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Garcinia kola Extract Potentiates a Pro-inflammatory Response While Not Affecting Type 1 Diabetes Incidence in an Experimental Mouse Model

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Garcinia kola Extract Potentiates a Pro-inflammatory Response While Not Affecting Type 1 Diabetes Incidence in an Experimental Mouse Model

by

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Abstract

Type 1 diabetes (T1D) is a non-preventable autoimmune disease where autoreactive T cells orchestrate the destruction of insulin-producing pancreatic beta cells. Individuals with T1D are committed to a life-long dependency on insulin, which it and T1D associated care is expensive, and in many regions around the world is unattainable. Thus, there is an imperative to find an accessible and efficient alternative. The seeds of *Garcinia kola* are commonly used for cultural and medicinal purposes in West Africa. Previous studies have shown that *Garcinia kola* seed extract (GKE) has hypoglycemic and anti-inflammatory effects which suit is as a candidate for preventing disease development. It was hypothesized that treatment with GKE would prevent T1D development through its anti-inflammatory action in an experimental mouse model. Male seven-week-old C57BL/6J mice were treated with aqueous or ethanol GKE (100 mg/kg) in their drinking water for five weeks and were chemically induced with T1D at eight weeks of age. Biweekly body weight and glycemia measurements were performed from nine to 13 weeks of age. Mice were euthanized at days 11 and 30 after the initial STZ injection to evaluate GKE's effects on T cells. Treatment with GKE had no effect on glycemia, diabetes incidence or prevalence. However, the immunomodulatory activity of GKE displayed a shift to a pro-inflammatory response, suggesting potential therapeutic benefits for other diseases. This study did not confirm the anti-diabetic potential of GKE, however, it did highlight the need for more consistent research on herbal compounds.

Acknowledgement

I am so deeply thankful for the abundance of help and support throughout my graduate school journey. I would like to give huge thanks to Brian Lorenz for all of the vivarium and technical support he provided, my committee members Cassidy Dobson and Oladele Gazal for providing me with their guidance and expertise throughout my project, and to all of the undergraduate students who have helped me in the lab. I am so proud of their effort and thankful for their help. I would like to thank a SCSU alumna, Sinduja Thinamany for her support and all the time she took to train me and answer all of my questions. These two years would have been so much more difficult without that! Lastly, I must give special thanks to my advisor and mentor, Dr. Cetkovic-Cvrlje. I will never be able to fully express my gratitude for her mentorship throughout my undergraduate and graduate career. She has instilled my passion for research, and if it were not for her, I would not realize my career calling in public health. I am so thankful for all of her continuous advice and the opportunities she has provided me throughout my time at SCSU. I feel so honored to have been a member in her lab and her graduate student. I would like to dedicate my thesis to two very special people in my life- my mom for her unconditional love and support, and to my godson Dax (aka chubs) as I hope he shares the similar joy and passion for science as I have had.

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Chapter I: Introduction

Over the past few decades, diabetes has become a global issue due to the increasing incidence, rate of inadequate access to treatment, and morbidity. There have been approximately 347 million deaths, and diabetes is anticipated to be the seventh leading cause of death worldwide by 2030 (Sharma, Petersen, Nazareth, & Coton, 2016). The mortality rate in 2017 from diabetes and its associated complications was approximately 11% among people aged 20-79, this is higher than the combined rate of deaths from HIV/AIDS, tuberculosis, and malaria (International Diabetes Federation [IDF], 2017). In 2017, there was a global estimate of 425 million people with diabetes and is projected to increase to 628 million people by 2045 (IDF, 2017). At least 90% of these individuals have type 2 diabetes (T2D), and type 1 diabetes (T1D) accounts for approximately 5-10%. Historically, T1D has been referred as juvenile-onset diabetes, and T2D affects adults, but T2D prevalence in youth has become significantly more frequent within the past two decades (D'Adamo & Caprio, 2011). However, T1D is still the most common form of diabetes seen in children, and there is an estimated 3-5% global increase in incidence annually; in 2015, over 540,000 children had T1D. Less developed countries have been experiencing the highest increased rate (IDF, 2015).

T1D related care, such as glucose monitoring tools, insulin treatment, and hospital visits- are very expensive; T1D related care costs the United States approximately $14.4 billion (Tao, Pietropaolo, Atkinson, Schatz, & Taylor, 2010). If there were to be a new therapy that would potentially eliminate this disease, an approximate $422 billion would be saved by existing T1D patients in their lifetime (Tao et al., 2010). An approximate $4,700 is the annual cost of T1D related expenses per child, and the bulk of the spending is related to glucose monitoring tools and insulin treatment (Bächle et al., 2013).

Type 1 Diabetes

T1D results with hyperglycemia induced by T lymphocyte (T cell)-mediated autoimmune attack of insulin-producing beta-cells within pancreatic islets (Atkinson, 2012). It has been proposed that a combination of genetic and environmental factors initiates disease development; environmental factors that are significantly associated with T1D development include viral infections, exposure to persistent organic pollutants, consumption of cow's milk, lack of Vitamin D, maternal age of childbirth over 35, and child delivery via cesarean section (Ahadi, Tabatabaeiyan, & Mozzami, 2011). Although, recently, environmental factors seem to be a far greater risk for T1D development (Krzewska & Ben-Skowronek, 2016). Particular alleles of, human leukocyte antigen (HLA) class II genes has high association with T1D development. HLA class II is located on antigen presenting cells (B lymphocytes, macrophages, and dendritic cells) which activate helper T cells (Dean & McEntyre, 2004). An additional theory explaining T1D development is molecular mimicry. It is thought that epitopes on viruses, such as the Coxsackie B4, are structurally similar to epitopes on beta cells, and can affect T cell maturation and activation (Cusick, Libbey, & Fujinami, 2012).

Clinical symptoms of hyperglycemia include polyuria, polyphagia, polydipsia, and weight loss. Poor management of hyperglycemia can lead to more serious health consequences including kidney disease, nerve damage, and retinopathy (IDF, 2015). People with T1D are dependent on life-long insulin therapy, and continuous glucose monitoring. Insulin therapy can be administered via injections or through a pump system (Chamberlain et al., 2017).

Immunology of Type 1 Diabetes

The immune system is divided into two branches- innate and adaptive. The innate branch is not specific, but has perfect self and non-self recognition due to the lack of specificity. Cells of the innate branch include phagocytes such as macrophages and non-phagocytes including natural killer (NK) cells (Turvey & Broide, 2010). The adaptive branch is very specific and elicits a more powerful response, problems of self recognition can arise; T cells and B cells are the cells of this branch. There are three T cell populations that can be identified by their surface markers called clusters of differentiation (CD): T_{helper} (Th), by CD4 marker, $T_{cytotoxic}$ (Tc), by CD8 marker, and $T_{regulatory}$ (Treg) cells by CD4 and CD25 markers. Th cells can be further divided in three subtypes: Th1, Th2, and Th17 (Bonilla & Oettgen, 2010). In a healthy immune system, there is a balance between Th1, Tc, and Th17 cells which release pro-inflammatory cytokines, and Th2 and Treg cells which release anti-inflammatory cytokines. During T1D development, it is thought that pathogenic T-cells including Th1, Th17 and Tc, potentiate beta cell destruction by the secretion of interferon-γ (IFNγ), interleukin 17a (IL-17a), and tumor necrosis factor-α (TNFα). In contrast, Th2 and Treg, which release interleukin 4 (IL-4) and interleukin 10 (IL-10) are believed to be protective cells, trying to prevent the destruction initiated by the pathogenic T cells (Wagner, 2011). The destruction of beta cells begins with the activation of Th cells by antigen presenting cells, followed by the differentiation into Th1 cells. Th1 cells then recruit Tc and macrophages by releasing cytokines, IL-2 and IFNγ, to propagate beta cell destruction (Yoon & Jun, 2005). Hallmarks of T1D include insulitis and chronic hyperglycemia; insulitis refers to the islet inflammation, characterized by accumulation of T cells and macrophages.

Experimental Mouse Models of Type 1 Diabetes

To understand the mechanism of T1D development, experimental mouse models have been used. There are two common models used to study experimental T1D: female NOD/LtJ mouse model with spontaneous T1D development, and chemically-induced T1D in male C57BL/6J mice. The spontaneous model of T1D development is a model that is most similar to how humans develop the disease and is regarded to be the top standard for studying how different compounds affect disease development (Shoda et al., 2005). However, this model requires a long time, usually about six months to a year (depends on the colony) for mice to become diabetic. On average, female NOD/LtJ mice start becoming diabetic from 12 weeks of age (Shoda et al., 2005). To ensure T1D development in C57BL/6J mice, multiple low-doses of Streptozotocin (STZ) (MLDSTZ) are administered intraperitoneally (Cetkovic-Cvrlje, Thinamany, & Bruner, 2017). STZ is an anti-cancer drug for pancreatic cancers due to its beta cell specific toxicity. STZ is classified as a glucosamine-nitrosourea compound, one of its mechanisms of action is DNA damage which induces poly ADP-ribosylation. Poly ADP-ribosylation results in the decline of cellular ATP, resulting in the inhibition of insulin synthesis (Szkudelski, 2001). Due to its similarities to glucose, STZ is transported to beta cells by the glucose transport protein, GLUT-2 (Wang & Gleichmann, 1998). MLDSTZ treatment is believed to initiate the release of autoantigens, such as glutamic acid decarboxylase, triggering beta cell autoimmunity, and inducing the T_{h1}-dependent inflammatory response (Van Belle, Taylor, & von Herrath, 2009). STZ can also be administered in a single high-dose. The single high-dose STZ does not trigger beta cell autoimmunity like the MLDSTZ. Instead, beta cells are rapidly destroyed, and hyperglycemia is immediate (King, 2012). Another common T1D chemical

inducer is alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil), which is generally injected in a single, high-dose, therefore not inducing the autoimmune development of T1D. Alloxan is structurally similar to glucose and is transported to beta cells by the GLUT-2 transporter. Beta cell destruction is induced by glucokinase inhibition and the generation of reactive oxygen species (ROS) (Lenzen, 2008). Alloxan also induces beta cell destruction by the oxidation of important thiol groups and disrupting intracellular calcium homeostasis (King, 2012).

