat and Fructose Diet

Fabian Dominicus Mihafu

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature…….Date……

Fabian Dominicus Mihafu

This thesis has been submitted for examination with our approval as the University Supervisors.

Signature…….Date……

Dr. Beatrice. N. Kiage, PhD

JKUAT, Kenya

Signature…….Date……

Dr. Andrew. K. Nyerere, PhD

JKUAT, Kenya

Signature…….Date……

Dr. Judith. K. Okoth, PhD

JKUAT, Kenya

DEDICATION

This work is dedicated to my parents, Mzee Dominicus Daudi Mihafu and mama Arjentina Zakaria Kihanga for laying a foundation of my education.

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ACRONYMS AND ABBREVIATIONS

AAS	Atomic Absorption Spectrophotometer
AI	Atherogenic Index
ALA	α-Linolenic Acid
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
AST	Aspartate Aminotransferase
BAS	Basophils
BCAT	Black Chia Among'ura-Teso
BCK	Black Chia Kigumba
BCM	Black Chia Molo
BCOT	Black Chia Ongariama-Teso
CHD	Coronary Heart Disease
CRI	Coronary Risk Index
CVDs	Cardiovascular Diseases
DAAD	Deutscher Akademischer Austauschdienst (German Academic Exchange Service)
DF	Degree of Freedom

DHA	Docosahexaenoic Acid
EFSA	European Food Safety Authority
EOS	Eosinophils
EPA	Eicosapentaenoic Acid
FAMEs	Fatty Acid Methyl Esters
FID	Flame Ionization Detector
GC	Gas Chromatography
GGT	Gamma-Glutamyltansferase
Hb	Blood Hemoglobin
HDL-C	High Density Lipoprotein Cholesterol
HFFD	High Fat and Fructose diet
IC_{50}	The concentration of extract required to inhibiting 50% of an enzyme activity
IR	Insulin Resistance
JKUAT	Jomo Kenyatta University of Agriculture and Technology
LA	Linoleic Acid
LDL-C	Low Density Lipoprotein Cholesterol
LYM	Lymphocytes
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration

MCV	Mean Corpuscular Volume
MON	Monocytes
MPV	Mean Platelet Volume
NEUT	Neutrophils
PCV	Packed Cell Volume
PLT	Platelets
PUFAs	Polyunsaturated Fatty Acids
RBC	Red Blood Cell
RDW	Red Cell Distribution Width
RUFORUM	Regional Universities Forum for Capacity Building in Agriculture
SAFARI	Small Animal Facility for Research and Innovation
T2DM	Type 2 Diabetes Mellitus
TC	Total Cholesterol
TCC	The Chia Company
TLC	Total Leukocyte Count
VLDL-C	Very Low Density Lipoprotein Cholesterol
WCB	White Chia Seeds
WHO	World Health Organization

ABSTRACT

Cardiovascular disease (CVD) is the world's biggest killer claiming about 17.9 million lives annually where 1/3 being prematurely under 70 years. The fundamental task of dealing with CVD epidemic is primary prevention of CVD risk factors using natural food sources to reduce side effects caused by synthetic drugs. Chia seeds *(Salvia hispanica L)* are among of the natural plant-based foods that contain the greatest amount of nutrients particularly omega-3 fatty acid (α-linolenic acid), fiber, protein, fats and minerals. They are considered a functional food with pronounced health benefits and deemed useful in cardiovascular health. The objectives of this study were to; determine the proximate composition, mineral contents, fatty acid profiles and phytochemical screening of chia seeds grown in East Africa (Kenya and Uganda); investigate the *in vitro* efficacy of chia seed extracts on the inhibition of α-amylase and pancreatic lipase activities; determine the effect of ground chia seeds/extracts on postprandial glycaemia, body weight, hematological parameters and cellular morphology in rats; and evaluate the dose effect of chia seed extracts on plasma glycaemia, body weight changes, lipemia, liver enzymes and hematological parameters in male Wistar rats fed with fructose and lard. Samples, black chia Among'ura-Teso (BCAT), black chia Kigumba (BCK), black chia Molo (BCM), Black chia Ongariama-Teso (BCOT) and White chia Bukembo (WCB) were collected from Kenya and Uganda. Official methods of analysis (AOAC International) were adopted for proximate analysis; minerals were determined by Atomic absorption spectrophotometer, and fatty acid profiles were analyzed by Gas chromatography. Phytochemicals and enzymatic assays for pancreatic lipase and α-amylase were conducted using standard methods. Acarbose and Orlistat were used as positive controls for enzymatic assays. For animal studies, 44 male Wistar rats were divided into two experimental groups (acute experiment-20 rats and dose effect experiment-24 rats). In acute experiment, 20 male Wistar rats were assigned into three experimental groups and a control (n =5). Each experimental group received 10 g/20 g fructose/lard. Group 1 and 3 were supplemented with 20 g chia seed extract while group 2 received ground chia seeds only. Control group received rat pellets only. Each rat received 15g of pellets while water were given *ad libitum* in all groups. All diets were fed for a period of 28 days. In a dose effect experiment, 24 male Wistar rats were randomly assigned into three experimental groups (low dose, medium dose and high dose of 6 animals each) and a control. The control group received 6g/12g fructose/lard; the experimental groups were given 6g/12g of fructose/lard and in addition received 12g/kg, 18 g/kg and 24 g/kg body weight of chia seed extract for low dose, medium dose and high dose respectively. Rat pellets (15g) were given to each rat while water was given *ad libitum* to all groups. The diets were fed daily for a period of 8 weeks. Statistical analysis was conducted using STATA version 14.1, a one-way analysis of variance (ANOVA) used Bonferroni multiple comparison test to determine the variability between groups, and significance was accepted at $p \leq 0.05$. Results on proximate composition indicated high contents of protein, fat and fiber. The fatty acid profiles revealed great amounts of α-linolenic acid (ALA) (45.29-56.99 %) followed by linoleic acid (15.9-20.28%) and oleic acid (6.88-11.58%). Although the amount of ALA was high compared to other fatty acids analyzed in all samples, the difference was not significant ($p = 0.7391$). Mineral determination (mg/100g) displayed high contents of potassium

(492.96-862.98), phosphorous (486.45-569.45), calcium (297.47-429.09) and magnesium (192.22-202.97) while considerable amount was observed for iron, zinc, manganese, and copper. There was a significant difference ($p = 0.0001$) in mineral content between BCK and WCB with exception of phosphorus. For the *in vitro* studies, BCAT displayed the highest inhibition on α-amylase activity with the IC_{50} value of 104.51 ± 0.24 μg/ml and 72.06 ± 0.12 μg/ml for ethanolic and methanolic extracts respectively, however they were significantly lower ($p = 0.0001$) than that of acarbose. On the other hand, BCAT exhibited the strong pancreatic lipase inhibitor with IC_{50} value of 90.02 ± 0.17 μg/ml and 96.19 ± 0.20 μg/ml) for ethanolic and methanolic extracts respectively, followed by BCM and BCK. Orlistat indicated a significantly higher ($p = 0.001$) IC_{50} (54.14 ± 0.15 μg/ml), establishing its relative potency as pancreatic lipase inhibitor than chia seed extracts. In an acute experiment, there was an increase in postprandial blood glucose levels in group 1 from week I to IV contrary to groups supplemented with chia seeds/extract indicating the ability of chia seeds in controlling blood glucose. Complete blood counts showed a significant increase ($p = 0.008$) in mean corpuscular hemoglobin concentration, basophils ($p = 0.035$), and platelets ($p = 0.0001$) in experimental groups compared to control. In a dose effect experiment, platelets were significantly lower ($p = 0.001$) in low dose group compared to a control and high dose groups. For liver enzymes, Gamma glutamyl transferase (GGT) decreased significantly ($p = 0.02$) in low and medium dose groups and bilirubin was significantly lower ($p = 0.005$) in experimental groups compared to a control. Serum lipids such as high density lipoprotein cholesterol (HDL-C) increased significantly ($p = 0.041$) in high dose group than the control while low density lipoprotein cholesterol (LDL-C) decreased significantly ($p = 0.035$) in high dose group as compared to control and medium dose groups ($p = 0.021$). The East African grown chia seeds, exhibited high amount of nutrients and fatty acids. The seeds inhibited the pancreatic lipase and α-amylase activities, these enzymes are responsible for the digestion of fats and carbohydrates respectively. Inhibition of these enzymes may help decrease hyperglycemia and hyperlipidemia the risk factors for CVD. The positive impact showed on LDL-C, HDL-C and triglycerides in rats fed on diet high in fat and fructose suggest the potential of chia seeds in reducing serum CVD risks. Generally, these results highlight that chia seeds could be among the preferred natural food sources for the primary prevention of CVD. Therefore, we suggest its moderate incorporation in diets as a healthy food ingredient.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Chia seeds (*Salvia hispanica L*) belong to the family lamiaceae native to Mexico and Guatemala (Ixtaina et al. 2021; Mohd et al. 2012; Ferreira et al. 2015; de Falco, Amato, & Lanzotti, 2017). The name salvia originates from a Latin word salvare meaning cure (Sosa, 2017). The seeds were initially cultivated by Aztec, Mayan and Incan tribes for their food source and production of medicine as well as paints ((Nitrayová et al. 2014). It was an important food for the Aztec warriors especially during the wars of conquest as they traveled great distances with just small amounts of this oilseed. In pre-Columbian times, the crop was cultivated in many areas of Mexico, Guatemala, Nicaragua and Honduras ((Sosa-Baldivia, Ruiz-Ibarra, de la Torre & López, 2018). Several studies have revealed the significance of using chia seed to enrich diets, as it is high in polyunsaturated fatty acids especially omega-3 [α-linolenic acid (ALA)], antioxidants, dietary fiber, proteins, phenolic compounds and other micronutrients (Segura-Campos *et al*. 2014b; Abdulrashed, Gazem, & Chandrashekariah, 2016).

Consumption of chia seeds is associated with lowering the risks of cardiovascular diseases (CVDs) (Ferreira et al. 2015). CVDs are the leading cause of deaths in the world and over three quarters of CVD deaths that take place in low- and middle-income countries are due to atherosclerosis, which in turn heightens heart attack and strokes (WHO, 2017b). Cardiovascular risk factors include central obesity, hypertension, dyslipidemia [elevated low density lipoprotein cholesterol (LDL-C), low high density lipoprotein cholesterol (HDL-C), elevated triglycerides (TG)] and diabetes particularly type two diabetes mellitus (T2DM) (WHO, 2013). These risk factors can be modified, controlled and treated, however choice of diet and lifestyle is of great concern (Laslett *et al*. 2012). Recently, there is a growing interest of using chia seeds as a functional food, food supplement and nutraceutical (Sargi et al.2013; Abdulrashed et al. 2016).

Experimental studies on the effect of chia seeds in lowering hypertension, elevated triglycerides, low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL-C), total cholesterol (TC) and postprandial glycaemia are encouraging (Ferreira et al. 2015). For instance, (Azcona et al. 2008), observed that, chia seeds reduced the amount of cholesterol in meat of pig and chicken as well as chicken eggs. Another study in rats revealed that chia seeds lowered the LDL-C and triglycerides while plasma high density lipoprotein cholesterol (HDL-C) was elevated (Ayerza & Coates, 2007). In this study, nutritional composition, fatty acid profiles, mineral and phytochemical contents as well as hypoglycemic and hypolipidemic effects of chia seed were assessed. Additionally, effect of chia seeds on body weight changes, postprandial glycaemia, hematological parameters, liver enzymes and inhibition of pancreatic lipase and α-amylase activities were investigated.

1.2 Statement of the Problem

CVDs refer to group of disorders of the heart and blood vessels, the most common form being coronary heart disease which is caused by atherosclerosis (WHO, 2017). Atherosclerosis is promoted by inflammation, elevated LDL-C and VLDL-C, diabetes mellitus and atherogenic diet (a diet high in fats especially saturated fats but low in vegetables, fruits and whole grain) (Swirski & Nahrendorf, 2013). Foods high in saturated fats have a strong effect on cholesterol levels, while diets high in carbohydrates especially those high in added sugars can raise blood triglycerides hence intensifying risks for CVDs (Rolfes, Pinna, & Whitney, 2012).

The magnitude of CVD is increasing all over the world in unmanageable scale (Figure 1.1). CVD is the world's biggest killer claiming about 17.9 million lives annually where 1/3 being prematurely under 70 years. About 80 % of all CVD deaths are from heart attack (7.4 million) and stroke (6.7 million) as reported by WHO (2021). Shockingly, more than 75 % of CVD deaths occur in developing countries (WHO, 2017a; Laslett et al. 2012).

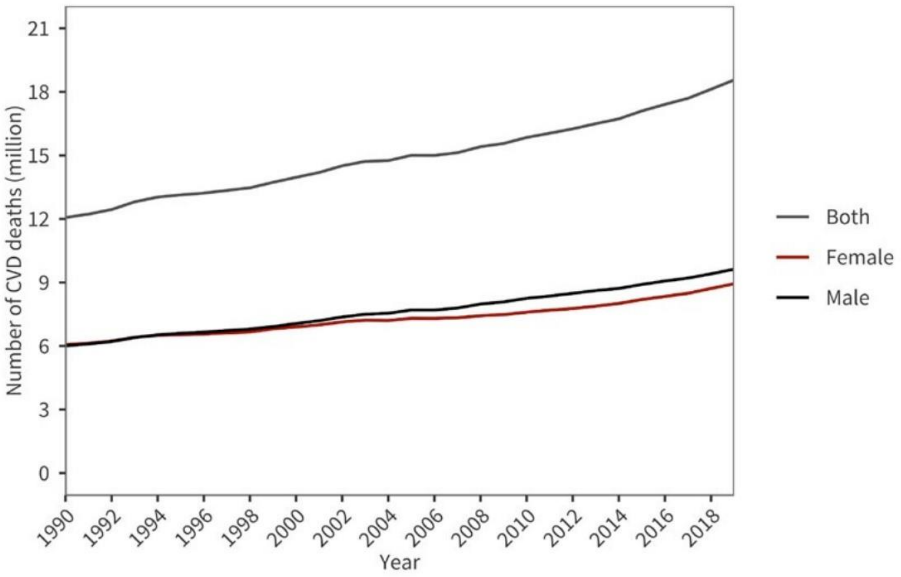

Figure 1.1: Prevalence of CVD Worldwide

Source: World Heart Report 2023.

The fundamental task of dealing with CVD epidemic is primary prevention of CVD risk factors (elevated LDL-C, low HDL-C, elevated triglycerides, and hyperglycemia) and understanding their interaction. Several synthetic drugs have been used to treat/reduce cardiovascular risk factors, for example fibrates (reduce elevated triglycerides and raise HDL-C), statins (reduce elevated LDL-C and triglycerides and increase HDL-C) and nicotinic acid (reduce elevated triglyceride and subsequent VLDL and LDL-C) (Rolfes, Pinna & Whitneys, 2003; Kiage-Mokua et al. 2018). However, frequent use of these drugs can cause adverse side effects like dyspepsia, abdominal pain, myopathy, blurred vision, elevated liver enzymes, abdominal bloating, flatulence and diarrhea (Kolovou *et al*. 2011; Kumar, Mazumder & Saravanan, 2008). Thus the need for alternatives from natural food sources such as chia seeds is indispensable.

1.3 Justification

The costs of medication for CVDs are high and estimated to reach US $1,044 billion by 2030 (Forum & Health, 2011), thus the need for alternative approaches that could be more effective, safer and affordable.

Chia seeds can be eaten directly as source of ALA hence considered safer, traceable and sustainable compared to fish, algae and flaxseed (Sosa, 2017). Omega 3-PUFAs are nutritionally important for good health and are beneficial to individuals suffering from heart disease, diabetes and immune response disorders (Coelho & Salas-Mellado, 2014). Experimental evidences demonstrate a strong correlation between regular dietary consumption of omega 3-PUFAs and lower incidence of CVD risks thus an increased demand for functional foods with varied health benefits (Mohd et al. 2012; Alissa and Ferns, 2012; Abdulrashed et al. 2016). On the other hand, high dietary fiber intakes are associated with reduced risk of developing CVD, and increasing intake has been shown to reduce blood pressure and other cardiometabolic risk factors (Reynolds et al. 2022), since chia seeds are rich in fiber it is likely to suit the role of preventing cardiovascular risk factors.

Indeed, the impact of chia seeds on hyperglycemia, hypertriglycerides, LDL-C and TC indicate future promise. However, most of the studies on the relationship between chia seed consumption and CVD risks are insufficient (Ferreira et al. 2015; Abdulrashed et al. 2016). For example, there is an inadequate protocol on effective dose that can suit human consumption, and an understanding of bioactive compounds and fatty acids responsible for biological activity in cell and animal models is needed before using chia seeds as functional food. This study therefore aimed at finding out the impact of chia seeds on cardiovascular risk factors in order to help prevention of CVD.

1.4 Objectives

1.4.1 General Objective

The main objective of this study was to investigate the efficacy of chia seeds in reducing cardiovascular risk factors in male Wistar rats fed a high fat and fructose diet.

1.4.2 Specific Objectives

i. To determine proximate composition, mineral content, fatty acid profiles and a qualitative phytochemical screening of chia seeds.
ii. To investigate *in vitro* effect of chia seed extracts on the inhibition of pancreatic lipase and α-amylase activities.
iii. To determine the effect of ground chia seed/extract on body weight, postprandial glycaemia and hematological parameters of male Wistar rats in an acute experiment.
iv. To evaluate the dose effects of chia seed extracts on plasma glycaemia, body weight changes, lipemia, liver enzymes and hematological parameters in male Wistar rats fed a high fat and fructose diet.

1.4.3 Research Questions

i. What are the nutritional composition and fatty acid profiles of chia seeds?
ii. Do chia seed extracts have an effect on *in vitro* inhibition of pancreatic lipase and α-amylase activities?
iii. Do ground chia seed/extracts have effect on body weight, postprandial glycaemia and hematological parameters of male Wistar rats in an acute experiment?
iv. Does a dose effect of chia seed extracts have an impact on plasma glycaemia, body weight changes, lipemia, liver enzymes and hematological parameters in male Wistar rats fed a high fat and fructose diet?

CHAPTER TWO

LITERATURE REVIEW

2.1 Background Information of Chia Seeds (Origin of Chia Seeds)

Chia seeds were one of the original foods of Mexicans and Guatemalans, traditionally used for beverage and flour production whereas its oil was used to increase the quality of paint production (Ixtaina, Nolasco & Tomas, 2008; Sosa, 2017). Chia was the third crop after corn and amaranth named as 'running foods'. The chia was classified by the Swedish botanist Carl Von Linneo in 1753, who named it *Salvia hispanica* (Spanish) that in Latin means Spanish plant to cure or save (Sosa, 2017). Chia plant can grow up to 1 m tall, has opposite arranged leaves and small purple flowers of about 3-4 mm with small corollas as shown in Figure 2.1 (Nitrayová et al. 2014). Chia seeds bear oval-shaped, multicolored seeds with predominant black and white colors (Figure 2.2). Chia plant is commercially grown for its seeds.

In Mexico, the word chia was derived from the Nahuati word 'chian' meaning oily; On Nahua language, the Chian word (today named chia) means oily, thus the Aztecs used the word chia to refer to all spices of Salvia genus whose main characteristic is their high oil content (for instance, *Salvia hispanica L, Salvia polystachya O*., *Salvia tiliifolia V.y* and *Salvia columbariae B*.) (Sosa, 2017)

Figure 2.1: Chia Plants

A B

Figure 2.2: Black Chia Seed (A) and White Chia Seeds (B)

Source: https://www.google.com/search?q=chia+plant+photos&tbm

2.2 Climatic Condition Suitable for Chia and its Growth Globally

Chia is a tropical specie of short day that can grow well in areas located between 20° 55’ N to 25° 05’ S (Figure 2.3). It can be grown across a variety of ecologies ranging from arid and semiarid climates to tropical rain forest (Gören, Kiliç, Dirmenci & Bilsel, 2006; Mohd et al. 2012). Currently, chia seed is widely grown in Mexico, Guatemala, Argentina, Bolivia, Ecuador, Nicaragua, Paraguay, Honduras, Colombia, Peru and United States (Segura-Campos et al. 2014a; Jamboonsri, Phillips, Geneve, Cahill & Hildebrand, 2012). Chia cultivation is further extended to Ghana, Australia, Chile and Southeast Asia (Jamboonsri et al. 2012). Today chia is grown in some parts of East African countries such as Uganda, Kenya and Tanzania (Lardizabal, 2014; Mihafu, Kiage, Okoth & Nyerere, 2019)

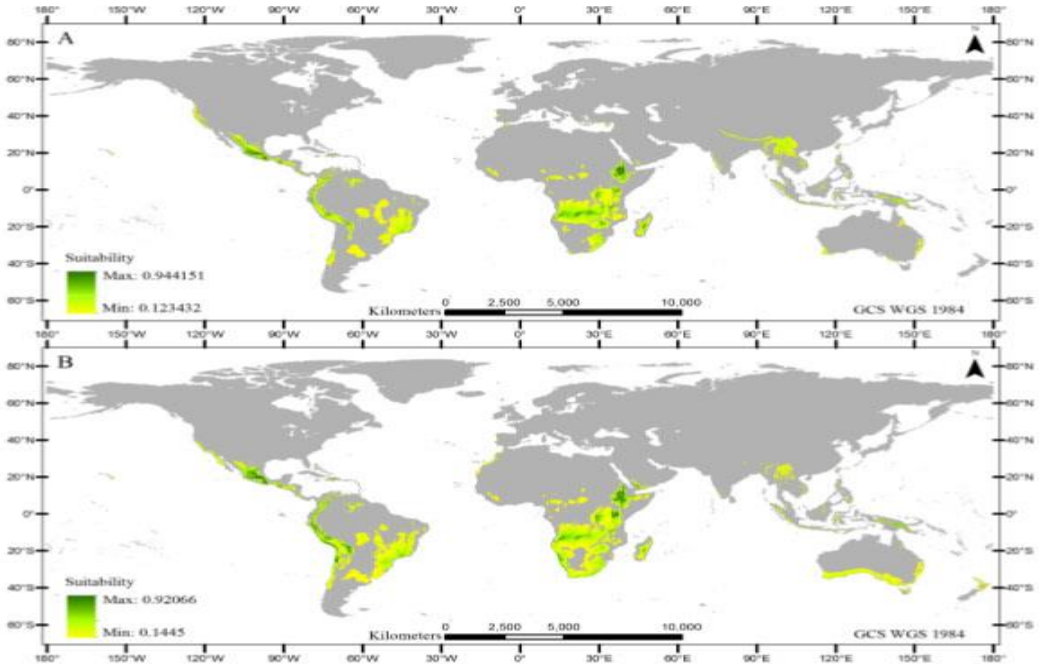

Figure 2.3: Suitable Climate for Growing Chia Seeds Globally

Source: (Cortés, Silva, Baginsky & Morales, 2017)

2.3 Prevalence of Cardiovascular Diseases Worldwide

CVDs are group of disorders of the heart and blood vessels (WHO, 2017). The different types of CVDs include coronary artery disease (e.g. heart attack),

cerebrovascular disease (e.g. stroke), diseases of the aorta and arteries (hypertension and peripheral vascular disease) (see section 2.4) , they are generally called CVDs due to atherosclerosis (Morris, Whitney & Cataldo, 2006). CVDs cause death of more than 17.9 million people per year (WHO, 2021), and are therefore the leading causes of death in the world representing about 31% of all deaths (World Health Organization, 2016). Coronary heart disease account for 7.4 million deaths while stroke account for 6.7 million deaths annually (WHO, 2017). The affected age group is between 30-69 years which is 10 years or younger than in more developed countries (Mbewu, 2008; WHO, 2021). This therefore shows that uncontrolled CVD can be epidemic.

An estimated 1 million deaths are attributable to CVD in sub-Saharan Africa (SSA) alone, which constitute 5.5% of all global CVD-related deaths and 11.3% of all deaths in Africa. CVD-related deaths contribute to 38% of all non-communicable disease-related deaths in Africa, reflecting the growing threat of both non-communicable disease and CVD (Keates et al. 2017). Lack of appropriate epidemiological data, coexistence of infectious and non-infectious diseases, under-nutrition/over-nutrition, and poor economic status are among the factors that influence CVD mortality in these countries (Roman et al. 2019). In Kenya, CVD contributes more than 13% of overall mortality (Ogeng'o, 2011). Trends of CVD death rates in SSA, including Tanzania, are highly driven by lifestyles changes, characterized by low levels of physical activity, excessive alcohol consumption, tobacco use and unhealthy eating. Poor management of these factors has resulted into intermediate risk factors such as raised blood pressure, raised blood cholesterol, diabetes, overweight and obesity that have direct linkage with CVDs (Roman et al. 2019). In fact, Africa is associated with greater loss of productive human resource hence affecting economic growth across the continent (Cappuccio & Miller, 2016). Previous studies highlight the role of diet on CVD, for example excessive consumption of foods that are calorie dense, nutritionally poor, highly processed, and rapidly absorbable can lead to systemic inflammation, reduced insulin sensitivity, and a cluster of metabolic abnormalities (obesity, hypertension, dyslipidemia, and diabetes) (Alissa & Ferns, 2012) .

These factors are the driving force for CVD (Medic, 2014; Ntandou et al. 2014). People with metabolic abnormalities have about 50-60% high CVD risk than those without it (Alshehri, 2010).

2.4 Definition of Terms Related to CVD

Coronary Heart Disease (Heart Attack): Refers to disease of blood vessels supplying the heart muscle. It is characterized by impaired blood flow through the coronary arteries that may lead to angina pectoris, heart attack or sudden death (WHO, 2017).

It is commonly caused by inflammatory conditions that cause narrowing of coronary arteries. Risk factors include high blood pressure, high blood cholesterol, tobacco use, unhealthy diet, physical inactivity, diabetes, advancing age, genetic disposition, poverty, low educational status, poor mental health (depression), inflammation and blood clotting disorders (WHO, 2021).

Cerebrovascular Disease (Stroke): Caused by disruption of the blood supply to the brain. This may result from either blockage (ischemic stroke) or rupture of a blood vessel (hemorrhagic stroke). Risk factors include high blood pressure, atrial fibrillation (a heart rhythm disorder), high blood cholesterol, tobacco use, unhealthy diet, physical inactivity, diabetes and advancing age (WHO, 2021).

Diseases of the Aorta and Arteries (Hypertension &Pulmonary Artery Disease) Hypertension are an initial risk factor for atherosclerosis and CVDs. In other words it is the leading cause of CVD and mortality (Rolfes et al. 2009). ***Hypertension*** means abnormal elevation of the blood pressure; specifically, a transitory or persistent elevation of the pressure of the blood in the arteries of the systemic circulation to a level that can induce cardiovascular damage. It has been reported that for every increase of 20 mmHg in systolic blood pressure and 10 mmHg in diastolic blood pressure, the risk of death from CVD doubles. In hypertensive condition the heart struggle to pump blood into arteries, the activity which weaken the heart muscles increasing the risk of heart failure, arrhythmias and sometimes sudden death (WHO, 2017; WHO, 2021).

Congenital Heart Disease- Malformations of heart structure occurring at birth. It may be caused by genetic factors or adverse exposure during gestation. The defects can involve the walls of the heart, the valves of the heart, and the arteries and veins near the heart. Examples of such defects include holes in the heart, abnormal valves and abnormal heart chambers. (WHO, 2016).

Rheumatic Heart Disease: Damage to heart muscles and valves from rheumatic fever caused by streptococcus bacteria. Rheumatic fever mostly affects children in developing countries, especially where poverty is widespread. Globally, about 2% of deaths from cardiovascular diseases are related to rheumatic heart disease (WHO, 2017).

Inflammatory Heart Disease: The inflammation of the heart muscles, such as myocarditis, the membrane sac which surrounds the heart called as pericarditis, and the inner lining of the heart or the myocardium, heart muscle as endocarditis are known as the inflammatory heart diseases. Inflammation of heart is caused by infectious agents, viruses, bacteria, fungi and by toxic materials from the environment, water, food, air, toxic gases, smoke and pollution (Lu et al. 2015).

Cardiac arrhythmias- are disturbances in the normal electrical activity of conduction system. Arrhythmias can be detected on a recording of the ***electrocardiogram (ECG).***

The electrical disturbances interfere with the ability of the heart to pump blood, and may cause angina pectoris or congestive heart failure. Severe arrhythmias can cause ventricular fibrillation and sudden death (Rolfes et al. 2009)

Cardiomyopathy- disorder of heart muscles or disease of heart muscle resulting into inability of heart to pump enough blood round the body. Cardiomyopathy can be acquired due to another disease or condition (Rolfes et al. 2009).

2.5 Proximate Composition, Mineral Content, Fatty Acid Profiles and Phytochemicals in Chia Seeds

Chia seeds contain about 21 % protein, a level greater than other nutritional grains such as wheat (14 %), corn (14 %), rice (8.5 %), oats (15.3 %), barley (9.2 %), and amaranth (14.8 %) (Sarasvathi & Many, 2017). Chia seeds have an oil content of approximately one third of its weight, about 60 % of which is α-linolenic acid, making this ingredient a source of omega-3 fatty acids. Once the oil has been extracted from the seeds, the material that remains contains 50-60 % fiber. The seeds alone possess about 5 % soluble fiber. Chia seeds are also a source of vitamin B-complex and minerals such as calcium, phosphorous, potassium, magnesium, iron, zinc, and copper (Amato et al. 2015).

The seeds also contain natural antioxidants (chlorogenic acid, caffeic acid and flavanol glycosides (Bresson et al. 2009; Turck et al. 2019). Average proximate composition of chia seeds have been highlighted in Table 2.1

Table 2.1: Average Proximate Parameters of Chia Seeds

Parameter	% Mean range
Proteins	15 – 25
Fats	21 – 40
Carbohydrates	14 – 45
Dietary fiber	18 – 35
Ash	3.5 - 5.5
Moisture	5.5 – 8.0

Source: Ixtaina, Nolasco, & Tomás, (2012); Segura-Campos, Ciau-Solís, Rosado-Rubio, Chel-Guerrero, (2014); Medic, (2014)

Dietary fiber: Chia seeds have high fiber content (Abdulrashed et al. 2016), dietary fiber comprises of non-structural carbohydrates such as starch, simple sugar and fructans that are easily hydrolyzed by enzymatic reactions and absorbed in the gastrointestinal tract (Anderson et al. 2009). On the other hand, complex carbohydrates are resistant to digestion but can be completely or partially fermented by microflora in the large intestine into short chain fatty acids which are used by

probiotics (Dhingra et al. 2012; Ötles & Ozgoz, 2014). Both soluble and insoluble fibers are important for healthy life. Soluble fiber forms a gel when mixed with liquid in the stomach, which in turn prolongs absorption of carbohydrates so that sugar/glucose is released slowly into the bloodstream, thus helping to normalize the blood glucose level (Weickert et al. 2005). Soluble fibers also bind with fatty acids and lower total cholesterol especially LDL-C, hence reducing the risk of heart diseases (Ötles & Ozgoz, 2014).

