8-2017

Garcinia Kola and Type One Diabetes: A Study on the Effects of Aqueous Garcinia Kola Extract on the Incidence and Severity of Type One Diabetes in a C57Bl/6J Mouse Model

Eryn Ebinger
St. Cloud State University, eber0901@stcloudstate.edu

Garcinia Kola and Type One Diabetes: A Study on the Effects of Aqueous *Garcinia*

Kola Extract on the Incidence and Severity of Type One

Diabetes in a C57Bl/6J Mouse Model

by

Eryn Ebinger

A Thesis

Submitted to the Graduate Faculty of

St. Cloud State University

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

in Biology

August, 2017

Thesis Committee:
Marina Cetkovic-Cvrlje, Chairperson
Heiko Schoenfuss
CassidyDobson

Abstract

Type 1 Diabetes (T1D) is an insulin-dependent autoimmune disease characterized by the T-cell mediated autoimmune destruction of insulin-producing pancreatic β-cells. This destruction results in hyperglycemia (elevated blood glucose levels), which causes metabolic imbalances, pathological changes in tissue, vascular complications and multi-organ damage. Pharmaceuticals used to treat T1D are expensive and not readily available in underdeveloped countries. It is therefore an imperative to study natural compounds with a potential hypoglycemic and anti-inflammatory properties, such as *Garcinia kola* seeds. The effect of aqueous *Garcinia kola* seed extract (GKE) on incidence and severity of T1D was studied in a multiple-low-dose streptozotocin (MLDSTZ)-induced mouse model of T1D. It is hypothesized that GKE treatment would decrease glycemia levels and reduce incidence of autoimmune mouse T1D. GKE was prepared by aqueous extraction, and C56Bl/6J male mice were exposed to 100 mg/kg/day of GKE via drinking water for a period of five weeks (from 7- to 12-weeks of age). At 8-weeks of age T1D was chemically induced with five consecutive injections of 40 mg/kg streptozotocin (STZ). Body weights and blood glucose levels were measured before STZ administration and bi-weekly from day 8 until day 29 post first STZ injection. The results showed that GKE treatment did not reduce body weights and glycemia levels; even a trend of elevated body weights and hyperglycemia levels was observed in GKE-treated mice. In addition, GKE exposure significantly increased T1D incidence in MLDSTZ-treated mice. In conclusion, this study, as the first examination of the anti-diabetogenic potential of aqueous GKE, did not confirm its potential in reducing hyperglycemia and preventing T1D.

Table of Contents

List of Tables

List of Figures

List of Images

Image   Page

Chapter 1: Introduction

Type 1 Diabetes (T1D)

Diabetes mellitus is a disease that negatively affects the body's ability to produce or respond to the hormone insulin. The United Nations recognizes diabetes as a chronic killer and considers it a global threat (Diabetes Care and Research in Europe, 1990). Per the 2012 regional estimates, by the National Diabetes Federation, 371 million people suffer from diabetes worldwide. There are multiple types of diabetes, affecting about 1 in 10 adults, the most common types being type 1 and type 2 (Center for Disease Control [CDC], 2014). Type 2 diabetes (T2D) is a dietary based disease that affects how the body responds to insulin, cells become insulin resistant. Type 1 diabetes (T1D) is the insulin dependent and more commonly known as juvenile diabetes, as it is usually diagnosed in children. Both T1D and T2D cause sever metabolic imbalances, pathological changes in tissue, vascular complications, chronic inflammation, oxidative stress, and multi-organ damage. In 2012, 4.8 million people died due to the complications caused by T1D and T2D. This high death toll adds to the growing concern over the increased number of individuals diagnosed with diabetes, especially T1D that, unlike T2D, is an unpreventable autoimmune disease. The exact causes of T1D are unknown, though it is believed to be a combination of genetic and environmental factors. Sadly, these factors are causing an increasing trend in T1D incidence in children, a trend that is predicted to double in new cases of T1D in children under 5 and have a 70% rise in prevalent cases in children under 15 by 2020 (Patterson, Dahlquist, Gyurus, Green, & Soltesz, 2009). Aside from the concern of increasing incidence of T1D, is providing therapies and treatments for those that have been and have yet to be diagnosed. T1D is an insulin dependent disease, its treatment heavily revolves around insulin injections. Insulin, among

other pharmaceuticals used for treating T1D, is expensive and unavailable in many under developed countries, such as Africa with an estimated 81.2% undiagnosed individuals in comparison to the United States 29.2%, a current statistic with the potential to increase as T1D incidence increases (CDC, 2014).

Immunology of T1D

T1D is a T-cell mediated autoimmune disease. There are two main branches of the immune system, being innate immunity and adaptive immunity. Innate immunity is the body's non-antigen-specific initial response to an invading pathogen. This type of immune response involves natural killer cells and phagocytes, being macrophages, neutrophils, and dendritic cells. These cells create an immune response by phagocytosis of the invading pathogen, leading to an inflammatory response via cytokine secretion, resulting in the recruitment of other immune cells. Adaptive immunity, on the other hand, is the bodies "acquired" immune response, as it is an antigen specific response. T-cells and B-cells are part of the body's adaptive immune system, B-cells being responsible for antibody immune responses and T-cells being responsible for cell-mediated immune responses. A T-cell mediated response requires the activation of T-cells and cytokine production. There are various types of T-cell subsets, the main one's being, T-helper, T-cytotoxic, and T-regulatory cells. Once activated these various types of T-cells work together with other cells of the immune system to clear an invading pathogen. Though, prior to activation T-cells must go through a maturation process. T-cells mature in the thymus, one of the bodies primary lymphoid organs. The T-cell repertoire is accomplished through negative and positive selection. Positive selection only permits the survival of T-cells with receptors that can recognize self-major histocompatibility complexes (self-MHC). Negative selection, on the

other hand, eliminates T-cells that react too strongly to self-MHC and self-peptide complexes. T-cells that fail these two types of selection die of apoptosis or neglect in the thymus, in-fact, only 2-5% of thymocytes exit the thymus as mature T-cells with the desired level of self-recognition (Owen, Punt, Stranford, & Jones, 2006). This process of positive and negative selection is known as self-tolerance and works to ensure T- and B-cell clones are deleted if their receptors have too high or low affinity for self-recognition. About 10% of the human population suffers from autoimmunity, which is the failure of this central tolerance process (Owen et al., 2006). When central tolerance fails, the body is no longer protected against self-reactive lymphocytes resulting in the destruction of self-proteins, cells, and organs by auto-antibodies, from autoreactive B-cells, or self-reactive T-cells. These destructive autoimmune diseases, such as T1D, autoimmune pancreatitis, or Crohn's, can be either systemic or organ specific.

T1D is an organ specific autoimmune disease that affects the pancreas. The pancreas contains insulin producing β-cells, the target of the overstimulated self-reactive T-lymphocytes. β-cells are located in tiny clusters of cells called the islets of Langerhans. In T1D islets start out with no inflammation and contain healthy β-cells, slowly islets become infiltrated by inflammatory cells followed by a slight decrease in β-cells before resulting in an islet with complete β-cell destruction and inflammation (Wilcox, Richardson, Bone, Foulis, & Morgan, 2009). The infiltration and destruction of pancreatic β-cells describes a histological lesion called insulitis. It is believed insulitis begins with cytotoxic lymphocyte infiltration and the activation of macrophages. The activation of macrophages is followed by the release of cytokines and the production of auto-antibodies, all mounting to a cell mediated response, to what the immune system thinks is a pathogen (Notkins, 2002). Essentially T-helper$_1$ cells call

in macrophages and T-cytotoxic cells to kill pancreatic beta cells, a response that is thought to be mediated by cytokines and lytic enzymes released by the activated macrophages (Owen et al, 2006).