Flavonoids

Flavonoids are a ubiquitous secondary metabolite present in plants that are often responsible for pigmentation, and also contribute to protection against microbial and environmental stressors. They are also common in fruits, vegetables, and herbs, where they are often referred as dietary flavonoids (Panche, Diwan, & Chandra, 2016). The structural skeleton of a flavonoid consists of 15 carbons with two aromatic rings that are connected together with a three-carbon bridge (Figure 1); structural differences within the B and C rings of the flavonoid skeleton yield subclasses of flavonoids, including flavanol, flavone, isoflavone, anthocyanidin, and flavanone (Del Rio et al., 2013). Flavonoids have been reported to exhibit anti-oxidant and anti-inflammatory properties that are influenced by modifications in the skeletal structure (Rengasamy et al., 2018). Structural requirements for anti-oxidant activity include an o-dihydroxy structure in the B ring, the presence of 3-OH and 5-OH groups, and a C2-C3 double bond (Rengasamy et al., 2018).

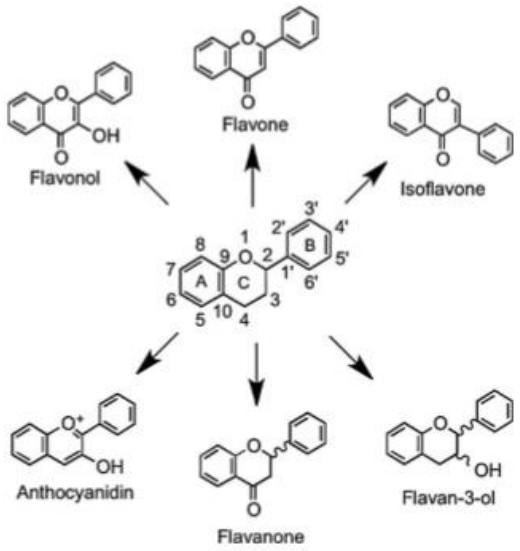


Figure 1. Structure of a flavonoid and the six subtypes (Del Rio et al., 2013).

Garcinia kola

Garcinia kola, a member of the *Guttiferae* family, is a large tree that is dominantly grown in Central and West Africa. The seeds (bitter kola), stem and bark act as an intricate staple in African culture and medicine, used for treatments of laryngitis, cold, liver disease, fertility complications, and hepatitis (Adedara, Awogbindin, Anamelchi, & Farombi, 2015; Iwu, Igboko, Okunji, & Tempesta, 1990). Garcinia kola seed extracts (GKE) contain Kolaviron (KV), a biflavonoid mixture consisting of Garcinia biflavonoid (GB)-1, GB-2, kolaflavanone, kolaflavone, and binarigenin (Oyenihi, Brooks, & Oguntibeju, 2015). KV has been reported to have beneficial effects, such as anti-microbial, anti-oxidative, anti-inflammatory, and hypoglycemic (Farombi & Owoeye, 2011).

Hypoglycemic Potential of *Garcinia kola*

Iwu et al demonstrated hypoglycemic properties of KV, extracted by light petroleum ether/acetone/chloroform Soxhlet method, in alloxan-induced T1D rabbits. Significant decreases

in fasting glycemia levels were observed in both diabetic and normoglycemic rabbits treated with KV (Iwu et al., 1990). Other studies done in Wistar rats, using a single-dose of STZ (50 mg/kg, 65 mg/kg, and 35 mg/kg) found a significant decrease in fasting glucose levels in both normal and diabetic rats treated with KV in a dose of 100 mg/kg (Adaramoye & Adeyemi, 2006; Adaramoye, 2012; Ayepola, Brooks, & Oguntibeju, 2014; Ayepola, Cheogou, Brooks & Oguntibeju, 2013). Interestingly, it has been shown that glycemia levels were significantly decreased by treatment with crude KV, and two KV fractions (FI and FII), but not fraction FIII (Adaramoye & Adeyemi, 2006). In a long-term diabetic rat model, hypoglycemic and neurodegeneration-preventative activities were demonstrated in rats treated with an aqueous GK extract. Diabetes was induced in rats with a single injection of 60 mg/kg of STZ. GK treatment of diabetic rats, via oral gavage lasted for 30 days and significantly decreased glycemia levels in diabetic rats (Farahna et al., 2017).

Anti-oxidative and Anti-inflammatory Properties of *Garcinia kola*

The anti-oxidative effects of KV were demonstrated in diabetic rats treated with KV by oral gavage at a dose of 100 mg/kg for 6 weeks. Anti-oxidative enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), and glutathione (GSH), as well as the effects of apoptosis and oxygen radical absorbance capacity (ORAC) were measured. Significant reductions in CAT, SOD, ORAC, and apoptosis activity were found in diabetic rats treated with KV (Oyenihi et al., 2015). Similarly, anti-oxidative enzymes, ORAC, pro and anti-inflammatory cytokines were measured in diabetic rats treated with KV by gastric gavage for 6 weeks. Diabetic rats treated with KV exhibited restorative levels of CAT and GSH, and a significant increase of ORAC. Similarly, diabetic rats treated with GK seeds at a dose of 250

mg/kg for 2 weeks demonstrated a significant restoration of antioxidant enzymes (Adedara et al., 2015). A reduction of pro-inflammatory cytokines, such as IL-1 and TNFα, with a slight increasing trend of IL-10, were also observed in diabetic KV-treated rats (Ayepola, Cerf, Brooks, & Oguntibeju, 2014). A study that measured anti-oxidant and anti-inflammatory markers in the blood of diabetic rats treated with 100 mg/kg KV by oral gavage for 6 weeks found an increasing trend of ferric-reducing antioxidant power (FRAP), and a reduction of IL-1, monocyte chemotactic protein-1 (MCP-1) and vascular endothelial growth factor (VEGF) (Ayepola et al., 2014).

Chapter II: Aim and Hypothesis

The goal of this study was to evaluate the effects of GKE on the development of autoimmune experimental T1D in MLDSTZ-treated C57BL/6J mice. Previous studies have described the hypoglycemic, anti-inflammatory, and anti-oxidant properties of GKE, however, GKE effects on immune cell populations, primarily T cell level and their function, have never been studied. In addition, all of the previous studies performed GKE treatment via oral gavage, and not through drinking water, which physiologically resembles the natural way how this seed is consumed in humans. Based on the previously described hypoglycemic and anti-inflammatory properties of GKE, it was hypothesized that GKE will decrease the incidence, prevalence, and severity of T1D through its anti-inflammatory action on pathogenic T cells. Thus, besides studies of glycemia levels and diabetes incidence, the effects on T cell populations/subpopulation levels, and their function, were studied in GKE-treated mice during the induction of experimental autoimmune diabetes in C57BL/6J mice.

Chapter III: Methods

Mice

The C57BL/6J and NOD/LtJ mouse strains were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in the St. Cloud State University (SCSU) Vivarium. All procedures performed on the mice were approved by the Institutional Animal Care and Use Committee before the start of this project (Protocol Number 5-98). BPA free cages were used for housing, and mice were allowed free access for water and food with casein-based phytoestrogen-free diet (AIN-93G Rodent Diets, Research Diet, Inc., Harlan research laboratories). Mice were exposed to a 12-hour light/dark cycle, and were kept in separate sexed cages, until chosen for the experiment based on age. Seven-week-old male C57BL/6J and four-week-old female NOD/LtJ mice were used for experiments. Mice were observed before and throughout the entire experimental period to ensure the absence of health issues or illnesses. During the experimental endpoints and/or upon detection of detrimental health issues, mice were euthanized via CO_2 asphyxiation.

GK Seeds and GK Extraction

Garcinia kola seeds were kindly provided by Dr. Oladele Gazal, and were purchased in Ijebu-Ode, Ogun State, Nigeria. The seeds were verified in the Department of Forestry and Wildlife Management, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria. For one experiment, new seeds were purchased from the vendor, Veroex, through Amazon.com. The outer brown layer *Garcinia kola* seeds were peeled, the seeds chopped, and the seed pieces dried at room temperature for 4-5 days. The dried seeds were pulverized into a fine powder. A stock GKE solution of 40 mg/ml was prepared via aqueous extraction per the methods of Ogunmoyole,

Olaekan, Fatai, Makun, and Kade, with modifications. A 1:5 *w:v* ratio of *Garcinia kola* seeds was added to autoclaved, DI water, followed by 48 hours of maceration at 4°C. This solution was filtered through Whatman filter paper, and the filtrate dried for 72 hours at 37°C. GKE was collected and weighed, and the appropriate amount of autoclaved-DI water was added to make a stock solution of 40 mg/ml. The solution was divided into aliquots which were placed in a -20°C freezer for further use. Ethanol extractions were prepared via the method described by Ogunmoyole, et al., with modifications (Ogunmoyole et al., 2012). A 1:5 (*w:v*) ratio of *Garcinia kola* seeds was added to 100% ethanol, followed by 24 hours of room temperature maceration. The solution was filtered with Whatman filter paper, and prior to collection, a 24-hour evaporation period was allowed to ensure there is no ethanol remaining. A GKE stock solution of 40 mg/ml was prepared with the addition of DMSO (Sigma-Aldrich, USA); GKE stock was divided into aliquots and stored at room temperature for further use.