Insoluble fiber passes through our intestines largely intact, promote regular bowel movements, reduce risks for developing colon cancer by keeping optimal pH (acidity) in the intestines and prevent the occurrence of hemorrhoids and constipation (Ötles & Ozgoz, 2014).

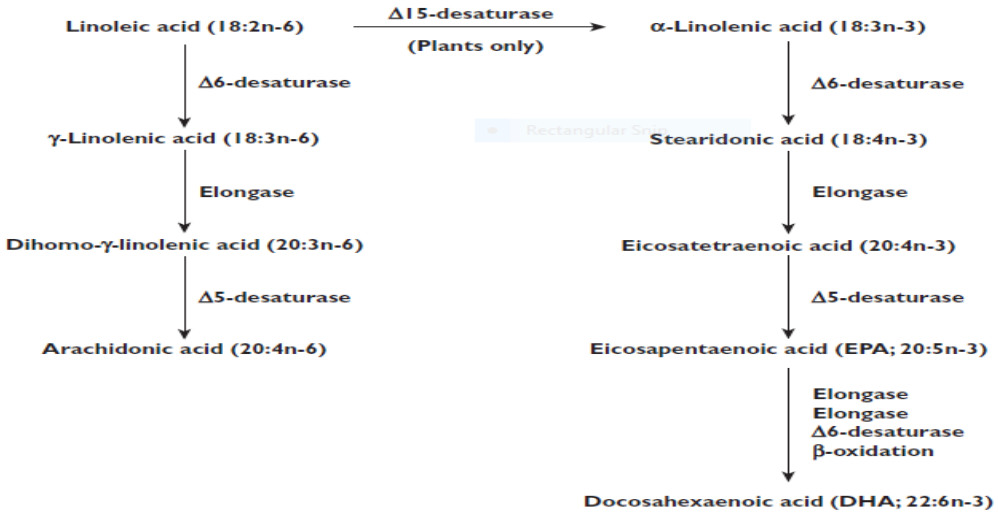

Figure 2.4: Conversion of ALA to EPA and DHA

Source: (Calder, 2013)

Fatty acids: About 60 % of the total lipids in chia seeds consist of omega 3-PUFAs that is ALA, 20 % consist of linoleic acid (LA), 6 % consist of monounsaturated fatty acids (MUFAs) and 14-16 % consist of saturated fatty acids (Mohd et al. 2012; Bresson et al. 2009). ALA is fatty acid that can also be converted to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Figure 2.4).

Its conversion starts with linoleic acid from plants by 15-dasaturase (Calder, 2013). Therefore, it is possible that, ALA from chia seeds can be substituted for fish and being useful to people allergic to fish (Ramzi & Sharada, 2014). Chia seeds contain up to 39 % of oil, which has the highest content of ALA (n-3 PUFA) up to 68 % (Gazem, Puneeth, Madhu & Sharada, 2016). PUFAs especially omega-3 and omega-6 can modulate immune and inflammatory responses (Fritsche, 2006).

Regular dietary supplementation with omega 3-PUFAs brings numerous health benefits such as prevention of cardiovascular diseases, hypertension and inflammatory diseases (Fritsche, 2006). Generally, n-3 PUFAs have cardioprotective, anti-inflammatory, and triglyceride-lowering properties, so they may help treat obesity and improve metabolic syndrome (Liu et al. 2023).

Phytochemicals: Phytochemicals are bioactive non-nutrient plant compounds that show various physiological effects on human body and also have been speculated to reduce the risk of major chronic diseases (Liu, 2013). Flavonoids are major group of phenolic compounds that have mostly been linked to reducing the risk of chronic diseases, such as heart disease, cancer, stroke, diabetes, and other age-related ailments (Zhang et al. 2015). Phytochemicals have been categorized into six classes as phenolics, alkaloids, nitrogen-containing compounds, organosulfur compounds, phytosterols and carotenoids (Figure 2.5). Research findings highlight more than 5000 individual flavonoids being isolated and categorized based on their structural differences as flavonols (quercetin, kaempferol, and myricetin), flavones (luteolin and apigenin), flavanols (catechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate), flavonones (naringenin and hesperitin), anthocyanidins (cyaniding, malvidin, delphinidin and pelargonidin), and isoflavonoids (genistein and daidzein) (Liu, 2013: Zhang et al. 2015). Therefore, presence of some classes of phytochemicals/antioxidants (chlorogenic acid, caffeic acid and flavanol glycosides) in chia seeds signifies their impact in cardiovascular risk control.

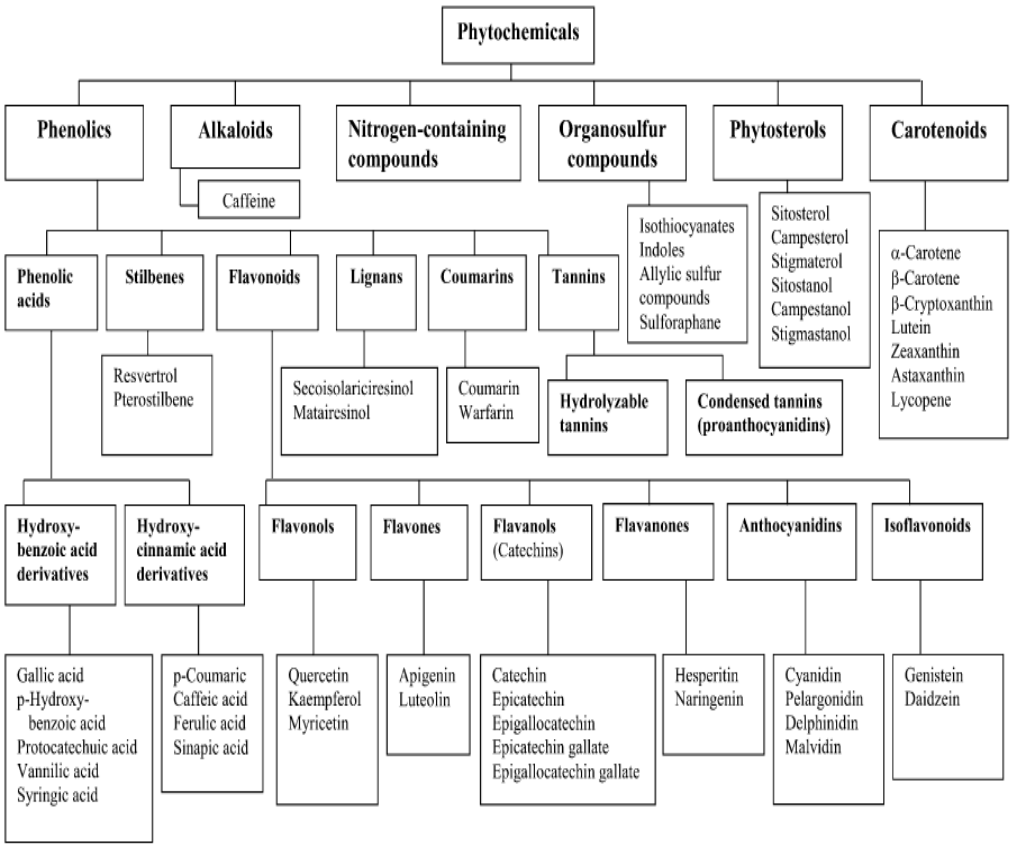

Figure 2.5: Categories of Phytochemicals

Source: (Liu, 2013)

Flavonoids have many beneficial effects on health, the most important being the antioxidant, anti-inflammatory, antiplatelet, antihypertensive, and anti-ischemic effects (Russo et al. 2019). They have also the ability to interfere with lipid metabolism, decrease platelet adhesion, and improve endothelial function. The ability of flavonoids to donate electrons to peroxynitrite, hydroxyl, and peroxyl radicals reduce the levels of reactive oxygen and nitrogen species by forming stable flavonoid radicals and the stabilization of hydroxyl group ($^{-}$OH), superoxide and peroxynitrite radicals (Kumar & Pandey, 2013).

The antioxidant effect of flavonoids is achieved by three mechanisms namely eliminating reactive oxygen species, preventing the production of reactive oxygen species, secondary to the interaction of flavonoids with enzymes that control the

production of free radicals, and increasing the protection of antioxidant systems (Ciumarnean, 2020).

Flavonols: The free forms of flavonols are called aglycones, they share a 3-hydroxyflavone backbone and differ by the presence and position of hydroxyl groups. The number of hydroxyl groups contribute to the bioactivity of these compounds (Popiolek-Kalisz & Fornal, 2022). The most important flavonols are quercetin, kaempferol, myricetin, and isorhamnetin (Figure 2.6). The chemical structure of flavonols modifies their activity and also impacts their bioavailability. Flavonols and flavanols are the most abundant flavonoids found throughout the plant kingdom (Kozłowska & Szostak-Wegierek, 2022), while quercetin is the most abundant flavonols.

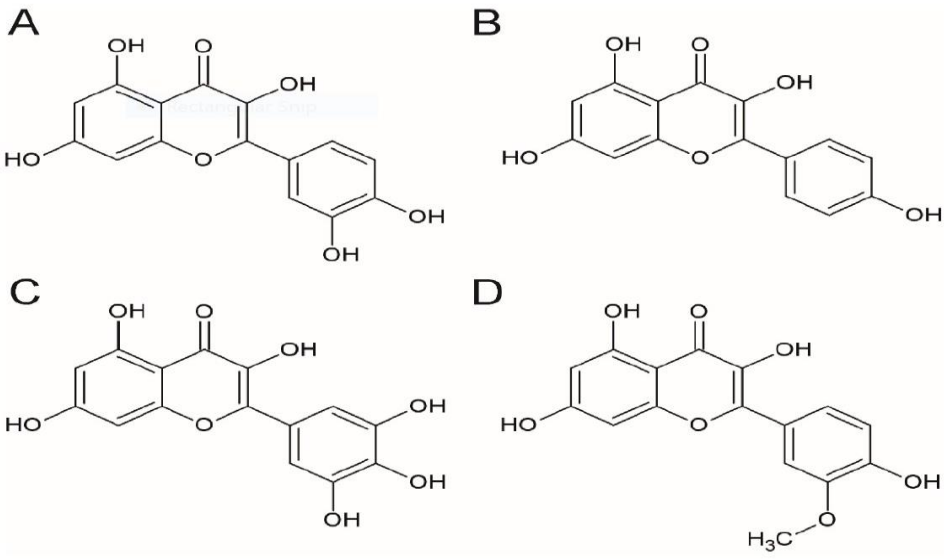

Figure 2.6: The Chemical Structure of the Selected Flavonols: A-quercetin, B-Kaempferol, C-Myricetin, and D- Isorhamnetin

Source: Khan et al. (2021).

Quercetin: Exerts its antihypertensive effect by improving endothelial function, modulating the mechanism of contraction of smooth muscles in blood vessels, decreases oxidative stress, and lowers blood pressure in patients with diabetes or metabolic syndrome (Pfeuffer et al. 2013; Li et al. 2021). A study conducted among

patients with dyslipidemia who received a quercetin supplement, revealed a reduction of TC, triglyceride and LDL-C with parallel increase in HDL-C (Talirevic & Sehovic, 2012). Another study in healthy men with different apolipoprotein E genotypes, who were daily administered with 150 mg quercetin, significantly decreased postprandial triacylglycerol concentrations and increased HDL-C level compared to placebo (Pfeuffer et al. 2013).

Kaempferol: Protects against cardiac disease through antiapoptotic, antioxidative, antiinflammatory, calcium regulatory, and antifibrotic mechanisms, as well as maintaining mitochondrial function, resulting in the improvement of cardiac structure and function (Vishwakarma et al. 2018). The capability of kaempferol in scavenging reactive oxygen species (ROS) plays a prominent part in its protective role (Kamisah et al. 2023).

Generally, flavonols (Figure 2.7) are associated with a cardioprotective potential and reduction of the risk of cardiovascular diseases. The most studied associations are flavonols' antioxidant and anti-inflammatory activities; favonols can impact the cardiovascular system by numerous pathways such as angiotensin converting enzyme inhibition, antiplatelet aggregation effects, and low density lipoprotein (LDL) receptor expression in the liver (Hettihewa et al. 2018; Stainer et al. 2019; Li et al. 2021).

Isoflavones; Exert its effect mainly by diminishing the damage caused by oxidative stress but also by increasing nitric oxide synthesis through reducing LDL oxidation or by increasing the production of prostaglandins (Munoz et al. 2009).

Flavanones (Figure 2.7), demonstrate antihypertensive effect and antioxidant properties by blocking the activity of free radicals, reducing blood pressure, modulating nitric oxide levels and protects against endothelial dysfunction (Khan et al. 2018).

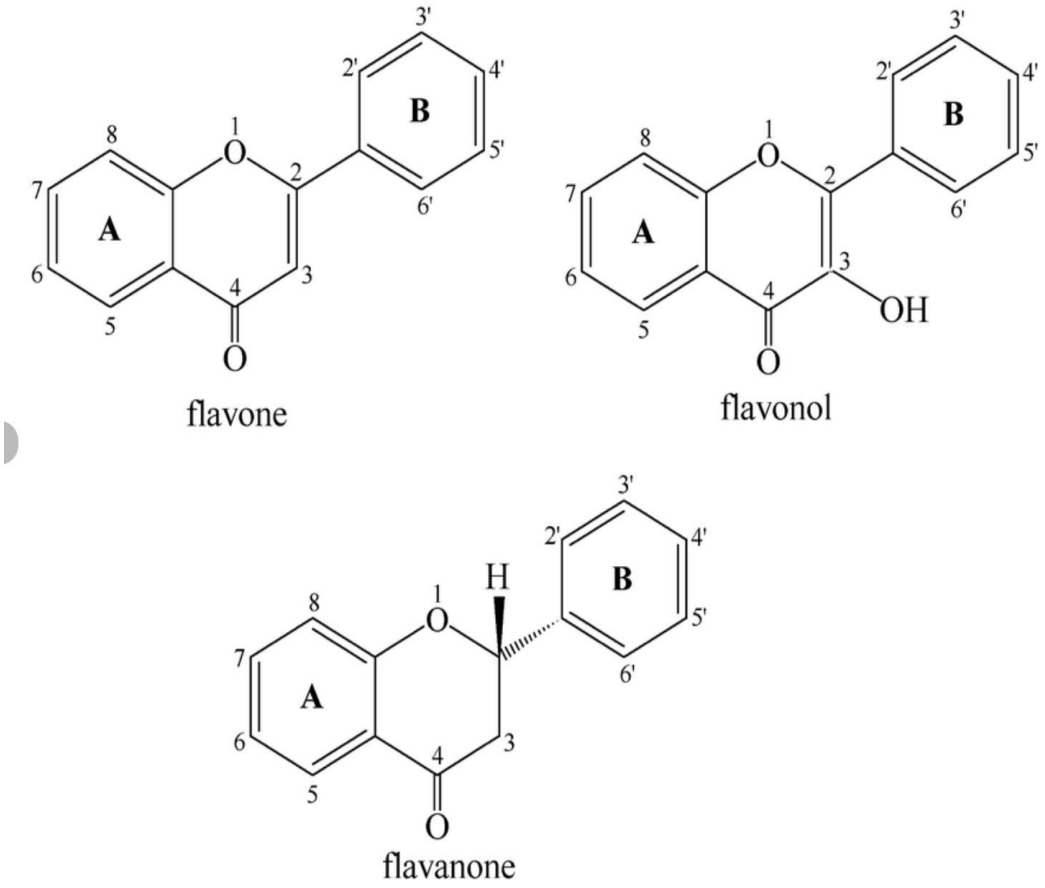

Figure 2.7: Structure of Flavone, Flavonol and Favanone

Source: Khan et al. (2021)

Table 2.2: Summary of Flavonoids with their Impact on CVD Risk Factors

Type of Flavonoids	Major antioxidants	Health benefits
Flavonols	Quercetin, Kaempferol Myricetin, Isorhamnetin	Regulates systolic blood pressure and glycemic levels
Flavones	Apigenin, Luteolin	Regulates blood glucose levels
Flavanones	Eriodictyol, Hesperetin, Naringenin	Lowers risk of ischaemic stroke
Flavanols	Catechins, Epicatechins	Reduces mean arterial pressure, improves insulin resistance, LDL-C, and HDL-C levels
Isoflavones	Daidzein, Genistein, Glycitein	Beneficial for type 2 diabetes mellitus
Anthocyanidins	Cyanidin, Delphinidin, Malvidin, Pelargonidin, Peonidin, Petunidin	-Improve insulin resistance, insulin production, and hepatic glucose uptake during type 2 diabetes mellitus -Lowers risk of Myocardial infarctions

Source: Kumar& Pandey, (2013); Khan et al. (2021)

2.6 Role of Natural Inhibitors on α-Amylase and Pancreatic Lipase Activities

In vitro tests can play a very significant role in the evaluation of antidiabetic activity of drugs in the preliminary screening process. They are believed to provide useful information on the mechanism of action of therapeutic drug in question (Sama, Murugesan & Sivaraj, 2012; Roy, 2013). The use of carbohydrate digestive enzyme inhibitors from natural sources such as chia is suggested to be a potential block to dietary carbohydrate absorption with reduced adverse effects compared to synthetic enzyme inhibitors (Thakre, Damodar, Khan & Mular, 2017).

The α-amylase is an enzyme responsible for the breakdown of monosaccharides, disaccharides and oligosaccharides. Acarbose, Miglitol and Voglibose are the common inhibitors used in the clinical practice. However, the major disadvantages of these drugs include various gastrointestinal side effects such as flatulence, abdominal pain and diarrhea in the patients (Kolovou et al. 2011). Thus, efforts are needed to identify and explore the α-amylase inhibitors from natural sources having fewer side effects (Bhutkar, 2018). In animals, α-amylase inhibitors reduce postprandial glycaemia by decelerating enzymatic conversion of starch into simple sugar (Nair, Kavrekar & Mishra, 2013a).

Inhibition of α-amylase help to reduce the rate of digestion of carbohydrates thus less amounts of glucose is absorbed in gastrointestinal tract (Nair et al. 2013b; Thakre *et al*. 2017). Whole grain chia has been reported to lower postprandial glycaemia when incorporated into carbohydrate rich meal, which indicates its ability in controlling hyperglycemia and hence type 2 diabetes (Lee, 2009). On the other hand, pancreatic lipase is a significant enzyme in the digestion of dietary fats (Gholemhoseanian 2010), its inhibition is thought to reduce dyslipidemia. The most common anti-obesity drug is orlistat, a potent inhibitor of gastric and pancreatic lipase, it has been proved to be effective for the treatment of human obesity by 35 % reduction in fat absorption (Original, 2016). Synthetic drugs such as orlistat have been used in experimental trials and demonstrated that long-term lipase inhibition may reduce postprandial triglycerides and fasting LDL-C (Sahib et al. 2012).

However, management of hyperlipidemia without any side effects is still a challenge (Heck, Yanovski & Calis, 2000; Filippatos et al. 2008). Consumption of synthetic drugs like orlistat may cause side effects such as nausea, gastric irritation, hyperuricemia, diarrhea, nyositis, flushing, dry skin, oily spotting, flatus with discharge and fecal incontinence (Kumar et al. 2008; Filippatos et al. 2008). Chia seeds like many other plant species have high phytochemical contents namely flavonoids, isoflavones, athocynins, proanthocyanidins, flavanols which are reported to exhibit anti-diabetic properties. Inhibitors from natural sources can provide less toxic effects compared to synthetic ones (Kumar et al. 2008).

2.7 Implication of Plasma Glucose, Triglycerides and Cholesterol in CVD Risk Factors

Epidemiological evidence suggests that postprandial glycaemia is an independent and modifiable predictor of cardiovascular disease (Lee, 2009). Studies show that, even after adjusting for other cardiovascular risk factors, the relationship between 2-hour postprandial glycaemia and cardiovascular risk remains direct and continuous, extending below and beyond the cut-off points for impaired glucose tolerance (7.8 mmol/L-11 mmol/L). There is growing evidence that whole grains may play an important role in the prevention of chronic disease. Since chia is an exceptionally

rich source of dietary fiber, vegetable protein, PUFAs, calcium, magnesium, iron and antioxidants, its incorporation in diets may lower CVD risk factors (Vuksan et al. 2007).

High triglyceride levels are markers for atherogenic lipoproteins, particularly important in patients with insulin resistant, type 2 diabetes mellitus and other metabolic syndrome commonly present with combined dyslipidemia characterized by fasting and postprandial hypertriglyceridemia, low HDL-C, small LDL and VLDLs) (Talayero & Sacks, 2011). These patients are at great risk for CVD even if LDL-C levels are within the borderline. High blood levels of LDL-C and VLDL promote atherosclerosis, especially when lipoproteins are oxidized. On contrary, HDL-C help prevent the oxidation of LDL-C and also remove cholesterol from circulation, thus low levels of HDL-C contribute to the development of atherosclerosis (Cai, Shi, Xue & Lu, 2017). To control CVD risk factors, understanding the coronary heart disease risk assessment is important (Table 2.2).

Table 2.3: Standards for Coronary Heart Disease Risk Assessment

Clinical measure	Desirable	Borderline	High risks
Body mass index (BMI)	18.5-24.9	25-29.8	≥ 30
Blood pressure (Systolic/Diastolic) (mmHg)	< 120/80	120-139/80-89	≥ 140/90
Total blood cholesterol (mg/dL)	< 200	200-239	≥ 240
LDL cholesterol (mg/dL)	< 100	130-159	160-189
HDL cholesterol (mg/dL)	≥ 60	40-59	< 40
Fasting triglycerides (mg/dL)	< 150	150-199	200-499

Source: (Rolfes *et al*. 2009)

Tenore et al. (2018), reported the significance of chia seed diet in lowering triacylglycerol levels, LDL-C and its derived fatty acids in rat serum. In another study, dietary chia seeds prevented the onset of dyslipidemia and insulin resistance (IR) in the rats fed with the sucrose-rich diet (Chicco, D'Alessandro, Hein, Oliva & Lombardo, 2009). Additionally, plasma cholesterol has been associated with cardiovascular risks; this is because the total plasma cholesterol correlates with the presence of lipoproteins (LDL-C and small dense LDL in particular) that may

become trapped in the intima of coronary and other arteries to form atherosclerotic plaque (Nordestgaard & Varbo, 2014). Saturated fats have strong effect on blood cholesterol levels so its replacement by omega 3-PUFAs from natural food sources such as that contained in chia seeds can generally lower LDL-C levels (Abdulrashed et al. 2016).

2.8 Effect of Plant Extracts on Hematological Parameters and Liver Enzymes

Hematological parameters are marked as good indicators of the physiological status of animals, they are of great significance in routine clinical evaluation of the state of health (Etim, 2014). Examining blood composition can offer significant information for the diagnosis and prognosis of diseases in animals. Afolabi et al. (2011) highlighted that, variations in hematological parameters are regularly used to determine various status of the body as well as stress due to pathological, nutritional and environmental factors.

Chia seeds are rich in omega 3 fatty acids that play a key role in preventing the formation of clots and plaques in the arteries and so help to prevent cardiovascular diseases (Prathyusha, Kumari, Suneetha & Sai, 2019). Hematological determination includes red blood cells (RBC) count, blood hemoglobin concentration (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), total leukocyte count (TLC), Neutrophils (NEUT), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), platelets (PLT) and mean platelet volume (MPV) (Andrew, Joseph, Daniel & Millicent, 2016).

Erythrocytes, leucocytes and platelets are of great concern. Erythrocytes are responsible for transporting oxygen and other dissolved substances in different parts of the body. Leucocytes are the main cells of the immune system that provide innate and specific adaptive immunity (Gitahi & Kiambi, 2015). Some leucocytes are atherogenic and some sustain inflammation, for instance hypercholesterolemia increases circulating monocyte counts and renders these cells more prone for emigration into atherosclerotic lesions (Soehnlein, 2012). On the other hand, platelets play a major role in hemostasis, thrombosis, clot retraction, vessel constriction and

repair (Maina & Kiambi, 2015). For liver enzymes, levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP) and bilirubin in the blood can be used to indicate hepatocellular injury, which in turn is associated with insulin resistance. These enzymes have been considered as important indicator of liver function (Yilmaz, Temel, Gürsoy & Dogan, 2013).

Gamma-glutamyl transferase circulates on the external surfaces of most cells, particularly hepatocytes and in serum, it is used as a common biomarker of liver injury and excessive alcohol intake (Park, Bae, Park, & Chae, 2020). ALT and AST are found within hepatocytes, their role is to catalyze the transfer of amino groups to generate products in gluconeogenesis and amino acid metabolism (Hall & Cash, 2012). Changes in ALT and AST reflect hepatocyte injury and liver function (Kim et al. 2019). ALP catalyzes the hydrolysis of inorganic pyrophosphate, which is a vascular calcification inhibitor. Serum levels of ALP are commonly used in clinical practice as a marker of liver or bone disease (Kunutsor, Apekey, Seddoh & Walley, 2014).

Bilirubin is a byproduct of the routine destruction of erythrocytes occurring in the liver. It is normally released as bile in the feces. It is generally regarded as a waste product with little or no physiological purpose and can be toxic if it accumulates (McArdle et al. 2012; Gupta et al. 2016). However, various studies reported an inverse association of elevated bilirubin with cardiovascular disease (McArdle et al. 2012; Kang et al. 2013). Recent studies have shown that, high bilirubin exhibit anti-oxidant, anti-inflammatory, vasodilatory, anti-apoptotic and anti-proliferative functions. It suppresses the oxidation of lipids and lipoproteins thus preventing plaque formation and subsequent formation of atherosclerosis (Gupta et al., 2016; Jayanthi et al. 2018).

Albumin is a common protein found in the blood with different functions. It is produced only in the liver; low levels of albumin can be a suggestive of chronic liver disease or liver cirrhosis (Thapa & Walia, 2007). The association between high ALT and CVD is mostly due to underlying hepatic inflammation or non-alcoholic fatty

liver disease (NAFLD) that are more prevalent in subjects with elevated levels of ALT (Ndrepepa & Kastrati, 2019). Accumulation of liver fat and elevated liver enzymes are associated with type 2 diabetes and hypertension both of which are representative risk factors for CVD. Previous studies have suggested a potential link between NAFLD and CVD. NAFLD is associated with vascular inflammation, which may reflect rupture-prone vulnerable atherosclerotic plaques (Ghouri, Preiss & Sattar, 2010).

Targher & Byrne, (2015), reported on the mechanism linking GGT and CVD risk. The GGT located in arterial atheromatous plaques has been shown to stimulate the low-density lipoprotein oxidation through a redox reaction leading to further development of atherosclerotic plaques. It has been proposed further that the hydrolysis of glutathione produces cysteinylglycine, which is a powerful reductant of $Fe^{3+,}$ which is present within the atherosclerotic plaque, this result into the production of Fe^{2+} and a free thiyl radical. Consequently, reactive oxygen species, produced from similar reaction, contribute to a pro-oxidant effect, leading to low-density lipoprotein oxidation (Targher & Byrne, 2015). Both visceral fat accumulation and risk of metabolic syndrome are considerably correlated with GGT, AST and ALT levels (Figure 2.6). GGT has pro-oxidant and proinflammatory activities, which play important role in GSH homeostasis, influencing the antioxidant capacity of glutathione hemocystine (Liu,Yeung, Lin, Leung & Schooling, 2016; Liu, 2013). In the presence of iron or other transition metals, reactive oxygen species are generated with the presence of GGT (Rahmani et al. 2019). Increased GGT levels predispose plaque formation in the atherosclerotic process. Thus the reason why GGT elevation is linked to an increased risk for CVD but also for T2DM and metabolic syndrome (Mirmiran, Gaeini, Bahadoran, & Azizi, 2019).

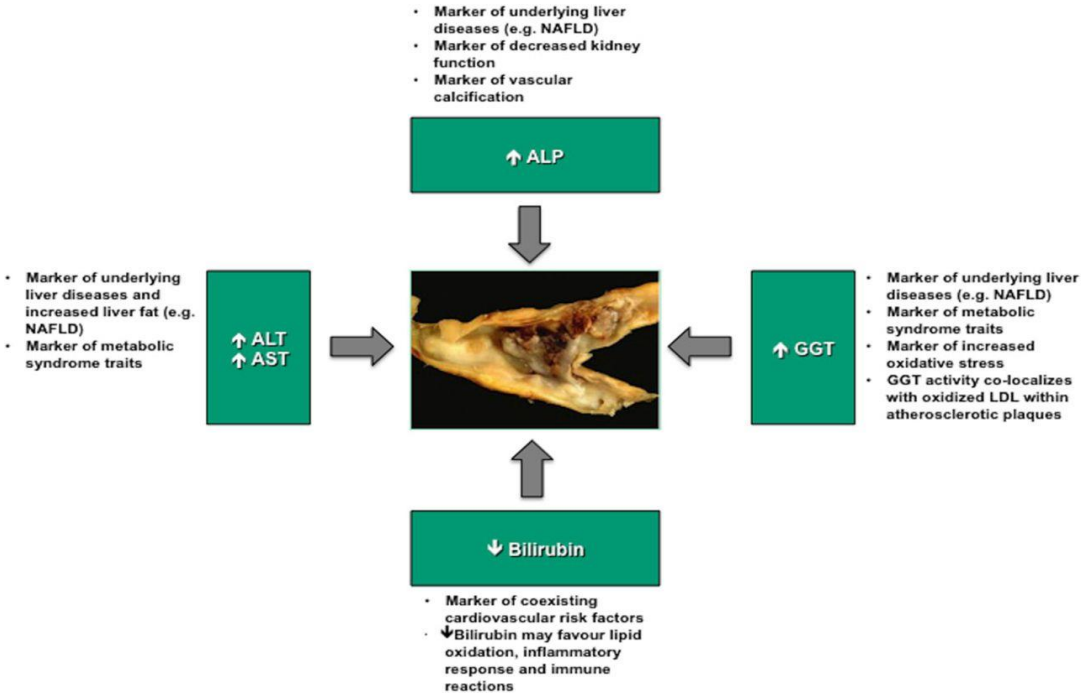

Figure 2.8: Lower and Higher Levels of Liver Enzymes are Associated with an Increased Risk of Future Cardiovascular Disease Events

Source: (Targher & Byrne, 2015)

2.9 Body Weight, Atherogenic Index and Coronary Risk Index as Related to CVDs

Weight change can be determined by multiple factors such as fat intake and total energy intake; all appears important for the successful long-term control of body weight. Weight loss interventions can prevent serious harm for people with obesity and CVD (Chenhan et al. 2017). There is evidence that even modest weight loss at least 5-10 % can reduce CVD risk even when the patient remains in the obese state (Brown, Buscemi, Milsom, Malcolm & O'Neil, 2016). Diets play a big a role in weight control. Preferences on lower fat and low sugary foods is considered the best alternative towards weight control. Alterations should also be made to decrease the amount of solid fats that contain saturated and trans fats to oils containing polyunsaturated and monounsaturated fats to improve diet quality and overall health (Brown et al. 2016). The diets rich in fiber such as cereals, nuts, fruits and vegetables have a positive effect on health since their consumption has been related

to decreased incidence of several diseases like diabetes, heart disease and bowel cancer (Dhingra, Michael, Rajput & Patil, 2012).