Destruction of the β-cells and lack of insulin production results in hyperglycemia, elevated blood sugar levels. Chronic hyperglycemia causes metabolic imbalances, chronic inflammation, and oxidative stress due to the generation of free radicals. Free radicals refer to reactive oxygen species (ROS), reactive nitrogen species (RNO), and oxidants. These unstable molecules are a product of normal cellular metabolism and in moderate levels can exert beneficial effects, they can aid in the maturation process of cellular structures, defense against pathogens, and the induction of a mitogenic response (Sachdev & Davies, 2008). Though high concentrations of free radicals can cause oxidative stress, damaging cell structures (Halliwell, 2007). Increased levels of oxidative stress and chronic inflammation affect insulin secretion and sensitivity, aiding the progression of T1D (Alexandraki et al., 2008).

Experimental Models of T1D

The non-obese diabetic mouse (NOD) is the preferred animal model to use when studying T1D. NOD mice spontaneously develop T1D similarly to that of humans; their pancreatic β-cells become infiltrated by lymphocytes and macrophages, resulting in insulitis and hyperglycemia. Insulitis is observed in both sexes of NOD mice post 3 weeks of age. For this reason, a mouse model involving the chemical induction of T1D was used for this pilot experiment in order to ensure all mice used in this experiment were exposed to GKE prior to diabetes onset. C57BL/6J mice do not spontaneously develop T1D, allowing for the exposure of GKE prior to diabetes onset. Therefore, this pilot experiment used a chemically-induced

C57BL/6J mouse model of T1D to investigate the hypoglycemic and anti-diabetic potential of GKE.

Multiple low-dose streptozotocin (MLDSTZ)-induced model of T1D. The diabetogenic agent streptozotocin (STZ) has been used to chemically induce T1D. STZ is the most commonly used drug for induction of T1D in rodents since the initial discovery of its diabetogenetic properties in 1963 (Rakieten, Rakienten, & Nardkarni, 1963). Diabetes can be induced in multiple different ways using STZ. STZ has glucose moiety, meaning it has a functional group on the molecule that looks similarly to glucose. This glucose moiety directs the chemical to the insulin-producing pancreatic β-cells where it recognizes the glucose transporter receptor (GLUT-2), which takes STZ into the cell (Wu & Yan, 2015). Once in the cell, STZ causes changes in the pancreatic β-cells DNA, resulting in β-cell death due to the alkylation of the DNA (Etuk, 2010). STZ also causes destruction of pancreatic islets due to the release of nitric oxide (NO) when being metabolized in the cell (Merzouk et al., 2000; Schein, Cooney, McMenamin, & Anderson, 1973). Due to excess NO, β-cells manifest changes that are characteristic for NO action, such as increased activity of guanylyl cyclase and enhanced formation of cyclic GMP (Turk, Corbett, Ramanadham, Bohrer, & Mcdaniel, 1993).

T2D can be induced by neonatal exposure of STZ resulting in loss of β-cell sensitivity to glucose (Portha, Picon, & Rosselin, 1979). T2D can also be induced by administering STZ after the administration of nicotinamide in combination with a high fat diet (Szkudelski, 2001). One high dose of STZ can cause a toxic form of diabetes (basically T1D without autoimmune etiology), where there is complete β-cell destruction. Autoimmune T1D can be chemically induced with STZ by administration of multiple consecutive sub-diabetogenic

doses, causing initially partial damage of β-cells. This partial destruction of the β-cells by STZ causes a cell-mediated autoimmune response. Macrophages begin releasing inflammatory cytokines which call in T-lymphocytes, and the autoimmune progression of T1D, previously described, begins. Once this progression begins a progressive hyperglycemia will develop; T1D has been induced and the disease will continue even after STZ has been metabolized from the body. Alloxan is another drug commonly used to study diabetes. Alloxan causes β-cell necrosis which leads to an insulin dependency. However, alloxan does not cause a cell-mediated immune response resulting in the autoimmune form of T1D (Etuk, 2010).

Garcinia kola. *Garcinia kola* (GK) is a fruiting plant found naturally in Sierra Leone, Nigeria, and Angola. The fruit contains 2-4 seeds that have a bitter taste, giving it the common name of bitter kola. In Nigeria, GK seeds are consumed as a natural part of the diet. GK has been used for multiple medical purposes in many parts of Africa, such as, a chewing stick for dental health (Agyili, Sacande, & Kouame, 2006) and as an adaptogen (Esimone, Nworu, Adikwu, Odimegwu, & Ezugwu, 2007), expressing anti-stress properties. Aside from chewing sticks, GK seeds have been used as folklore medicine to treat conditions from colds to liver disease. In fact, a recent study on GK seed extract found that the biflavonoid complexes from the seed actually abolished the expression of COX-3 and iNOS proteins in dimethyl nitrosamine treated rat liver, alleviating liver inflammation (Farombi & Owoeye, 2011.)

Garcinia *kola* and diabetes. Recent studies have looked into the GK seed extract hypoglycemic properties (Adaramoye, 2012; Adaramoye & Adeyemi, 2005; Ayepola, Chegou, Brooks, & Oguntibeju, 2013; Ayepola, Cerf, Brooks, & Oguntibeju, 2014). The GK

seed contains multiple biflavonoids, some of which being, kolaviron (containing GB-1, GB-2, kolaflavonone, and kolaflavone), xanthones, oleoresin alkaloids, and tannins (Ebana, Madunagu, Ekpe, & Otung, 1991; Hussain, Owegby, Parimoo, & Eatomam, 1982; Onayade, Looman, Scheffer, & Gbile, 1998; Terashima, Aqil, & Niwa, 1995). These biflavonoids, in particular kolaviron, are believed to express hypoglycemic potential by inhibiting specific enzymes, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), scavenging free radicals, and possibly, acting as an anti-inflammatory agent (Haskins et al., 2003; Havsteen, 2002; Farombi, Akanni, & Emerole, 2002). Hyperglycemia leads to multiple medical complications, many of which revolve around issues related to oxidative stress, like damage to islets and vascular tissues, due to the excess free radicals that stimulate the release of pro-inflammatory factors. Recent animal studies showed that GK seed extracts inhibited oxidative stress, protecting against the damage it causes in various tissues (Olajide, Enaibe, Bankole, Akinola, & Laoye, 2016; Oyenihi, Brooks, & Oguntibeju, 2015; Ayepola, Brooks, & Oguntibeju, 2014.) One study found GK extract to be an effective hydrogen donor and a primary antioxidant to react with lipid radicals (Olatunde et al., 2008). Other studies have shown *Garcinia* kola GK extracts' anti-inflammatory properties through reduction of pro-inflammatory molecules (Ayepola, Brooks et al., 2014; Ayepola, Cerf et al., 2014; Ren & Torres, 2009).

Based on the anti-inflammatory and oxidative stress-relieving properties displayed in animal studies, GK extract might show promise as an anti-diabetic compound, or a complementary therapy for T1D. Therefore, the present study investigated the effects of *Garcinia kola* extract (GKE) on the development and severity of T1D. Previous studies that tested GK extract in animals used ethanol/methanol extract of the seeds, called kolaviron, that

is not soluble in the water; the oral gavage of ethanol- or DMSO-dissolved kolaviron was a method of its administration. However, people in Africa do chew GK seeds. Therefore, we aimed to use an aqueous extract of the GK seeds, in order to test GK effects on T1D when it has been consumed naturally, via drinking water. It is hypothesized that aqueous GKE would decrease glycemia levels and reduce T1D incidence in a MLDSTZ-induced C57BL/6J mouse model of T1D.