GKE Treatment

Treatment mice received a dose of 100 mg/kg of either aqueous or ethanol GKE. To ensure mice received the proper dosage, the amount of GKE stock solution was altered per the current average body weight. Control mice for aqueous GKE extract groups received cold, autoclaved DI water, and control mice in ethanol extracted GKE groups received autoclaved DI water with the addition of 1.8% DMSO. Water levels were monitored daily and were filled accordingly; water bottles were replaced weekly, and mice received new GKE-treated and control water every 4-5 days.

Liquid-Chromatography Mass Spectrophotometry (LC-MS) Analysis of GKE

Three aqueous and two ethanol samples of GKE, each obtained during a separate extraction procedure, were sent to the University of Iowa for LC-MS analysis. 100ul of each sample was diluted into a ml of 90/10 H2O/ACN. The liquid chromatogram column used was a Zorbax Eclipse C18 (2.1 x 150 mm). The gradient started at 95% water that ramped up to 100% ACN over 20 minutes. The instrument used was a Waters QTOF Premier with an Acquity UPLC and Acquity TUV; wavelengths of 254 nm and 240 nm were used on the TUV. Mass spectrophotometry data was collected using positive ion electrospray ionization (ESI).

Induction of MLDSTZ-T1D in C57BL/6 Mice

STZ was administered intraperitonially for five consecutive days at a low dose of 40 mg/kg to both control and GKE treated mice. STZ was prepared daily before each injection by dissolving it in 0.05 M sodium citrate buffer (147.1 mg sodium citrate in 10 mL cold DI H_2O). The buffer pH was set to 4.5, and was properly adjusted by the addition of either 1 M HCl or 1 M NaOH. This solution was vortexed to create a drug suspension. Injections were done at a volume of 6.52 uL/g of body weight, as previously described (ter Veld et al., 2008). STZ administrations were completed within 15 minutes to avoid drug denaturation.

Glycemia Measurements

Initial glycemia and body weight measurements were done prior STZ injections and continued biweekly at 9 weeks of age until the experimental endpoint. Glycemia measurements were done with the Accu-Chek Aviva glucometer (Roche Diagnostics, Indianapolis, IN, USA). Mice were held in 50 mL Falcon tubes with breathing holes drilled on the sides and on the cap; the tails were placed into a warm water bath to induce vasodilation of the lateral vein. A single

blood drop, approximately 0.6 uL, was obtained by poking a vein, and was placed on the Accu-Chek Aviva glucose strips. Mice were considered diabetic when there were two consecutive glycemia measurements of 250 mg/dL or greater, with the first one considered as the day of diabetes onset.

Preparation of a Single Cell Suspension from the Spleen

Mice were euthanized via CO_2 asphyxiation at the determined experimental endpoints, and spleens were aseptically harvested. Single cell suspensions were prepared by mashing the spleens with a plunger from a 10-mL syringe and a passage through 40 um nylon strainers (BD Falcon, USA). Suspensions were centrifuged at 1200 rpm for 5 minutes. The supernatant was decanted, and the cell pellet was resuspended in 750 uL of ACK lysis buffer (NH4Cl 8.29g/L, KHCO3 1.0g/L, EDTA Na2· 2H2O 0.0375g/L; 18 Lonza Bio Whittaker, Walkersville, MD, USA) for 1-minute for complete hemolysis. An additional 5 mL of 1X PBS was added to stop the action of the lysis buffer, and the suspensions were centrifuged. Three additional washes with 5 ml 1X PBS and subsequent centrifugation were done to remove cell debris and washout the ACK buffer. Cells counts, and viability were done via Trypan Blue (Lonza Bio Whittaker Walkersville, MD, USA) exclusion method. Briefly, the isolated splenocytes were prepared in a 1:20 dilution with Trypan Blue and were loaded in a hemocytometer; the loaded hemocytometer was put under 10x magnification objective of a microscope to determine live and dead cell by counting all cells in two opposing quadrants. Dead cells appear blue and alive cells yellow.

T Cell Proliferation Assay

Proliferation assays were done to determine the *ex vivo* effects of GKE treatment on T cell function. Single cell suspensions, obtained from the spleens of mice post *in vivo* treatment

by aqueous or ethanol GKE, were diluted in the culturing medium [RPMI-1640 with 5 units Penicillin and Streptomycin and 10% Fetal Bovine Serum (Sigma, St. Louis, MO,USA)]. Splenocytes were plated in a 96 well plate in a concentration of $4x10^5$ in a total volume of 100 uL. Selected wells received the addition of the T-cell mitogen Concanavalin A (3 ug/mL) [(Con A) Sigma, St. Louis, MO, USA], non-stimulated wells received additional complete media. Plates incubated for 72 hours at 37°C with 5% CO_2. ConA specifically stimulates the proliferation of T cells. At the end of the culturing period, the Alamar blue assay was utilized to quantify T cell proliferation (Invitrogen, Grand Island, NY, USA). Alamar blue is a colorimetric assay which measures reducing capacity of cells. The active ingredient, resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) is reduced to fluorescent pink resorufin (Rampersad, 2012). Ten uL of Alamar blue was added to the wells. The plate incubated for an additional 5-6 hours under the conditions previously described and read by a spectrophotometer at 570 nm to obtain the optical density.

Characterization of Immune Cell Populations (Immunophenotyping)

Flow cytometry was done to determine immune cell composition (T cells and respective subtypes, B cells, macrophages, and NK cells) post GKE treatment. Isolated splenocytes from single cell suspensions were used at a concentration of $1x10^6$ and were transferred to FACS tubes with the addition of 1 ml FACS buffer (1% FBS, PBS, 0.1% NaN3). Splenocytes were centrifuged for 5 minutes at 1200 rpm at 4°C, supernatant was discarded, and tubes were blotted to rid excess buffer. To determine immune cell populations, splenocytes were stained with fluorochrome-conjugated antibodies that bind to specific CD markers Antibodies were prepared with FACS buffer in a ratio of 1:100, and splenocytes incubated with the addition of antibodies

for 30-45 minutes at 4°C protected from light. The CDs that classify all T cells, Th, Tc, Treg, B cells, macrophages, and NK cells are respectively: CD3, CD4, CD8, CD4/CD25, CD45R/B220, NK1.1b, and CD11b. Antibodies were conjugated with the following fluorochromes: fluoroscein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll (PerCP), and allophycocyanin (APC). The following fluorochrome-conjugated antibodies were used for flow cytometry: anti-CD4 PerCP (clone RM4-5), anti-CD8 FITC (clone 53-6.7), anti-CD25 APC (clone 3C7), anti-B220 APC (clone RA3-6B2), anti-NK 1.1 FITC (clone PK 136), anti-CD 11b PerCP (clone M1/70), and anti- CD3 PE (clone 145-2C11) (BD Biosciences). After incubation, cells were washed three times with FACS buffer; an additional 300 uL of FACS buffer was added to the samples before an acquisition of cells by the flow cytometer.

Cytokine Collection and Analysis

Isolated splenocytes were cultured in a 24-well plate at a concentration of $2x10^6$ in 500 uL Selected wells received Con A, and other wells received additional complete media, for a total volume of 1 mL. The plates were incubated for 48 hours under conditions previously described. Once the incubation period was completed, the plate was spun down in a centrifuge for 10 minutes at 1200 rpm, 4°C the supernatant was collected in 250 uL aliquots, and stored at -80°^{c}. Cytokine quantification was done via the CBA Mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, USA); cytokines of interest included IL-2, IL-4, IL-6, IL-10, IL-17, IFNγ, and TNFα. Briefly, lyophilized mouse standards were reconstituted with 2.0 mL of assay diluent, followed by a serial dilution that was performed in a ratio of 1:2 through the final dilution of 1:256. The total bead capture solution was prepared by the addition of 10 uL of each cytokine, 50 uL of this solution was added to each dilution tube, with the addition of 50 uL of cell

supernatant. 50 uL of the fluorochrome, PE, was also added, and the samples incubated for 2 hours at room temperature, protected from light. Acquisition was done by flow cytometer, and samples were analyzed using FCAP array software (Softflow, New Brighton, MN, USA).

Assessment of T Cell Proliferation and Cytokine Levels

The effects of GKE on T-cell proliferation and cytokine secretion were tested *in vitro*. Single cell suspensions were prepared from the spleens of non-treated C57BL/6J mice as previously described. Proliferation assays were done to investigate the effects of aqueous GKE on *in vitro* T-cell proliferation. Cultured splenocytes with suitable cell counts in a concentration of $4x10^5$ were plated in a 96-well plate in a total volume of 100 ul. Selected wells were cultured in ConA and the addition of GKE from 2 ug/mL to 1 mg/mL. Select non-stimulated wells received additional complete media and GKE (1 mg/mL). When investigating the effects of ethanol extracted GKE, appropriate DMSO controls were included to account for the immunosuppressive activity of DMSO at high concentrations. The culture plates incubated for 72 hours, 37˚C with 5% CO_2, and received 10 uL of Alamar blue with an additional 5-6 hours of incubation as previously described. The optical density was determined by reading the plate at 570 nm. Isolated splenocytes were cultured in a 24-well plate at a concentration of $4x10^5$ in 500 ul. Selected wells received Con A, and GKE at low concentrations (2 ug/mL and 4 ug/mL) and high concentrations (250 ug/mL, 500 ug/mL, and 1 mg/mL) and other wells received additional complete media, for a total volume of 1 mL; the culture plate incubated for 48 hours under conditions previously described. Once the incubation period was completed, the plate was spun down in a centrifuge for 10 minutes at 1200 rpm, 4˚C the supernatant was collected in 250 uL

aliquots and were stored at -80°C. Cytokine quantification was done via the CBA Mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, USA).

Statistical Analysis

Diabetes incidence of control and GKE treated mice were analyzed using a survival analysis statistical method (JMP, SAS, Cary, NC, USA). Glycemia and body weight measurements were analyzed using a one-way ANOVA with repeated measures (significance was considered as $p<0.05$ for both measures). Differences in T cell proliferation (both non-stimulated and stimulated), and immune cell composition between control and GKE treated mice were analyzed with a two-tailed, unpaired student's T-test (significance defined as $p<0.05$).