Dietary fiber promotes the feeling of fullness by increasing transit time, promoting stomach expansion and hence decreasing energy intake by slowing down absorption efficiency (Clark & Slavin, 2013). On the other hand, Coronary risk index (CRI) and Atherogenic Index (AI) have been reported by previous studies as the marker to predict the risk of atherosclerosis and coronary heart disease. An AI value of less than 0.11 is associated with low risk of CVD while values ranging from 0.11 to 0.21 are associated with intermediate CVD risks and those above 0.21 are associated with increased risks. AI is calculated as a ratio of LDL-C/HDL-C that is often used to estimate cardiovascular disease risk. On the other hand, high level of TG has been related with an increased LDL-C particles and increased cardiovascular risk, so CVD risk can be estimated by calculating CRI, which is the ratio of TG/HDL-C and/or TC/HDL-C (Adeneye & Olagunju, 2009).

2.10 The Use of Chia Seeds in Food Industry

Chia seeds have been used as a food supplement particularly to vegetarians and gluten-free diets. It has also been used as an ingredient in breads, confectionary items (cakes, muffins, breakfast cereals and nut mixes). Additionally, the seeds can be used as thickeners for sauces and puddings, or a substitute for jellying agents in jam and jellies due to its ability of forming a jelly like substance in water (Coorey, Grant & Jayasena, 2012). Chia seeds have also been used as a nutritional supplement (Pintado, Herrero, Jiménez-Colmenero & Ruiz-Capillas, 2016). Athletes use chia seeds as natural form of energy that enhances stamina. People with health discomforts use chia as a supplement to improve digestive system, regulate blood sugar for diabetics and improve cardiovascular health and healthy weight regulations.

2.11 The Use of Rat Model in Experimental Studies

Rats continue to be used as experimental models in a wide range of scientific and medical disciplines, including neurosciences, nutrition studies, pharmacology, genetics, behavioral psychology and medical sciences (Kuramoto et al. 2012).

Rats have been used in many experimental studies for understanding diseases, genetics, the effects of drugs and other subjects in health and medicine. The Albino Wistar rat was first bred at the Wistar Institute in 1906 for use in biological and medical research, hence the origin of its name (Krinke, 2000). The male Wistar rat (Figure 2.7) is a good model for studying effects of diet on disease, since it develops all aspects of metabolic abnormalities when put on a high fat and fructose diet (HFFD) (Reuter, 2007). Rats can be used to simulate human disease when exposed to similar conditions to humans, they allow the study of chronic illnesses such as diabetes and obesity, they are able to produce symptoms which are predictable and controllable, they satisfy economical, technical, and animal welfare considerations and they allow the measurement of relevant biochemical and hematological parameters (Srinivasan & Ramarao, 2012).

Figure 2.9: Male Wistar Rats

Although rats can be either model (be diet induced or genetically engineered models of disease), it is recommended to use diet induced models of disease rather than the genetically modified models (Reuter, 2007). The major reason is that, the genetically engineered models differ in many aspects with a typical patient whose disease develops slowly and only under certain environmental conditions. Therefore, the diet-induced models represent the phenotypic developments of a patient who is developing for example from obesity to insulin resistance and type 2 diabetes under

conditions of high fat and energy consumption. Moderate consumption of fructose, may contribute to the onset of metabolic syndrome which may further be aggravated by consumption of a high fat diet (Kiage-Mokua et al. 2018).

Previous findings highlight that a combination of a HFFD may exert deviating effects of the metabolism of glucose in rats (Huang, Chiang, Yao & Chiang, 2004). However, it is not easy to compare metabolic alterations in rats fed a HFFD due to differences in study designs. These differences include: rat strains used such as Albino Wistar rat and Sprague-Dawley; the age of the animals at the beginning of the experiment; the amount of fat/fructose given, some give fructose with drinking water while others mix in the diets; The duration of experiment for fructose and fat administration is also not standardized because it may differ from 4-8 weeks to even months; the control diet is also not standardized. Therefore, due to all these experimental variations, they result in varied physiological responses.

There are number of animal models (rat models) used for various human diseases, however, no model is able to exactly mimic human illnesses in all its aspects, this may be due to the fact that not all changes in human disease are fully understood hence cannot be reflected in rats (Srinivasan & Ramarao, 2012). Moreover, many rat models produce pathophysiological changes that are rare in human disease states (Ghosh, Golbidi, Werner, Verchere & Laher, 2010). It is therefore important to understand well the advantages and limitations of each model before extrapolating the results to humans (Srinivasan & Ramarao, 2012; Kiage-Mokua et al. 2018).

CHAPTER THREE

PROXIMATE COMPOSITION, MINERAL CONTENTS, FATTY ACIDS PROFILE AND QUALITATIVE PHYTOCHEMICAL SCREENING OF CHIA SEEDS GROWN IN EAST AFRICA

3.1 Introduction

There is a growing need for functional foods worldwide. Functional foods are foods thought to have physiological benefits beyond their basic nutritional functions (Alissa & Ferns, 2012). Chia (*Salvia hispanica L*) is an example of a functional food that has been linked to various health benefits due to its nutrient composition. It belongs to a species of flowering plants placed in family lamiceae, originating from Mexico and Northern Central America (Ixtaina, Nolasco & Tomás, 2008) (Sosa-Baldivia et al. 2018). It is an annual plant commercially grown for its seeds with black chia being dominant compared to white ones (Mohd et al. 2012). Chia is a drought-resistant crop and can grow well in arid environments. Its minimum and maximum growth temperatures are between 11-36 ^{o}C, with an optimum range between 16 ^{o}C and 26 ^{o}C. Chia plant grows well in sandy, well-drained soils with moderate salinity and a pH ranging from 6-8.5 (Antruejo et al. 2011; Yeboah et al. 2014; Lovelli et al. 2015).

With time, several studies have revealed the significance of using chia seeds to enrich diets, as they are high in omega-3 polyunsaturated fatty acids (n-3 PUFA) especially α-linolenic acid (ALA), antioxidants, dietary fiber, proteins and phenolic compounds (Segura-Campos et al. 2014). The ALA is essential for maintaining a healthy cholesterol level, brain development and immune system (Creus, Ferreira, Oliva & Lombardo, 2016). The seeds also contain vitamins (vitamin E and B_1) and minerals such as potassium, calcium, phosphorus, magnesium, zinc and iron (Yeboah et al. 2014; Amato et al. 2015; Giaretta, Lima & Carpes, 2018). Production and marketing of chia has spread in various parts of the world like Paraguay, Argentina, Bolivia, Peru, Australia, Colombia, Nicaragua, Africa and Europe (Lovelli et al. 2015; Julio et al. 2016).

In East Africa, The Chia Company (TCC) officially introduced chia in 2012, targeting farmers from Kenya, Tanzania and Uganda. Recently, the crop has become popular in Uganda and Kenya (Mihafu et al. 2019). Chia seeds play an important role as a functional food and nutritional supplement. However, its nutritional composition and concentration of bioactive compounds depend on several factors such as climatic conditions, soil type, geographical area, agricultural practices and to some extent the extraction methods (Ayerza & Coates, 2009; Ayerza, 2010; Suri, Passi & Goyat, 2016). Based on previous studies, chia seeds do not contain anti-nutrients, toxic compounds and gluten thus considered a safe ingredient for gluten-free diets (Menga et al. 2017). There is also a conscious hesitation for marine foods among some people due to xenobiotic chemicals and bio-magnification of heavy metals in fish. Hence, in this case, chia seeds may be the best substitute for dietary fish. This study is therefore aimed at determining the nutrient and phytochemical composition of chia seeds grown in East Africa and further adding to the body of knowledge on using chia for human health.

3.2 Material and Methods

3.2.1 Sample Collection, Preparation and Storage

The dried black chia seeds were purchased from farmers based at Molo in Nakuru County, and Among'ura and Ongariama-Teso in Busia County all in Kenya. Other black seeds and white seeds were purchased from Dr. Chia, the Kenyan company pioneering the seed's commercialization in East Africa based in Nairobi Central Business District, who outsource the seeds from Bukembo and Kigumba in Uganda. The purchased chia seeds were placed in khaki bags and packed in ziplock bags and stored at JKUAT Food Science and Technology Analysis Laboratory. The seeds were cleaned by removing all the impurities and damaged seeds. Thereafter, the seeds were crushed using a grinder (Moulinex grinder, AR11, China) and the flour packed in khaki bags (Paper bags Ltd, Kenya) and stored in a cool dry place until use.

3.2.2 Chemicals and Reagents

All reagents and chemicals used in the study were of analytical grade and purchased from Sigma Aldrich, USA and Loba Chemies PVT Ltd, India.

3.2.3 Proximate Analysis

All proximate analyses and qualitative phytochemical screening were conducted at JKUAT Food Science and Technology Analysis Laboratory.

3.2.3.1 Moisture

The moisture content was determined by the Oven drying method (AOAC, 2005). About 5 g of samples (W1) were weighed on pre-weighed aluminum dishes and placed in an oven (Memmert GmbH + Co.KG, Germany) set at 105^{o}C for 4 hr. The samples were removed from the oven and cooled in a desiccator for 30 minutes. The dried samples (W2) were weighed and percentage moisture content calculated as follows;

$$\% \, Moisture = \frac{W1 - W2}{W1} \, x \, 100$$

3.2.3.2 Ash

Total ash was determined by incineration of 3 g sample in an Electric Muffle Furnace (Advantec KL-420, Japan) set at 550^{o}C for 12 hr. The percentage ash was calculated as;

$$\% \, Ash = \frac{Weight \; after \; ashing}{Weight \; of \; sample} \, x \, 100$$

3.2.3.3 Crude Fiber

The crude fiber content was determined by boiling 2 g of the samples in 1.25 % H_2SO_4 and 1.25 % NaOH. The samples were first boiled in 1.25 % H_2SO_4 for 30

min, filtered using glass wool and the filtrate discarded. The residue together with glass wool were boiled in 1.25 % NaOH for 30 min, filtered and the residue retained. Hot water passed through the residue followed by ethanol and petroleum ether to remove pigments and lipids. The residue was transferred into clean dry crucibles and dried in an oven at 105^{o}C for 30 min to remove water and volatile materials. The samples were cooled in a desiccator and then weighed (W1). The cooled content in crucibles was incinerated in Muffle furnace (Advantec KL-420, Japan) at 550^{o}C for 30 min. After incineration, the contents were cooled in a desiccator and weighed (W2). The percentage crude fiber calculated as;

$$\%\ Crude\ fiber = \frac{W1}{W2}\ X\ 100$$

3.2.3.4 Crude fats

Crude fat was determined using Soxhlet apparatus (Memmert Soxhlet extractor and a Water bath (Ikeda Scientific Co. Ltd, Germany). Approximately 2 g of sample (W1) was weighed into extraction thimbles and covered with glass wool. Round bottom flasks were weighed (W2) and 100 ml of petroleum ether was added to each flask. The flasks containing samples were fitted to the extractor connected to the condenser. Cold water for cooling was allowed to run at a steady rate throughout the condenser. The heater turned on and adjusted so that the solvent boiled gently and its vapour condensed back into the thimbles with samples. After 8 hr reflux, the samples in the flasks were cooled and connected to rotary evaporator (Bibby sterilin Ltd) and evaporated to dryness. The samples in flasks were dried in an air oven at 70^{o}C for 1 hr, then cooled in a desiccator and weighed (W3). The percentage fats calculated as follows;

$$\%\ Crude\ fat = \frac{W3 - W2}{W1}\ x\ 100$$

3.2.3.5 Crude Proteins

About 1.5 g of samples were placed in the digestion tubes and 15 ml H_2SO_4, 0.5 g $CuSO_4$ and 5 g K_2SO_4 were added into the digestion tubes and fixed on the digester (Heating Digester DK 6, Velp Scientifica, Italy). The digester was switched on to start the digestion process for 1.5 hr. After that, the digester was switched off, samples cooled and diluted to 100 ml distilled water. The diluted samples were put under distillation process using 40 % NaOH, 4 % Boric acid (H_3BO_3) and a mixed indicator (Bromocresol green + methyl red + thymol blue). During distillation, 60 ml was collected for each sample and used for titration. Hydrochloric acid (0.02N) and a mixed indicator were used for titration process. Titre values were recorded and used for calculations as follows; % N = (V-B) x C (mol/L) x MwN/L x f x 100/S

Where, V- volume of titrant, B- volume of blank titre, C- concentration of acid, MwN/L – molecular weight of Nitrogen per liter, f- dilution factor, S- Sample weight.

% N= [(V-B) x 0.02 x 0.014 x 10 x 100]/S; % Crude protein = % N x Protein factor (6.25).

3.2.3.6 Carbohydrates

The total carbohydrate was calculated by difference as a remainder percentage of sums of all the other components (moisture, ash, fats, fiber and proteins) (Hart & Fisher, 2012). All proximate determinations were done in duplicates except moisture and ash that were done in triplicates.

3.2.4 Mineral Determination

The ash of each sample was dissolved in 100 ml 1N nitric acid, the solution mixture filtered through Whatman paper no. 42 and stored in 150 ml plastic bottles with tight lids. All minerals determination was done in triplicate. Calcium, magnesium, potassium, iron, zinc, manganese and copper were analyzed by Atomic absorption spectrophotometer-AAS (AA-7000 Shimadzu, Japan) with appropriate standards (García & Báez, 2012). Total phosphorus was determined by a pH adjustment

method using ammonium molybdate/ammonium vanadate mixed reagent (Okalebo, Gathua & Woomer, 2002).

3.2.5 Determination of Fatty Acid Profile

The fatty acid methyl esters (FAMEs) were prepared from the extracted lipids by the esterification reaction according to a modified method described by (Bligh & Dyer, 2010). The analysis was done by the Gas chromatography (GC-2010 PLUS AF 230V, Shimadzu, Japan). Condition of GC: Injector AOC -20i; autosampler AOC-20S. Mobile phase- nitrogen gas, detector- FID (Hydrogen + compressed air). Column Supelco-UK, Size 30 m x 0.53 mm x 0.5 µL; column initial and final temperature was 170^{o}C and 230^{o}C respectively. Column injector temperature was 240^{o}C; column detector emperature was 260^{o}C, and column program rate was 5^{o}C/min. Individual fatty acids were expressed as the mass of fatty acid in 100 g of oil. These were conducted at JKUAT Food Science and Technology Instrumental Laboratory

3.2.5.1 Extraction of Total Lipids

Approximately 2 g of milled chia was weighed and placed into 45 ml centrifuge tubes and denatured over boiling water (100^{o}C) for 3 min. Then 4 ml of water and 15 ml of 2:1 (v/v) methanol-chloroform mixture was added and the mixture shaken thoroughly on a shaker at room temperature for 2 hours. The contents were centrifuged at 3000 rpm for 10 min, and the supernatant decanted into 50 ml centrifuge tube, the residue was re-suspended in 9.5 ml of methanol-chloroform-water (2:1:0.2 v/v) and shaken to homogenize and centrifuged, again the supernatant was removed and combined with the first extract followed by addition of 7.5 ml of chloroform and water, shaken and centrifuged. The chloroform phase was withdrawn using a Pasteur pipette and put into a pre-weighed pear-shaped 50 ml flask and evaporated to dryness on a vacuum rotary evaporator (Bibby sterilin Ltd, UK) at low temperature.

3.2.5.2 Preparation of Fatty Acids Methyl Esters (FAME)

Approximately 40 g of the extracted oil was weighed in conical fask, 8 ml of methanolic HCl (95 ml: 5 ml) solution was added and heated under reflux for 1.5 hours and cooled under tap water. The solution was transferred into a separating funnel and 8 ml of hexane added, shaken vigorously and left to stand. The hexane layer was collected and the aqueous layer returned to repeat the extraction one more time. The hexane fractions were combined and washed with 4 portions of distilled water to remove acid and then filtered using defatted cotton wool and anhydrous sodium sulphate to remove water. Thereafter, the filtrate was concentrated on a rotary evaporator at 40^{o}C and about 1 ml of the concentrate was placed in vials and stored in a freezer before analysis by gas chromatography (GC). This was conducted at JKUAT Food Science and Technology Analysis Lab.

3.2.6 Qualitative Phytochemical Screening of Chia Seeds

About 20g of ground chia seeds were mixed with 100 ml of absolute ethanol and the mixtures shaken for 60 minutes in a shaker (Kika Labortechnik, KS 250 basic, Germany) at room temperature. The mixtures were centrifuged (Hettich zentrifugen, Universal 16A, Germany) at 4000 rpm for 20 minutes while the supernatant was collected and concentrated in a Rotary Evaporator RE 100 (Bibby sterilin Ltd, UK). The concentrate was kept at 40^{o}C in the fridge (Hisense, RD-28DR4SA, China). Phytochemicals were determined by modified methods (Prashant Tiwari et al. 2011; Thakur & Sidhu, 2014; Banu & Cathrine, 2015) as described below;

3.2.6.1 Test for Flavonoids

NaOH test: To 3 ml of crude seed extract, 1 ml of 10 % aqueous NaOH solution was added. When a slight intense yellow color was observed which turned colorless on the addition of dilute HCl, it indicated the presence of flavonoids.

3.2.6.2 Test for Alkaloids

Mayer's reagent test: To 3 ml of crude seed extract in a test tube, 1 ml of 1% HCl was added. The mixture was then heated gently for 20 min, cooled and filtered. Then

2 drops of Mayer's reagent were mixed with 1ml of the filtrate. Occurrence of turbidity/creamy precipitate indicated the absence of alkaloids.

3.2.6.3 Test for Gums and Mucilage

To 1 ml crude seed extract, 2.5 ml of absolute ethanol was added with constant stirring. The formation of precipitates indicated the presence of gums and mucilage.

3.2.6.4 Test for Glycosides

$FeCl_3$ test: To 2 ml of crude seed extract, 5 ml of concentrated H_2SO_4 was added and boiled for 15 minutes in a water bath. This mixture was cooled and neutralized with 10 % NaOH followed by the addition of 3 drops of 5 % $FeCl_3$ to one half of the mixture. The formation of green to black precipitates indicated the presence of glycosides.

3.2.6.5 Test for Phenol

$FeCl_3$ test: To 3 ml of crude seed extract, 2 drop of 10% $FeCl_3$ was added. The appearance of blue-green color indicated the presence of phenol.

3.2.6.6 Test for Phlobatannins

To 1ml of crude seed extract, 2 drops of 2 % aqueous HCl was added and boiled for 2 min. The appearance of white color instead of red precipitates indicated the absence of phlobatannins.

3.2.6.7 Test for Saponins

Froth test: 2 ml of crude seed extract was taken in a test tube and shaken vigorously; froth formation indicated the presence of saponins.

3.2.6.8 Test for Steroids

H_2SO_4 test: To 1 ml of crude seed extract, 6 drops of concentrated H_2SO_4 were added along the sidewalls of the test tube. The appearance of red color indicated the presence of steroids.

3.2.6.9 Test for Tannins

$FeCl_3$ test: To 1 ml of crude seed extract, 2 drops of 5 % $FeCl_3$ was added, occurrence of pale dark green color indicated the presence of tannins.

3.2.6.10 Test for Terpenoids

Salkowaski test: To 1 ml of crude seed extract, 2 ml of chloroform was added and mixed thoroughly, followed by addition of 3 ml of concentrated H_2SO_4 along the sides of test tube. The appearance of reddish-brown at interface indicated the presence of terpenoids.

3.2.7 Statistical Analysis

STATA 12.0 Corp LP was used for statistical analysis of samples. Determinations for moisture and ash were done in triplicate while for fat, crude fiber and proteins were done in duplicate. A one-way analysis of variance (ANOVA) used Bonferroni multiple comparison test to determine the variability between group means. Significance was accepted at $p \leq 0.05$.

3.3 Results and Discussion

3.3.1 Proximate Composition

The average moisture, ash, protein, fat and fiber contents ranged from (6.17 -7.10 %); (2.79-4.19 %); (20.10-21.87 %); (27.51-33.10 %) and (24.56 -33.44 %) respectively (Table 3.1). White chia Bukembo (WCB) had the highest moisture content than black chia Among'ura-Teso (BCAT), black chia Kigumba (BCK), black chia Molo (BCM) and Black chia Ongariama-Teso (BCOT) as shown in Table 3.1.

The moisture, ash and protein contents were close to 7.86 %, 3.63 %, and 21.52 %, respectively as reported previously by Sargi et al. (2013). High fiber (33.44 %) and low-fat contents (27.51 %) were observed in WCB. Carbohydrate that was obtained by a difference ranged from (7.24 -14.07 %). This amount is lower compared to 19.24 % and 34.57 % reported by Marineli et al. (2014) and Owaga, Kibui & Mburu, (2018). There was no significant difference between the samples for moisture content, ash, protein, and carbohydrates respectively.

However, this is not surprising because there are many factors that can affect nutritional composition and concentration of bioactive compounds in chia seeds such as climatic conditions, soil type, geographical area, agricultural practices and to some extent the extraction methods (Ayerza & Coates, 2009; Suri et al. 2016). Although the fat content of WCB was not statistically significant different from that of BCAT, BCK, and BCM, it was significantly different ($p = 0.023$) with BCOT. Moreover, fiber content of WCB was significantly different ($p = 0.005$) with that of BCM and ($p = 0.003$) for BCOT (Table 3.1). Again, in this case, since the seeds were collected from Kenya and Uganda, factors like variety, climatic conditions and agricultural practices might have contributed to their differences in composition too. Indeed, results on proximate composition support other findings that chia seeds are rich in proteins, fats and fiber particularly insoluble fiber (Medic, 2014; Coelho & Salas-Mellado, 2014).

Table 3.1: Proximate Composition of Milled Chia Seeds

Sample	Proximate composition (%)					
	Moisture	Ash	Fat	Protein	Fiber	Carbohydrate
BCAT	6.27 ±0.98^{a}	3.67 ± 0.22^{a}	29.83 ± 1.10^{ab}	20.41± 0.48^{a}	31.15± 2.13^{b}	8.67 ± 2.52^{a}
BCK	6.77 ± 0.08^{a}	2.79 ± 0.59^{a}	30.99 ± 0.51^{a}	21.87± 0.28^{a}	28.01±0.15^{cb}	9.56 ± 1.11^{a}
BCM	6.17 ± 0.08^{a}	3.24 ± 0.68^{a}	30.86 ± 1.22^{a}	20.10± 0.05^{a}	25.57± 0.05^{c}	14.07 ± 2.22^{a}
BCOT	6.25 ± 0.06^{a}	3.71 ± 0.20^{a}	33.10 ± 0.26^{a}	20.60± 1.02^{a}	24.56± 0.39^{c}	11.78 ± 1.53^{a}
WCB	7.10 ± 0.37^{a}	4.19 ± 0.16^{a}	27.51 ± 1.32^{b}	20.53± 0.81^{a}	33.44±0.13^{ab}	7.24 ± 2.05^{a}

Values are means ± SD (standard deviation) of triplicate (ash and moisture) and duplicate determinations; Means with the same superscripts within the same column are not significantly different ($p \leq 0.05$). BCM- Black chia Molo, BCOT- Black chia Ong'ariama-Teso, BCAT- Black chia Among'ura-Teso, BCK- Black chia Kigumba and WCB-White chia Bukembo.

3.3.2 Mineral Content of Chia

The concentration of minerals in the chia samples is shown in Table 3.2. All samples indicated high contents of potassium (492.96-862.98 mg/100g), phosphorous (486.45-569.45 mg/100g), calcium (297.47- 429.09 mg/ 100g) and magnesium (192.22-202.97 mg/100g. Furthermore, the amount of iron, zinc, manganese, and copper was also considerable high. Notably, BCM had higher contents of K, Mg, Fe, Zn, and Cu compared to other tested samples and the difference was statistically significant ($p = 0.0001$). Generally, WCB had low mineral contents compared to BCAT, BCK, BCM and BCOT. However, the content of phosphorous between samples was not significantly different at $p \leq 0.05$.

Previous studies have reported that the amount of phosphorus, calcium and potassium in chia seeds is 6-9 times more than that contained in wheat, rice, oats and corn, while iron content is higher than that of spinach and lentils (Ullah et al. 2016). Of interest was that the mineral content between BCM and WCB was significantly different ($p = 0.0001$) except for phosphorus. Therefore, this indicates that seed variety can mark a significant difference in nutrient composition.

Since calcium, phosphorus, and magnesium are important for bone formation, chia seeds and especially the black chia may be a good source of minerals responsible for maintaining bone health.

Table 3.2: Mineral Contents of Chia Seeds

Mineral (mg/100g)	Sample				
	BCAT	BCK	BCM	BCOT	WCB
P	493.3 ± 0.14^{a}	486.2 ±0.42^{a}	515.1 ± 0.28^{a}	563.4 ± 0.14^{a}	569.5 ± 0.14^{a}
K	649.05 ± 0.28^{b}	644.05 ± 0.21^{b}	862.10 ± 0.35^{a}	492.05 ± 0.21^{b}	523.1 ± 0.28^{b}
Ca	304.15 ± 0.35^{b}	297.05±0.36^{b}	429.02± 0.55^{a}	393.21 ± 0.35^{a}	300.85 ± 0.35^{b}
Mg	195.15 ± 0.35^{b}	200.05 ± 0.36^{b}	295.22 ± 0.42^{a}	192.1 ± 0.28^{b}	202.15 ± 0.35^{b}
Fe	3.63 ± 0.09^{b}	3.82 ± 0.42^{b}	5.45 ± 0.29^{a}	3.23 ± 0.07^{b}	2.1 ± 0.04^{c}
Zn	2.78 ± 0.17^{b}	2.70 ± 0.26^{b}	4.37 ± 0.04^{a}	3.16 ± 0.14^{b}	2.79 ± 0.03^{b}
Mn	2.69 ± 0.05^{b}	2.67 ± 0.02^{b}	3.11 ± 0.06^{b}	1.89 ± 0.17^{c}	3.98 ± 0.29^{a}
Cu	0.81 ± 0.02^{b}	0.79 ± 0.01^{b}	1.37 ± 0.21^{a}	0.92 ± 0.04^{b}	0.49 ± 0.06^{b}

Values are means± SD (standard deviation) of triplicate determinations; Means with the same superscripts within the same row are not significantly different ($p \leq 0.05$). P- phosphorous, K- potassium, Ca- calcium, Mg- magnesium, Fe- iron, Zn- zinc, Cu- copper and Mn- manganese.

3.3.3 Fatty Acid Profiles

Results on fatty acid profile indicated the presence of lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, α-linolenic and arachidic in all tested samples (Table 3.3). However, α-linolenic (45.29-56.99 %), linoleic (15.9-20.28 %) and oleic (6.88-11.58 %) were abundant (Table 3.3) while low content of saturated fatty acids palmitic (6.21-8.53 %) and stearic (2.94-5.36 %) was observed in all samples. The amount of ALA was similar to that reported by Ding et al. (2018). Furthermore, the amount of LA and ALA in all tested samples was close to (17-26 %) and (50-57 %) respectively as previously cited by (Saphier, Kamer, Tavor, Silberstein & Ben-Abu, 2017). Although the amount of ALA was high compared to other fatty acids analyzed in all samples, the difference was not statistically significant ($p = 0.7391$).

The BCK had the highest amount of ALA (56.99 %) but this was lower than 63.10-68.5 % previously reported by Ayerza, (2013); Segura-Campos et al. (2014) and Owaga et al. (2018). However, in these studies, the contents of linoleic and palmitic acids were comparable to what observed in this study.

Table 3.3: Fatty Acids Profile of Chia Seeds

Fatty acids (%)	Sample				
	BCAT	BCK	BCM	BCOT	WCB
Lauric	2.8 ± 0.95[a]	0.12 ± 0.16[a]	0.17 ± 0.10[a]	8.93 ± 0.16[a]	0.23 ± 0.02[a]
Myristic	0.35 ± 0.26[a]	0.17 ± 0.15[a]	0.35 ± 0.26[a]	1.07 ± 0.08[a]	0.50 ± 0.04[a]
Palmitic	6.33 ± 0.55[a]	6.21 ± 0.11[a]	7.48 ± 0.13[a]	8.53 ± 0.36[a]	7.15 ± 0.60[a]
Palmitoleic	0.21 ± 0.03[a]	0.15 ± 0.83[a]	0.16 ± 0.04[a]	0.90 ± 0.03[a]	0.18 ± 0.01[a]
Stearic	5.36 ± 0.38[a]	4.26 ± 0.76[a]	4.54 ± 0.79[a]	2.94 ± 0.44[a]	4.81 ± 0.40[a]
Oleic	10.76 ± 0.11[a]	7.17 ± 0.36[a]	8.79 ± 0.17[a]	11.58 ± 0.24[a]	6.88 ± 0.57[a]
Linoleic	20.28 ± 0.6[a]	18.30 ± 0.33[a]	18.03 ± 0.87[a]	16.55 ± 0.30[a]	15.90 ± 0.29[a]
α-linolenic	51.14 ± 0.11[a]	56.99 ± 0.41[a]	51.01 ± 0.32[a]	45.29 ± 0.25[a]	49.12 ± 0.39[a]
Arachidic	0.39 ± 0.11[a]	0.63 ± 0.5[a]	0.69 ± 0.53[a]	0.52 ± 0.23[a]	0.30 ± 0.02[a]

Values are means ± SD (standard deviation) of duplicate determinations; Means with the same superscripts within the same row are not significantly different ($p \leq 0.05$). The percentages are not absolute content but they are relative to the total fatty acid profile. BCM-Black chia Molo, BCOT-Black chia Ong'ariama-Teso, BCAT- Black chia Among'ura-Teso, BCK- Black chia Kigumba and WCB-White chia Bukembo.