Chapter 2: Materials and Methods

Animals

C57BL/6J breeding pairs, purchased from the Jackson Laboratories (Bar Harbor, ME), were bred in the St. Cloud State University vivarium animal care facilities. The mice were housed in Optimimice® caging systems (Animal Care systems, Centennial, CO) with temperature (22.2°C), relative humidity (40-60%), and 12-hour light/dark cycle-controlled conditions. All mice had continuous access to food (Harlan Tekland 18% Global Protein Diet 2018) and water. At 21 days of age mice were sexed and separated based on gender. Seven-week-old male mice were randomly selected for experimental use. All procedures performed on the mice were approved by the SCSU Animal Care and Use Committee (protocol # 5-98) before the start of the project.

Garcinia *kola* seed extraction. GK seeds (obtained from Nigeria, gifted from Dr. Oladele Gazal) were peeled, sliced, and air-dried (room temperature, 20-25° C). GK seed extract was obtained using the aqueous extraction method of Iwu, Igboko, Okunji, and Tempesta (2013). An aqueous extraction method was chosen to allow administration of the extract via drinking water. Briefly, 20 or 40 grams of dried seeds were pulverized into a powder, added to 200 mL deionized water, and stirred in a cold room at 4°C for 48 hours. Filtration was then performed by using 4.0 cm diameter filtration paper, filtrate was dried in an incubator for 48 hours at 30° C. The extract, called GK extract (GKE), was collected by scrapping, and prepared in a stock solution of 40 mg/ml of autoclaved deionized water. Dried extract easily went into a water solution at that concentration by vortexing for 3 minutes. The GKE stock was aliquoted in 5.0 ml volume in sterile centrifuge tubes, and stored at -20° C until further dilution in mouse drinking water.
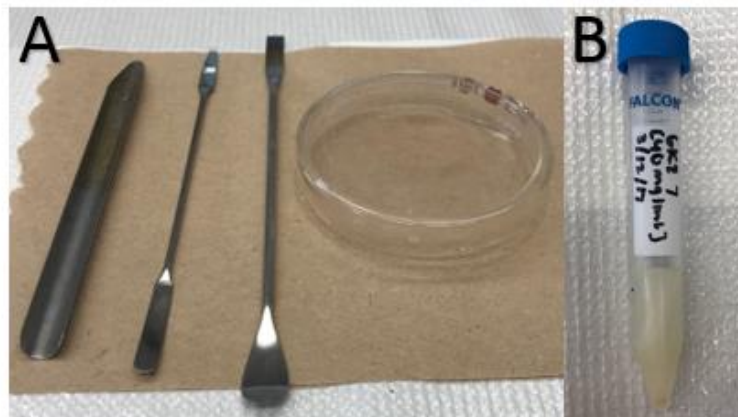
Image 1. Petri dish containing GKE extract after being filtered and dried (image A); GKE stock solution of 40 mg/ml (image B).

Determination of daily water intake in control and GKE-exposed non-diabetes and diabetic mice. A preliminary water intake experiment was performed determining a) the amount of water that 7-week-old mice drink, and b) whether mice drink the same amount of water in which GKE is added in concentration that allows mice to receive 100 mg GKE/kg/day. This was used to calculate the dose of extract added to drinking water to ensure mice were receiving the desired dose of 100mg/kg each day. In short, an empty water bottle and stopper were weighed and then filled with autoclaved water and re-weighed before giving to mice (7-wk-old C57BL/6 mice; n = 5 per cage) for water consumption. The following day, after 24 hours, the water bottle with remaining autoclaved water was weighed, then refilled and re-weighed before giving again to mice. This process was repeated over a course of 3 days. The weight of the water bottle and stopper was subtracted from the total weight, and water consumption for each day was calculated by subtracting the weight of the water recorded at 8:00am from the weight of the water after being refilled. After this initial data was obtained, GKE was added in the drinking water at desired concentration of 100 mg/kg/day;

3.4 ml of GKE stock of 40 mg/ml was added to 196.6ml of drinking water in a bottle, which was added to a mouse cage containing five 8-wk-old C57Bl/6J males. Furthermore, the water intake in control diabetic (n = 4, 12-week-old C57BL/6 males) and GKE-exposed diabetic mice (n = 4, 12-week-old C57BL/6 males) was determined in the same manner described for non-diabetic mice in order to evaluate how much water intake increased because of hyperglycemia.

Exposure of C57BL/6 male mice to GKE. Seven-week old male C57Bl/6J mice were randomly separated into control and treatment groups. Treatment mice were exposed to GKE (100 mg/kg/day) via autoclaved drinking water, based on their average water intake of 3.1 ± 0.1 ml/day; control mice received only autoclaved water. Mice were exposed to GKE until 12-weeks of age.

Induction of T1D in C57BL/6 mice. At 8-weeks of age, control and treatment mice received five consecutive multiple low-doses (40 mg/kg/day) of STZ (MLDSTZ) via intraperitoneal injections. Before the administration, STZ was dissolved in a citrate buffer solution (pH 4.5). STZ ~~is~~ was dissolved in the buffer through vortexing and was administered within 15 minutes of being added to the buffer. Post STZ injections, glucose measurements and body weights were recorded bi-weekly until the experimental endpoint, e.g., day 29 post first injection of STZ (STZ1) (Figure 1).

Body weight and blood glucose measurements. Body weights and glucose measurements were recorded on the first day of GKE exposure, 3 days prior STZ1, and afterwards on a bi-weekly basis from day 8 to day 29 post STZ1. Glucose measurements were performed using a 26-gage needle to prick the lateral tail vein. Tails were immersed in warm water 43.0° C to induce vasodilation of blood vessel prior to venipuncture. 0.6µL of blood

was collected, administered to an Accu-Check Aviva® blood glucose meter strip, and

glycemia recorded by Accu-Check Aviva® blood glucose glucometer (Roche Diagnostics,

Indianapolis, IN). Mice with two or more consecutive readings of glycemia level at or above

250 mg/dl were considered diabetic, with first one considered as the beginning of clinical

disease.

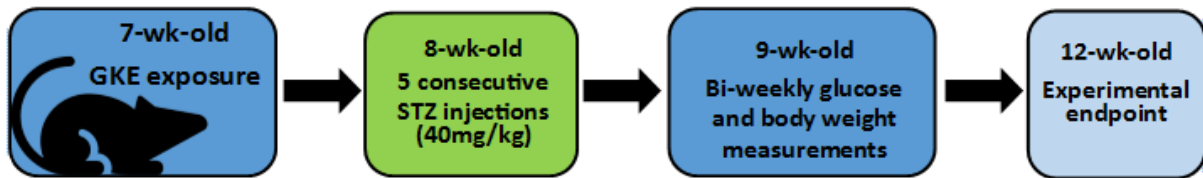**Figure 1.** Experimental timeline for GK seed extract (GKE) exposure and induction of T1D
in C57BL/6 mice.

Statistical analysis. A life-table analysis was used for the statistical analysis of

diabetes incidence ($p < 0.05$ was considered as statistically significant). An analysis of

variance was performed by Excel for the analysis of glycemia levels and body weights

($p < 0.05$ was considered as statistically significant).

Chapter 3: Results

GK Extraction

A total of 5 GK seed aqueous extractions were performed in order to provide adequate amount of GKE for mouse treatment (Table 1). Twenty or 40 grams of pulverized GK seeds provided an average of 44.3± 4.0 mg GKE per gram of GK seeds.