Chapter IV: Results

In Vitro Effects of Aqueous GKE on T Cell Proliferation and T Cell Cytokine Profiles

The potential direct effects of aqueous GKE on T cells were studied through analysis of T cell function and cytokine profiles using splenocytes of male and female C57BL/6J mice. A serial dilution of aqueous GKE, concentrations ranging from 2 ug/mL to 1 mg/mL, was added to C57BL/6 splenocyte cultures stimulated with Con A (3 ug/mL). There was no significant effect observed at any GKE concentration on T cell proliferation (Figure 2). In addition, cytokine analysis was performed in cultured Con A-stimulated splenocytes exposed to low and high concentrations of GKE. High concentrations of GKE ranged from 250 ug/mL to 1 mg/mL, low concentrations were 2 ug/mL and 4 ug/mL; selected splenocyte cultures did not receive GKE and served as a control. There were no significant differences in IL-10, IL-2, or IFNγ secretion amongst all concentrations of GKE tested (Figures 3B, 3D, 3F). IL-17A was significantly increased amongst the two highest concentrations of GKE compared to the two lowest concentrations and the control (Figure 3A). TNFα concentration obtained in T cell cultures exposed to GKE at 1 mg/mL was significantly increased compared to those exposed to GKE at 250 ug/mL, 4 ug/mL, 2 ug/mL and the control (Figure 3C). IL-4 was significantly increased in cultures treated by GKE 1 mg/mL compared to 4 ug/mL (Figure 3E). IL-6 level was significantly increased in GKE 1 mg/mL-treated splenocytes compared to those cultured with addition of GKE in the concentration of 4 ug/mL, 2 ug/mL and the control (Figure 3G).

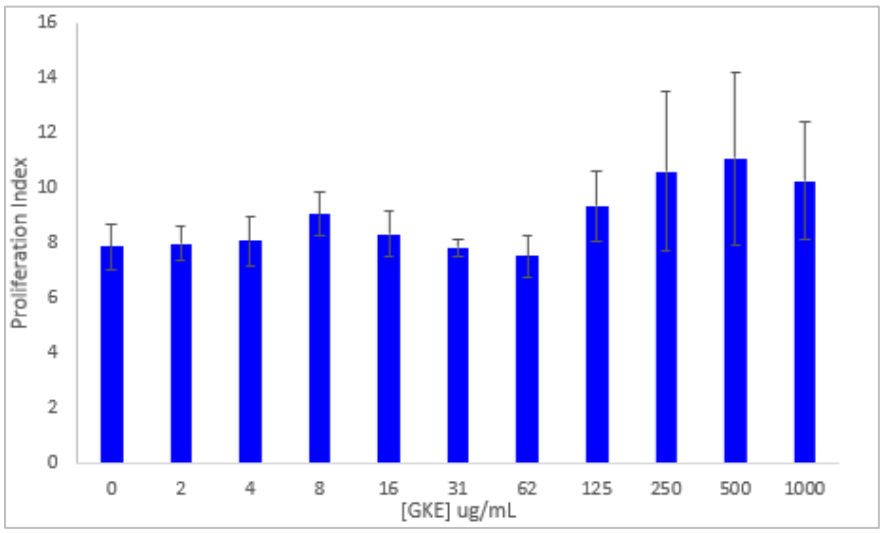
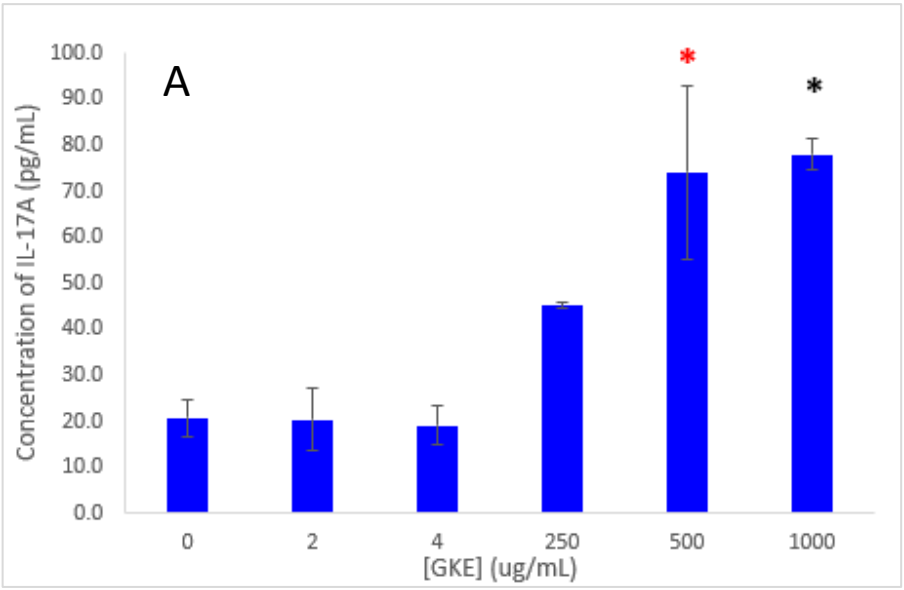
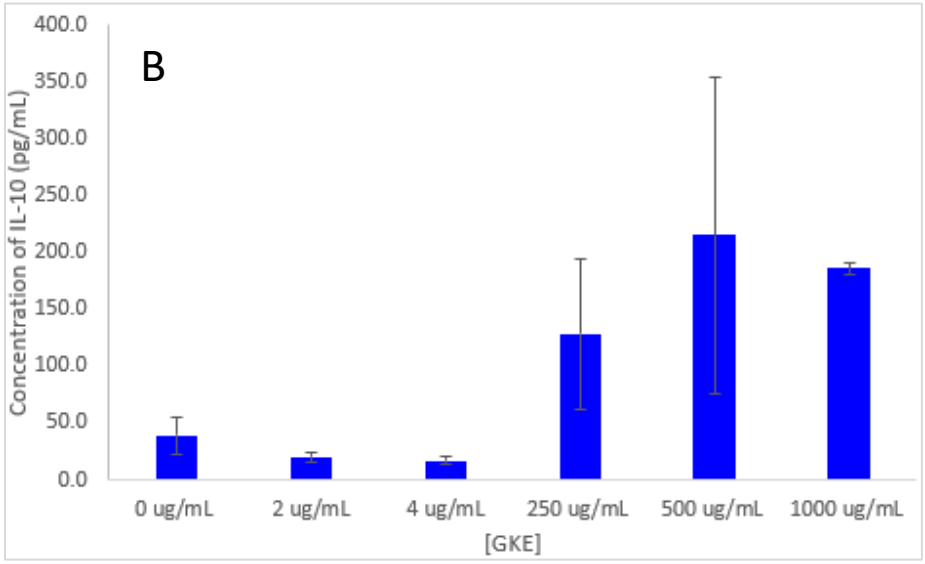
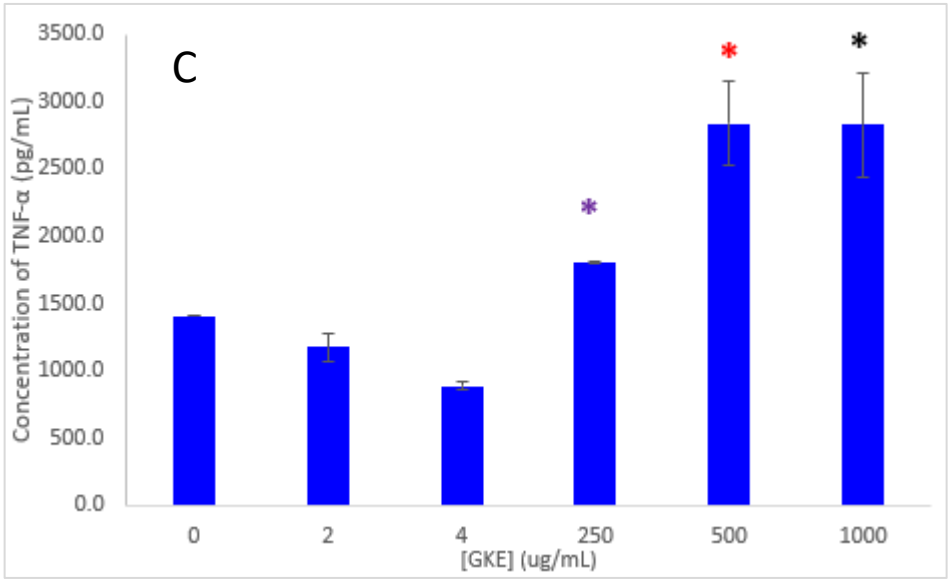
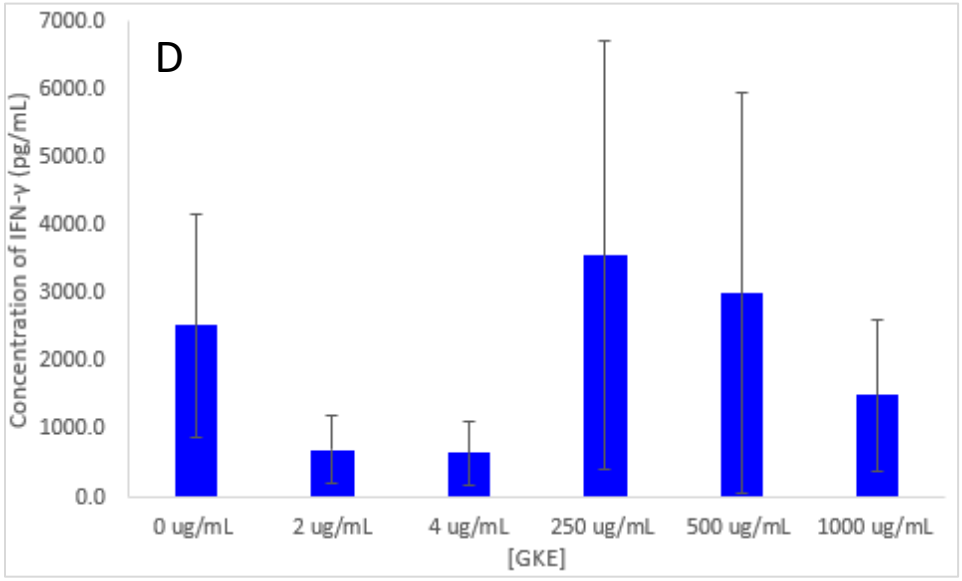