In fact, the presence of high levels of ALA implies the beneficial effects of chia seeds have on human health; for example, the anti-hyperglycemia, anti-hyperlipidemia, anti-hypercholesterolemia, anti-inflammatory and anti-cancer properties (Mohd et al. 2012; Gazem & Sharada, 2014; Ferreira et al. 2015). On the other hand, the average ratios of n-6 PUFA to n-3 PUFA were 1:3. Indeed, this observation is an important hint towards lowering the risk of coronary heart disease (CHD). According to Mozaffarian, Appel & Horn, (2011), diets with high omega-6 polyunsaturated fatty acids (n-6 PUFA) to n-3 PUFA ratio, and those high in total fat, saturated fatty acids and trans fatty acids have been directly related to increased CHD risk.

3.3.4 Qualitative Phytochemical Screening of Chia Seeds

Phytochemical screening indicated the presence of flavonoids, terpenoids, phenols, saponins, steroids, tannins, glycosides, gum and mucilage (Table 3.4). Indeed, the presence of these compounds determine the importance of plant material nutritionally and medicinally (Mohd et al. 2012). Phytochemicals are bioactive non-nutrient plant compounds that may provide desirable health benefits beyond basic nutrition to reduce the risk of developing chronic diseases such as CHD, diabetes and cancer (Liu, 2004; Liu, 2013). For example, saponins have been reported to have hypotensive and cardio depressant properties; glycosides have a protective role

against cardiac-related problems and tannins are important in the treatment of ulcerated/inflamed tissues while isoflavones have been reported to prevent cancer and symptoms of menopause, post-menopause, osteoporosis and estrogen-related disorders (Vijay, Sharmila & Pushpalatha, 2014).

Table 3.4: Qualitative Determination of Phytochemical Components of Chia Seed Extracts

	Phytochemical	BCAT	BCK	BCM	BCOT	WCB
1	Flavonoids	+	+	+	+	+
2	Alkaloids	-	-	-	-	-
3	Glycosides	+	+	+	+	+
4	Terpenoids	+	+	+	+	+
5	Phenol	+	+	+	+	+
6	Phlobatannins	-	-	-	-	-
7	Saponins	+	+	+	+	+
8	Steroids	+	+	+	+	+
9	Tannins	+	+	+	+	+
10	Gums and mucilage	+	+	+	+	+

Present: +; Absent/not detected: −; Results are average of duplicate determination. BCAT- Black chia Among'ura-Teso, BCK- Black chia Kigumba, BCM-Black chia Molo, BCOT- Black chia Ong'ariama-Teso and WCB-White chia Bukembo.

The most studied groups of dietary phytochemicals linked to human health are phenolics and carotenoids. Phenolics are generally classified as subgroups of phenolic acids, flavonoids, stilbenes, coumarins, lignans, and tannins (Liu, 2004; Liu, 2013). Flavonoids are further classified into flavonols (quercetin, kaempferol and myricetin), flavones (apigenin, luteolin), flavanols (catechins), flavanones (hesperitin, naringenin), anthocyanidins (cyanidin, delphinidin, malvidin) and isoflavonoids (genistein, daidzein) (Liu, 2013). Previous studies on the consumption of flavonoids in humans indicated the significant inverse correlation with mortality from CHD. In fact, the total intake of flavonoids was also inversely associated with LDL cholesterol and plasma total cholesterol concentrations (Liu, 2004). Phenolics (caffeic acid, quercetin, myricetin and kaempferol) have expressed their carcinogenic and antihypertensive potential in various experimental studies also protective role against diabetes and osteoporosis (Mohd et al. 2012; Saphier et al. 2017).

Chia seeds have a high content of gums and mucilage, these form a gel in water which can be easily extracted and have potential as a thickener, emulsifier and

stabilizer in food product development (Felisberto et al. 2015). Moreover, Chia gum has high water holding capacity and is stable at high temperatures up to 244^{o}C hence regarded suitable for high-value food formulations (Segura-Campos et al. 2014; de Falco et al. 2017). Therefore, the detection of these phytochemicals in chia seeds suggests its potential as a functional food and nutraceutical product.

3.4 Conclusion

The East African grown chia seeds indicated a high content of proteins, fats, fiber, and n-3 PUFA ALA. Black chia (BCM) from Kenya had high minerals compared to BCK from Uganda that had high alpha-linolenic acid. On the other hand, white chia (WCB) had high moisture, ash, fiber and phosphorous compared to other black chia seeds but the difference between them was not significant. Climatic conditions, agricultural practices, and variety might have contributed to the difference in nutrient composition.

Generally, the results from this study support other findings that chia seeds are the best-known plant source with the highest content of nutrients and are suggested to be among the functional foods with pronounced health benefits. The seeds can be an appropriate alternative to n-3 PUFA sources for vegetarians, people allergic to fish, and gluten-containing diets. Presence of phytochemicals highlights the significance of the seeds in the prevention and management of several non-communicable diseases such as CHD, diabetes, inflammatory disorders, and cancer. However, there is need for more clinical and animal trials on the use of chia seeds to establish the required quantity for human consumption.

CHAPTER FOUR

EFFICACY OF CHIA SEED EXTRACTS ON THE INHIBITION OF PANCREATIC LIPASE AND ALPHA AMYLASE ACTIVITIES *IN VITRO*

4.1 Introduction

Type 2 diabetes mellitus (T2DM) and obesity are chronic conditions of public health importance because of their increasing prevalence globally. T2DM is a chronic metabolic disorder, whose hallmark is characterized by hyperglycemia. T2DM occurs because of defects in insulin secretion combined with progressive increase in insulin resistance and is mainly due to excess body weight and physical inactivity (Merovci et al. 2014; WHO, 2017a). Obesity on the other hand, is defined as a condition characterized by the accumulation of body fat, which increases risk to health (Oliveros, Somers, Sochor, Goel & Lopez-Jimenez, 2014). Many causes of obesity are complex in nature. However, studies suggest that there is a complex interaction (which is poorly understood) between genetics, dietary intake, physical activity, lifestyle and environmental factors.

These interactions cause a long-term positive energy balance and eventually an increase in body fat mass and therefore, obesity is a multi-factorial condition (Yumuk, Frühbeck, Oppert, Woodward & Toplak, 2014). Furthermore, Obesity is also confirmed as a risk factor for the development and progression of several chronic diseases including T2DM. Both obesity and T2DM have a strong association as obesity accounts for 80–85 % of the risk of developing T2DM, which has led to the term ‘diabesity’(Golay & Ybarra, 2005; Zambad et al. 2013). Indeed, studies suggest that a 5–10 % reduction in weight is beneficial to health and may significantly improve insulin sensitivity in T2DM *(*Wing et al. 2011). One of the viable therapeutic approaches to combating obesity and T2DM is the inhibition of carbohydrate and fat digestive enzymes such as α-amylase and pancreatic lipase, respectively (Patil, Mandal, Tomar & Anand, 2015; Otieno, Altuwaijri & Kang, 2019).

By inhibiting these digestive enzymes, amounts of calories and glucose that are absorbed into the body are reduced. In fact, tested pharmacological approaches include the use of synthetic compounds (drugs) such as Acarbose and Orlistat to inhibit these digestive enzymes. However, consumption of these synthetic inhibitors causes gastrointestinal side effects such as abdominal pain, hyperuricemia, diarrhea, nausea, nyositis, gastric irritation, flushing, dry skin, oily spotting, flatus with discharge, flatulence, abnormal liver function, and fecal incontinence (Kolovou et al. 2011; Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012).

Considering that the prevalence of obesity and T2DM are increasing globally, there is an urgent need to identify alternative enzyme inhibitors with less negative side effects. Hence, compounds of natural sources (such as dietary components like Chia seeds) may be more desirable as they are thought to have less negative side effects when compared with the synthetic inhibitors (Patil et al. 2015). Chia seeds are high in phytochemical contents particularly flavonoids such as flavones, flavonols, flavanones, flavanols, and anthocyanidins which are said to have anti-diabetic properties (Liu, 2013; Vijay et al. 2014).

In addition, they have fewer reported toxic effects than the synthetic ones (Kumar et al. 2008). Therefore, the consumption of chia seeds may be a more acceptable source of natural inhibitors due to their low cost and relative safety, including a low incidence of serious gastrointestinal side-effects and allergenicity (Turck et al. 2019). Thus, the objective of this study was to produce ethanolic and methanolic extracts from Chia seeds and test their inhibitory activities against α-amylase and pancreatic lipase enzymes.

4.2 Material and Methods

4.2.1 Sample Collection and Preparation

The samples were purchased and prepared as outlined in section 3.2.1

4.2.2 Preparation of Chia Seed Extract

Chia seeds were extracted with 95 % ethanol and/or methanol according to the method described by (Thakur & Sidhu, 2014) with some modifications. Ground Chia seeds (20 g) were mixed with 200 ml of ethanol and/or methanol, the mixtures were kept for 12 hours in a shaker (Kika Labortechnik, KS 250 basic, Germany) at room temperature. The mixtures were centrifuged (Hettich zentrifugen, Universal 16A, Germany) at 4000 rpm for 20 minutes and the supernatant was collected and concentrated in a Rotary Evaporator RE 100 (Bibby sterilin Ltd, UK). The ethanolic and/or methanolic extracts of chia seeds were kept at 4 ^{o}C in the fridge (Hisense, RD-28DR4SA, China) until use.

Both ethanol and methanol can extract polar and non-polar secondary metabolites. Ethanol is better in extracting polyphenols whereas methanol is commonly used for extraction of bioactive compounds. However, for human and animal safety of exposure and consumption, ethanol is safer than methanol. In this study both solvents were used to test their effectiveness in extraction which in turn may impact the enzyme inhibitory activities. However, it has to be noted that, the optimal solvent for extraction depends on the particular plant materials and the compounds that are to be isolated (Truong et al. 2019).

4.2.3 α-Amylase Inhibitory Assay

α-amylase inhibitory assay adopted a method by Bhutkar et al. (2018). An aliquot of 20 µL ethanolic/methanolic extracts of chia seeds and α-amylase enzyme were premixed in a concentration range of 20 -100 µg/ml. 200 µL of 0.02 M potassium hydrogen phosphate (KHPO4) buffer was added, the assay mixture was incubated for 10 minutes at room temperature. 200 µL of 0.4 % starch was added in all test tubes, the reaction was terminated by adding 400 µL of 3,5-dinitrosalicyclic acid (DNS) reagent and placed in a boiling water bath for 5 minutes, the contents in test tubes allowed to cool and diluted with 10 ml distilled water.

The absorbance was measured at 540 nm using Uv/Vis Spectrophotometer (UV-1601PC Shimadzu, Japan). Acarbose was prepared as positive control in the same concentration range as for the samples.

$$\% \text{ Inhibition} = \frac{\text{Abs Control} - \text{Abs Extract}}{\text{Abs Control}} x\ 100$$

4.2.4 Pancreatic Lipase Inhibitory Assay

This colorimetric assay was based on the measurement of pancreatic lipase activity using methylresorufin method as described by (Möller et al. 2009) with modifications. This lipase colorimetric assay is based on the release of methylresorufin from a chromogenic lipase substrate. 25 µL of Lipase (Type II, Sigma, USA) and 25 µL of extract were pre-incubated for 20 min at 37 °C, and maintained with 200 µL of BICIN buffer with pH 8.0 (Sigma, USA), and about 1 % gum arabic was added (Aldrich, USA). 25 µL of pure extraction solvent was used as a non-inhibited enzyme activity control. Orlistat (tetrahydrolipstatin) was used as a positive control.

$$\% \text{ Inhibition} = \frac{\text{Abs Control} - \text{Abs Extract}}{\text{Abs Control}} x\ 100$$

4.2.5 Calculation of IC_{50} Values

Individual values for enzymatic activity are the mean of triplicate determinations. Values for the samples and the controls were expressed as % enzyme inhibition in relation to the non-inhibited enzyme activity control. The extract dilution producing 50 % of the enzyme activity compared with the non-inhibited enzyme control was designated as IC_{50}. The value was calculated from the parametric regression curves using the following equation: y = mx + c, % y = 50; for each sample extract. The values in the equation were obtained after plotting the graph of % Inhibition versus Concentration.

4.2.6 Statistical Analysis

STATA 14.0 Corp LP was used for statistical analysis of samples. The experiments were performed in triplicates and the results were expressed as the mean ± standard deviation (SD). A one-way ANOVA used Bonferroni comparison test to determine the variability between group means. Significance was accepted at $p \leq 0.05$.

4.3 Results

4.3.1 Inhibition of α-Amylase by Chia Seed Extracts

The inhibitory activity of ethanolic and methanolic extracts of chia seeds (BCAT- Black chia Among'ura-Teso, BCOT- Black chia Ong'ariama-Teso, BCK- Black chia Kigumba, BCM-Black chia Molo and WCB-White chia Bukembo) on α-amylase was investigated in this study and the results are shown in Table 4.1. Acarbose indicated the highest IC_{50} (51.16 ± 0.15 and 51.28 ± 0.27 μg/ml) for ethanolic and methanolic extracts respectively, establishing its relative potency as α-amylase inhibitor. BCAT was a strong inhibitor of α-amylase, exhibiting an IC_{50} of 104.02 ± 0.24 μg/ml and 72.06 ± 0.12 μg/ml for ethanolic and methanolic extracts respectively, but it was significantly lower ($p = 0.0001$) than that of acarbose (Table 4.1). The percent α-amylase inhibition of the five chia seed extracts was plotted as a function of concentration in comparison with acarbose as shown in Figure 4.1 and 4.2. Ethanolic extract of chia seeds (BCAT and BCK) and methanolic extract of BCAT, BCOT and BCM showed inhibition of above 50 %.

Table 4.1: IC_{50} Values for Ethanolic and Methanolic Extracts of Chia Seeds on Inhibition of α-Amylase Activities

Sample	Ethanolic extract of chia seeds (µg/ml)	Methanolic extract of chia seeds (µg/ml)
Acarbose	51.16 ± 0.15^{a}	51.28 ± 0.27^{a}
BCAT	104.02 ± 0.24^{b}	72.06 ± 0.12^{b}
BCOT	116.18 ± 0.27^{c}	90.97 ± 0.28^{c}
BCK	102.24 ± 0.42^{bd}	101.01 ± 0.18^{d}
BCM	111.50 ± 0.52^{e}	88.89 ± 0.29^{ce}
WCB	109.76 ± 0.33^{e}	99.57 ± 0.75^{df}

Values are means ± SD (standard deviation) of triplicate determinations. Means with the same superscripts within the same column are not significantly different ($p \leq 0.05$). BCAT- Black chia Among'ura-Teso, BCOT- Black chia Ong'ariama-Teso, BCK- Black chia Kigumba, BCM-Black chia Molo and WCB-White chia Bukembo

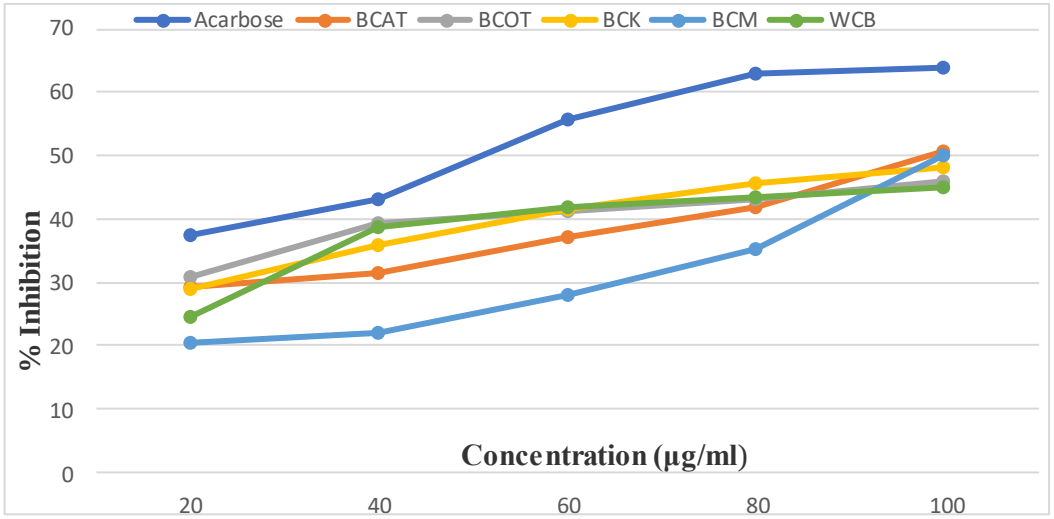

Figure 4.1: Percentage Inhibition of Alpha Amylase by Ethanolic Extract of Chia Seeds

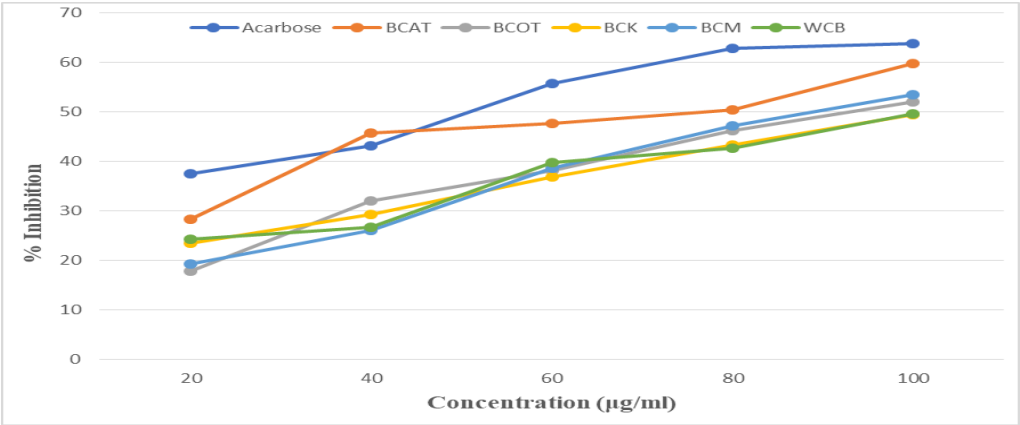

Figure 4.2: Percentage Inhibition of Alpha Amylase by Methanolic Extract of Chia Seeds

4.3.2 Inhibition of Pancreatic Lipase by Chia Seed Extracts

Results on pancreatic lipase inhibitory activity by ethanolic and/or methanolic extracts of chia seeds are shown in table 4.2. Although BCAT demonstrated the highest inhibitory activity with IC_{50} value of 90.02 ± 0.17 µg/ml and 96.19 ± 0.20 µg/ml, it was significantly lower ($p = 0.001$) for both ethanolic and methanolic extracts compared to the control. The percentage inhibition of pancreatic lipase by the five chia seed extracts were plotted as a function of concentration in comparison with orlistat (Figure 4.3 and 4.4).

Table 4.2: IC_{50} Values for Ethanolic and Methanolic Chia Seed Extracts on Pancreatic Lipase Inhibition Activity

Sample	Ethanolic extract of chia seeds (µg/ml)	Methanolic extract of chia seeds (µg/ml)
Orlistat	54.14 ± 0.15^{a}	54.12 ± 0.18^{a}
BCAT	90.02 ± 0.17^{b}	96.19 ± 0.20^{b}
BCOT	111.05 ± 0.09^{b}	115.20 ± 0.31^{b}
BCK	104.15 ± 0.20^{b}	109.16 ± 0.21^{b}
BCM	101.24 ± 0.28^{b}	104.17 ± 0.22^{b}
WCB	114.13 ± 0.20^{b}	115.06 ± 0.10^{b}

Values are means ± SD (standard deviation) of triplicate determinations. Means with the same superscripts within the same column are not significantly different ($p \leq 0.05$). BCAT- Black chia Among'ura-Teso, BCOT- Black chia Ong'ariama-Teso, BCK- Black chia Kigumba, BCM-Black chia Molo and WCB-White chia Bukembo

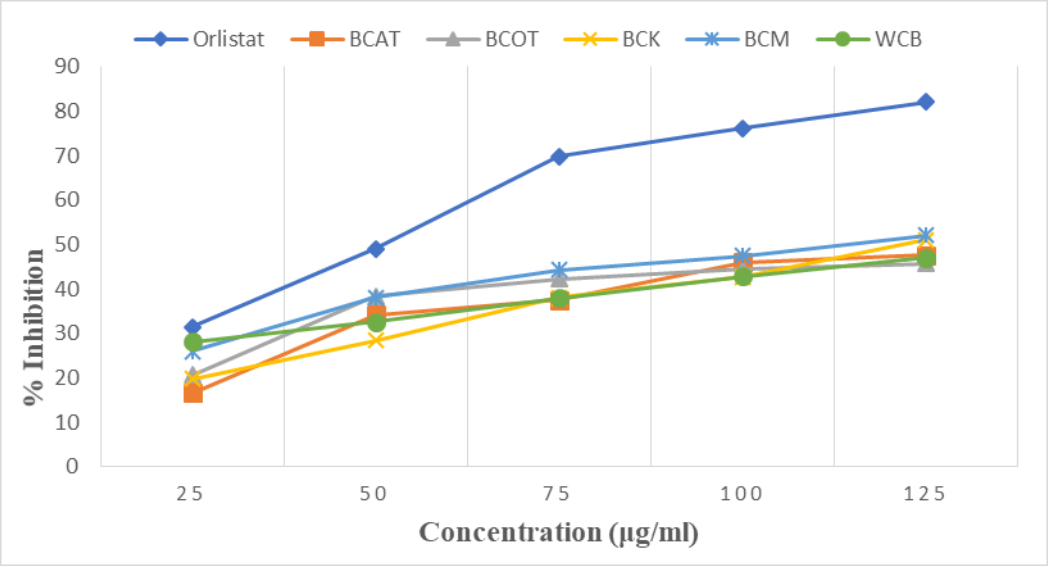

Figure 4.3: Percentage Inhibition of Pancreatic Lipase by Ethanolic Extract of Chia Seeds

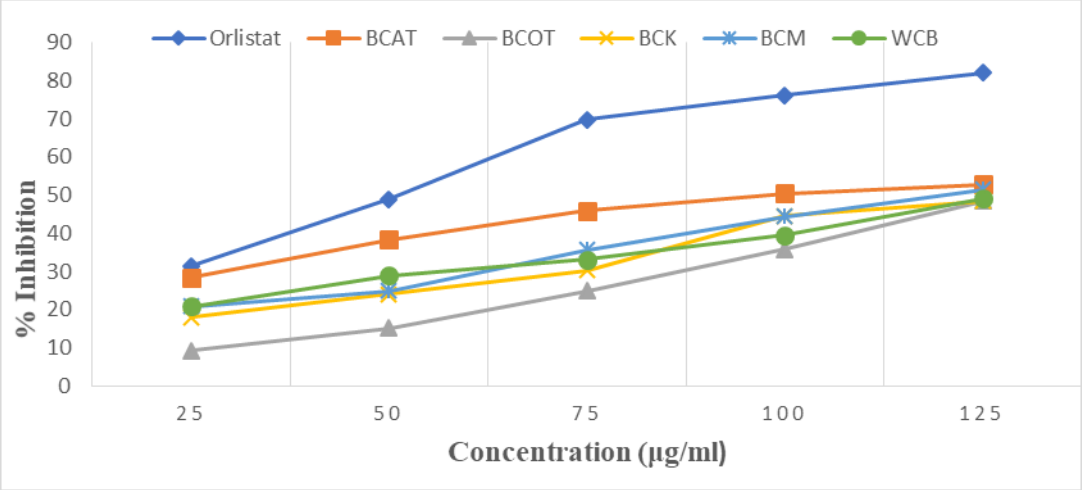

Figure 4.4: Percentage Inhibition of Pancreatic Lipase by Methanolic Extract of Chia Seeds

4.4 Discussion

Ethanolic and methanolic extracts of chia seeds inhibited α-amylase and pancreatic lipase *in vitro* by 50.6 % and 50.1 %, and 52.1 % and 51 % respectively (Figure 4.1 and 4.3). The α-amylase catalyzes the hydrolysis of starch, glycogen and various oligosaccharides into simple sugars which can be readily available for absorption in

small intestine, so inhibition of α-amylase by chia seeds extracts is a hint that, it may be effective in controlling hyperglycemia and hence type 2 diabetes mellitus (T2DM) (Nair, Kavrekar & Mishra, 2013b; Bhutkar et al. 2018). On the other hand, inhibition of pancreatic lipase is an indication that, chia seeds can be a good candidate in weight management and can be a means of controlling obesity (Rahman, 2017). Pancreatic lipase is an essential enzyme in the hydrolysis of fats in human gastrointestinal tract (Zhu, Fang, Zhu, Huang & Yang, 2021). Foods rich in fats especially saturated fats are linked to overweight and obesity, thus the use of natural inhibitors such as chia seeds are considered potential therapeutic agent for controlling diet-induced obesity with less side effects (Jang & Kim, 2009; Tucci, 2010; Yun, 2010).

A study by Möller et al. (2009), revealed that, high fat containing seeds develop lipase inhibitory substances as a protection against lipolysis of lipid deposits, thus their strong inhibitory activities. The findings are in line with Rahman, de Camargo, & Shahidi, (2017) who reported on the inhibition of pancreatic lipase activities by phenolic and polyphenolic profiles of chia seeds.

Generally, there is scarcity of information on the ability of chia seeds in inhibiting the activities of α-amylase and pancreatic lipase. Since these enzymes are primarily responsible for the release of caloric substrates from foods, the use of an agent that can inhibit activities of these enzymes could provide a more effective means of controlling T2DM and obesity.

4.5 Conclusion

Chia seed extracts demonstrated an effective α-amylase and pancreatic lipase inhibitory activities. These enzymes are present in the digestive tract and are responsible for the digestion of carbohydrates and fats respectively. The findings of this study provide supporting information that chia seeds may control T2DM and obesity. Additionally, the use of inhibitors from chia seeds may help reduce intestinal side effects caused by synthetic inhibitors (drugs). Therefore, efforts are needed to explore the α-amylase and pancreatic lipase inhibitors from natural sources having fewer side effects.

CHAPTER FIVE

EFFECT OF CHIA SEEDS ON POSTPRANDIAL GLYCAEMIA, BODY WEIGHT AND HEMATOLOGICAL PARAMETERS IN RATS FED A HIGH FAT AND FRUCTOSE DIET

5.1 Introduction

Excessive consumption of high-fat diets and foods that are calorie-dense, highly processed and quickly absorbable can lead to systemic inflammation and a cluster of cardiometabolic disorders, including obesity, hypertension, dyslipidemia, glucose intolerance and insulin resistance (Crescenzo, Bianco, Mazzoli & Giacco, 2015; Alissa & Ferns, 2014). These factors increase the risks of chronic diseases such diabetes and cardiovascular diseases. There is growing interest in identifying novel therapeutic approaches including functional foods towards preventing and controlling chronic diseases and other health risks (Creus et al. 2016). Typical functional food contains bioactive compounds including dietary fiber, polyunsaturated fatty acids, phytochemicals and antioxidants that have potential roles in reducing the risk of chronic diseases (Abdulrashed et al. 2016). Chia seed is among the growing functional food that have been reported to protect the cardiovascular system, exhibit anti-inflammatory properties, anti-oxidative properties and control blood sugar and lipid metabolism (Abdulrashed et al. 2016; Marcinek & Krejpcio, 2017; Asgary, Rastqar & Keshvari, 2018).

Postprandial glycaemia (PPG) refers to blood glucose concentrations after a meal (Bernardo et al. 2015). It is a useful predictor of diabetes and cardiovascular events and its regulation is key to controlling metabolic diseases (Blaak et al. 2012). Normally, in nondiabetic conditions, plasma glucose concentrations rise approximately 30 to 60 minutes after a meal, rarely exceed 140 mg/dL and returning to pre-prandial levels 2 to 3 hours after. The extent and time of rise of plasma glucose concentration depends on a number of factors such as meal timing, quantity and composition.

Furthermore, the rate of gastric emptying affects the absorption rate of meal components and is related to the postprandial blood glucose level after meals (Marathe, Rayner, Jones & Horowitz, 2013). The PPG profile is determined by carbohydrate absorption, secretion of hormones (insulin and glucagon) and their corresponding effects on glucose metabolism in the liver and peripheral tissues (Marathe et al. 2013; Bernardo et al. 2015).

Reactive oxygen species (ROS) can stimulate PPG, which in turn aggravates inflammation, endothelial dysfunction and other complications associated with hyperglycemia (Nwaoguikpe, 2010; Blaak et al. 2012; Bernardo et al. 2015). There is growing evidence that whole grains may play a central role in the prevention of chronic diseases (Vuksan et al. 2007). Epidemiological studies suggest a strong association between increased consumption of whole-grain foods and reduced risk of diabetes and cardiovascular disease (Ho et al. 2013; Alissa & Ferns, 2014; Asgary et al. 2018).

Chia seeds are currently consumed by varied populations due to their protective, functional and antioxidant properties attributed to the presence of dietary fibre, lipids, protein, phenolic compounds, minerals and omega-3 fatty acids (α-linolenic acid) (Abdulrashed et al. 2016; de Falco, Amato & Lanzotti, 2017; Prathyusha et al. 2019). A study by Ho et al. (2013), demonstrated that both ground and whole chia seeds equally attenuated blood glucose levels in a dose-dependent manner when incorporated into bread and tested in healthy volunteers. Costantini, Molinari & Merendino, (2019), reported a significant decrease in endothelin-1 (ET-1) a biomarker of cardiometabolic diseases when spontaneously hypertensive rats were supplemented with chia seeds. Further, Vuksan et al. (2010) reported a reduced postprandial glycemia in individuals fed with bread containing up to 24 g chia seeds per day. Therefore, a therapeutic approach to treat diabetes is to decrease postprandial hyperglycemia.