Table 1

GKE Yield Obtained from Five Aqueous GK Seed Extractions

Extraction trial	1	2	3	4	5
GK seed (g)	20	20	40	40	40
GKE (g)	0.65	0.95	2.16	2.11	1.39
GKE (mg) per 1.0 g seed	32.9	47.3	54	52.7	34.6

Determination of water and GKE-enriched water intake in non-diabetic and diabetic C57Bl/6J males. A preliminary water consumption experiments were performed prior to GKE exposure experiments to determine how much water mice drank per day, and to calculate a daily dose of GKE, based on this information. Mice (n = 5/cage) were drinking autoclaved water for three days. The weight of the water bottle was checked daily to determine water intake. As shown in Table 2, the average water consumption of control and GKE-treated mice did not differ; both groups consumed 3.1 ± 0.1 water per day/mouse, for both control and GKE-treated groups. Next, the water and GKE-treated water consumption was evaluated in diabetic mice, as it was expected that diabetic mice would drink excessively compared to non-diabetic ones. Control diabetic mice (n = 5, 12-week-old) drank on average 11.3 ± 0.6 ml water per day/mouse. Interestingly, it was observed that treatment group (n = 5,

12-week-old) drank more than twice as much as control mice, e.g., 23.9 ± 0.6 ml/day/mouse (Table 2).

Table 2

Average Water Consumption per Day in Non-diabetic (ND) and Diabetic (D) Control (C) and GKE-treated (GKE) Mice. Non-diabetic (7-week-old) and diabetic (12-week-old) C57BL/6J male mice were exposed to either water alone or water enriched with GKE in the concentration of 100 mg/kg/day over the period of three days. Data presented as average ± SEM.

	ND-C (n=5)	ND-GKE (n=5)	D-C (n=4)	D-T (n=4)
Daily water intake (ml)	3.1+0.1	3.1+0.1	11.5+0.6	23.9+0.6

Body weights and glycemia levels in GKE-exposed MLDSTZ-treated mice.

Extract collected from the GK aqueous extraction was mixed into a 40 mg/ml stock solution, which was then added to mice' drinking water in the concentration of 100 mg/kg of GKE per day. Water bottles were filled with 196.6 ml of water with addition of 3.4 ml of 40 mg/ml GKE stock. The bottles were changed once a week, when a new autoclaved bottle filled with GKE-enriched water was added.

Water opacity was observed in a GKE-enriched drinking water, usually towards the end of a week with a same bottle (Image 2). After the water opacity was observed, we attempted to find a cause of such a change in water property, speculating about contamination with mouse saliva or possible GKE solubility problems. A water bottle was filled with GKE-enriched water, and placed into an empty cage without presence of mice. After 6 days, water transparency changed towards opacity. Thus, a mouse saliva was excluded as a potential reason for opacity. The GKE coming out of solution has possibly been a cause of such a phenomenon, as suspension went back into solution after manually swirling the water bottle.

Following this observation, treatment water was manually swirled during animal care once

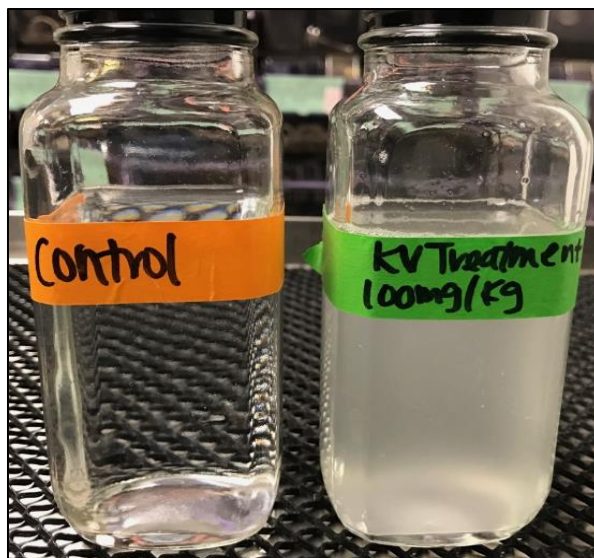daily to ensure that GKE stayed in solution for the remainder of the experiment.

Image 2. Opacity observed in GKE-enriched water in comparison to control water.

Exposure of C57BL/6J male mice to GKE-treated water started at 7-weeks of age and

lasted up to 12-weeks of age, e.g., 29 days post first STZ injection (STZ1). At 8-weeks of age

T1D was chemically induced with five consecutive injections of low-dose STZ. Body weights

and glycemia were measured before (day -3), and then bi-weekly until day 29 after the STZ1.

Overall, 16/22 (73%) of control and 21/25 (84%) of GKE-treated mice became diabetic.

Noticing that all of the MLDSTZ-treated mice did not develop clinical disease, we decided to

analyze data obtained for studied parameters (body weights, glycemia levels and diabetes

incidence) in two ways: a) in entire cohort of MLDSTZ-treated mice (control and treatment

mice who did or did not develop disease), and b) only in those control and GKE-treated mice

who did develop T1D. Whereas body weights between the control and treatment groups were

not significantly different in all mice treated with MLDSTZ, it is worth noticing that GKE-

treated mice expressed a trend of increased body weights throughout the entire experimental period (Figure 2). A similar trend in body weights was observed in GKE-treated compared to control mice, if only mice who developed obvious clinical T1D were analyzed (Figure 3). Mice exposed to 100 mg/kg of GKE daily displayed no statistically significant differences in glycemia levels when compared to controls, either when all MLDSTZ-treated (Figure 4) or diabetic only mice were analyzed (Figure 5). However, Figure 4 shows that there is a trend of increased glycemia levels in GKE-treated mice compared to controls, from day 15 to day 29 post STZ1, when all MLDSTZ-treated mice were analyzed. Similarly, there was a trend of increased hyperglycemia, but less pronounced, in GKE treatment group compared to controls in the cohort of diabetic mice (Figure 5).