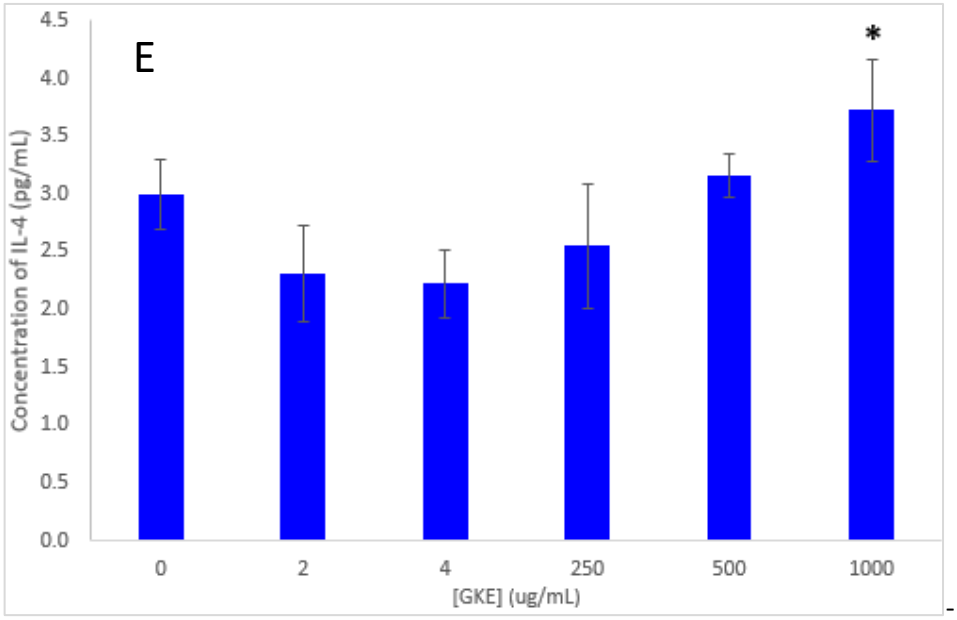
Figure 2. T cell proliferative index of C57BL/6J splenocytes cultured with a serial dilution of aqueous GKE. Proliferation index was calculated by dividing the optical densities of Con A-stimulated by optical densities of non-stimulated T cells. Data presented as average ± SEM, n=3.




B
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
Concentration of IL-10 (pg/mL)
0 ug/mL
2 ug/mL
4 ug/mL
250 ug/mL
500 ug/mL
1000 ug/mL
[GKE]


C
3500.0
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
Concentration of TNF-α (pg/mL)
*
*
*
0
2
4
250
500
1000
[GKE] (ug/mL)


D
Concentration of IFN-γ (pg/mL)
7000.0
6000.0
5000.0
4000.0
3000.0
2000.0
1000.0
0.0
0 ug/mL
2 ug/mL
4 ug/mL
250 ug/mL
500 ug/mL
1000 ug/mL
[GKE]


E
*
Concentration of IL-4 (pg/mL)
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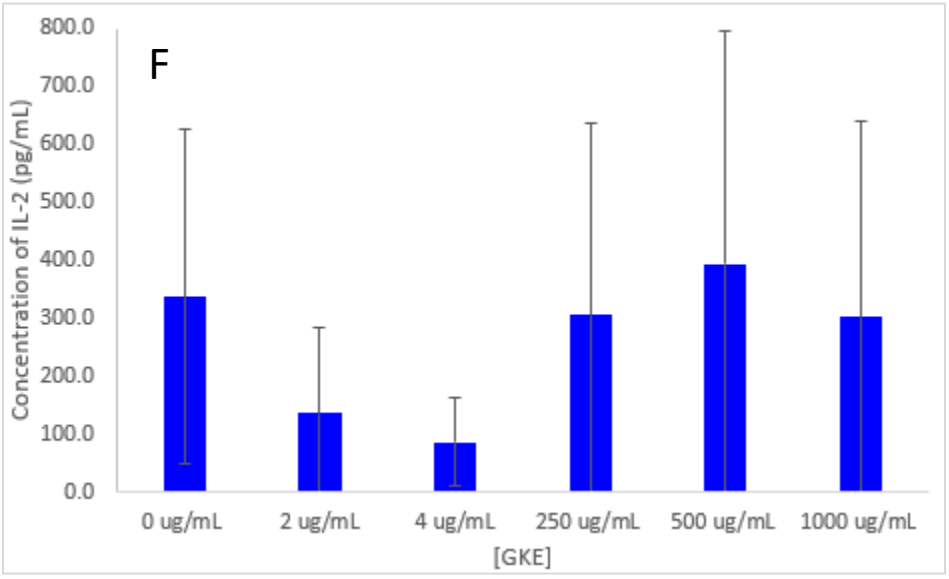
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
250
500
1000
[GKE] (ug/mL)



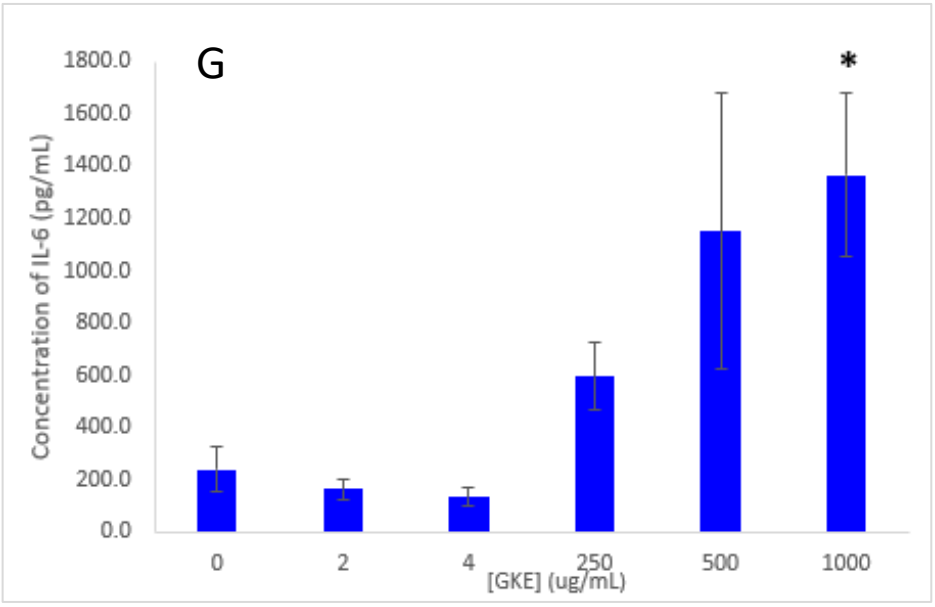


Figure 3. Concentration of cytokines obtained in Con A-stimulated C57BL/6 splenocytes cultured with different concentrations of GKE. A. IL-17; B. IL-10; C. TNFα; D. IFNγ; E. IL-4; F. IL-2; G. IL-6. Data presented as average ± SEM; n=2; * $p < 0.05$ compared to 0, 2, and 4 ug/mL GKE (A); compared to 0, 2, 4, and 250 ug/mL GKE (C); compared to 4 ug/mL GKE (E). * $p < 0.05$ compared to 0, 2, and 4 ug/mL (A); compared to 0, 2, 4, 250 ug/mL GKE (C). *$p < 0.05$ compared to 4 ug/mL GKE.

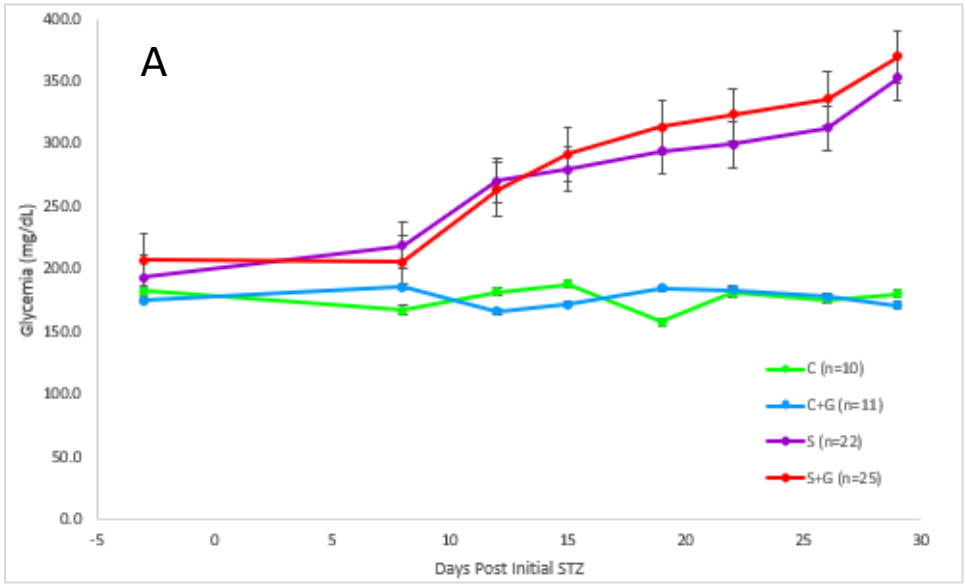
In Vivo Examination of Aqueous GKE Treatment Effects on Incidence and Glycemia Levels in MLDSTZ Model of T1D

Seven-week-old male C57BL/6J mice were treated with aqueous GKE (100 mg/kg) in their drinking water until 13 weeks of age. At eight weeks of age, mice were intraperitoneally injected with multiple, low-doses (40 mg/kg) of streptozotocin (MLDSTZ) for five days. Body weight and glucose levels were measured on the third day before STZ injections, and biweekly from the eighth day after the first STZ injection, until the experimental endpoint on day 30 (or 13 weeks of age). Glycemia and body weight measurements were also done in mice that were treated with aqueous GKE but did not receive STZ injections (STZ-free mice). GKE treatment did not exhibit significant effects on glycemia levels in MLDSTZ mice (Figure 4A). However, GKE had significantly increased body weight ($p= 0.0091$) in treated mice compared to their controls (Figure 4B). In addition, GKE treatment did not slow down diabetes development in STZ-exposed mice (an average day of diabetes occurrence ± SEM for GKE treatment group was 14.6 ± 1.0 vs. 17.1 ± 1.5 days for control group), nor reduced disease incidence, as 84% of the aqueous GKE-treated mice and 73% and of the control mice were diabetic by the experimental endpoint (Figure 4C).