Hematological parameters have been associated with health indices and are of great significance in routine clinical evaluation of the state of health (Etim, 2014; Kensa & Neelamegam, 2014). Therefore, examining blood composition can offer significant

information for the diagnosis and prognosis of diseases in animals (Budzianowski, Pieszko, Burchardt, Rzeźniczak & Hiczkiewicz, 2017). This is because the destruction mediated by free radicals results in the disruption of membrane fluidity, lipid peroxidation, protein denaturation and alteration of platelet functions that heighten chronic health problems such as diabetes, inflammation, atherosclerosis and cancer (Kensa & Neelamegam, 2014). Hence, variations in hematological parameters are regularly used to determine various statuses of the body as well as stress due to pathological, nutritional and environmental factors (Nwaoguikpe, 2010).

Chia seeds are rich in omega 3 fatty acids that play a key role in preventing the formation of clots and plaques in the arteries and so help to prevent cardiovascular diseases (Ullah et al. 2016; Prathyusha et al. 2019). The present study was therefore aimed at determining the effects of ground chia seeds and its extracts on postprandial glycaemia, body weight, hematological parameters and cellular morphology in male Wistar rats.

5.2 Materials and Methods

5.2.1 Sample Collection and Preparation

5.2.1.1 Collection and Preparation of Chia Seeds

The samples were purchased and prepared as outlined in section 3.2.1

5.2.1.2 Preparation of Chia Seed Oil Extract

The extract was prepared according to the method described by Scapin et al. (2015) with modification of the ratios. Ground chia seeds and ethanol were used in the preparation of extract. Ethanol solution (95%) at a ratio of 20:200 (20 g ground seeds:200 mL 95% ethanol solution) was added. The mixture was subjected to constant stirring using a shaker (Kika Labortechnik, KS 250 basic, Germany) for 2 hours.

The mixture was then sieved with a sieve cloth to get the liquid portion. The liquid portion was then filtered with a whatman filter paper number 1 in order to get a clear

filtrate. The filtrate was then concentrated in a Rotary Evaporator RE 100 (Bibby sterilin Ltd, UK). The crude ethanolic extract of chia seeds was then dried in an air oven at 50^{o}C for 1 hour and later cooled in a desiccator. Then the volume was completed with distilled water to keep the initial concentration, and packaged in amber flasks which were kept at 4^{o}C in the fridge (Hisense, RD-28DR4SA, China) until use.

5.2.1.3 Preparation of Lard and Fructose

The pork fat was sliced into small pieces and put into a saucepan and heat applied on it. The pork fat melted into oil and continued boiling until the oil achieved a golden color (lard). All preparations were conducted at JKUAT Food Science and Technology Laboratory (Meat processing workshop). Fructose (Fructofin C, Danisco Sweeteners Oy, Finland) was prepared by dissolving 20 g of fructose granules in 100 ml distilled water (w/v).

5.2.2 Animal Experiment

5.2.2.1 Experimental Animals

Male Wistar rats aged 8 to 10 weeks and weighing between 140 g to 250 g were used. The breeds were obtained from Small Animal Facility for Research and Innovation (SAFARI), College of Health Science, JKUAT. The rats were acclimatized on rat pellet (Unga farm Care (EA) Ltd, Nairobi) for two weeks before commencement of the experiment. The lard (4 g/rat), fructose (2 g/rat) and chia seed extracts (4 g/rat) were orally administered by a gavage for a period of 28 consecutive days. The animals were housed in plastic cages. About 15g of rat pellets were given to each rat, and water were given *ad libitum* during the experiment. The composition of experimental diets and the control were shown in Table 5.1.

Table 5.1: Nutrient Composition of the Diets

Nutrient	Control (rat pellet) (%)	Experimental diet (ground chia seed) (%)	Experimental diet (chia seed extract) (%)
Crude protein	18.1	20.1	4
Crude fat	8	30.9	26.0
Crude fiber	7	25.6	18.0
Carbohydrates	-	14.1	12.0
α-linolenic	-	51.0	57.0
Linoleic	-	18.0	18.3

5.2.2.2 Experimental Design

Twenty (20) male Wistar rats were randomly assigned into three experimental groups and a control (n = 5). Control group received 75g rat pellets only, group 1 received 10g fructose, 20g lard and 75g rat pellets; group 2 received 10g fructose and 20g lard supplemented with 75g ground chia seeds; group 3 received 10g fructose, 20g lard and 75g rat pellets supplemented with 20g chia seed extract. Water was given *ad libitum* to all groups. All these diets were given for 28 days.

5.2.2.3 Sample Size

Sample size was calculated based on 'Group comparison-one-way ANOVA' using degree of freedom (DF) as previously reported by (Charan & Kantharia, 2013; Arifin & Zahiruddin, 2017) . n = DF/k + 1; N = n x k; where n = number animals per group, k = number of groups, N = total number of animals. The power analysis approach (ANOVA) is normally between 10 to 20, so any sample that keeps DF (E) between 10 and 20 is considered adequate. The total number of animals obtained for this experiment was 20.

5.2.2.4 Blood Collection

At the beginning of the experiment the blood was collected by making a small cut on a tip of a tail, the tail was slowly milked to release blood on a strip inserted in glucometer (Accu-Check Active, Roche, Mannheim, Germany), the blood sugar levels were read and recorded. Postprandial glycaemia was determined 2 hours after

feeding on weekly basis while fasting blood glucose was determined at the end of the experiment using handhold glucometer. At the end of the intervention, rats were anaesthetized with carbondioxide and blood collected from them by cardiac puncture. About 4 ml of blood was collected into EDTA-anticoagulated tubes (BD, Belliver Industrial Estate, Plymouth, UK) from each animal and used for determining the parameters of interest. The blood in tubes were stored at 8^{o}C for 12 hours and used for hematological analysis.

5.2.2.5 Hematological Assays

The blood in tubes were transported in cool box maintained at 4^{o}C. Hematological analysis was conducted at the Institute of Primate Research (IPR) Laboratory, Nairobi, Kenya. Whole blood with anticoagulant in tubes were homogenized by slowly shaking the tubes, then they were put into Hematology Analyzer (Ac.T 5diff CP, Beckman Coulter, France) for analysis. Hematological parameters determined were red blood cells (RBC) count, blood hemoglobin concentration (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), total leukocyte count (TLC), Neutrophils (NEUT), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), platelets (PLT) and mean platelet volume (MPV).

5.2.2.6 Weight Records and Faeces Collection

The body weight of each rat was recorded daily until the end of the experiment. Body weight results were expressed as mean ± standard deviation. % Weight gain was calculated between weeks [(eg. Week II-Week I/Week II) x 100]. Faeces from each cage were collected daily and at the end of the experiment lipids were extracted.

5.2.2.7 Extraction of Lipids from Faeces

Soxhlet Official Method was used to extract lipids from faeces. Approximately 2 g of dry faeces (W1) was weighed into extraction thimbles and covered with glass wool. Round bottom flasks were weighed (W2) and 100 ml of petroleum ether was

added to each flask. The flasks containing samples were fitted to the extractor connected to the condenser. Cold water for cooling was allowed to run at a steady rate throughout the condenser. The heater turned on and adjusted so that the solvent boiled gently and its vapour condensed back into the thimbles with samples. After 8 hr reflux, the samples in the flasks were cooled and connected to rotary evaporator (Bibby sterilin Ltd) and evaporated to dryness. The samples in flasks were dried in an air oven at 70^{o}C for 1 hr, then cooled in a desiccator and weighed (W3). The percentage lipids calculated as follows;

$$\% \, Lipids = \frac{W3 - W2}{W1} \; x \; 100$$

5.2.3 Statistical Analysis

STATA Corp 14.0 was used in data analysis. Primary data were analyzed and presented as the mean +/- standard deviation (SD). To determine the level of significance, the data were further analyzed using ANOVA-Bonferroni multiple comparison test to determine the variability between groups. The difference in the test values versus the control values was considered significant at $p \leq 0.05$.

5.2.4 Ethical Approval

All aspects of animal care and experimentation were performed according to the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The JKUAT Institutional Ethics Review Committee (IERC) approved the study (Appendix 1).

5.3 Results

5.3.1 Effect of Chia Seeds/Extract on Body Weight Changes in Rats

Results on body weight changes in relation to the initial assessment were compared between the control and experimental groups based on the percentage weight gain (% Wt gain). There was a decrease in % Wt gain of the rats in experimental group 2&3

from week I to IV, this indicates that chia seeds and chia seed extract may have the ability to reduce body weight. These results cannot really tell the differences between groups since the animals were of different weight at the beginning of the experiment. They can only tell the differences in weight gain per group in a period of four weeks.

Table 5.2: Body Weight (g) Changes of Wistar Rats Fed with Fructose/Lard and Ground Chia Seeds/Extract

Time	Experimental groups			
(Week)	Control	Group 1	Group 2	Group 3
I	186.40 ± 0.28	229.21± 0.52	196.92± 0.54	189.41 ± 0.31
% Wt gain	2.02	13.99	6.95	8.17
II	190.25 ± 0.37	266.50± 0.55	211.62± 0.52	206.26 ± 0.43
% Wt gain	2.62	5.40	2.62	4.82
III	195.38 ± 0.40	281.70± 0.27	217.31± 0.46	216.71 ± 0.41
%Wt gain	8.95	6.15	0.93	2.87
IV	214.60 ± 0.44	300.18± 0.32	219.35± 0.35	223.11 ± 0.23

Values are means ± SD (standard deviation) (n = 5); Wt-Weight; The difference in % Wt gain was compared within the same column. Control (received 90 g rat pellet only); Group 1 (received 10 g/20 g fructose/lard and 90 g rat pellet); Group 2 (received 10 g/20 g fructose/lard supplemented with 90 g ground chia seeds); Group 3 (received 10g/20 g fructose/lard, 90 g rat pellet supplemented with 20 g chia seed extract).

5.3.2 Effect of Ground Chia Seeds and Its Extract on Postprandial Glycaemia

Postprandial glycaemia (PPG) in group 3 reduced significantly as compared to group I that was fed fructose and lard for 60, 90 and 120 minutes in week I (Table 5.3). In week II there was no significant difference in blood glucose levels between groups. In week III, there was an increase in PPG for group 1 and the difference was significant ($p = 0.0001$) for 30, 60, 90 and 120 minutes. Likewise, in week IV, group 1 differed significantly ($p = 0.0001$) from other groups as PPG increased for 30, 60, 90 and 120 minutes. On the other hand, fasting blood glucose ranged from 4.94 ± 0.35 - 6.42 ± 0.36, 3.84 ± 0.50 - 9.26 ± 0.88, 3.76 ± 0.47 - 5.54 ± 0.49 and 4.12 ± 0.24 - 5.08 ± 0.38 for the control, group 1, 2 and 3 respectively.

Table 5.3: Postprandial Glycaemia of the Rats Fed with Fructose/Lard and Ground Chia Seeds/Extract

Period(Week)	Time	Postprandial glycaemia (mmol/L)			
	(min)	Control	Group 1	Group 2	Group 3
I	30	6.02 ± 0.48^{a}	6.22 ± 0.48^{a}	6.20 ± 0.48^{a}	5.34 ± 0.56^{a}
	60	5.76 ± 0.64^{ab}	6.06 ± 0.37^{ab}	6.22 ± 0.59^{a}	5.08 ± 0.53^{b}
	90	5.62 ± 0.55^{ab}	6.28 ± 0.86^{a}	5.88 ± 0.60^{ab}	4.84 ± 0.77^{b}
	120	5.42 ± 0.39^{b}	6.32 ± 0.65^{a}	5.12 ± 0.68^{b}	4.44 ± 0.56^{b}
II	30	7.20 ± 0.86^{a}	7.14 ± 0.88^{a}	6.22 ± 0.22^{a}	6.32 ± 0.94^{a}
	60	6.14 ± 0.51^{a}	6.98 ± 1.14^{a}	6.32 ± 0.60^{a}	5.82 ± 0.73^{a}
	90	6.16 ± 0.41^{a}	7.42 ± 1.35^{a}	6.28 ± 0.38^{a}	6.06 ± 1.46^{a}
	120	6.10 ± 0.84^{a}	7.06 ± 1.41^{a}	5.82 ± 0.63^{a}	5.40 ± 0.10^{a}
III	30	6.04 ± 0.22^{b}	8.08 ± 0.45^{a}	5.88 ± 0.56^{b}	5.42 ± 0.56^{b}
	60	5.72 ± 0.33^{b}	8.60 ± 0.94^{a}	5.28 ± 0.52^{b}	5.84 ± 0.44^{b}
	90	5.22 ± 0.47^{b}	8.56 ± 0.97^{a}	6.16 ± 0.38^{b}	5.94 ± 0.50^{b}
	120	5.26 ± 0.32^{b}	8.40 ± 0.81^{a}	5.80 ± 0.87^{b}	5.90 ± 0.40^{b}
IV	30	6.22 ± 0.30^{b}	9.50 ± 0.45^{a}	6.56 ± 0.55^{b}	6.48 ± 1.15^{b}
	60	5.20 ± 0.25^{b}	9.54 ± 0.70^{a}	5.92 ± 0.39^{b}	6.08 ± 1.26^{b}
	90	5.70 ± 0.29^{b}	9.82 ± 0.46^{a}	5.16 ± 0.57^{b}	5.54 ± 1.07^{b}
	120	5.26 ± 0.28^{b}	9.30 ± 0.79^{a}	4.86 ± 0.04^{b}	5.34 ± 1.22^{b}

Values are means ± SD (n = 5). Means with the same superscript within the same row are not significantly different ($p \leq 0.05$). Control (received 90 g rat pellet only); Group 1 (received 10 g/20 g fructose/lard and rat pellet); Group 2 (received 10 g/20 g fructose/lard supplemented with 90 g ground chia seeds; Group 3 (received 10g/20 g fructose/lard, rat pellet supplemented with 20 g chia seed extract).

5.3.3 Effect of Ground Chia Seeds and its Extract on Hematological Parameters and Cellular Morphology

Results on complete blood counts showed no significant difference ($p = 1.000$) in red blood cells (RBC) count, blood hemoglobin concentration (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), total leukocyte count (TLC), Neutrophils (NEUT), lymphocytes (LYM), monocytes (MON), eosinophils (EOS) and mean platelet volume (MPV) between experimental groups and a control (Table 5.4).

Table 5.4: Hematological Parameters of Rats Fed with Fructose/Lard and Ground Chia Seeds/ Extract

Parameter	Control	Group 1	Group 2	Group 3
RBC (10^6/µL)	7.85 ± 0.49^a	7.81 ± 0.21^a	8.14 ± 0.40^a	7.93 ± 0.37^a
Hb (g/dL)	14.16 ± 0.60^a	13.66 ± 0.63^a	14.62 ± 0.36^a	14.24 ± 0.24^a
PCV (%)	45.08 ± 0.24^a	43.34 ± 0.25^a	46.36 ± 0.39^a	44.64 ± 0.25^a
MCV (ft)	57.80 ± 0.45^a	56.20 ± 0.44^a	57.20 ± 0.47^a	56.20 ± 0.45^a
MCHC (%)	31.04 ± 0.27^b	31.34 ± 0.53^b	31.58 ± 0.44^b	32.80 ± 0.25^a
MCH (pg)	18.06 ± 0.53^a	17.52 ± 0.56^a	18.24 ± 0.24^a	18.18 ± 0.28^a
RDW (%)	12.64 ± 0.34^{ab}	13.42 ± 0.69^a	11.94 ± 0.86^b	11.96 ± 0.34^b
TLC ($x10^3$/µL)	9.20 ± 0.23^a	10.16 ± 0.26^a	11.20 ± 0.24^a	9.20 ± 0.28^a
NEUT (%)	1.97 ± 0.32^a	3.29 ± 0.30^a	2.44 ± 0.40^a	1.63 ± 0.05^a
LYM (%)	7.2 ± 0.20^a	5.20 ± 0.23^a	8.22 ± 0.24^a	6.45 ± 0.42^a
MON (%)	0.67 ± 0.24^a	1.28 ± 0.26^a	0.87 ± 0.40^a	0.48 ± 0.02^a
EOS (%)	0.07 ± 0.02^a	0.08 ± 0.04^a	0.09 ± 0.02^a	0.08 ± 0.01^a
BAS (%)	0.1 ± 0.05^{ab}	0.08 ± 0.06^b	0.12 ± 0.04^{ab}	0.18 ± 0.02^a
PLT ($x10^3$/µL)	543.2 ± 0.45^a	521.2 ± 0.47^a	710.8 ± 0.45^b	678.68 ± 0.46^b
MPV	7.86 ± 0.52^a	6.94 ± 0.67^b	6.96 ± 0.32^b	6.72 ± 0.24^b

Values are means ± SD (n =5). Means with the same superscripts within the same row are not significantly different ($p \leq 0.05$). Control (received 90 g rat pellet only); Group 1 (received 10 g/20 g fructose/lard and 90 g rat pellet); Group 2 (received 10 g/20 g fructose/lard supplemented with 90 g ground chia seeds; Group 3 (received 10g/20 g fructose/lard, 90 g rat pellet supplemented with 20 g chia seed extract). Red blood cells (RBC) count, blood hemoglobin concentration (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), total leukocyte count (TLC), Neutrophils (NEUT), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), platelets (PLT) and mean platelet volume (MPV).

There was a significant increase ($p = 0.008$) in mean corpuscular hemoglobin concentration (MCHC) in group 3, and basophils (BAS) also increased significantly ($p = 0.035$) in experimental group 3 (Table 5.4). Mean platelet volume (MPV) increased significantly ($p = 0.04$) in a control group unlike experimental groups. Platelets (PLT) increased significantly ($p =0.0001$) in experimental group 2 and 3 compared to a control.

Blood smears indicated morphological changes of cells from the experimental group fed with high fat high fructose diet compared to those fed with chia seeds and chia seeed extract (Figure 5.1). Most cells observed to be creanated and some exhibited leptocytosis.

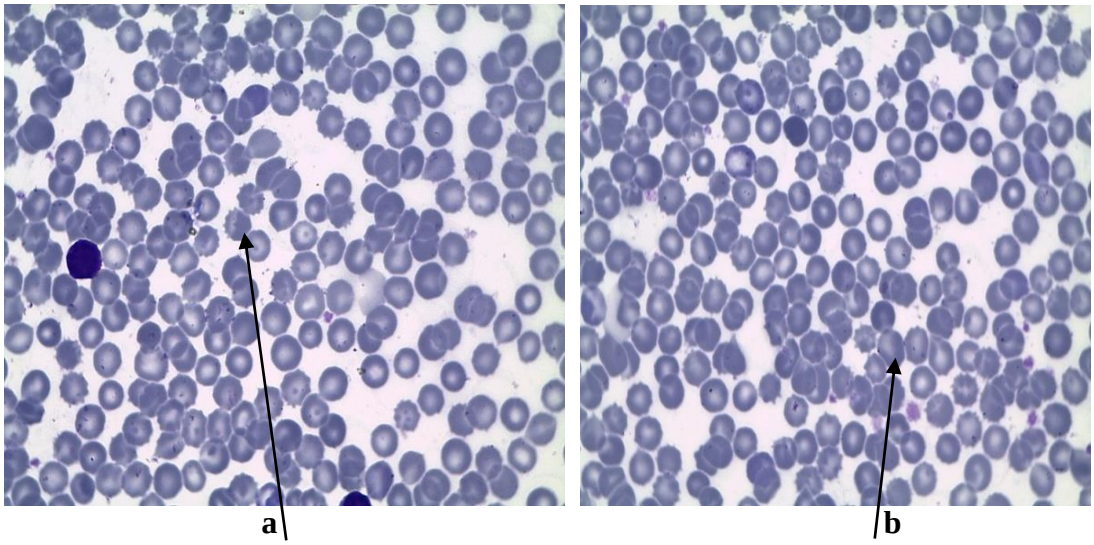

Figure 5.1: Leptocytosis (a) and Creanated Cells (b)

5.3.4 Extracted Lipids from Faeces

Results on lipids (%) extracted from faeces indicated an increased amount of lipids in chia seed extract fed rats (2.92 ± 0.27), followed by ground chia fed rats (1.90 ± 0.70) as compared to the control (1.77 ± 0.23). The amount of lipids in chia seed extract fed rats differed significantly ($p = 0.004$) from the group that was fed with fructose/lard and rat pellets only.

5.4 Discussion

Foods containing high fats and sugars impose high risk of diabetes and cardiovascular disease. Low intake of saturated fatty acids with increased intake of omega-3 polyunsaturated fatty acids can lessen food related health risks (Nettleton, Jebb, Risérus, Koletzko & Fleming, 2014). Chia seeds have been reported by several studies (Ullah et al. 2016; Abdulrashed et al. 2016) to be useful in controlling blood glucose levels and offering cardioprotective effects because they contain high levels of omega 3 fatty acids.

Overweight and obesity are modifiable risk factors of some of these metabolic diseases like type 2 diabetes (Ramzi & Sharada., 2014). This study showed a decrease in weight gain in experimental groups supplemented with ground chia seeds and its extracts (Table 5.2). It is an indication that, chia seeds can be a good

candidate for weight loss if executed with other therapeutic life changes such as reducing intake of foods and food products rich in saturated fats and trans-fatty acids; increasing intake of foods high in omega-3 fatty acids and dietary fiber including fruits and vegetables, reducing intake of sodium to less than 2400 milligrams per day and reducing physical inactivity *(*Rolfes et al. 2003; Anand et al. 2015; Toscano, Tavares, da Silva & Silva, 2015).

There was an increase in blood glucose levels in group 1 from week I to week IV contrary to group 2 and 3. There was also a significant decrease in PPG in experimental groups that included chia seeds and its extract (Table 5.3). Indeed, it has been reported that controlling PPG independently activates homeostasis of thrombotic-prone conditions, mediated by brief overproduction of reactive oxygen species (Zheng et al. 2010; Blaak et al. 2012). These may stimulate inflammation, hypercoagulability and endothelial dysfunction that favors vasoconstriction and hypertension, causing metabolic diseases such as type 2 diabetes mellitus and cardiovascular disease (Kensa & Neelamegam, 2014).

Lower PPG is presumed to reduce ROS that may damage proteins, lipids and DNA hence upsurge resistance in insulin sensitive tissues especially under the absence of adequate antioxidants (Jenkins et al. 2006). Therefore, decreased PPG in groups supplemented with ground chia seeds/extracts are promising for primary prevention of type 2 diabetes and cardiovascular diseases. Hence, the economic cost of diabetes could be reduced through the control of postprandial glycaemia.

High red cell distribution width (RDW) is an indication of nutrient deficiency particularly iron, folate and vitamin B_{12} and can also show macrocytic anaemia (Agiang, Dongo, Williams & Utu-Baku, 2017). In the study, the experimental group that was supplemented with fructose/lard had a significant increase ($p < 0.008$) in RDW compared to the groups that were supplemented with ground chia seeds/extract only (Table 5.4). This can be an indication that chia seeds may boost micronutrients in body and especially iron (Amato et al. 2015). However, it was noted that, PCV in experimental group 1 was low compared to group 2 that was supplemented with

ground chia seeds; this may be an indication of cell destruction, since low packed cell volume indicates cellular damage.

The decline observed in the platelet counts in group 2 highlight possible effects on blood clotting when fructose/lard are supplemented to normal rat pellet. Platelet count is one of the essential screen tests of platelet function (Monteomo et al. 2018). An increase in RBC count led to the corresponding increase in Hb and PCV in groups supplemented with chia seeds/extract compared to the control. However, erythrocyte indices, MCH and MCHC were increased in experimental groups 1 and 2 compared to a control. These parameters are used to measure the size and weight per unit volume of red blood cells. The size of the cells may be normal (normocytic), below normal (microcytic) and above normal (macrocytic). This is comparable to what is experienced in iron deficiency anaemia (Agiang et al. 2017).

The study revealed low levels of MCV and MCH in fructose/lard supplemented group compared to those of ground chia seed/ extract fed groups, which may indicates microcytic anaemia, a condition in which red blood cells become too small than normal and usually characterized by a low MCV.

Iron deficiency is the most common cause of microcytic anemia. The rats might have developed this condition because this group was only fed fructose, lard and rat pellets in which iron content was not guaranteed. This study also showed an increase in the concentration of TLCs in group supplemented with ground chia seeds (Table 5.4). This implies that chia seeds are capable of boosting the immune system (Monteomo et al. 2018). Generally, low levels of hematological parameters were observed in group 1 as compared to the groups supplemented with ground chia seeds/extract (Table 5.4).

On the other hand, cellular morphology indicated crenation and leptocytosis, these were much observed in a control group and group 1 that were fed with fructose/lard and rat pellets only, and most cells in experimental groups fed with chia seeds were greatly normal. Crenation implies formation of abnormal notched surfaces on cells as result of water loss through osmosis (Figure 5.1). In absence of gastrointestinal bleeding, uremia or any other medical conditions, crenation may indicate aged cells

or may occur *in vitro* when blood stays for a prolonged period of time in the collection tubes. In this study, the blood was kept at 4^{o}C for 24 hours before analysis, it might be one of the reason for crenation. Leptocytosis is a condition in which red blood cells become thinner than normal and may contain less hemoglobin (Figure 5.1). It is a feature of iron deficiency anemia, thalassemia and liver disease (Youngson, 2005).

Increased level of lipids in faeces from ground chia seeds/extract fed rats may be an indication that, they are capable of inhibiting pancreatic lipase an enzyme responsible for digestion of dietary fat. There is possibility of slowing down the digestion of fats after consumption of chia seeds/extract hence their release through feces. Inhibition of this enzyme is thought to improve dyslipidemia. Pancreatic lipase inhibitors are considered valuable therapeutic agent for treating diet-induced obesity (Aruna et al. 2014).

5.5 Conclusion

In conclusion, findings in this study provide the evidence that chia seeds can be the good candidate for lowering postprandial glycaemia and improving hematological parameters which are marked as good indicators of the physiological status of animals. Results on body weight revealed the significance of chia seeds in controlling weight gain. In addition, the seeds can be used to slow down the digestion of fats by inhibiting pancreatic lipase. It is therefore believed that, the next generation of functional foods, supplements and processed foods will have the chia seeds as their primary ingredient.

CHAPTER SIX

EVALUATION OF DOSE EFFECT OF CHIA SEED EXTRACTS ON PLASMA GLYCAEMIA, BODY WEIGHT CHANGES, LIPEMIA, LIVER ENZYMES AND HEMATOLOGICAL PARAMETERS IN MALE WISTAR RATS FED A HIGH FAT AND FRUCTOSE DIET

6.1 Introduction

Cardiovascular disease (CVD) risks have been associated with a cluster of metabolic abnormalities (high triglycerides, low high density lipoprotein cholesterol, elevated low density lipoprotein cholesterol, hyperglycemia and central obesity) (Ghosh, Mishra, Rao & Aggarwal, 2006; Medic, 2014; Pistrosch, Schaper, & Hanefeld, 2013; World Health Organization, 2014). Some studies concur that impaired fasting glycaemia is well perceived when fasting blood glucose is above normal between (110 - 126 mg/dl) while postprandial glycaemia is within normal ranges (< 140 mg/dl). In practice, normal fasting blood glucose concentrations are kept within a narrow physiological range of 3.5-5.5 mmol/L (Tirosh et al. 2005). Raised serum triglycerides especially mild to moderate concentrations (2-10 mmol/L or 178-890 mg/dL), have long been related with an increased risk for CVDs (Nordestgaard & Varbo, 2014; Han, Nicholls, Sakuma, Zhao & Koh, 2016). Total cholesterol correlates with the presence of lipoproteins mostly low density lipoprotein cholesterol (LDL-C) and small dense very low density lipoprotein cholesterol (VLDL-C) that may become trapped in the intima of coronary and other arteries to form atherosclerotic plaque (Nordestgaard & Varbo, 2014).

Atherogenic index (AI) and coronary risk index (CRI) are considered strong markers for predicting the risk of atherosclerosis and coronary heart disease and can reveal the presence of LDL-C or triglycerides (TGs) in the serum of related patients (Kazemi, Hajihosseini, Moossavi, Hemmati & Ziaee, 2018). In practice, AI value of less than 0.11 is normally associated with low risk of CVD while values ranging from 0.11 to 0.21 are associated with intermediate CVD risks and those above 0.21 are associated with increased risks (Niroumand et al. 2015). At times the ratio of total cholesterol (TC) to high density lipoprotein cholesterol (HDL-C) is used to

predict coronary heart disease (CHD) risk, a high TC value suggests elevated LDL-C levels, and a low HDL-C value is associated with the multiple risk factors of the metabolic syndrome such as high TGs and small, dense LDL-C(Rolfes et al. 2003; Rolfes et al. 2012).

The variations in serum lipid such as an increase in TC, LDL-C and TGs, and a decrease in HDL-C heighten cardiovascular disease progression (Kiage-Mokua et al. 2018). High TG levels are markers for atherogenic lipoproteins, particularly important in patients with insulin resistant, type 2 diabetes mellitus and other metabolic syndrome commonly present with combined dyslipidemia characterized by fasting and postprandial hypertriglyceridemia (Talayero & Sacks, 2011).

Liver enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) are useful biomarkers of liver and cardiac injury (Kunutsor et al. 2014; Hasan, Tamanna & Haque, 2018; Choi et al. 2018). Liver enzymes are predominantly found in the liver but also in other tissues like heart, muscles, pancreas, intestines, kidneys and brain (Hall & Cash, 2012). ALT is produced in hepatocytes, and it is a very specific marker of hepatocellular damage. When body tissues are injured, ALT and AST are released into the bloodstream causing an increased serum enzyme level, thus ALT and AST activity increase with liver injury (Lin et al. 2019). Indeed, AST level in serum is elevated in heart attacks or with muscle injury (Hall & Cash, 2012; Kim et al. 2019). ALP is produced in the membranes of cells lining bile ducts and canaliculi, released in response to the accumulation of bile salts or cholestasis (Hall & Cash, 2012). The ALP test is used to detect blocked bile ducts, liver damages or bone disorders (Kunutsor et al. 2014).