Diabetes incidence in GKE-exposed MLDSTZ-treated mice. Diabetes incidence was also analyzed either in all MLDSTZ-treated mice, regardless of their diabetic status (Figure 6), or only in mice who became diabetic (Figure 7). An increased diabetes incidence ($p < 0.05$) was observed in GKE-exposed mice in comparison to controls in all MLDSTZ-treated animals (Figure 6). Thus, on day 12, 15, 19, and 22 post STZ1 there were 56%, 64%, 76%, and 84%, and 14%, 37%, 59%, and 64% diabetic mice in GKE-exposed and control groups, respectively. By the endpoint of the experiment (day 29 post STZ1), 84% became diabetic in GKE group, in comparison to 73% of controls. By analyzing only those mice who became diabetic (Figure 7), there was a similar trend of increased diabetes incidence observed in GKE-treated mice. However, a statistical significance was not reached. All mice in GKE treatment group became diabetic by day 22, while 100% of controls became diabetic on day 29.
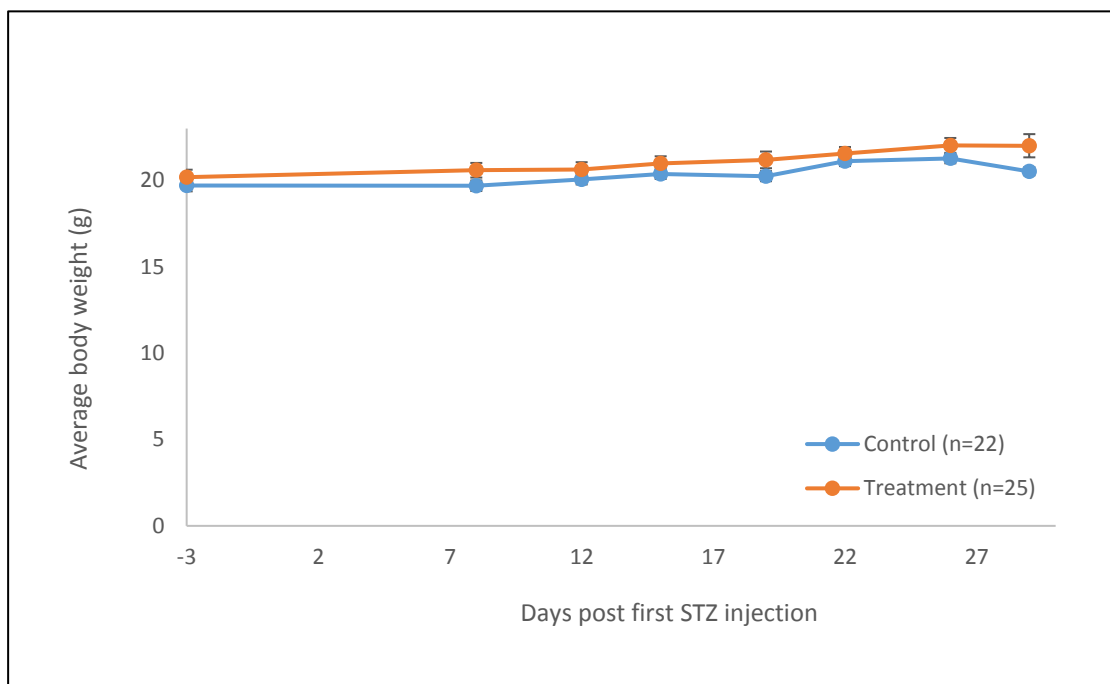
Figure 2. Body weights of control and GKE-exposed MLDSTZ-treated C57BL/6J male mice. Body weights measured from day -3 before until day 29 post STZ1. Data shown as average $\pm$ S.E.M.
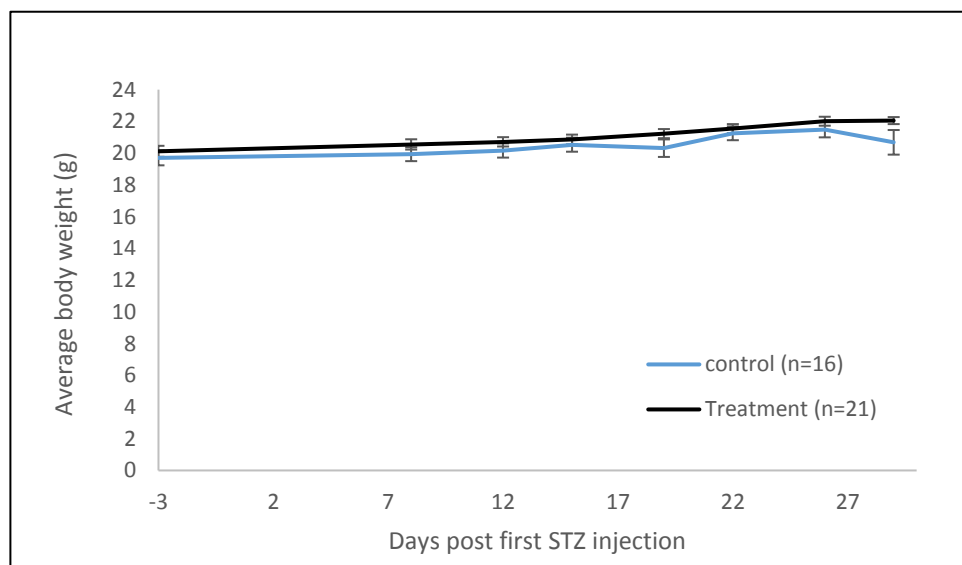

Figure 3. Body weights of <u>diabetic</u> control and GKE-exposed MLDSTZ-treated C57BL/6J male mice. Body weights measured from day -3 before until day 29 post STZ1. Data shown as average $\pm$ S.E.M.

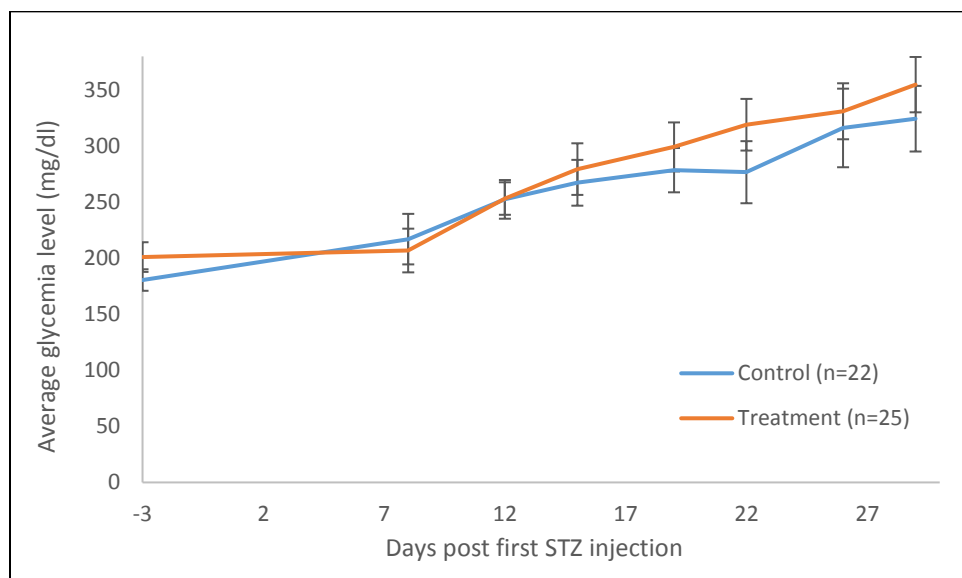**Figure 4**. Glycemia levels (mg/dl) in control and GKE-exposed MLDSTZ-treated C57BL/6J male mice. Glycemia levels measured from day -3 before until day 29 post STZ1. Data shown as average $\pm$ S.E.M.
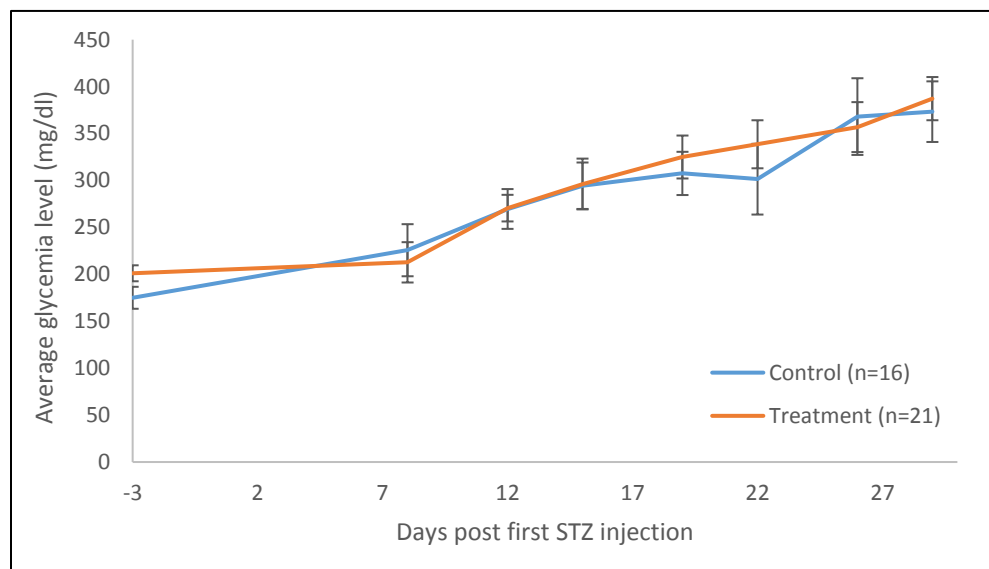

Figure 5. Glycemia levels in <u>diabetic</u> control and GKE-exposed MLDSTZ-treated C57BL/6J male mice. Glycemia levels measured from day -3 before until day 29 post STZ1. Data shown as average $\pm$ S.E.M.