In STZ-free, non-diabetic mice, GKE had no effect on glycemia levels (Figure 4A); STZ-free mice treated with GKE had a trend of decrease in body weight compared to their controls (Figure 4B).

Since no effect on glycemia or diabetes incidence was observed in our initial experiments with aqueous GKE, we questioned the quality and freshness of *Garcinia kola* seeds we used. Thus, fresh seeds were purchased from the other source. The aqueous GKE was extracted and administered a week before the STZ injections to mice, as described previously.

This aqueous GKE treatment, which lasted for 15 days post first STZ injection, did not exhibit significant effect on body weight, glycemia levels (data not shown), or diabetes incidence of treated mice compared to controls (an average day of diabetes occurrence ± SEM for GKE treatment group was 10.5 ± 0.6 vs. 11.0 ± 0.1 days for control group). In addition, we also questioned our GKE administration protocol, in which fresh GKE treatment water was prepared every five days. Thus, a pilot experiment was performed in which the aqueous GKE treatment water was prepared daily from a GKE stock solution, and given a week before the STZ administration to MLDSTZ mice for drinking. The mice were followed until day 22 post first STZ injection. This treatment had no effect on either body weight and glycemia levels (Figure 5A and 5B), nor on diabetes development (an average day of diabetes occurrence ± SEM for GKE treatment group was 10.5 ± 0.5 vs. 11.8 ± 1.1 days for control group).



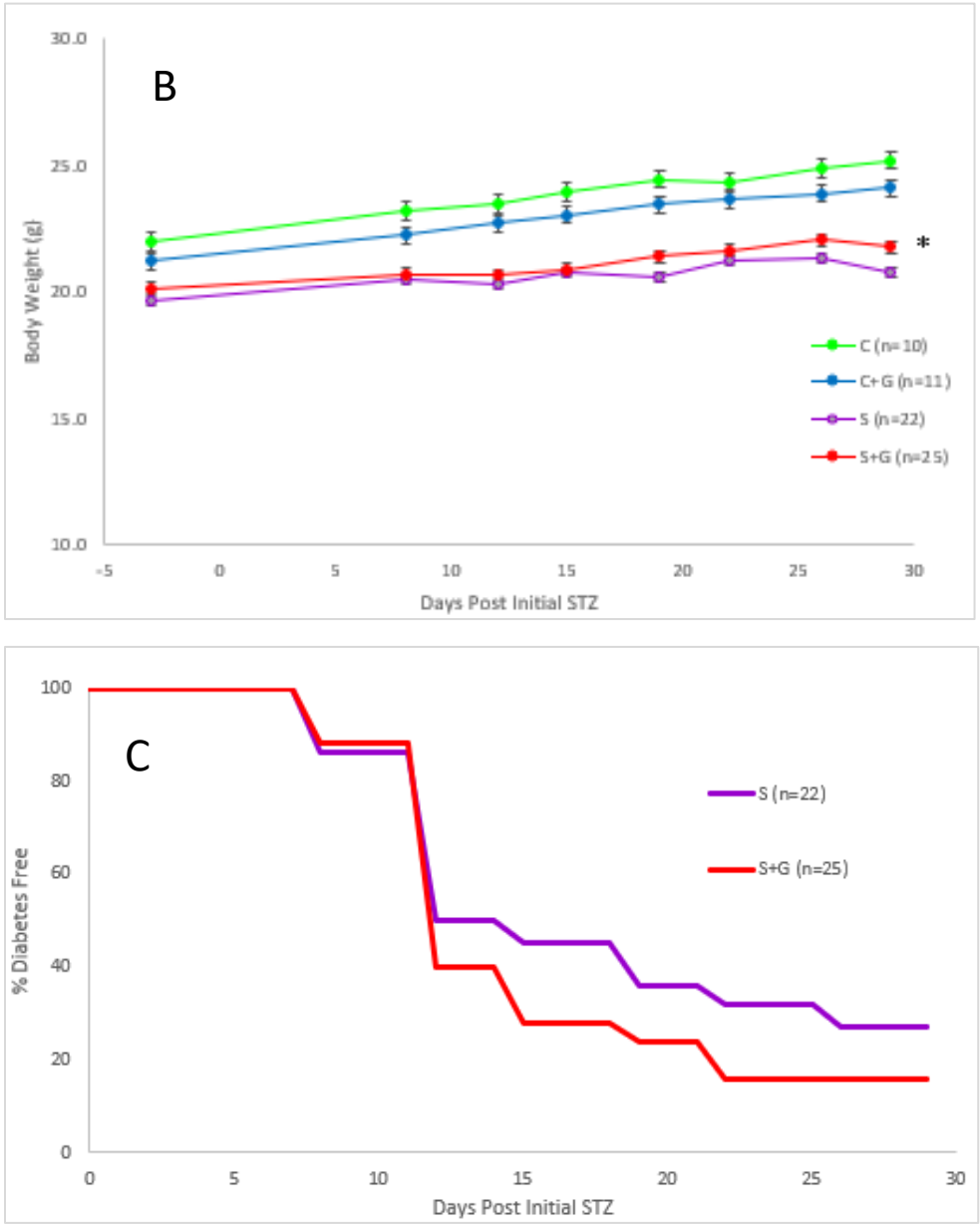


Figure 4. Average glycemia (A), body weight (B), and diabetes incidence (C) of MLDSTZ and STZ-free C57BL/6J mice treated with aqueous GKE for six weeks. (A) Glycemia measurements started three days before and lasted until 30 days after the initial STZ injection. Data presented as average ± SEM. (B) Body weights measurements started three days before the initial STZ injection and lasted until the experimental endpoint. Data presented as average ± SEM, *$p<0.05$ compared to control mice. (C) Diabetes incidence presented as the percentage of diabetes-free mice. Diabetes was defined as having two consecutive measurements of glycemia of 250 mg/dL or greater. C and C+G groups were not graphed, as they stayed 100% diabetes-free throughout the experiment; C-control, STZ-free mice; C+G-control, STZ-free mice treated by GKE; S-control MLDSTZ mice, and S+G-MLDSTZ mice treated by GKE.