Gamma glutamyl transferase is found in the microsomes of hepatocytes and biliary epithelial cells; its rise in association with an increase in ALP is highly suggestive of a biliary tract obstruction and is known as a cholestatic picture (Hall & Cash, 2012). Bilirubin is a known potent antioxidant, unconjugated bilirubin being a stronger antioxidant than conjugated bilirubin (Jayanthi et al. 2018). Bilirubin modulates signaling pathways regulating inflammation and affects apoptosis, cell proliferation and immune responses (Targher & Byrne, 2015). Furthermore, bilirubin is a

catabolic product of hemoglobin, and it is released in unconjugated form that enters into the liver and converted to conjugated bilirubin.

The serum bilirubin levels more than 17μmol/L are suggestive of liver diseases (Gupta et al. 2016). Liver enzymes have been implicated with risk of CVD and type 2 diabetes mellitus (T2DM) (Choi et al. 2018). For instance, elevated serum GGT has been associated with various cardiometabolic diseases, including coronary heart disease, atrial fibrillation and congestive heart failure, while increased ALT and AST levels are associated with increased oxidative stress and are predictive of future cardiovascular risks (Choi et al. 2018; Fujii et al. 2020). ALT has been reported to worsen diabetes through insulin resistance with hepatic steatosis (Ndrepepa & Kastrati, 2019).

Hematological parameters are relatively inexpensive and widely used in clinical practice; they have also demonstrated their diagnostic and prognostic value in a number of cardiovascular diseases (Budzianowski et al. 2017). They include red blood cells count (RBC), blood hemoglobin concentration (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), total leukocyte count (TLC), neutrophils (NEUT), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), platelets (PLT) and mean platelet volume (MPV) (Andrew et al. 2016; Budzianowski et al. 2017).

The linkage of hematological parameters with cardiovascular risk factors has been reported by a few studies, for example Crescente et al. (2009) revealed that platelet aggregation contributes to both the development of atherosclerosis and to acute platelet thrombus formation, followed by embolisation of stenosed arteries. On the other hand, Quercetin have been reported to inhibit platelet aggregation independently of arachidonic acid or ADP. It also inhibits platelet activation through the blockade of activity of the proto-oncogene tyrosine protein kinase Fyn and the tyrosine phosphorylation of spleen tyrosine kinase and phospholipase C gamma 2 (Andriantsitohaina et al. 2012). Since chia seeds are rich in Quercetin, it may be used to control hematological abnormalities particularly platelet aggregation.

High fructose, sucrose and/or fat diets which may lead to various metabolic changes such as reduced lipolytic activity in fat tissue, reduced leptin secretion, dyslipidemia, insulin resistance and obesity, have been used to induce metabolic and physiological alterations in small animals, and simulating several aspects of the metabolic abnormalities in humans (Kiage-Mokua et al. 2018). In this study, chia seeds which are rich in polyunsaturated fatty acids (α-linolenic acid), fiber, protein and minerals, were used to investigate their dose effects on plasma glycaemia, body weight changes, serum lipids, liver enzymes and hematological parameters in male Wistar rats fed with fructose and lard.

6.2 Material and Methods

6.2.1 Preparation of Chia Seed Extracts

Chia seed extracts were prepared from black seeds by extracting the ground seeds with 99 % ethanol (Loba Chemie Pvt. Ltd) according to the method described by Thakur and Sidhu (2014) with some modifications. Ground chia seeds (20 g) were mixed with 200 ml of ethanol, the mixture was kept for 2 hours in a shaker (Kika Labortechnik, KS 250 basic, Germany) at room temperature. The mixture was centrifuged (Hettich zentrifugen, Universal 16A, Germany) at 4000 rpm for 20 minutes while the supernatant was collected and concentrated in a Rotary Evaporator RE 100 (Bibby sterilin Ltd, UK). The volume of ethanolic extracts was completed with distilled water to keep the initial concentration, and packaged in amber flasks which were kept at 4^{o}C in the fridge (Hisense, RD-28DR4SA, China) until use.

6.2.2 Preparation of Lard and Fructose

The lard and fructose were prepared as indicated in section 5.2.1.3

6.2.3 Experimental Animals, Housing and Interventions

Animals. Male Wistar rats weighing 110 g- 250 g were used for the experiments. The breeds were obtained from the stock in the Small Animal Facility for Research and Innovation (SAFARI), College of Health Science, JKUAT. After breeding in the animal house, they were placed in plastic cages in groups of 6 animals for seven days

for acclimatization and fed on rat pellets (Unga farm Care (EA) Ltd, Nairobi) and water *ad libitum.* Throughout the experiment, the animals were maintained on a 12-hour light/dark cycle at a constant temperature and humidity. All aspects of animal care and experimentation were approved by the ethical committee of JKUAT and was in accordance with the EEC directive of 1986 (86/609/EEC). (Directive 86/609/EEC on the Protection of Animals Used for Experimental and Other Scientific Purposes)

6.2.4 Experimental Design

Twenty-four (24) male Wistar rats were randomly assigned into three experimental groups (low dose, medium dose and high dose rats of 6 animals each) and a control (n = 6). The control group received 6g fructose and 12g lard; the experimental groups received 6g fructose and 12g lard and in addition received 12g/kg, 18 g/kg and 24 g/kg body weight of chia seed extract for low dose, medium dose and high dose respectively. About 15g of rat pellets was given to each rat in all experimental groups and a control, water was given *ad libitum* to the rats in all groups. The lard, fructose and chia seed extracts were orally administered by a gavage. The diets were given daily for a period of 8 weeks.

6.2.5 Sample Size

Sample size was calculated based on 'Group comparison-one-way ANOVA' using degree of freedom (DF) as previously reported by (Charan & Kantharia, 2013; Arifin & Zahiruddin, 2017).

n = DF/k + 1,
N = n x k, where
n = number animals per group,
k = number of groups, and
N = total number of animals.

The power analysis approach (ANOVA) being between 10 to 20. So any sample that keeps DF (E) between 10 and 20 is considered adequate.

In this experiment

DF = 20; k = 4+1=5;

n= 20/5 = 4

Number of animals per group = 4 (before considering % attrition)

Total number = n x k = 4x5 = 20 (Before including % attrition)

However acceptable % attrition is 10%

10% of N = 0.1x20 = 2

N= (nxk) + % attrition

N= 20+2 = 22, but animals per group will be 22/4 = 5.5 = 6; 6x4 = 24

Therefore, the total number of animals obtained for this experiment was 24.

6.2.6 Blood Collection

At the beginning of the intervention, the blood was collected by making a small cut on a tip of a rat tail, and the blood allowed to drain into a strip inserted in a glucometer (Accu-Check Active, Roche, Mannheim, Germany) in order to measure blood sugar levels (day 1), and at the end of the intervention (day 56) fasting blood glucose was determined using hand hold glucometer. Additionally, the rats were sacrificed after a 12 hour fast. Before termination, the rats were anaesthetized with carbon dioxide and blood collected from them by cardiac puncture.

About 4 ml of blood was collected into EDTA-anticoagulated tubes (BD, Belliver Industrial Estate, Plymouth, UK) from each animal and used for determining hematological parameters. Another 4 ml of blood was collected in Heparin-anticoagulated tubes from each animal and was subjected into centrifugation (5000 × g for 10 min) to get serum that was stored at -20°C for biochemical analyses. The blood in tubes were transported to IPR in cool box maintained at 4^{o}C for enzymatic and serum lipids analyses.

6.2.7 Hematological Assays and Biochemical Indices

6.2.7.1 Hematological Assays and Blood Smears

The hematological analysis was conducted at the Institute of Primate Research (IPR) Laboratory, Nairobi, Kenya using Hematology Analyzer (Ac.T 5diff CP, Beckman Coulter, France). Parameters determined were red blood cells (RBC) count, blood hemoglobin concentration (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), total leukocyte count (TLC), Neutrophils (NEUT), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), platelets (PLT) and mean platelet volume (MPV). Blood smears were taken on microscope slides and read on microscope to determine cellular morphological changes.

6.2.7.2 Biochemical Indices

6.2.7.2.1 Liver Enzymes

6.2.7.2.1.1 Aspartate Aminotransferase, Alanine Aminotransferase and Gamma Glutamyl Transferase

About 1ml oxoglutarate and tris-buffer containing aspartate (GOT/AST) or alanine (GPT/ALT) and or L-γ-Glutamyl-3-carboxy-4-nitranilide (GGT) dispensed into a labeled test tube for each enzyme. Approximately 0.1ml (100µl) of each serum sample added into each tube and mixed well and the absorbance was read immediately at 365 nm using Chemistry analyzer (Humalyzer 2000®).

6.2.7.2.1.2 Alkaline Phosphatase

About 1ml of prepared reagent dispensed into a labeled test tube. About 20µl of each serum sample added into each tube and mixed well and the absorbance was read immediately at 405 nm using Chemistry analyzer (Humalyzer 2000®).

6.2.7.2.1.3 Bilirubin

About 1 ml of bilirubin working reagent was added into each tube followed by a drop of total bilirubin reagent. About 0.1 ml of serum was added into each tube, incubated at room temperature before reading absorbance at 546 nm.

6.2.7.2.2 Serum Lipids

6.2.7.2.2.1 Total Cholesterol (TC)

About 1000 µl of the working reagent consisting of Phosphate buffer (pH 6.5), 4-Aminophenazone, Phenol, Peroxidase, Cholesterolesterase and Cholesteroloxidase was dispensed into blank standard and test sample tubes. Approximately 10µl of serum/standard was added into respective tubes and the absorbance read at 546 nm within 60 min.

6.2.7.2.2.2 High-Density Lipoprotein Cholesterol (HDL-C)

About 750 µl of the working reagent consisting of Good´s buffer (pH 7.0), Cholesterol esterase, Cholesterol oxidase, Catalase, and N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (HDAOS) was dispensed into blank standard and test sample tubes. About 10 µl of serum/standard was added into respective tubes and incubate for 5 min at 37^{o}C. Approximately 250µl of substrate (Peroxidase, 4-Aminoantipyrin (4-AA) and Good´s buffer, pH 7.0) was added into tubes containing serum/standard, then mixed gently and further incubated for 5 min at 37^{o}C before reading the absorbance at 555 nm.

6.2.7.2.2.3 Low-Density Lipoprotein Cholesterol (LDL-C)

Approximately 750µl of the working reagent consisting of Cholesterol esterase, Cholesterol oxidase, Catalase and N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS) and Good´s buffer, pH 7.0) was added into blank standard and test sample tubes. About 10µl of serum/standard was added into respective tubes and incubate for 5 min at 37^{o}C. About 250µl of substrate (Peroxidase, 4-Aminoantipyrin (4-AA) and Good´s buffer, pH 7.0) was added, mixed gently and further incubated

for 5 min at 37ºC before reading the absorbance of standard/sample against the reagent blank at 593 nm.

6.2.7.2.2.4 Triglycerides (TG)

About 1000 µl of the working reagent (magnesium Salt, 4-Aminoantipyrine,4-Chlorophenol, Glycerol-3-phosphate Oxidase, Lipases, Glycerol Kinase, Peroxidase and Pipes Buffer pH 7.5) was added into blank standard and test sample tubes. About 10µl of serum/standard was added into respective tubes. The absorbance of standard/sample against the reagent blank was read at 546 nm within 60 min.

6.2.7.3 Determination of Atherogenic Index (AI) and Coronary Risk Index (CRI)

AI was calculated as a ratio of (LDL-C /HDL-C) while CRI was calculated as ratio of (TC/HDL-C and TG/HDL-C) (Adeneye & Olagunju, 2009).

6.2.8 Weight Records

The body weight of each rat was recorded daily until the end of the experiment. Weight gain was calculated between weeks as %Weight gain [(eg. Week II-Week I/Week II) x 100; (Week III-Week II/Week III) x 100].

6.2.9 Statistical Analysis

STATA 14.0 Corp LP was used for statistical analysis of samples. The experiments were performed in duplicates and the results were expressed as the mean ± standard deviation (SD). ANOVA applied Bonferroni multiple comparison test to determine the variability between groups. Statistical significance was accepted at $p \leq 0.05$.6.3 Results and Discussion

6.3.1 Results

6.3.1.1 Effect of Chia Seed Extracts on Fasting Blood Glucose

There was a decrease in blood glucose levels in experimental groups, however the difference was not significant in low dose ($p = 0.936$), medium dose ($p = 0.687$) and high dose group ($p = 0.354$) compared to a control (Table 6.1).

Table 6.1: Fasting Blood Glucose Levels of Male Wistar Rats Fed With Fructose, Lard and Chia Seed Extract

Group	Fasting glucose at the beginning of intervention (mmol/L)	Fasting glucose at the end of intervention (mmol/L)	P-value
Control	4.0 ± 0.44	6.48 ± 0.53	0.985
Low dose	4.0 ± 0.39	6.22 ± 0.40	0.936
Medium dose	3.92 ± 0.50	6.08 ± 0.32	0.687
High dose	3.97 ± 0.40	4.97 ± 0.52	0.354

Values are expressed as mean ± SD. The difference in the test values versus control values were considered significant at $p \leq 0.05$. Control group received 6g/12 g fructose/lard only, low dose group received 6g/12g of fructose/lard plus 12g/kg body weight of chia seed extract, medium dose group received 6g/12g of fructose/lard plus 18 g/kg body weight of chia seed extract and high dose group received 6g/12g of fructose/lard plus 24 g/kg body weight of chia seed extract.

6.4.1.2 Effect of Chia Seed Extract on Body Weight Changes in Rats Fed with Fructose and Lard

The differences in weight was calculated as percentage weight gain between groups (Table 6.2). The rats gained weights from week 1 to week VIII in control group, low dose group and medium group. However the % weight gain was much reduced between week VII and VIII in a low dose group. For a high dose group, there was a negative %weight gain between week VI &VII and between week VII&VIII (Figure 6.2). This is an indication that chia seeds may be effective in controlling weight gain.

Table 6.2: Body Weight Changes of Male Wistar Rats Fed With Lard, Fructose and Chia Seed Extract

Time	Experimental groups			
(Week)	Control	Low dose	Medium dose	High dose
I	197.47 ± 0.36	196.78 ± 0.17	253.22 ± 0.94	253.23 ± 0.58
% Wt. gain	0.12	10.51	10.89	10.25
II	197.2 ± 0.30	219.23 ± 0.31	284.17 ± 0.35	282.25 ± 0.39
% Wt. gain	13.9	8.6	0.91	5.93
III	228.47 ± 0.52	240.75 ± 0.79	286.25 ± 0.35	300.83 ± 0.34
% Wt. gain	4.52	6.22	6.21	1.38
IV	239.13 ± 0.75	256.30 ± 0.74	305.68 ± 0.80	305.32 ± 0.44
% Wt. gain	6.88	5.96	5.04	5.52
V	257.22 ± 0.35	272.37 ± 0.45	322.35 ± 0.49	322.17 ± 0.51
% Wt. gain	5.4	3.78	3.59	0.62
VI	272.25 ± 0.37	283.75 ± 0.41	334.30 ± 0.77	324.10 ± 0.73
% Wt. gain	3.2	4.71	1.69	- 3.49
VII	281.61 ± 0.40	297.28 ± 0.44	339.37 ± 0.49	313.37 ± 0.41
% Wt. gain	8.12	0.56	2.69	- 0.29
VIII	306.65 ± 0.49	298.20 ± 0.37	330.53 ± 0.43	313.53 ± 0.43

Values are means ± SD (n = 6). Wt-Weight. % Wt gain was compared between groups within the same column. Control group received 6g/12 g fructose/lard only, low dose group received 6g/12g of fructose/lard plus 12g/kg body weight of chia seed extract, medium dose group received 6g/12g of fructose/lard plus 18 g/kg body weight of chia seed extract and high dose group received 6g/12g of fructose/lard plus 24 g/kg body weight of chia seed extract.

6.3.1.3 Effect of Chia Seed Extracts on Hematological Parameters

Table 6.3: Hematological Parameters of Male Wistar Rats Fed with Fructose, Lard and Chia Seed Extract

Blood Parameters	Experimental groups			
	Control	Low dose	Medium dose	High dose
RBC ($x10^3/m^3$)	6.53 ± 0.24^a	6.88 ± 0.31^a	6.95 ± 0.32^a	6.85 ± 0.36^a
Hb (g/dl)	15.35 ± 0.24^a	15.08 ± 0.31^a	15.20 ± 0.23^a	14.76 ± 0.19^a
PCV (%)	37.26 ± 0.27^a	38.25 ± 0.24^a	38.36 ± 0.42^a	37.36 ± 0.35^a
MCV (fl)	57.83 ± 0.41^a	56.17 ± 0.40^{ab}	54.83 ± 0.40^b	53.80 ± 0.45^b
MCHC (%)	41.33 ± 0.90^a	39.73 ± 0.37^b	39.75 ± 0.28^b	39.82 ± 0.25^b
MCH (Pg)	23.90 ± 0.86^a	$22.43 \pm 0 .95^b$	21.90 ± 0.53^b	21.82 ± 0.58^b
RDW (%)	10.87 ± 0.67^a	10.80 ± 0.55^a	10.58 ± 0.39^a	10.96 ± 0.30^a
TLC($x10^3/mm^3$)	10.18 ± 0.29^a	8.32 ± 0.32^a	11.10 ± 0.14^a	10.48 ± 0.46^a
NEU	14.38 ± 0.33^a	13.45 ± 0.32^a	15.39 ± 0.29^a	13.18 ± 0.19^a
LYM	$79. 25 \pm 0.37^a$	66.30 ± 0.29^a	76.15 ± 0.23^a	76.14 ± 0.32^a
MON	5.27 ± 0.27^a	6.28 ± 0.32^a	5.25 ± 0.21^a	7.36 ± 0.32^a
EOS	0.67 ± 0.18^a	0.70 ± 0.26^a	0.52 ± 0.07^a	0.84 ± 0.27^a
BAS	1.02 ± 0.23^a	0.80 ± 0.24^a	1.28 ± 0.90^a	0.94 ± 0.26^a
PLT($x10m^3/mm^3$)	521.83 ± 0.41^a	345.00 ± 0.43^b	469.83 ± 0.41^{ab}	495.20 ± 0.45^a
MPV (fl)	6.93 ± 0.08^{ab}	6.98 ± 0.25^a	6.78 ± 0.41^{ab}	6.32 ± 0.38^b

Values are expressed as mean ± SD. Means with the same superscripts within the same row are not significantly different ($p \leq 0.05$). RBC- Red blood cells count, Hb-blood hemoglobin concentration, PCV- packed cell volume, MCV- mean corpuscular volume, MCH- mean corpuscular hemoglobin, MCHC-mean corpuscular hemoglobin concentration, RDW- red cell distribution width, TLC- total leukocyte count, NEUT-Neutrophils, LYM-lymphocytes, MON-monocytes, EOS-eosinophils, BAS-basophils, PLT-platelets, MPV-mean platelet volume. Control group received 6g/12 g fructose/lard only, low dose group received 6g/12g of fructose/lard plus 12g/kg body weight of chia seed extract, medium dose group received 6g/12g of fructose/lard plus 18 g/kg body weight of chia seed extract and high dose group received 6g/12g of fructose/lard plus 24 g/kg body weight of chia seed extract.

There was a significant increase ($p = 0.027$) in mean platelet volume (MPV) in low dose compared to other groups. However, there was a significant decrease in mean corpuscular volume (MCV) ($p = 0.0001$), mean corpuscular hemoglobin concentration (MCHC) ($p = 0.001$) and mean corpuscular hemoglobin (MCH) ($p = 0.001$) between experimental groups and a control (Table 6.3).

Platelets were significantly lower ($p = 0.0001$) in low dose group compared to a control and high dose groups. Other hematological parameters such as total white blood cells (WBC), red blood cells (RBC) count, blood hemoglobin concentration (Hb), packed cell volume (PCV), Neutrophils (NEUT), lymphocytes (LYMP), monocytes (MON), eosinophils (EOS) and basophils (BAS) exhibited no significant differences between experimental groups and a control.

Blood smears indicated cellular morphological changes of the rats from the experimental group fed with high fat high fructose diet, whereas most cells in experimental groups supplemented with chia seed extract were normal (Figure 6.1).

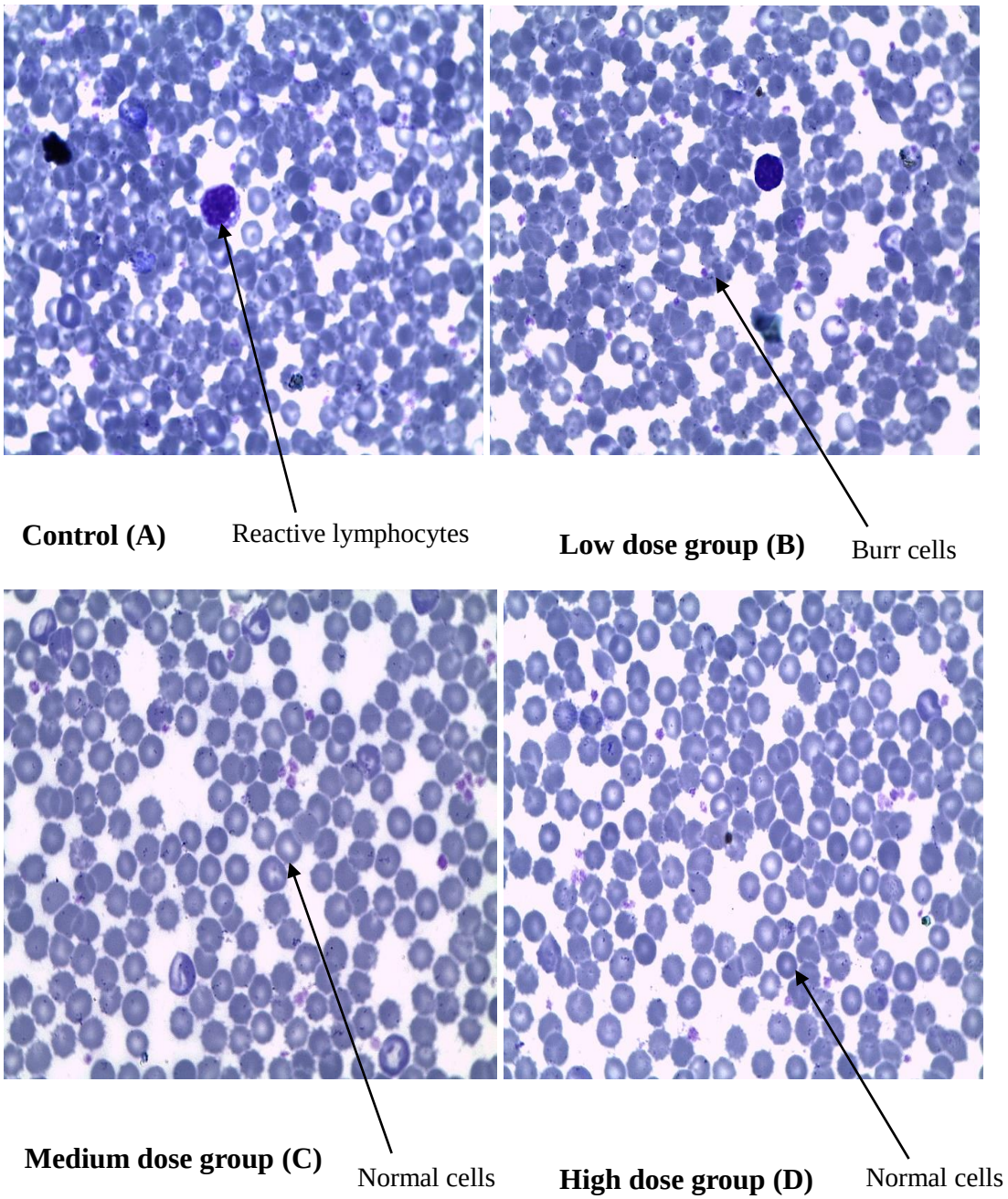

Figure 6.1: Cellular Mophological Changes of Rats (A, B,C And D) Fed A HFFD and Chia Seed Extracts

Reactive lymphocytes in a control group (A) may be a sign of a chronic condition or injuries in body cells. Burr cells in B are abnormally shaped red blood cells with notched surfaces, they are also known as echinocytes. Morphology of cells in C and D observed to be normal an indication that chia seed extract contributed to an improvement of cellular morphology. The presence of burr cells in B may indicate abnormally high level of nitrogen waste products in the blood (Kimang'a et al. 2016). Additionally, a non-uniform color of erythrocytes (anisochromasia) was observed in A, this is caused by a non-uniform distribution of hemoglobin. Excessive anisochromasia can lead to forms of anemia that are caused by a deficiency of iron. That means HFFD may impair the distribution of hemoglobin in blood, hence affecting its role of transporting iron in the body of animals.

6.3.1.4 Effect of Chia Seed Extract on Biochemical Indices of Liver Function

Aspartate aminotransferase (AST) decreased significantly ($p = 0.001$) in low dose group compared to other groups. The level of alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin and total protein (TP) in all groups was not significant (Table 6.4). Gamma glutamyl transferase (GGT) decreased significantly ($p = 0.02$) in medium and low dose groups likewise bilirubin was significantly lower in experimental groups ($p = 0.005$) compared to a control (Table 6.4).

Table 6.4: Effect of Chia Seed Extract on Biochemical Indices of Liver Function

Liver Enzyme	Experimental groups				
	Control	Low dose	Medium dose	High dose	P-value
AST (U/L)	106.25 ± 0.64	88.30 ± 0.28	112.00 ± 0.42	114.95 ± 0.21	0.001
ALT (U/L)	57.85 ± 0.49	48.25 ± 0.78	51.20 ± 0.57	51.35± 0.78	0.058
ALP (U/L)	204.00 ± 0.14	181.10 ± 0.57	202.80 ± 0.21	196.40 ± 0.42	0.227
GGT (U/L)	6.20 ± 0.27	4.80 ± 0.28	4.1± 0.28	4.35 ± 0.64	0.020
Bil (µmol/L)	7.19 ± 0.30	4.9 ± 0.14	4.4 ± 0.28	3.80 ± 0.18	0.005
Albu (g/dl)	35.75 ± 0.35	36.75 ± 0.35	38.45 ± 0.64	37.2 ± 0.28	0.448
TP (g/dl)	68.87 ± 0.48	67.33 ± 0.82	68.55 ± 0.65	70.25 ± 0.49	0.413

Values are expressed as mean ± SD. Statistical significance was accepted at $p \leq 0.05$ within the same row. AST-Aspartate aminotransferase; ALT-Alanine aminotransferase; ALP-Alkaline phosphatase; GGT- Gamma glutamyl transferase; Bil- Bilirubin; Albu- Albumin; TP- Total protein.

6.3.1.5 Effect of Chia Seed Extract on Serum Lipids

Table 6.5: Effect of Chia Seed Extract on Serum Lipids (mmol/L) of Rats Fed Fructose and Lard

Serum lipids	Experimental groups			
	Control	Low dose	Medium dose	High dose
TC	2.23 ± 0.70^{a}	1.94 ± 0.35^{a}	2.18 ± 0.43^{a}	1.54 ± 0.15^{a}
HDL-C	0.53 ± 0.05^{b}	0.63 ± 0.06^{ab}	0.92 ± 0.11^{ab}	1.11 ± 0.62^{a}
LDL-C	0.83 ± 0.03^{a}	0.89 ± 0.33^{a}	0.66 ± 0.05^{ab}	0.37 ± 0.05^{b}
TG	1.60 ± 1.01^{a}	1.43 ± 0.42^{a}	0.87 ± 0.03^{a}	0.77 ± 0.10^{a}
LDL/HDL	1.57 ± 0.60^{a}	1.41 ± 0.18^{a}	0.72 ± 0.45^{ab}	0.33 ± 0.08^{b}
TG/HDL	3.02 ± 0.07^{a}	2.27 ± 0.14^{a}	0.95 ± 0.27^{a}	0.69 ± 0.16^{a}
TC/HDL	4.21 ± 0.07^{a}	3.08 ± 0.17^{a}	2.37 ± 0.12^{b}	1.39 ± 0.24^{b}

Values are expressed as mean± SD. Means with the same superscripts within the same row are not significantly different ($p \leq 0.05$). TC-Total cholesterol; HDL-C- High density lipoprotein cholesterol; LDL-C- Low density lipoprotein cholesterol; TG- Triglycerides. Control group received 6g/12 g fructose/lard only, low dose group received 6g/12g of fructose/lard plus 12g/kg body weight of chia seed extract, medium dose group received 6g/12g of fructose/lard plus 18 g/kg body weight of chia seed extract and high dose group received 6g/12g of fructose/lard plus 24 g/kg body weight of chia seed extract.

Although total cholesterol (TC) and triglycerides (TG) decreased in high dose group as compared to other groups, their differences were not significant. High density lipoprotein cholesterol (HDL-C) increased significantly ($p = 0.041$) in high dose group than the control while low density lipoprotein cholesterol (LDL-C) decreased significantly ($p = 0.035$) in high dose group as compared to control and medium dose groups ($p = 0.021$) (Table 6.5).

Atherogenic index (AI) (LDL-C/HDL-C) decreased as the dose increased, however, the difference among the groups were not significant. There was a significant decrease in coronary risk index (CRI) (TC/HDL) in high dose group compared to a control.

6.3.2 Discussion

In this study, there was a decrease in blood glucose levels in medium and high dose groups at the end of intervention (Table 6.1), this tells us that consumption of chia seeds can slow down elevated levels of blood glucose hence controlling hyperglycemia/diabetes. These results are consistent with Enes et al. (2020) who

observed that chia oil improved glucose and insulin tolerance. It is also supported by Fonte-Faria et al. (2019), who found that chia oil restored glucose and insulin tolerance in mice fed high fructose diet. Nevertheless, Marineli et al. (2014) reported that, both chia oil and flour improved glucose and insulin tolerance in rats fed high fat high fructose diet. Effect of chia seeds on blood glucose shows that, they can be among the ideal choice of natural food sources for the primary prevention of hyperglycemia.

Body weight changes revealed a pronounced decrease in body weight of rats fed with a medium and high doses of chia seed extract (Figure 6.2). Although there was a gain in body weight of rats from week I to week VIII in all groups, the medium and high dose groups exhibited the gradual increase in body weight. This tells us that chia seeds may control body weight gain when consumed sufficiently. These results match with the previous findings by (Toscano et al. 2015) who reported a decrease in weight among obese men when consumed 35 g of chia flour for 12 weeks.