Figure 6. Diabetes incidence in control and GKE-exposed MLDSTZ-treated C57BL/6J male mice. GKE administered at a dose of 100 mg/kg via drinking water. Glycemia levels measured from day -3 before until day 29 post STZ1. Diabetes occurrence defined by first day of two consecutive measurements of glycemia level of 250 or more mg/dl. Life table analysis; *p < 0.05.
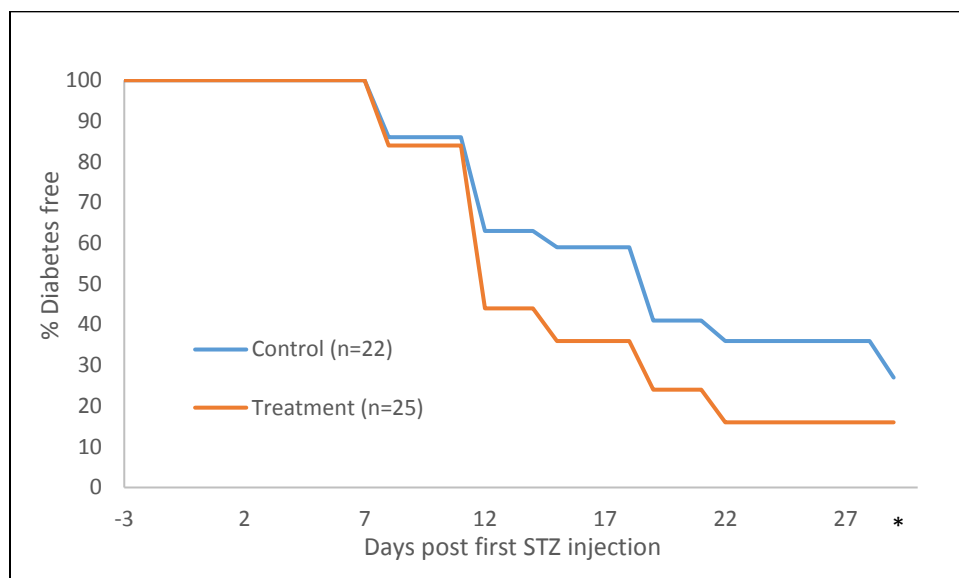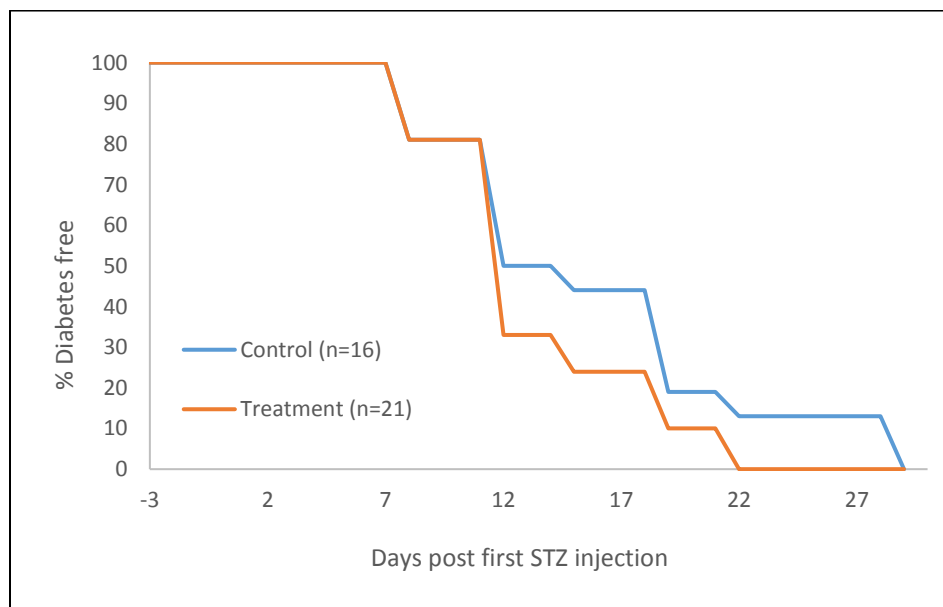

Figure 7. Diabetes incidence in diabetic control and GKE-exposed MLDSTZ-treated C57BL/6J male mice. GKE administered at a dose of 100 mg/kg via drinking water. Glycemia levels measured from day -3 before until day 29 post STZ1. Diabetes occurrence defined by first day of two consecutive measurements of glycemia level of 250 mg/dl or more.

In summary, the effect of aqueous GKE treatment on glycemia levels and diabetes incidence was studied in a MLDSTZ mouse model of T1D. Seven-week-old C57BL/6 male mice were pre-treated with 100 mg/kg/day GKE in a drinking water and diabetes induced by low-doses of STZ at 8-weeks of age. GKE treatment lasted permanently from 7-weeks of age until 12-weeks of age (e.g., until day 29 post STZ1). Glucose and body weight measurements started 3 days prior STZ injections and continued from day 8 until day 29 post STZ1. It was hypothesized that aqueous GKE would decrease T1D incidence and glycemia levels in treated mice. This hypothesis has been rejected, as neither glycemia levels nor diabetes incidence were reduced by GKE treatment. In contrast, even a trend of higher glycemia, as well as a statistically significant increase in diabetes incidence have been observed in MLDSTZ-treated mice exposed to GKE. Thus, our data do not support hypoglycemic and anti-diabetic properties of aqueous GKE.

Chapter 4: Discussion

The aim of this study was to examine aqueous GKE as a possible anti-diabetogenic compound. GK extract was chosen, and its anti-diabetogenic properties hypothesized in this study, as its complexes were described to display hypoglycemic (Adaramoye, 2012; Adaramoye & Adeyemi, 2005; Ayepola et al., 2013; Ayepola, Brooks et al., 2014; Ayepola, Cerf et al., 2014), anti-inflammatory (Ayepola et al., 2013), and oxidative stress-relieving properties (Ayepola, Brooks et al., 2014). Previous studies have used the ethanol extraction of GK seeds, originally described by Iwu et al. (1990). This extraction method allows for the separation of complexes found in GK seeds, which can be administered and examined separately. However, for administration of these complexes need to be dissolved in dimethylsulphoxide (DMSO) or ethanol and given via gastric gavage (Adaramoye, 2012; Adaramoye & Adeyemi, 2005; Ayepola et al., 2013; Ayepola, Brooks et al., 2014; Ayepola, Cerf et al., 2014). The GK seed complex that has previously been examined the most is a biflavonoid Kolaviron (KV). KV has been found to reduce oxidative stress by scavenging free radicals by enhancing catalase activity, which increases the removal of hydrogen peroxide in living cells, as well as, protects against hydrogen radical toxicity (Oyenihi et al., 2015). KV has also been found to have anti-inflammatory properties by inhibiting production of LPS-induced tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine (Olaleye, Onasamwo, Ige, Wu, & Cho, 2010).