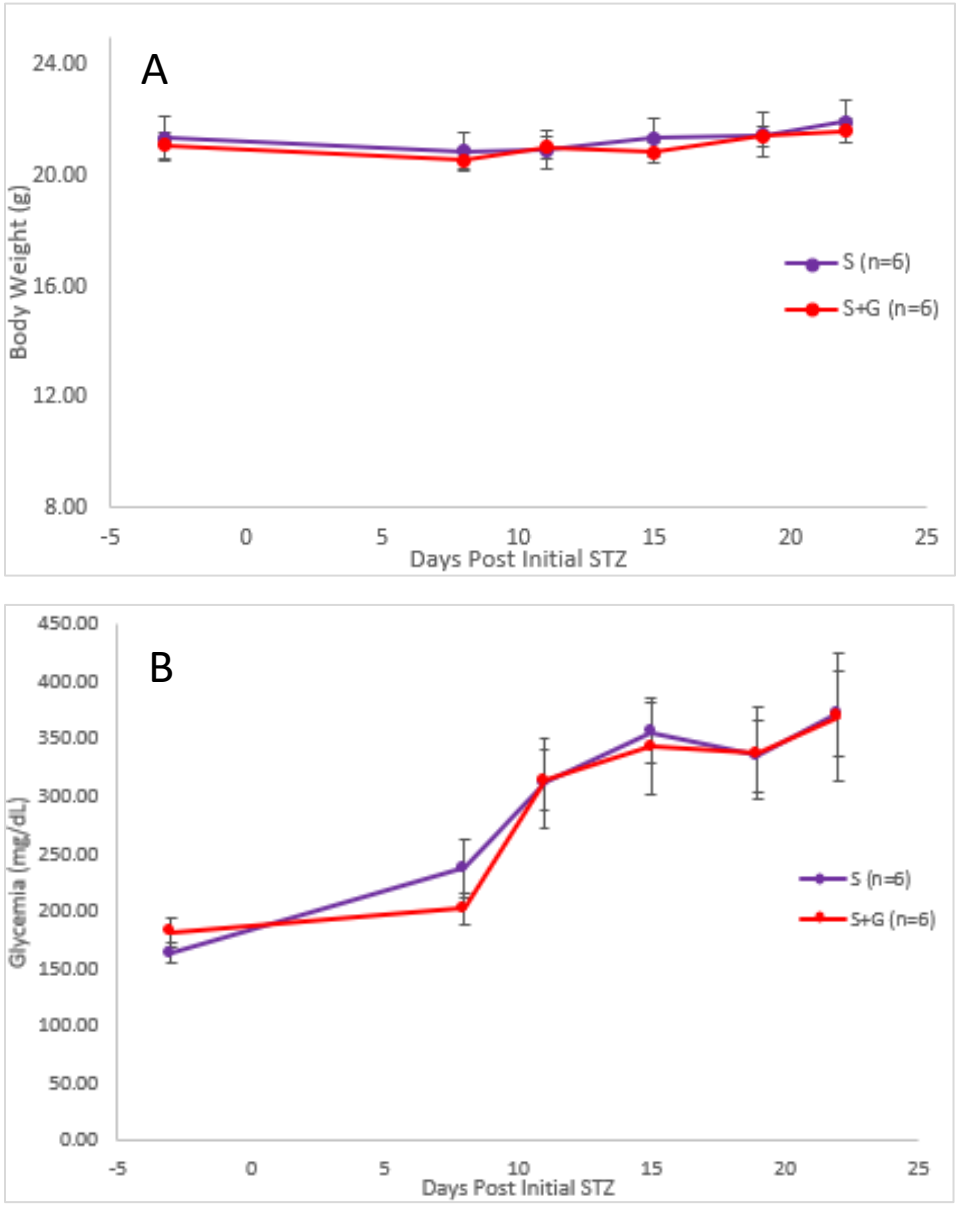


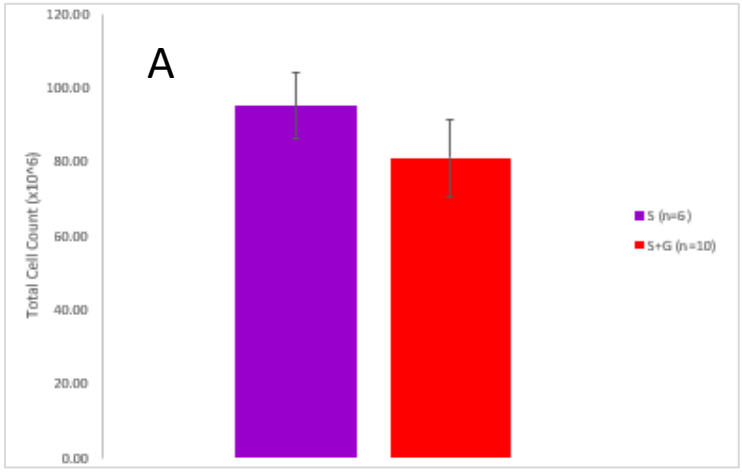
Figure 5. Average body weight (A) and glycemia (B) for MLDSTZ mice treated with fresh, daily aqueous GKE for four weeks. (A) Body weight measurements started three days before STZ injections and continued biweekly until 22 days after the first STZ injection. Data presented as average ± SEM. (B) Glycemia measurements started three days before STZ injections and continued biweekly until 22 days after the first STZ injection. Data presented as average ± SEM.

Effects of Aqueous GKE on Immune Parameters During T1D Development

Mice were sacrificed at two time points, on days 11 and 30 post the initial STZ injection, to evaluate aqueous GKE's effects on immune parameters, such as: splenic cell counts and

viability, T cell proliferation (presented as proliferation index), T cell sub-populations, and cytokine profiles.

The following data were obtained from the spleens of MLDSTZ mice treated with aqueous GKE and their controls on day 11. GKE treatment did not significantly affect the cell counts or cell viability (Figures 6A and 6B). There was a trend of decrease in T cell proliferation in GKE-treated mice compared to controls (Figure 7). Immunophenotyping of spleens obtained from mice treated with GKE, presented as a percentage, showed significant decreases in T, Th, Tc, Treg, and MAC cells ($p= 0002$, $p< 0.0001$, $p= 0.001$, $p= 0.001$, and $p=0.001$, respectively), whereas B cells were significantly increased ($p= 0.0001$), compared to the controls (Figure 8A). Immunophenotypes, in terms of total cell counts, showed that GKE treatment significantly decreased MAC and NK cells ($p= 0.04$ and $p= 0.04$) and induced a trend of decrease in T cells and T cell subtypes, compared to controls (Figure 8B). Cytokine measurements showed significant increases in TNFα, IFNγ, and IL-6 ($p= 0.003$, $p= 0.03$, and $p= 0.02$, respectively), and a significant decrease in IL-2 ($p= 0.02$) in mice treated with GKE compared to their controls (Figure 9).



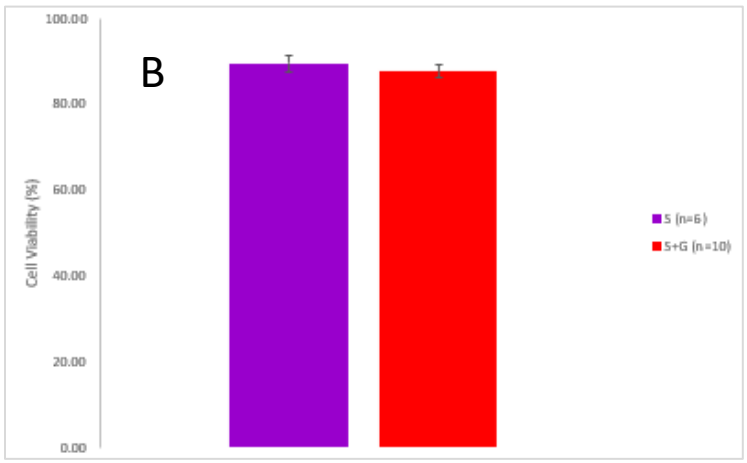


Figure 6. Total splenocyte counts (A) and cell viability (B) obtained on day 11 from MLDSTZ C57BL/6J mice treated with aqueous GKE. (A) Cell counts, and viability were measured using the Trypan blue exclusion method. Data presented as average ± SEM. (B) Cell viability was calculated by dividing alive cells by total cells x 100. Data presented as average ± SEM. The treatment groups' legend described in Figure 3.

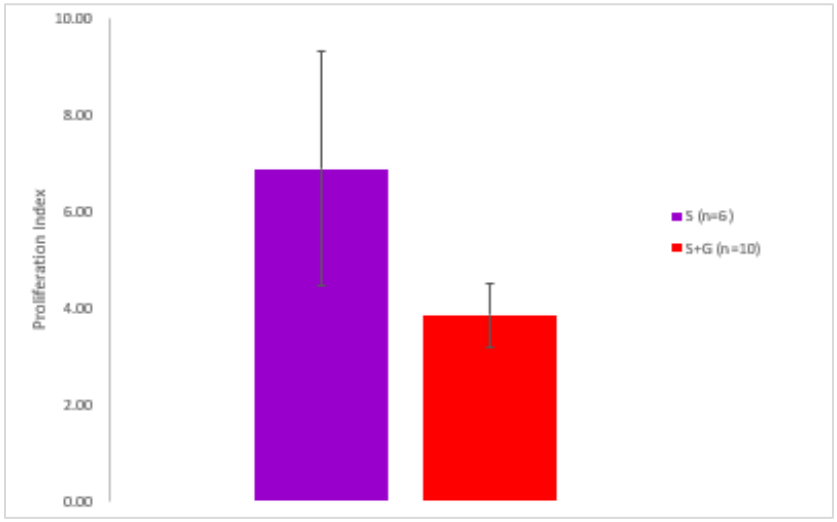


Figure 7. T cell proliferative index obtained on day 11 from the spleens of MLDSTZ C57BL/6J mice treated with aqueous GKE. T cell proliferation was induced by Con A stimulation during a 72-hour culture period and optical density was measured by an Alamar Blue assay. Proliferation index was obtained by dividing optical densities measured in ConA-stimulated by non-stimulated cells. Data presented as average ± SEM. The treatment groups' legend described in Figure 3.