Hematological parameters provide significant information for the diagnosis and prognosis of diseases in animals (Agiang et al. 2017; Budzianowski et al. 2017). They have been associated with health indices and are of great importance in routine clinical evaluation of the state of health. They include RBC, Hb, PCV, MCV, MCH, MCHC, RDW, TLC, NEUT, LYM, MON, EOS, BAS, PLT and MPV. An increase in MPV in low dose group may be attributed by low platelet count, since MPV is inversely proportional to platelet count. MPV can provide important information on the course and prognosis of many pathological conditions such as CVDs, diabetes mellitus, rheumatoid arthritis and a number of neoplastic diseases (Budzianowski et al. 2017).

MCV, MCHC and MCH are used to measure the size and weight per unit volume of red blood cells. The size of the cells may be normocytic (normal average red blood cell size), microcytic (small average red blood cell size) and macrocytic (large average red blood cell size). In this study MCV, MCHC and MCH decreased in experimental groups compared to a control, this indicates microcytic anemia, a condition in which red blood cells become too small than normal, iron deficiency is

the most common cause of microcytic anemia (Agiang et al. 2017). Many factors are responsible for iron deficiency and iron deficiency anemia including acute and chronic infections, parasite infestations and poor bioavailability of dietary iron, these can lower blood hemoglobin concentration leading to anemia (Rodríguez, Hotz & Rivera, 2007). In this study, we controlled parasites and infections, so poor bioavailability of dietary chia might be the cause for microcytic anemia. Although MCV, MCHC and MCH decreased per dose, they were within the reference values of male Wistar rats aged 8-12 weeks.

PLT was high in a control group that was fed with lard and fructose, implying the likelihood of a variety of prothrombotic and inflammatory diseases. Presence of large and reactive platelets increase the risk of thrombus formation after atherosclerosis plague rupture. A decrease in the circulating platelets less than 50 % of the normal value may cause bleeding (Monteomo et al. 2018), which was not the case for the chia seed extract fed groups. PLT dropped in low dose group and started to increase as per increase in dose (Table 6.3), that means chia seed extract did not have detrimental effects on PLT and other blood components.

Liver enzymes are commonly used in the evaluation of liver diseases and other tissue damages; they are used to determine whether a primary disorder is hepatitic or cholestatic in origin (Hall & Cash, 2012). Liver enzymes have been associated with risk of CVD and T2DM (Liu et al. 2016; Kim et al. 2019). In this study, elevated levels of AST were observed in medium and high dose groups (Table 6.4), which may be an indication of liver or muscle injury, but the mechanism is not known.

Alanine aminotransferase (ALT), GGT and ALP decreased in experimental groups as compared to the control group that was fed with fructose and lard (Table 6.4). This indicates the possibility of chia seeds in protecting the hepatocytes and biliary epithelial cells from injuries and obstruction. Bilirubin was significantly lower in high dose as compared to control group which is not generally harmful. However, lower serum bilirubin levels have been associated with endothelium and microvascular malfunction (Jayanthi et al. 2018).

Hypobilirubinemia might be a possible risk factor of arteriosclerosis, coronary artery disease and inflammation (Gupta et al. 2016; Jayanthi et al. 2018).

Although there is limited information on the effect of chia seeds on liver enzymes, these findings may provide a ground for establishing relevant information on the impact of chia seeds consumption on liver enzymes.

Results on serum lipids showed a decreased level of triglycerides and a significant decrease in LDL-C and an increase in HDL-C in high dose group (Table 6.5). Various components of chia seeds such as n-3 PUFAs, phytosterols, fiber, antioxidants, minerals and their combined effects could be responsible for this reduction. These results are in line with the previous study by Ayerza & Coates, (2007), who reported a decrease in serum triglycerides and an increased HDL-C in rats fed with ground chia seed, whole chia seed and chia seed oil. Another study by Chicco, D'Alessandro, Hein, Oliva & Lombardo (2009) stated that dietary chia seed normalized hypertriglyceridemia and prevented the onset of dyslipidemia and insulin resistance in the rats fed with sucrose rich diets. Results found by Guevara-Cruz et al. (2012) revealed that the amount of serum triglycerides reduced when standard diet containing chia seeds and other grains were consumed by individuals with metabolic syndrome. Our findings on serum lipids also match with Toscano et al. (2015) and the colleagues who observed a decrease in total cholesterol and an increase in HDL-C in men and women that ingested 35 g of chia flour/day for 12 weeks. These results support the evidence that, a decrease in HDL-C and an increase in LDL-C, total cholesterol (TC) and triglyceride (TG) may contribute to the progression of atherosclerosis (Cai et al. 2017). Atherogenic and coronary risk indices are strong predictors of the risk of atherosclerosis and coronary heart disease and can disclose the presence of LDL-C or TG in the serum (Kazemi et al. 2018). In this study, atherogenic and coronary risk indices decreased as the dose increased. Low levels of AI and CRI suggest the possibility of chia seeds in lowering CVD risks when consumed in adequate amount.

6.4 Conclusion

This study demonstrated that an 8-weeks dietary intervention with chia seed extract was associated with reduced blood glucose levels, triglycerides, LDL-C, TC and an increase in HDL-C in rats fed with fructose and lard. An improvement of hematological parameters and less effect on liver enzymes except AST was also observed. Although chia seeds appear safe on face value, there are injuries that may result out of its overconsumption, thus food scientists and nutritionists need to do more to determine safe amounts that can be consumed without the resultant biological injury. Therefore, we recommend the inclusion of moderate amounts of chia seeds in diets targeting the primary prevention of cardiovascular diseases.

CHAPTER SEVEN

SUMMARY, CONCLUSION, RECOMMENDATIONS, LIMITATION AND SUGGESTIONS FOR FUTURE WORK

7.1 Summary

The fundamental task of dealing with CVD is primary prevention of serum CVD risk factors [elevated triglycerides, low density lipoprotein cholesterol (LDL-C), decreased high density lipoprotein cholesterol (HDL-C) and hyperglycemia/diabetes] and other associated risk factors (central obesity, atherogenic diets etc), and more importantly understanding their interaction. Several synthetic drugs have been used to treat/reduce cardiovascular risk factors, for example fibrates, statins and nicotinic acid. However, frequent use of these drugs is implicated with adverse side effects like dyspepsia, abdominal pain, myopathy, blurred vision, elevated liver enzymes, abdominal bloating, flatulence and diarrhea (Kolovou et al. 2011). Additionally, the costs of medication for CVDs are high and estimated to reach US $1,044 billion by 2030 (Forum & Health, 2011). Therefore, alternative approaches especially the use of pharmacological components from natural sources such as chia seeds should be adapted.

In this study, chia seeds indicated high content of fiber, fats, proteins, minerals (phosphorous, potassium and magnesium) and polyunsaturated fatty acids particularly the omega-3 (α-linolenic acid), omega-6 (linoleic acid), and omega-9 (oleic acid). Phytochemical screening highlighted the presence of several bioactive compound such as flavonoids that are beneficial in the prevention of cardiovascular risk factors. The ethanolic and methanolic extracts of chia seeds inhibited α-amylase and pancreatic lipase by 50 %. There was a dose dependent increase in percentage inhibitory activity against α-amylase and pancreatic lipase by all five chia seed extracts. Since α-amylase catalyzes the hydrolysis of starch, glycogen and various oligosaccharides into simple sugars, its inhibition in the digestive tract can decrease the absorption of glucose from carbohydrates, thus being considered effective in controlling hyperglycemia and hence type 2 diabetes mellitus (T2DM) (Bhutkar, 2018). On the other hand, inhibition of pancreatic lipase is considered best strategy in

weight management and obesity (Rahman, 2017). Decreased PPG and fasting blood glucose in groups supplemented with ground chia seeds/extracts is promising for primary prevention of type 2 diabetes and cardiovascular diseases. Hematological parameters were improved in groups supplemented with ground chia seeds/extract. The influence of chia seed extract on lipid and glucose metabolism was assessed in a dose effect experiment with male Wistar rats that were fed a high fat and fructose diet to induce hyperglycemia and dyslipidemia. Chia seed extract significantly lowered low-density lipoprotein cholesterol (LDL-C) and significantly increased high density lipoprotein cholesterol (HDL-C) in high dose group. Blood glucose, triglycerides (TG) and total cholesterol (TC) decreased in high dose group but the difference was not significant. Atherogenic and coronary risk indices are strong predictors of cardiovascular disease risk. In this study, atherogenic index (AI) was significantly lower in high dose group compared to a control while coronary risk index decreased as the dose increased. Low levels of AI suggest the possibility of chia seeds in lowering CVD risks when consumed in adequate amounts. These results point out the ability of chia seeds in lowering elevated blood glucose, triglycerides and cholesterol levels since they are high in polyunsaturated fatty acids and fiber (Mohd et al. 2012; Abdulrashed et al. 2016).

On the other hand, elevated levels of alanine aminotransferase (ALT), gamma glutamyl transferase (GGT) and alkaline phosphatase (ALP) were observed in a control group that was fed with fructose and lard, this mirrors the negative effect of dietary fat and sugars which are likely to promote dyslipidemia. Bilirubin was significantly lower in high dose as compared to control group which is not generally harmful. These findings may provide a ground for establishing relevant information on the impact of chia seeds consumption on liver enzymes.

7.2 Conclusion

The East African grown chia seeds indicated a high content of nutrients and fatty acids. Phytochemical screening highlighted the presence of several bioactive non-nutrient plant compounds. *In vitro* studies on chia seed extract indeed demonstrated its ability in inhibiting pancreatic lipase and α-amylase activities. On the other hand,

animal studies revealed a significant reduction of LDL-C and a significant increase of HDL-C. Triglycerides and total cholesterol were also reduced in experimental groups compared to a control. The atherogenic and coronary risk indices in a dose effect experiment were reduced. An improvement of hematological parameters and liver enzymes except AST in a dose effect experiment was observed. Generally, chia seeds can be an appropriate alternative to n-3 PUFA sources for vegetarians and people allergic to fish and gluten-containing diets. Presence of phytochemicals highlights the significance of the seeds in the prevention and management of several non-communicable diseases such as coronary heart disease, diabetes, inflammatory disorders and cancer. Therefore, we suggest incorporation of appropriate amounts of chia seeds in the regular diets.

7.3 Recommendations

In this study, experiments on cardiac enzymes were not conducted due to budget constraints. Therefore, more studies may be carried out to consider the determination of cardiac enzymes, which may be linked with liver enzymes so as to get the broader picture of chia effects on them.

White chia seeds were not used in animals studies because they were found to have low fatty acid profile and minerals (chapter three), so in this study they were screened based on that. However this cannot be a justification of ignoring the use of chia seeds. More studies may be conducted to justify the comparability of white chia seeds and black chia seeds in terms of nutrient composition and their effectiveness in preventing cardiovascular risk factors.

In a situation where enough funds are available, it would be better to conduct clinical trials and more dose dependent experiments to ascertain the proper dosages of chia seeds for humans.

7.4 Limitations

7.4.1 Characterization of Active Compounds

In our studies, we did not characterize the active compounds in the used extracts; it was beyond the scope of the concentration that were responsible for the observed effects. Thus, the results should be considered exploratory and hence further studies are needed to validate the findings. Furthermore, the dose per animal per day was administered based on body weight, may be the effective dose could be higher than what we used in our experiment. The extraction method may have also resulted in low yields of potentially active components. Ethanol is considered safe compared to other diluents like methanol in animals and humans. However, evaluation and selection of pre-extraction preparation and extraction methods depend on the study objectives, samples and target compounds as observed by (Nn, 2015).

7.4.2 Sample Size

Sample size is the number of observations or replicates to include in a statistical sample, in this case, it is the number of animals in each group. The size of sample should be neither excessively large, nor too small. It should be optimum that is it should fulfill the requirements of efficiency, representativeness, reliability and flexibility (Kothari, 2004). The sample size is an important feature of any empirical study in which the objective is to make inferences about a population from a sample. The size of a sample influences two statistical properties: (a) the precision of our estimates and (b) the power of the study to draw conclusions. If the sample size is too small, then there is a reduced likelihood of detecting significances where they really exist (less power) and hence an increased chance of type 1 error.

Contrary, if the sample size is too large, this will be a waste of animals and resources. Our experimental groups in this study had unequal sample sizes meaning that power decreased. Additionally, in some cases, our sample sizes may not have been large enough to detect real significances where they existed. We suggest that, future experiments on chia seed extracts should include equal and larger sample sizes in order to increase the experimental power.

7.5 Future Research

Taking into account the prior-mentioned limitations, future studies should focus on increasing sample size so as to gain more power; proper isolation and characterization of the extracts so as to be able to know which bioactive compound(s) is responsible for the observed effects; using other plant extraction procedures and diluents so as to maximize the amount of bioactive components in the extracts for sound results; lastly but not least is clinical trials with chia seed extracts, this helps to validate the results.

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APPENDICES

Appendix I: Ethical clearance approval letter

JOMO KENYATTA
UNIVERSITY

OF

AGRICULTURE AND
TECHNOLOGY

P. 0. Box 62000-00200 Nairobi, Kenya Tel
0675870225 OR Extn 3209

Institutional Ethics Review
Committee

May 21st, 2019 REF: JKU/2/4/8968

Fabian Dominicus Mihafu,

Department of Human Nutrition Sciences.

Dear Mr. Mihafu,

RE: **EFFICACY OF CHIA SEEDS *(Salvia hispanica L)* ON CARDIOVASCULAR RISK FACTORS IN WISTAR RATS FED A HIGH FAT AND FRUCTOSE DIET**

The JKUA T Institutional Ethics Review Committee has reviewed your responses to issues raised regarding your application to conduct the above mentioned study with you as the Principal Investigator.

The is to inform you that the IERC has approved your protocol. The approval period is from May 21st, 2019 to May 21st, 2020 and is subject to compliance with the following requirements:

a) Only approved documents (informed consent, study instruments, study protocol, etc.) will be used.

b) All changes (amendments, deviations, violations, etc.) must be submitted for review and approval by the JKUAT IERC before implementation.

c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the IERC immediately.

d) Any changes, anticipated or otherwise that may increase the risks to or affect the welfare of study participants and others or affect the integrity of the study must be reported immediately.

e) Should you require an extension of the approval period, kindly submit a request for extension 60 days prior to the expiry of the current approval period and attach supporting documentation.

f) Clearance for export of data or specimens must be obtained from the JKUAT IERC as well as the relevant government agencies for each consignment for export.

g) The IERC requires a copy of the final report for record to reduce chances for duplication of similar studies.

Should you require clarification, kindly contact the

JKUAT IERC Secretariat. Yours Sincerely,

J.K.U.A.T
DIRECTOR,
RESEARCH DEPARTMENT (RPE)
P O Box 62000-00200
NAIROBI

Dr. Patrick Mbindyo

SECRETARY, IERC

Setting Trends in Higher Education, Research, Innovation and Entrepreneurship

Appendix II: Animal Ethics Application Form with Questionnaire

JOMO KENYATTA UNIVERISTY OF AGRICULTURE & TECHNOLOGY

ANIMAL ETHICS COMMITTEE [AEC]

APPLICATION FOR ANIMAL ETHICAL REVIEW

1.0 SUBMISSION PROCEDURE

Complete the application form in **English** and submit **FOUR copies** as indicated below:

Three printed copies *(Signed)*

One electronic copy *(electronic copy does not need to be signed)*

Send to:

AEC Secretary

Jomo Kenyatta University of Agriculture & Technology

P. O. Box 62000- 00200

Nairobi

Kenya

Tel: +254-

Fax: +254-

E-mail: aecsecretary@jkuat.ac.ke

Note: i) JKUAT-AEC meets on the second Tuesday of each month. Applications need to reach the AEC Secretary at least TWO weeks prior to a meeting to ensure inclusion on the Agenda.

ii) Incomplete forms will not be processed

iii) Administrative fee of $ 200 will be charged for each proposal

FOR OFFICIAL USE ONLY

Date Received……. AEC#.....

2.0. GENERAL PROJECT INFORMATION

2.1 Project title: EFFICACY OF CHIA SEEDS (*Salvia hispanica L*) ON CARDIOVASCULAR RISK FACTORS IN WISTAR RATS FED A HIGH FAT AND FRUCTOSE DIET.

2.2 Investigators (fill table)

Name	**Affiliation and address**	**Role in the project (PI, CO-PI, collaborators, student, Technicians/Research assistants**	**Highest Level of Education**
Fabian .D. Mihafu	Department of Human Nutrition Sciences	PI	MSc
Dr. Beatrice. N. Kiage	Department of Human Nutrition Sciences, JKUAT P.O. Box 62000-00200, Nairobi, Kenya.	Supervisor	PhD
Dr. Andrew. K. Nyerere	Department of Medical Microbiology,	Supervisor	PhD

	JKUAT, P.O. Box 62000 – 00200 Nairobi, Kenya.		
Dr. Judith. K. Okoth	Department of Human Nutrition Sciences, JKUAT P.O. Box 62000-00200, Nairobi, Kenya.	Supervisor	PhD

2.1.1 No. of students involved...……. PhD: ONE……. MSc: N/A……Others: N/A.

2.1.2 Name (s) of students involved in the project…FABIAN DOMINICUS MIHAFU.

2.3 Funding agency... DAAD/RUFORUM.

2.3.1 Address of funding agency... RUFORUM, Plot 151/155 Garden Hill, Makerere University Main Campus, P.O Box 16811 Wandegeya, Kampala, Uganda.

2.3.2 Total project amount in KSh. 450,000/=

2.3.3 Date funding approved……June…2018

2.4 Project duration in months/years…5 months (For laboratory animal study).

2.4.1 Proposed project start date…April, 2019……Project end date…August, 2019.

3.0 APPLICATION TYPE SELECTION

3.1 Experimental (lab based) (research or teaching) – Start the Laboratory Research Questionnaire section

3.2 Field Research utilizing domestic & wild animals (research or teaching) – Start the Field Research Questionnaire section

4.0 LABORATORY RESEARCH QUESTIONNAIRE SECTION

4.1- Please state the scientific or educational aims clearly and if appropriate, include an outline of how the project relates to an overall program of work. [250 words]

Chia seeds (*Salvia hispanica* L.) belong to family lamiaceae, native to Mexico and Guatemala. They have been used as a functional food and also to produce supplements and nutraceutical products due their health benefits associated with high amounts of α-linolenic acid (ALA), phytochemicals, dietary fiber, proteins and other micronutrients. These benefits stimulate the search for its efficacy in controlling risks for cardiovascular disease (CVD). The overall objective is to investigate the efficacy of chia seeds in reducing cardiovascular risk factors in Wistar albino rats (WRs) fed a high fat and fructose diet (HFFD). Specific objectives will be to: determine the effect of chia seeds on postprandial glycaemia and lipemia in WRs in an acute (short term) experiment; and evaluate the dose effect of chia seed extracts in blood glucose, lipemia, liver enzymes and hematological parameters in WRs fed a HFFD.

High omega-3 ALA is essential for cardiovascular system while high fiber and phytochemicals help to lower cholesterol. Therefore, chia seeds may be a safer alternative to CVD synthetic drugs as they have been reported to cause various adverse side effects such as dyspepsia, myopathy, abdominal pain and flatulence.

4.2- Provide detailed plan of activities using the table below for entire project duration

Activity	**Year 2019**				
	April	May	June	July	August
Animal studies					
1.1 Purchase of animals and acclimatization					
1.2 Acute experiment (weights measurement, postprandial glycaemia and triglycerides determination)					

1.3 Long-term and dose effect experiment (weight measurement and blood collection)					
1.4 Hematological and biochemical analysis					
1.5 Statistical analyses					

4.3 List the procedures/experiments to be done at JKUAT. For each procedure indicate the laboratory/Animal facility room where it will be conducted.

- Animals will be kept at Small Animal Facility for Research and Innovation (SAFARI) house in the College of Health Sciences (COHES).
- Blood collection for glucose, hematological and other biochemical analysis will be done at SAFARI. Blood glucose will be determined using a hand glucometer.
- Blood will be collected into EDTA tubes before treatment with chia seed extracts, in order to measure baseline parameters and also it will be collected at the end of the treatment.
- At the end of treatment with extract, three (3) rats from each group will be anaesthetized with carbon dioxide (CO_2) and blood collected from them by cardiac puncture. The other three (3) rats will be sacrificed then heart, liver and kidney harvested for histological analysis.
- Biochemical parameters will include liver enzymes, blood glucose, triglycerides, total cholesterol, high density lipoprotein cholesterol and low density lipoprotein cholesterol.

4.4. List the procedures/experiments to be done at the collaborating Institution. For each procedure indicate the laboratory/Animal facility room where it will be conducted.

- Biochemical and hematological parameters will be analyzed at the Institute of Primate Research (IPR) Laboratory.

4.5. List all the records to be collected during the experimental period

 Food consumption.

 Body weight.

 Clinical and diagnostic information

-Appetite (eating or drinking), body condition, body posture, hair coat, respiration, behavior and discharge from orifices or surgical wounds

 Blood. This will be collected at the beginning of the experiment and at the end of experiment/treatment for biochemical analysis (blood glucose, triglycerides, total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol, liver enzymes) and hematological parameters [packed cell volume (PCV), Red blood cells (RBC) count, White blood cells (WBC) count, mean corpuscular Hemoglobin concentration (MCHC), mean corpuscular volume (MCV), Lymphocytes, Monocytes and Neutrophils].

4.6. Where will the experimental data be kept?

- The experimental data will be kept in Lab book, data sheets, notebook and Computer Microsoft Excel.

5.0 Animal Care and Use

5.1 - Please describe the potential benefits of the outcomes of your study, and the evidence that supports the use of animals.

- The study will explore the impact of chia seeds in reversing elevated triglycerides, glucose and LDL-C, also raising HDL-C in Wistar rats fed a HFF diet in order to add to the body of knowledge on incorporating chia seeds in human diets.
- Enhanced understanding that α-linolenic acid from chia can substitute that of fish hence being useful to people with limited access to fish
- The study involves different treatments aimed at establishing the required dose that can be extrapolated for human consumption
- Most studies on the effect of high fats, high sugar foods on human health have been conducted using lower animals; this is because there is not enough data in

humans. Animals therefore provide the best model of study where important information concerning blood glucose control, lipid lowering, cardio-protective and pharmacological properties of the extracts is obtained and applied for clinical trials.

5.2 Please provide details of why the use of animals is essential to achieve all the stated aims. Provide details of the potential alternatives that are available to replace the use of animals in all or part of the project and why these alternatives are not suitable.

- Animal models (Wistar rats) are suitable in establishing the minimum required dose of extracts (chia seed extract) which can be extended for clinical trials. Wistar rats, biologically are relatively similar to humans. They are susceptible to many of the same health problems, and they have short life-cycles so they can easily be studied throughout their whole life-span or across several generations, additionally, environments are easily controlled around the animal (diet, temperature, lighting, etc.) which would be difficult to do with people.
- Cardiovascular disease is an important chronic disease and animals are the best subjects to study the efficacy of chia seed extracts on cardiovascular risk factors (LDL-C, HDL-C, elevated triglycerides and hyperglycemia). These will be monitored/studied in rats fed a high fat and fructose diet. Therefore, lower animals are preferred to humans when it comes to testing disease treatments, breeding, food supplements, and toxicology including cosmetics testing.
- Potential alternatives for studying CVD risk factors may include humans, in vitro systems (cell or tissue culture) and computer simulation.
- Research involving human subjects is littered with a history of scandal that often shapes people's views of the ethics of research (ref. Nazi experiment, Edward Jenner's development of the small pox vaccine 1796, Tuskegee syphilis trial, UK-run Porton Down chemical experiments etc.). There is hesitation of exposing humans to unforeseeable health risks in order to observe the course of a disease. For example, this study involves provision of high fat and high fructose diets which with time may cause hyperglycemia, hyperlipidemia etc.

5.3. Provide a justification for use of the selected species, strain or breed of animal.

- The research will require blood (1ml-3ml) from each rat and so WR will give the amount of blood required.
- The breeds are easily available at JKUAT and are cost-effective in terms of maintenance during experimentation.

5.4 Provide a justification for the numbers of animals including evidence that the numbers are minimal, but statistically robust to achieve the aims of the research. Where appropriate, information must be provided on:

a) Experimental design and statistical considerations.

Sample size calculation based on 'Group comparison—one-way ANOVA' using degree of freedom (DF) as previously reported by DF (E) = Total number of animals – total number of groups.

Expected attrition or death of animals: Final sample size should be adjusted for expected attrition. Corrected sample size = Sample size/ [1− (% attrition/100)].

E = degree of freedom of ANOVA, the value of E should be between 10 and 20. The power analysis approach (ANOVA) is between 10 to 20, so any sample that keeps DF (E) between 10 and 20 is considered adequate.

In this study there will be an acute experiment (a short time experiment) and a dose effect experiment.

For acute experiment: 4 groups will be used each with 5 animals, then E = (5x4) – 4 = 16.

For 10% attrition, corrected sample size = 5/0.9 = 5.5 = 6, so sample size = 6.

Total number of animals = 6x4 = 24 per experiment. For dose effect experiment the total number of animals will be 24.

Alternatively; “n = DF/k + 1, so for minimum sample, n = 10/k + 1 and maximum sample, n = 20/k + 1Total number N: for minimum N = minimum n x k and maximum N = maximum n x k; where k = number of groups, n = number of animals per group, N= total number of animals”

b) For teaching projects, the ratio of students to animals, and the number of times that each animal will be used in each class, and/or handled per day and/or per week.
- N/A

5.5. Is randomization being used as a method of experimental control? Please describe how and why or why not.

- Randomization will be used by assigning numbers with special marking to animals. For example, 1-mark on right hind limb, 2- mark on right forelimb,3-mark on right hind limb and right forelimb, 4-mark a strip on right side of belly, 5- mark on left hind limb, 6- mark on left hind limb and right hind limb, 7- mark on left hind limb and right forelimb, 8-mark on left hind limb, right hind limb and right forelimb, 9-mark on left hind limb and right side of belly, 10-mark on left forelimb. After marking all animals, using weight they will be randomly placed in the group of 6 per cage.

5.6 Please provide a clear description of the steps taken to consider and apply the 3Rs [Replacement, Reduction and Refinement]

- Replacement – this refers to methods that avoid using of animals, can either be absolute replacements or relative replacements. In this study relative replacement is preferred because lower animals (rats) will be used instead of humans. Rats are cost-effective in terms of maintenance during experimentation.
- Reduction – refers to methods that minimize the number of animals used per experiment. A considerable sample size of 48 rats will be used in order to get sound results.
- Refinement – refers to methods that minimize the pain or suffering of experimental animals. In this study, animals will be kept in groups to maintain their company, also sufficient space and proper facilities will be given to avoid

stress. At the end of treatment, animals will be sacrificed and they will be protected from pain and distress by applying CO_2 anesthesia.

5.7 Provides a concise description of experimental protocol(s) to be used in your study. This section should include information on what happens to the animal(s) from the time you obtain them until the time the project is completed. A flow chart or sequence of events table may assist in making this information clear. Identify all factors and procedures that may have an impact on an animal's wellbeing including handling, husbandry, housing as well as specific experimental procedures. Include a table showing the number of animals to be used in treatments (and controls).

-Forty-four (44) male Wistar albino rats 7 - 8 weeks old (weighing 150 g -220 g) will be purchased from SAFARI animal facility at Jomo Kenyatta University of Agriculture and Technology (JKUAT) and they will be housed during the duration of the experiment. The animal and cage identification system will be used to identify the experimental rats. Each group will individually be housed in cages with access to food (mice pencils, milled chia, fructose and lard) and *ad libitum* access to water. Shredded papers will be used as beddings. The room temperature and humidity will be controlled with a 12:12 hour light/dark cycle. Food spillage will be collected on spill papers put under each individual cage and changed daily. Food consumption will be recorded thrice weekly while weight will be recorded on weekly basis. All aspects of animal care and experimentation to be performed will conform to the Guide for Care and Use of Laboratory Animals of the National Institutes of Health (NIH) and will be in accordance with the EEC directive of 1986 (86/609/EEC). The study protocol will be approved by the JKUAT Institutional Ethics Review Committee (IERC).

- Experimental Wistar albino rats (n = 44) will be acclimatized to normal diet (rat pellets) for 2 weeks. Then they will be divided into two groups (**acute experiment** (n = 20)-they will be fed with fructose and lard plus chia seeds and (**dose effect experiment** (n = 24)-they will be fed with high fat and fructose diet (HFFD) plus chia seed extracts)]. Rat pellets and water will be given *ad libitum* in all groups (figure 1).

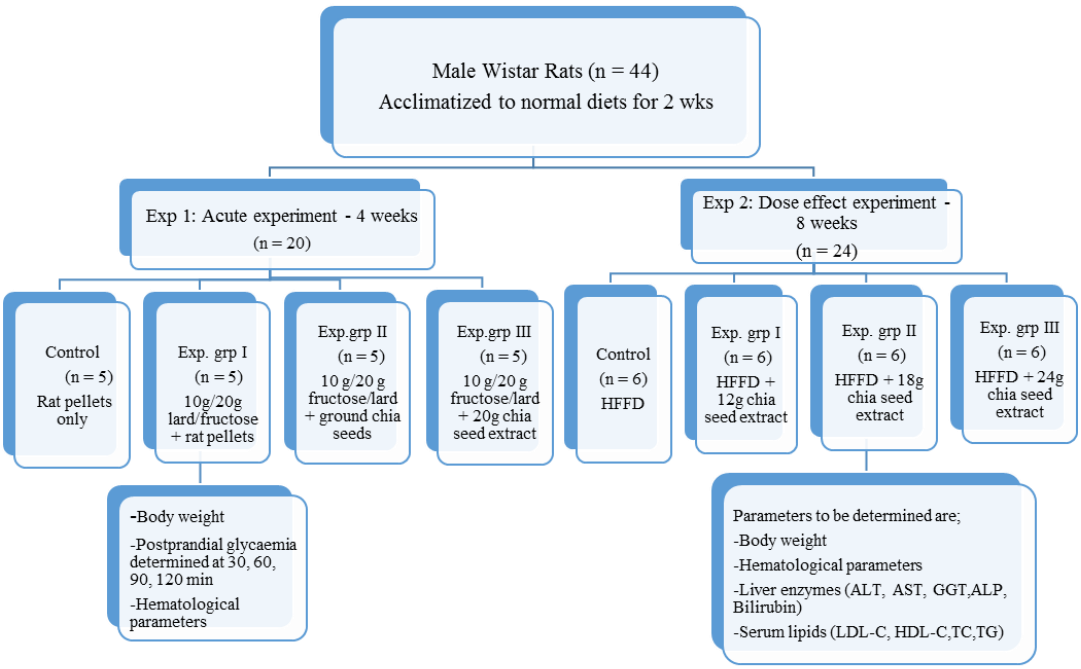

Figure 1: Animals' experimental design

-During the intervention, blood (2 ml) will be collected by tail snip in Bijou bottles. Postprandial glycaemia will be determined by glucometer twice per week for experiment I while fasting blood glucose, triglycerides, total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, hematological parameters and liver enzymes will be determined at the end of the experiment II.