Our study aimed to keep the exposure to GK seed extract as natural as possible. Therefore, an aqueous extraction of GK seeds was performed that allowed dilution of GKE in water, and GKE administration via drinking water. In this study, C57BL/6 mice were treated by GKE, dissolved in their drinking water, constantly over the period of 5 weeks, from 7- to

12-weeks of age. The dose of GKE of 100 mg/kg/day was chosen based on previously published data, showing KV extract efficacy in doses range of 100 mg/kg to 200 mg/kg (Adaramoye, 2012; Ayepola et al., 2013; Ayepola, Brooks et al., 2014; Ayepola, Cerf et al., 2014). Diabetes was induced by MLDSTZ in the second week of GKE treatment and diabetes incidence and severity of disease (glycemia levels) were analyzed throughout the experimental period. It is found that GKE treatment significantly potentiated diabetes development and even induced a trend of increased hyperglycemia in exposed mice. In contrast, previous studies, that investigated the effect of GK extract on amelioration of complications caused by hyperglycemia, also described its hypoglycemic properties (Adaramoye, 2012; Adaramoye & Adeyemi, 2005; Ayepola et al., 2013; Ayepola, Brooks et al., 2014; Ayepola, Cerf et al., 2014). Speculation of the differences in the results obtained in this study versus previously published observations might be attributed to different approaches used for GK extraction, T1D induction, administration of extract, and choice of timepoints and blood collection method for checking glycemia levels.

Firstly, as mentioned above, all the previous studies that studied hypoglycemic effects of GK seed extract (Adaramoye, 2012; Adaramoye & Adeyemi, 2005; Ayepola et al., 2013; Ayepola, Brooks et al., 2014; Ayepola, Cerf et. al., 2014) have used the ethanol extraction of GK seeds, originally published by Iwu et al. (1990). Thus, they investigated KV's effects on hyperglycemia, whereas we exposed mice to aqueous GKE. At this point, as we did not attempt to chemically analyze GKE, we could not provide data about composition differences of aqueous GKE versus KV.

Secondly, the model of diabetes used by these researchers was toxic T1D, induced by a single high-dose of STZ (usually 50-65mg/kg) in male Wistar rats, whereas this study used multiple-low-dose injections of STZ to induce the autoimmune form of T1D.

Aside from one, all previous studies only examined hyperglycemia at a maximum of two timepoints; once to confirm hyperglycemia and secondly after the exposure to KV, at the endpoint of experiment. Once hyperglycemia was confirmed (required levels being either 250 or 350 mg/dl), KV was administered by gastric gavage five times a week for either 3 or 6 weeks, collecting only on glycemia level timepoint at the endpoint of the experiment (Adaramoye, 2012; Ayepola et. al, 2013; Ayepola, Brooks et al, 2014; Ayepola, Cerf et al., 2014). One study did measure multiple hyperglycemia timepoints, though these timepoints were followed only hours after exposure to KV. The hypoglycemic effect was found four hours after the exposure and was not monitored more than nine hours from the beginning of the experiment (Adaramoye & Adeyemi, 2005). In contrast to previously published methodology, our study examined glycemia levels in GKE-exposed mice prior to T1D induction and throughout entire experimental period; such an experimental design provided a reliable amount of data to analyze glycemia levels throughout the progression of T1D and not simply before and after. Furthermore, all blood for glycemia levels analysis was obtained from live animals, whereas previous studies collected blood after the sacrifice of rat, using either cervical dislocation or drug euthanasia. As stated earlier, it has been found that KV exhibited oxidative stress-relieving and hepatocyte-protective properties. Hepatocytes are cells of the liver that can both store and produce glucose (Dong & Woo, 2001). Thus, the hypoglycemic effect observed previously could be attributed to the hepatoprotective properties of KV.

In our studies, GKE was administered via drinking water, whereas previous experiments used gastric gavage. This could possibly have cause differences in the digestion or metabolism of the given extract. Digestion starts with cephalic reflexes, or reflexes that start in the brain (central nervous system), these feedforward reflexes begin with sight, smell, sound, or thought of food and prepare the digestive system for ingestion of either food or liquids. This preparation includes processes such as salivation in the mouth, or secretion of hormones, such as the glucagon-like peptide-1, a feedforward signal responsible for insulin release (Silverthorn et al, 2012). Gastric or oral gavage could be disrupting these initial digestive steps.

To the best of our knowledge, this study for the first time examined GKE effects on the T1D development, and found a significant increase in T1D incidence ($p < 0.05$) in GKE-treated mice compared to controls. Previous studies observed anti-inflammatory properties of KV (Ayepola et al, 2013; Ayepola, Brooks et al., 2014; Ayepola, Cerf et al., 20014), meaning that GK seeds might be able to act on T-cells, and therefore preventing T-cell-mediated T1D development. However, KV is not the only compound found in GK seeds. GK seeds contain multiple flavonoids, possibly not all of them being anti-inflammatory and potentially anti-diabetogenic. It has been proposed that some flavonoids act more as signaling molecules than antioxidants or anti-inflammatory compounds. In fact, some dietary flavonoids have been found to affect the protein kinase C (PKC) pathway, a pathway that mediates hyperglycemic effects in vascular cells (Koya & King, 1998; Williams, Spencer, & Rice-Evans, 2004). This example does not imply an effect of a flavonoid found in GKE, but provides a clue that there are flavonoids that potentiate hyperglycemia. Therefore, we speculate that some of the other flavonoids found in GKE could possibly overact the effects of KV, causing increased

glycemia levels and potentiating the development of the disease. In order to address this possibility, a further study should be done using both aqueous (providing GKE) and ethanol extraction (providing KV) products in the same animal model of autoimmune T1D, analyzing glycemia levels, as well as further evaluating T-cells types and subtypes affected by these extracts. As T1D is a T-cell mediated autoimmune disease, analyzing T-cell composition, function (proliferation) and cytokine profiles, will help clarify whether and how GKE/KV affect the development and severity of T1D.

Chapter 5: Conclusion

The results of this study reject the hypothesis that aqueous GKE would decrease glycemia levels and reduce the incidence of MLDSTZ-induced T1D in GKE-treated mice, and suggests that aqueous GKE has hyperglycemic potential and acts as a diabetogenic compound. Future studies should focus on exploring the T-cell repertoire present in GKE-treated mice with MLDSTZ-induced T1D to further understand how aqueous GKE affects the incidence and severity of experimental T1D.

References

Adaramoye, O. (2012). Antidiabetic effect of kolaviron, a biflavonoid complex isolated from
garcina kola seeds, in wistar rats. *African Health Sciences, 12*(4), 498-506.

Adaramoye, O., & Adeyemi, E. (2005). Hypoglycaemic and hypolipidaemic effects of
fractions from kolaviron, a biflavonoid complex form garcinia kola in streptozotocin-
induced diabetes mellitus rats. *Journal of Pharmacy and Pharmacology, 58*, 121-128.

Agyili, J., Sacande, M., & Kouame, C. (2006). Garcinia kola Heckel. *Seed Leaflet No. 113*.
Millennium Seed Bank Project, Ghana.

Alexandraki, K. L., Piperi, C., Ziakas, P. D., Apostolopoulos, N. V., Makrilakis K, Syriou V,
Diamanti-Kandarakis, E., Kaltsas, G., & Kalofoutis, A. (2008). Cytokine secretion in
long-standing diabetes mellitus type 1 and 2: Associations with low-grade systemic
inflammation. *Journal of Clinical Immunology, 28*(4), 314-321.

Ayepola, O., Brooks, N., & Oguntibeju, O. (2014a). Kolaviron improved resistance to
oxidative stress and inflammation in the blood (erythrocyte, serum, and plasma) of
streptozotocin-induced diabetic rats. *Scientific World Journal*. Retrieved from
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3982470/.

Ayepola, O., Chegou, N., Brooks, N., & Oguntibeju, O. (2013). Kolaviron, a garcinia
biflavonoid complex ameliorates hyperglycemia-mediated hepatic injury in rats via
suppression of inflammatory responses. *International Society for Complementary
Medicine Research, 13*, 363.

Ayepola, O., Cerf, M., Brooks, N., & Oguntibeju, O. (2014b). Kolaviron, a biflavonoid
complex of garcinia kola seeds modulates apoptosis by suppressing oxidative stress

and inflammation in diabetes-induced nephrotoxic rats. *Phytomedicine, 21*(14), 1785-1793.

Center for Disease Control [CDC]. (2014). *Nation diabetes 2014 statistic report*. National Center for Chronic Disease and Prevention and Health Promotion Centers for Disease Control and Prevention.

Diabetes care and research in Europe: The Saint Vincent Declaration. (1990). *Diabetic Medicine, 7*(4), 1464-5491. Blackwell Publishing.

Dong, H., & Woo, S. (2001). Hepatic insulin production for type 1 diabetes. *Trends in Endocrinology and Metabolism, 12*(10), 441-446.

Ebana, R. U., Madunagu, B. E., Ekpe, E. D., & Otung, I. N. (1991). Microbiological exploitation of cardiac glycosides and alkaloids from Garcinia Kola, Borrereria Ocymoides, kolanitida and citrus auratifolia. *Journal of Applied Microbiology, 71*(5), 398-401.

Esimone, C. O., Nworu, C. S., Adikwu, M. U., Odimegwu, D. C., & Ezugwu, C. O. (2007). The effect of a new adaptogen, Garcinia kola seed, on the bioavailability of ofloxacin in humans. *Science Research Essays, 2*(11), 482-485.

Etuk, E. (2010). Animal models for studying diabetes mellitus. *Agriculture and Biology Journal of North America, 1*(2), 130-134.

Farombi, E. O., Akanni, O. O., & Emerole, G. O. (2002). Antioxidant and scavenging activities of flavonoid extract (kolaviron) of Garcinia Kola seeds. *Pharmaceutical Biology, 40*(2), 107-116.

Farombi, E. O., & Owoeye, O. (2011). Antioidative and chemopreventative properties of vernonia amygdalina and garcinia biflavonoid. *International Journal of Environmental Research and Public Health, 8*(6), 2533-2555.

Halliwell, B. (2007). Biochemistry of oxidative stress. *Biochemical Society Transactions, 35*(5), 1147-1150.

Haskins, K., Bradley, B., Powers, K., Fadok, V., Flores, S., Ling, X., Pugazhenthi, S., Reusch, J., & Kench, J. (2003). Oxidative stress in type 1 diabetes. *Immunology of Diabetes II: Pathogenesis from Mouse to Man, 1005*, 43-54.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology and Therapeutics, 96*(2-3), 67-202.

Hussain, R. A., Owegby, A. G., Parimoo, P., & Eatomam, P. G. (1982). Kolanone, a novel polyisoprenylated benzophenone with antimicrobial properties from fruit of Garcinia kola. *Journal of Medical Plant Research, 44*, 78-81.

Iwu, M., Igboko, O., Okunji, C., & Tempesta, M. (1990). Antidiabetic and aldose reductase 248 activities of biflavanones of garcinia kola. *Journal of Pharmacy and Pharmacology, 42*(4), 290-292.

Koya, D., & King, G. (1998). Protien kinase C activation and development of diabetic complications. *Diabetes, 47*(6), 859-866.

Merzouk, H., Madani, S., Chabane, D., Prost, J., Bouchenak, M., & Belleville, J. (2000). Time course of changes in serum glucose, insulin, lipids and tissue lipase activities in macrosomic offspring of rats with streptozotocin-induced diabetes. *Science, 98*(1), 21-30.

Notkins, A. L. (2002). Immunologic and genetic factors in type 1 diabetes. *Biological Chemistry, 277*, 42545-43544.

Olaleye, S., Onasanwo, S., Ige, A., Wu, K., & Cho, O. (2010). Anti-inflammatory activities of Kolaviron-inhibitition of nitric oxide, prostaglandin E2 and tumor necrosis-alpha production in activated macrophage-like cell line. *African Journal of Medicine, 39*, 41-46.

Olajide, O. J., Enaibe, B. U., Bankole, O. O., Akinola, O. B., Laoye, B. J. (2016). Kolaviron was protective against sodium azise (NaN3) induced oxidative stress in the prefrontal cortex. *Metabolic Brain Disease, 31*(1), 25-35.

Olatunde, F. E., Olayide, O. A., & Godwin, O. E. (2008). Antioxidant and savaging activities of flavonoid extract (Kolaviron) of garcinia kola seeds. *Journal of Pharmaceutical Biology, 40*(2), 107-116.

Onayade, O. A., Looman, A. M. G., Scheffer, J. J. C., & Gbile, Z. O. (1998). Lavender lactone and other volatile constituents of the oleoresin from seeds of Garcinia Kola Hechel. *Flavour Frangrance Journal, 13*(6), 409-412.

Owen, J., Punt, J., Stranford, S., & Jones, P. (2006). *Kuby immunology* (7th ed.). New York: W. H. Freeman and Company.

Oyenihi, O. R., Brooks, N. L., & Oguntibeju, O. O. (2015). Effects of Kolaviron on hepatic oxidative stress in streptozotocin induced diabetes. *National Institutes of Health [NIH], 15*, 236.

Patterson, C., Dahlquist, G., Gyurus, E., Green, A., & Soltesz, G. (2009). Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 200-20: A multicenter prospective registration study. *The Lancet, 373*(96680), 2027-2033.

Portha, B., Picon, L., & Rosselin, G. (1979). Chemical diabetes in the adult rat as the spontaneous evolution of neonatal diabetes. *Diabetologia, 17*(6), 371-377.

Rakienten, N., Rakienten, M. L., & Nardkarni, M. R. (1963). Studies on the diobetogenetic action of streptozotocin. *Cancer Chemotherapy, 29*, 91-98.

Ren, K., & Torres, R. (2009). Role of interleukin-1 beta during pain and inflammation. Brain Research Reviews, *60*(1), 57-64.

Sachdev, S., & Davies, K. J. (2008).Production, detection, and adaptive responses to free radicals in exercise. *Free Radical Biological Medicine, 44*(2), 215-223.

Schein, P., Cooney, D., McMenamin, M., & Anderson, T. (1973). Streptozotocin diabetes-further studies on the mechanism of depression of nicotinamide adenine dinucletide concentrations in mouse pancreatic islets and liver. *Biochemical Pharmacology, 22*(20), 2625-2631.

Silverthorn, D. U., Johnson, B. R., Ober, W. C., Ober, C. E., & Silverthorn, A. C. (2012). *Human physiology, and integrated approach* (7th ed.). Pearson Education, Inc.

Szkudelski, T. (2001). The mechanism of alloxan and streptozotocin action in B-cells of the rat pancreas. *Physiology Research, 50*, 536-546.

Terashima, K., Aqil, M., & Niwa, M. (1995). A novel biflavonoids from the roots of Garcinia Kola. *Heterocytes, 41*, 2245-2250.

Turk, J., Corbett, J. A., Ramanadham, S., Bohrer, A., & Mcdaniel, M. L. (1993). Biochemical evidence for nitric oxide formation from streptozotocin in isolated pancreatic islets. *Biochemical and Biophysical Research Communications, 197*(3), 1458-1464.

Willcox, A., Richardson, S. J., Bone, A. J., Foulis, A. K., & Morgan, N. G. (2009). Analysis of islet inflammation in human type 1 diabetes. *The Journal of Translational Immunology, 155*(2), 173-181.

Williams, R., Spencer, J., & Rice-Evans, C. (2004). Flavonoids: antioxidants or signaling molecules. *Free Radical Biology and Medicine, 36*(7), 838-849

Wu, J., & Yan, L. (2015). Streptozotocin-induced type 1 diabetes in rodents as a model for studying mitochondrial mechanisms of diabetic beta cell glucotoxicity. Diabetes Metabolic Syndrome and Obesity: Targets and Therapy, 8, 181-188.