-At the end of treatment (day 56) with chia seed extract, rats will be anaesthetized with CO_2 and blood (3ml-5ml) collected from them by cardiac puncture. 2 mL of blood will be dispensed into EDTA tubes while another 2 mL will be dispensed into clean Bijou bottles without anticoagulant and left to clot for biochemical studies.

Histological analysis of the three organs heart, liver and kidney will be conducted as well. Heart, liver and kidney will be removed from an animal and transferred into a fixative normally formalin. This will allow most tissues to become adequately fixed within 24-48 hours. After that the tissues will be processed into thin microscopic sections.

- Biohazard waste including blood stained tissues will be discarded as biohazard waste. Glass and sharps will be discarded in the sharps bins located in procedure room.

5.8 Will test substances administered to the animals cause pain, discomfort, distress or lasting harm? - No

5.9 Explain the measures that will be taken to reduce pain and discomfort of the animals to a minimum. - Handling animals gently.

5.10. State and justify the end point for the experiment and indicate measures to be taken to minimize pain and distress of animals.

- Euthanasia- at the end of treatment, rats will be anaesthetized with CO_2 and blood collected from them by cardiac puncture.
- Tissue collection and carcass disposal- Heart, liver and kidney will be harvested from the 3 sacrificed rats per group for histological analysis.
- Carcasses will be double-wrapped and frozen until disposal by standard practices.

5.11 (a) Are invasive procedures used to collect tissues, body fluids or any other samples from the live animal (e.g. bleeding, biopsies of tissues or lymph nodes etc.)?

-No

5.11 (b) Explain the measures that will be taken to reduce pain and discomfort of the animals to a minimum.

- Environmental enrichment (shredded paper).

- Proper animal handling.

5.12 Explain how pain, suffering, distress or lasting harm be avoided, recognized, alleviated and managed?

- Animal's health status will be monitored at least once daily, and those with specific health problems will be attended by a Veterinarian who will be available during the experimental period.
- Animals will be handled by well trained personnel.
- Rooms will be well ventilated, kept clean, quiet and uncluttered.
- Pharmacological interventions or euthanasia will be considered.

5.13 What type of anesthesia and/ or analgesia will be used?

- CO_2 may be administered to induce anesthesia when sacrificing the animals.

5.14 How are the animals acclimatized to, or trained to co-operate with the procedures?

- The animals will be left in the cages for one week to acclimatize. Mice pencil (Unga Farm Care (EA) Ltd, Nairobi) and water *ad libitum* will be provided on a regular basis. Well-trained personnel will help in handling the animals to reduce stress. Environmental enrichment and other activities will enhance the animals to co-operate with the procedures.

5.15 What are the precise clinical signs that will be monitored to safeguard the animals' welfare?

- Hair coat, body weight, body condition, appetite, body temperature, normal behavior, respiration, discharges and locomotion. Any deviation from the normality will be considered an illness and the animal will be carefully monitored.

5.16 How frequently and by whom will the animals be monitored?

- The animals will be monitored on daily basis throughout the week. The PI and a trained animal attendant will be taking care of the animals. The PI have attended a special training on handling small animals at SAFARI facility in JKUAT (2018).

5.17 What humane endpoints have been established to limit suffering (i.e. what are the criteria for euthanizing the animal or removing it from the experimental protocol)?

- CO_2 will be used when sacrificing the animals, so that no pain gets inflicted to them.

- Criteria for euthanizing the animals may include; rough hair coat, distended abdomen, weight loss ≥ 15% within 1 week, lesions interfering with eating or drinking, coughing, wheezing or nasal discharge, prolonged diarrhea ≥ 3 days.

5.18 From where will the experimental animals be sourced?

– The experimental animals will be sourced from SAFARI animal facility at JKUAT.

5.19 What are the husbandry and welfare standards at the source?

- SAFARI is well equipped to meet all animal ethical requirements and the welfare of the animals is well catered for.

5.20 If it will be necessary to transport the experimental animals, indicate approximate journey times and the measures that will be taken to minimize the potential stress during transport. N/A

5.21 (a) Does this experiment pose any health risk to staff or other animals? If so, how will this health risk be minimized?

– There is no foreseeable health risk to the staff.

5.21 (b) Describe the enrichment which will be provided to the animals during the study

- Animals will be kept in groups to allow them socialize or express normal behaviors.

- Provision of nesting materials eg, shredded papers.

5.22 What is the fate of the experimental animals at the end of the experiment (e.g. euthanasia, re-use, re-homing)?

– The animals will be euthanized with carbon dioxide and blood drawn for hematological and biochemical analysis. The carcasses will be disposed in a separate biohazard bag; this will be double-wrapped and sealed with autoclave tape, then stored in a freezer until disposal by standard university practices.

5.23 If the animals are to be humanely killed, what method will be used?

A chemical method will be used preferably CO_2.

5.24 List the qualifications and experience of the people who will be taking care and using the animals.

- **Mr. … Kimathi** … Animal husbandry

Experience – he is an animal attendant at the SAFARI animal house, he has greater experience in working with laboratory animals and all matters concerning with animal husbandry.

- **Ms. Mary** … Animal husbandry

Experience - she has done a lot of work in animal model research as well as biochemical analysis, she has attended various trainings in animal husbandry abroad.

5.25 Are there any other ethical issues that the Committee should be aware of?

– No

5.26 What toxic chemicals, carcinogens, radioisotopes, infectious agents or Genetically Modified Organisms will be used?

- None

5.27 Explain the safety measures in handling and disposal of toxic chemicals, carcinogens, infectious agents, radioisotopes or Genetically Modified Organisms stated above

– N/A

6.0 DECLARATION

I... FABIAN DOMINICUS MIHAFU……. Principal Investigator (PI) certify that;

- All information provided is correct.
- The research will be conducted in accordance with the National Guidelines on animal care and use stated above and those of the collaborating organizations involved.
- I will immediately report to the JKUAT-AEC anything which might warrant review of the ethical approval of the proposal.
- I will inform the JKUAT-AEC if the research project is discontinued before the expected date of completion
- I will adhere to the conditions of approval stipulated by the JKUAT-AEC and will cooperate with the AEC monitoring requirements.

PI Signature……. Date ……2nd March, 2019……..

7.0 SUPPORTING DOCUMENTS

7.1 Attach full proposal (including references) for the proposed study

- Yes

7.2 If available, attach any other relevant clearances obtained for the study. Any studies involving humans MUST attach all relevant clearances from the National Ethical Review Board.

- No

7.3 Describe in detail if there are conflicts of interests

- No

7.4 Attach 1-page CV of PI in the format provided

CURRICULUM VITAE of PI(s) *(1 page each)*
1.Surname: MIHAFU First name(s):FABIAN DOMINICUS Date of birth: 21/01/1977 Nationality: TANZANIAN
2. Degree(s) *(subjects, university or school, year)*
MSc - Food and Nutritional Sciences -Tuskegee University, USA - 2014 BSc - Agricultural Education and Extension - Sokoine University of Agriculture,Tz - 2011
3. Recent publications: Fabian Mihafu, Henry S. Laswai, Peter Gichuhi, Stewart Mwanyika, Adelia C. Bovell-Benjamin. Influence of Soaking and Germination on the Iron, Phytate and Phenolic Contents of Maize used for Complementary Feeding in Rural Tanzania. *International Journal of Nutrition and Food Sciences*. *Vol*. 6, No. 2, 2017, pp. 111-117. doi: 10.11648/j.ijnfs.20170602.18. Mihafu F.D, Bovell-Benjamin A.C, Laswai H.S. "Potent inhibitors for iron bioavailability in germinated maize used for complementary feeding in Tanzania''. Paper presented at the RUFORUM fifth Biennial Conference, Cape Town, South Africa, October 17-21, 2016. RUFORUM Working Document Series (ISSN 1607-9345) No 14(2). Available at **http://repository.ruforum.org**

FOR OFFICIAL USE

8.0 APPROVAL/REJECTION

The undersigned have evaluated the care and use of animals as described in this protocol in accordance with national and international guidelines for care and use of laboratory animals and found the procedures appropriate / not appropriate.

Project approved / Project rejected

…….

Signature of JKUAT-AEC

Chairperson……..Date…….……

…...**References**

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Ali, NM., Yeap, S. K., Ho, W. Y., Beh, B. K., Tan, S. W., & Tan, S. G. (2012). The promising future of chia, Salvia hispanica L. Journal of Biomedicine and Biotechnology. https://doi.org/10.1155/2012/171956

Appendix III: Postprandial Glycaemia of Rats Fed with Fructose, Lard and Chia Seed Extract for 28 Days

Rat no	Week	T1	T2	T3	T4
1	1	6.0	6.6	6.2	5.8
2	1	6.3	6.3	5.8	5.6
3	1	6.3	5.4	6.0	5.6
4	1	5.2	5.2	4.9	5.3
5	1	6.3	5.3	5.2	4.8
6	1	6.6	6.5	7.4	6.9
7	1	8.0	6.7	7.2	7.2
8	1	6.5	6.4	6.4	5.5
9	1	8.2	9.0	9.7	9.3
10	1	6.4	6.3	6.4	6.4
11	1	5.8	6.8	6.4	4.4
12	1	5.7	6.9	6.6	6.1
13	1	6.3	5.6	5.5	4.9
14	1	6.3	5.8	5.7	4.7
15	1	6.9	6.0	5.2	5.5
16	1	5.9	5.0	5.1	5.0
17	1	5.0	6.0	6.0	5.1
18	1	5.0	4.7	4.1	4.0
19	1	6.0	4.9	4.2	4.0
20	1	4.8	4.8	4.8	4.1
Mean		6.175	6.01	5.94	5.51
SD		0.873	0.997	1.25	1.26
Rat no	**Week**	**T1**	**T2**	**T3**	**T4**
1	2	6.5	7.2	6.1	6.2
2	2	6.3	6.5	6.5	6.4
3	2	6.3	6.3	6.5	5.7

4	2	6.0	5.6	5.7	4.8
5	2	6.0	6.0	6.6	6.0
6	2	5.9	6.0	6.3	6.1
7	2	6.8	6.0	6.9	6.4
8	2	5.6	6.4	4.8	5.4
9	2	6.5	6.4	6.8	7.2
10	2	6.3	5.5	6.6	6.5
11	2	6.9	5.8	6.1	6.0
12	2	8.2	6.8	6.3	6.2
13	2	6.5	6.2	6.3	6.4
14	2	8.0	6.4	6.6	7.1
15	2	6.4	5.5	5.5	4.8
16	2	5.8	5.2	4.8	4.5
17	2	8.7	9.8	11.2	10.5
18	2	7.9	7.9	7.3	6.8
19	2	6.2	5.7	5.2	4.9
20	2	5.6	5.5	4.9	4.6
Mean		6.62	6.34	6.35	6.13
SD		0.89	1.04	1.35	1.31
Rat no	**Week**	**T1**	**T2**	**T3**	**T4**
1	3	5.9	6.1	5.8	4.8
2	3	6.4	6.0	5.5	5.3
3	3	5.9	5.3	4.8	5.7
4	3	6.1	5.5	4.7	5.2
5	3	5.9	5.7	5.3	5.3
6	3	5.5	6.4	6.5	5.8
7	3	5.8	5.3	5.7	6.0

8	3	4.8	6.1	6.4	6.5
9	3	6.1	5.9	5.8	5.8
10	3	4.9	5.5	5.3	5.4
11	3	5.4	4.6	5.7	4.3
12	3	6.5	5.8	6.5	6.5
13	3	6.0	5.0	5.8	5.9
14	3	5.2	5.2	6.4	6.0
15	3	6.3	5.8	6.4	6.3
16	3	6.0	5.7	5.1	5.0
17	3	6.4	5.9	6.9	5.9
18	3	7.9	7.0	8.2	7.0
19	3	5.8	5.4	5.4	5.0
20	3	5.5	5.1	4.7	4.1
Mean		5.92 0.67	5.67	5.85	5.59
SD			0.54	0.85	0.74
Rat no	**Week**	**T1**	**T2**	**T3**	**T4**
1	4	5.8	4.8	5.8	5.4
2	4	6.4	5.4	5.9	5.4
3	4	6.1	5.3	5.9	5.5
4	4	6.2	5.1	5.2	4.8
5	4	6.6	5.4	5.7	5.2
6	4	5.4	7.7	6.3	6.0
7	4	7.1	6.4	6.4	6.8
8	4	6.0	5.9	5.7	5.3
9	4	5.8	6.3	6.3	7.2
10	4	6.6	5.6	6.3	6.8
11	4	6.5	5.8	5.4	4.6

12	4	7.3	5.8	6.0	6.0
13	4	6.9	6.4	5.0	5.9
14	4	6.1	6.2	4.5	3.9
15	4	6.0	5.4	4.9	3.9
16	4	5.1	4.6	4.2	3.8
17	4	7.3	7.8	7.0	6.9
18	4	7.6	6.5	6.0	6.0
19	4	5.4	5.1	4.9	4.5
20	4	7.0	6.4	5.6	5.5
Mean		6.36	5.90	5.65	5.47
SD		0.69	0.85	0.70	1.02

Rat no 1-5 :Control ; Rat no 6-10 :Exp. Group I ; Rat no 11-15 :Exp. group 2 and Rat no 16-20 : Exp. Group 3 ; T1= 30 minutes; T2= 60 minutes; T3= 90 minutes; T4= 120 minutes; SD= standard deviation

Appendix IV: Hematological Parameters of Male Wistar Rats Fed with Fructose, Lard, Ground Chia Seeds and Chia Seed Extract For 28 Days

Rat no	RBC	HB	PCV	MCV	MCHC	MCH	RDW	TLC	NEUT	LYM	MON	EOS	BAS	PLT	MPV
1	8.63	15.1	49.1	57	30.9	17.6	13.0	13.7	2.16	10.61	0.80	0.04	0.14	641	8.7
2	7.93	13.9	44.5	56	31.2	17.5	12.4	9.4	2.33	9.11	0.63	0.06	0.15	543	7.9
3	7.44	14.0	44.5	60	31.4	18.8	12.5	9.0	1.98	6.81	0.48	0.08	0.07	465	7.7
4	7.42	13.5	43.9	59	30.7	18.2	12.3	8.4	1.90	5.34	1.02	0.09	0.08	483	7.7
5	7.85	14.3	46.0	59	31.0	18.2	13.0	7.2	1.47	5.21	0.42	0.07	0.04	584	7.3
6	7.80	13.7	44.3	57	30.9	17.5	14.6	7.5	1.57	5.37	0.45	0.07	0.05	566	6.0
7	7.76	13.2	42.0	54	31.5	17.1	13.2	7.1	1.88	4.16	0.90	0.06	0.06	539	6.8
8	7.68	14.0	45.4	59	31.0	18.3	13.4	20.3	8.87	8.24	2.86	0.14	0.18	645	7.3
9	7.65	12.9	40.3	53	32.2	16.9	13.1	7.5	1.49	5.22	0.63	0.1	0.08	481	6.8
10	8.17	14.5	46.7	57	31.1	17.8	12.8	9.3	2.46	6.37	0.40	0.03	0.05	378	7.8
11	7.61	12.4	39.9	52	31.1	16.3	13.2	11.8	2.93	7.91	0.82	0.08	0.11	906	6.5
12	7.81	14.9	48.1	62	31.1	19.1	12.1	14.2	3.65	8.64	1.57	0.1	0.20	760	7.0
13	8.53	15.7	49.3	58	31.8	18.4	11.7	9.8	1.85	7.03	0.73	0.09	0.09	783	6.8
14	8.33	15.8	49.4	59	31.9	18.9	10.8	10.2	1.58	7.77	0.64	0.08	0.10	592	7.2
15	8.41	14.9	46.5	55	32.0	17.7	11.9	12.2	2.11	9.27	0.62	0.12	0.12	516	7.3
16	7.75	14.4	44.3	57	32.5	18.6	11.7	9.1	1.66	6.74	0.46	0.09	0.17	711	6.9
17	7.60	13.9	44.2	56	33.0	17.9	12.1	10.5	1.56	5.90	0.51	0.08	0.18	680	6.7
18	8.10	14.5	44.5	55	32.6	18.1	11.8	9.5	1.60	6.10	0.49	0.07	0.19	760	7.0
19	7.70	14.3	44.1	57	33.1	18.3	11.7	9.0	1.70	6.80	0.48	0.09	0.15	650	6.6
20	8.50	14.1	45.4	58	32.8	18.0	12.5	8.9	1.65	6.70	0.45	0.08	0.19	598	6.4
Mean	7.93	14.2 0.85	45.12 2.64	57	31.69	17.96	12.49	10.23	2.32	6.97	0.77	0.08	0.12	614.05	7.12
SD	0.37			2.43	0.76	0.69	0.83	3.09	1.63	1.63	0.56	0.03	0.06	127.77	0.62

RBC= red blood cells count; Hb= blood hemoglobin concentration; PCV= packed cell volume; MCV= mean corpuscular volume; MCH= mean corpuscular hemoglobin; MCHC= mean corpuscular hemoglobin concentration; RDW= red cell distribution width; TLC= total leukocyte count; NEUT= Neutrophils; LYM= lymphocytes; MON= monocytes; EOS= eosinophils; BAS= basophils; PLT= platelets; MPV= mean platelet volume; SD= standard deviation. Rat no 1-5 : Control ; Rat no 6-10 :Exp. Group I ; Rat no 11-15 :Exp. group 2 and Rat no 16-20 : Exp. Group 3.

Appendix V: Hematological Parameters of Male Wistar Rats Fed with Fructose, Lard and Chia Seed Extract for 56 Days

Rat no	RBC	HB	PCV	MCV	MCHC	MCH	RDW	TLC	NEU	LYM	MON	EOS	BAS	PLT	MPV
1	6.22	14.5	35.3	57	41.2	23.3	10.4	14.0	13.5	79.7	5.1	0.6	1.1	584	6.9
2	6.88	17.0	39.7	58	42.9	24.7	11.6	11.7	17.5	77	4.1	0.4	1.0	377	7.0
3	6.38	15.1	36.1	57	41.8	23.6	10.7	11.3	14.1	76.2	7.4	0.9	1.4	559	7.0
4	6.61	15.6	38.1	58	41.0	23.6	10.8	7.5	12.5	81.7	4.3	0.8	0.7	530	7.0
5	6.70	16.9	41.8	62	40.5	25.2	10.0	8.1	16.1	78.1	4.2	0.6	1.0	539	6.1
6	6.41	14.7	36.2	57	40.6	23.0	11.7	9.9	11.5	81.3	5.6	0.7	0.9	548	6.9
7	6.61	14.7	37.0	56	39.7	22.2	10.1	10.9	12.0	77.5	8.5	1.2	0.8	404	6.7
8	6.59	14.7	37.0	56	39.7	22.3	10.6	11.8	12.3	79.4	6.8	0.6	0.9	540	6.7
9	7.07	16.3	40.6	57	40.2	23.1	11.3	5.8	15.7	78.9	4.0	0.5	0.9	316	7.0
10	7.26	15.9	40.6	56	39.1	21.9	10.7	6.7	14.6	81.0	3.3	0.7	0.4	304	7.3
11	6.62	15.8	39.6	60	40.0	23.9	10.5	4.7	16.3	79.0	3.1	0.5	1.1	161	7.0
12	7.13	15.1	38.1	53	39.7	21.2	12.6	9.9	12.7	74.8	11.1	0.7	0.7	349	7.9
13	6.74	15.2	38.4	57	39.5	22.5	12.2	13.0	14.3	73.7	8.4	0.5	3.1	435	6.9
14	7.08	15.6	38.8	55	40.1	22.0	10.1	10.8	13.6	78.7	6.0	0.6	1.1	413	6.4
15	7.38	15.9	40.3	55	39.5	21.6	11.9	12.2	15.0	76.4	6.9	0.6	1.1	490	7.3
16	6.91	15.2	38.2	55	39.9	22.1	10.7	10.3	17.9	76.1	4.7	0.5	0.8	479	6.5
17	7.12	15.0	37.9	53	39.5	21.0	11.1	11.8	16.4	76.4	5.8	0.5	0.9	446	7.2
18	6.47	14.3	35.9	55	40.0	22.2	9.5	8.6	18.7	76.1	4.1	0.4	0.7	559	6.4
19	6.68	15.1	37.6	56	40.0	22.6	10.7	9.2	12.9	77.1	8.5	0.7	0.8	516	6.3
20	6.90	14.7	36.8	53	39.8	21.1	11.1	11.2	14.1	72.9	10.5	1.2	1.3	524	5.9
22	7.27	15.7	39.4	54	39.9	21.6	11.1	11.8	14.2	77.9	6.4	0.5	1.0	528	6.4
23	6.33	13.7	34.7	55	39.4	21.6	11.3	7.0	12.9	78.9	6.8	0.8	0.6	401	6.9
24	7.06	15.7	39.3	56	40	22.2	10.6	12	14.3	76.1	7.6	1.0	1.0	506	6.1
Mean	**6.80**	**15.32 0**	**38.15**	**56.13**	**40.17**	**22.54**	**10.93**	**10.01 2.4**	**14.48**	**74.51**	**6.23**	**0.67**	**1.01**	**456.87**	**6.77**
SD	**0.33**	**.79**	**1.86**	**2.14**	**1.11**	**0.86**	**0.74**	**2**	**1.95**	**14.70**	**2.19**	**0.22**	**0.51**	**104.14**	**0.46**

RBC= red blood cells count; Hb= blood hemoglobin concentration; PCV= packed cell volume; MCV= mean corpuscular volume; MCH= mean corpuscular hemoglobin; MCHC= mean corpuscular hemoglobin concentration; RDW= red cell distribution width; TLC= total leukocyte count; NEUT= Neutrophils; LYM= lymphocytes; MON= monocytes; EOS= eosinophils; BAS= basophils; PLT= platelets; MPV= mean platelet volume; SD= standard deviation. Rat no 1-6:Control; Rat no 7-12:Low dose group; Rat no 13-18: Medium dose group; Rat no 19-24: High dose group.

Appendix VI: Mean Weekly Weight (g) Records of Male Wistar Rats Fed with Fructose, Lard,

Rat no	Week 1	Week 2	Week 3	Week 4
1	188.44	178.42	174.61	181.04
2	175.96	166.31	163.00	167.73
3	186.96	176.09	180.97	186.83
4	187.76	177.49	181.54	188.67
5	210.99	200.53	203.66	207.99
6	196.09	202.43	225.90	240.04
7	176.41	189.59	190.97	187.63
8	242.91	262.81	272.47	272.97
9	169.21	181.11	187.34	185.56
10	203.30	210.61	213.20	209.73
11	154.53	156.61	156.61	177.27
12	193.66	224.74	224.74	232.06
13	277.13	321.04	321.04	375.61
14	154.16	157.37	157.37	162.01
15	166.56	174.13	174.13	169.19
16	243.14	274.69	301.13	320.74
17	212.81	238.51	262.33	279.49
18	243.14	272.73	289.91	314.06
19	226.50	256.67	284.04	306.23
20	219.49	244.59	268.93	283.40
Means	201.46	213.32	221.70	232.41

Rat no 1-5 :Control ; Rat no 6-10 :Exp. Group I ; Rat no 11-15 :Exp. group 2 and Rat no 16-20 : Exp. Group 3

Appendix VII: Mean Weekly Weight Records of Male Wistar Rats Fed with Fructose, Lard and Chia Seed Extract for 56 Days

Rat no	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
1	189.4	191	228.7	237.2	246.9	259	262.5	307.4
2	198	199.2	233.8	253.9	279.2	295.5	307.4	303.7
3	190.6	201.2	246.7	254.9	279.6	298.8	311.1	306.7
4	202.5	193.7	196.7	209.1	232	243.4	251.4	304.8
5	209	202.2	235.1	245.9	261.8	276.7	288.6	306.4
6	194.3	195.1	232.7	237.8	245.6	260	266.3	307.4
7	194.2	257.1	287	302.1	321.7	329	340.2	302.5
8	192	232.7	251.5	263.6	270.9	272.3	280.2	297.8
9	190.2	227.5	243.1	254.9	266.9	266.8	284.5	295.1
10	202.2	231.1	247.9	268.8	288	300.5	320.1	298.1
11	204.1	168	195.4	214.2	242.6	273.2	285.7	300.2
12	195.4	200	216.5	233.3	244.1	256.6	271.7	298.7
13	246	254.9	189.2	208.8	241.6	260.2	338.2	310.6
14	248.1	287.9	308	333.8	350.2	363.2	340.5	370.1
15	250.2	276.8	297.7	323.6	343.2	353.2	337.6	265
16	258	287	307.7	320.6	331.5	345.5	342.2	354.2
17	257.2	334.2	355.7	373.4	387.3	393.5	341.1	396.4
18	260.1	264.5	262.7	274.8	278.6	288.8	339.2	289.1
19	252.3	283.7	325.2	262	375	377.1	383.4	351.4
20	255.4	282.1	318.8	318.6	292.8	259.2	251.1	313.4
21	254.5	284.5	188.7	188.6	172.8	189.5	200.8	193.2
22	256.2	282.8	311	335	346.7	351.4	360.7	324
23	252.1	281.5	322.3	340.8	345.9	347.9	252.3	332
24	253.3	283.3	339	385.3	404	424.1	435.1	364
Mean	225.22	245.92	264.21	276.71	293.70	303.56	308.00	312.18

Rat no 1-6: Control; Rat no 7-12: Low dose group; Rat no 13-18: Medium dose group; Rat no 19-24: High dose group

Appendix VIII: Abstract of the Published Manuscript 1

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RESEARCH ARTICLE

Nutritional Composition and Qualitative Phytochemical Analysis of Chia Seeds (*Salvia hispanica* L.) Grown in East Africa

Fabian D. Mihafu[1,*], Beatrice N. Kiage[1], Judith K. Okoth[1] and Andrew K. Nyerere[2]

[1]*Department of Human Nutrition Sciences, School of Food and Nutrition Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya;* [2]*Department of Medical Microbiology, College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya*

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Abstract: ***Background*:** Chia seed (*Salvia hispanica* L.) is becoming one of the most popular plant-based foods that contain the greatest amount of nutrients particularly omega-3 fatty acid, α-linolenic acid. It is therefore considered a functional food with pronounced health benefits.

***Objective*:** The purpose of this study was to determine the proximate composition, minerals, fatty acid profiles and phytochemical contents of chia seeds grown in East Africa (Kenya and Uganda).

***Methods*:** Official methods of analysis, 2005 were adopted, minerals determined by Atomic absorption spectrophotometer, phytochemicals were determined by standard methods and fatty acid profiles were analyzed by Gas chromatography.

***Results*:** Proximate composition indicated high contents of protein, fat and fiber. The fatty acid profiles revealed great amounts of α-linolenic acid (45.29-56.99%) followed by linoleic acid (15.9-20.28%) and oleic acid (6.88-11.58%). However, the difference in the content of α-linolenic acid between samples was not significant ($p = 0.7391$). Mineral determination (mg/100g) showed high contents of potassium (492.96-862.98), phosphorous (486.45-569.45), calcium (297.47-429.09) and magnesium (192.22-202.97) while considerable amount was observed for iron, zinc, manganese, and copper. There was a significant difference ($p = 0.0001$) in mineral content between black chia Molo and white chia Bukembo with the exception of phosphorus.

***Conclusion*:** Both black and white chia seeds grown in East Africa, observed to have high amounts of α-linolenic acid, proteins, fats, fiber, and minerals. These findings support the evidence that chia is rich in nutrients that are beneficial to human health. Therefore, we suggest its incorporation in diets as a healthy food ingredient.

Keywords: Fatty acids, functional food, minerals, phytochemicals, proximate composition, vitamins.

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Appendix IX: Abstract of the Published Manuscript 2

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Effect of chia seeds (*Salvia hispanica*) on postprandial glycaemia, body weight and hematological parameters in rats fed a high fat and fructose diet

Fabian Dominicus MIHAFU[1,3*], Beatrice Nyanchama KIAGE[1], Andrew Nyerere KIMANG'A[2] and Judith Kanensi OKOTH[1]

[1]*Department of Human Nutrition Sciences, School of Food and Nutrition Sciences, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200, Nairobi, Kenya.*
[2]*Department of Medical Microbiology, College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200, Nairobi, Kenya.*
[3]*Department of Applied Sciences, Mbeya University of Science and Technology, P.O. Box 131, Mbeya, Tanzania.*
[*] *Corresponding author; E-mail: fmihafu21@gmail.com; Phone: +254 796233804/ +255 765426643*

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ABSTRACT

Chia seeds *(Salvia hispanica)* are currently consumed by varied populations as superfoods due to their protective, functional and antioxidant properties. The aim of this study was to determine the effect of ground chia seeds/extracts on postprandial glycaemia, body weight, hematological parameters and cellular morphology in rats. Twenty male Wistar rats were assigned into three experimental groups and a control (n =5). Each experimental group received 10 g/20 g fructose/lard. Additionally, 90 g rat pellet was fed to group 1 and 3 which was supplemented with 20 g chia seed extract, group 2 received ground chia seeds only. Control group received 90 g rat pellet only for 28 days. The results on body weight changes indicated a gradual increase in body weight of chia seeds/extract fed rats as compared to fructose/lard group. There was an increase in postprandial blood glucose levels in group 1 from week I to IV contrary to groups supplemented with chia seeds/extract. Complete blood counts showed a significant increase ($p = 0.008$) in mean corpuscular hemoglobin concentration, basophils ($p = 0.035$), platelets ($p = 0.025$) and red cell distribution width ($p = 0.008$) in experimental groups compared to control. These results pinpoint the benefits of chia seeds.
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Keywords: Blood composition, functional food, glucose concentration, metabolic diseases, omega-3 fatty acids