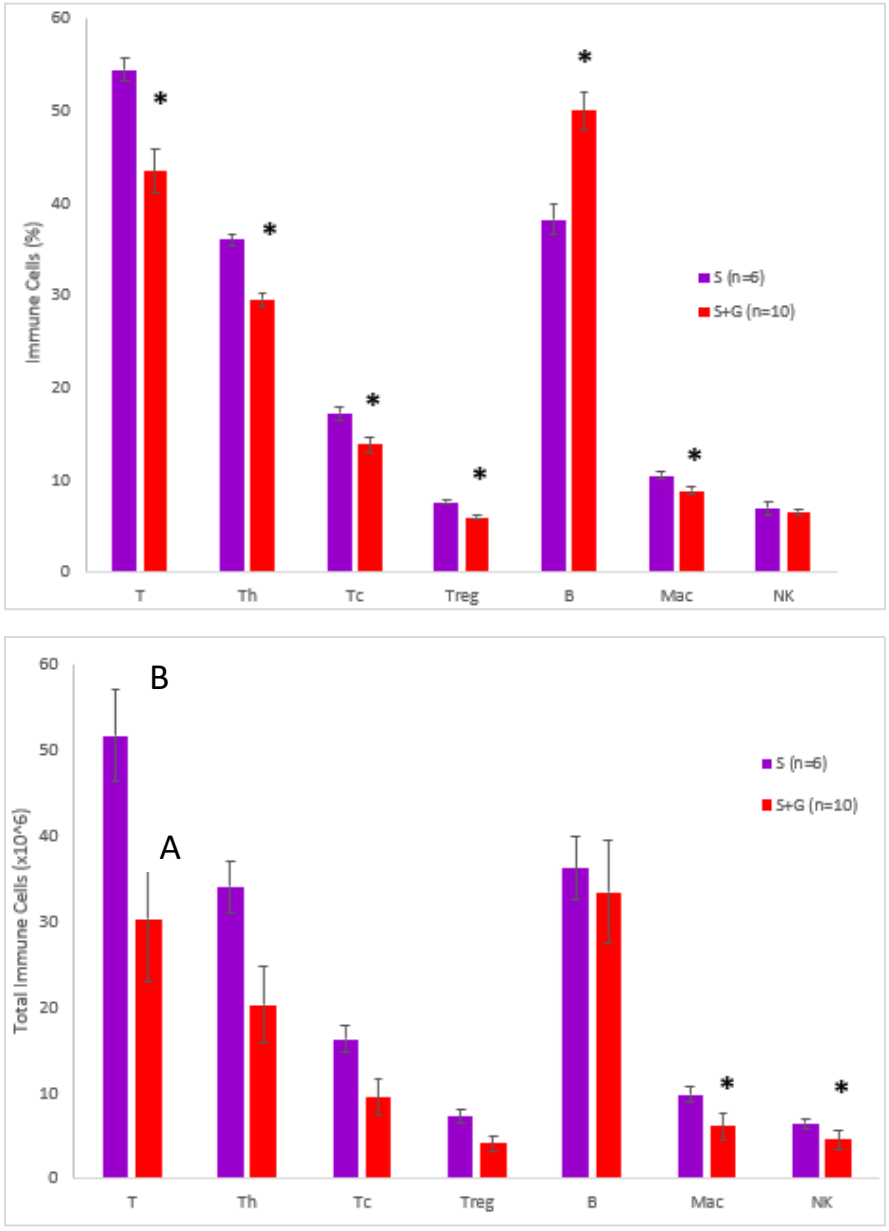


Figure 8. Immunophenotyping of splenocytes obtained on day 11 from MLDSTZ C57BL/6J mice treated with aqueous GKE. Immunophenotypes presented as percentages (A) and total cell counts (B). Data presented as average ± SEM, *p<0.05 compared to own, respective control group of mice for each particular cell type. The treatment groups' legend described in Figure 3.

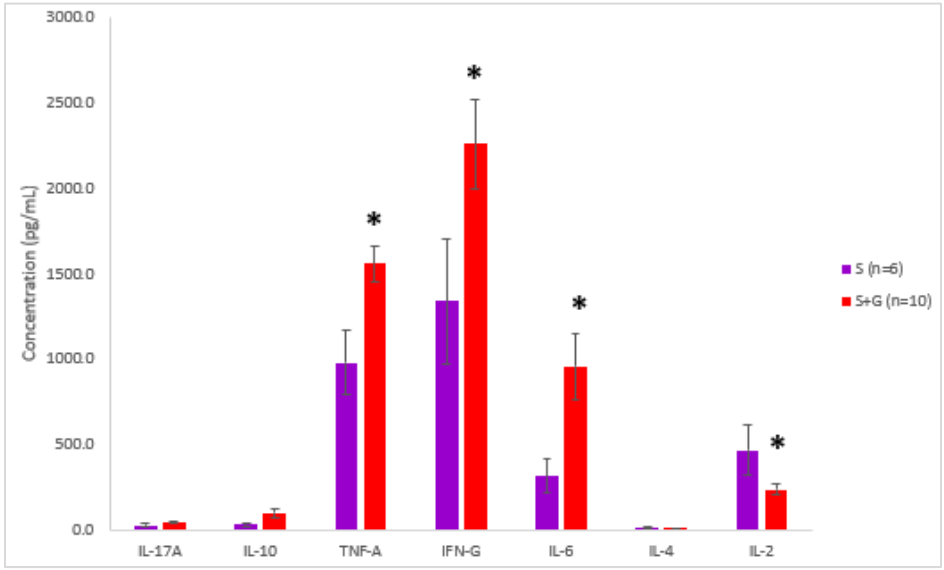


Figure 9. Cytokine profiles of splenocytes, obtained on day 11, from MLDSTZ C57BL/6J mice treated with aqueous GKE. Data presented as average ± SEM, *p<0.05 compared to own control group of mice. The treatment groups' legend described in Figure 3

The following data were obtained in MLDSTZ mice, treated with aqueous GKE and controls, on day 30. There were no significant effects on cell counts, nor cell viability in aqueous GKE-treated mice compared to their controls (Figures 10A and 10B). There was a trend of decrease in T cell proliferation of GKE-treated mice compared to controls (Figure 11). Immunophenotyping of MLDSTZ mice treated with GKE, presented as percentages, showed significant decreases in T, Th, Tc, and NK cells, respectively (p= 0.004, p= 0.02, p= 0.02, and p= 0.0007, respectively), and a significant increase in B cells (p= 0.003), compared to controls (Figure 12A). In terms of total cell counts, immunophenotyping showed trends of decrease in T cells and respective subtypes, and a trend of increase in B cells, in mice treated with GKE (Figure 12B). The cytokine profiles, obtained from GKE-treated mice, revealed a significant increase (p= 0.03) in IL-10, a trend in elevated levels of TNFα and IL-6, and a non-significant reduction of IL-2, compared to controls (Figure 13).

Next, we asked whether GKE treatment per se would induce the effects on immune parameters observed in MLDSTZ model. Thus, non-diabetic, STZ-free C57BL/6 mice, were treated with aqueous GKE from seven- to 13 weeks of age (end point was equivalent to day 30 endpoint post first STZ injection in MLDSTZ mice). GKE treatment did not change the splenic cell counts or cell viability (Figures 10A and 10B). There was a significant decrease ($p= 0.025$) in T cell proliferation observed in STZ-free mice treated with GKE compared to their controls (Figure 11). Immunophenotyping of spleens obtained from mice treated with GKE, presented as percentages, showed significant decreases in T, Th, Tc, and MAC cells ($p= 0.01$, $p= 0.003$, $p= 0.03$, and $p=0.02$, respectively); while B cells were significantly increased ($p= 0.01$), compared to controls (Figure 12A). There were no significant effects of GKE observed on immunophenotypes in terms of total cell counts, but there were trends of decrease observed among T cells and their subtypes, and a trend of increase in B cells (Figure 12B). The cytokine measurements showed significant increases in IL-10, and IFNγ ($p= 0.001$ and $p= 0.001$), and a significant decrease in IL-2 ($p= 0.01$). Though not statistically significant, there was an increase of TNFα and IL-6 (Figure 13).

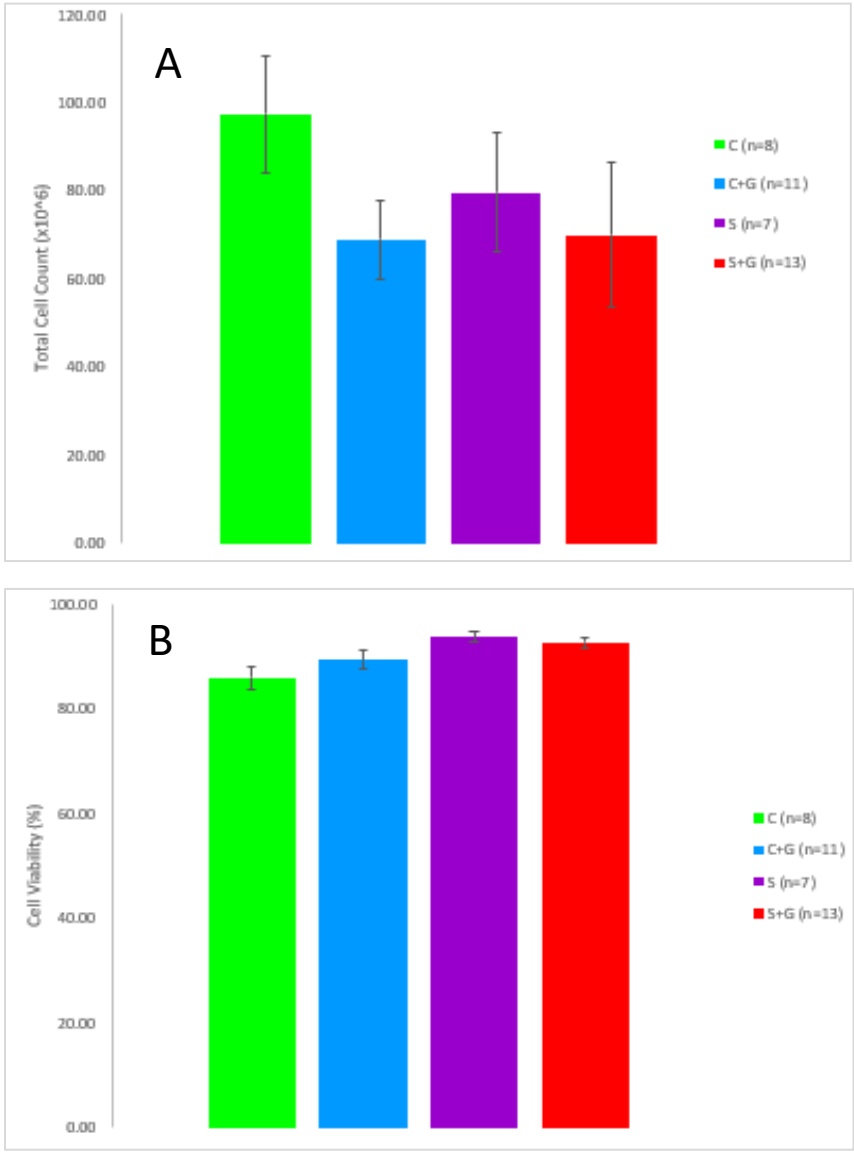


Figure 10. Total splenocyte counts (A) and cell viability (B) obtained on day 30 from MLDSTZ and STZ-free C57BL/6J mice treated with aqueous GKE from seven to 13 weeks of age. (A) Cell counts and viability were measured using the Trypan blue exclusion method. Data presented as average ± SEM. (B) Cell viability was calculated by dividing alive cells by total cells x 100. Data presented as average ± SEM. The treatment groups' legend described in Figure 3.

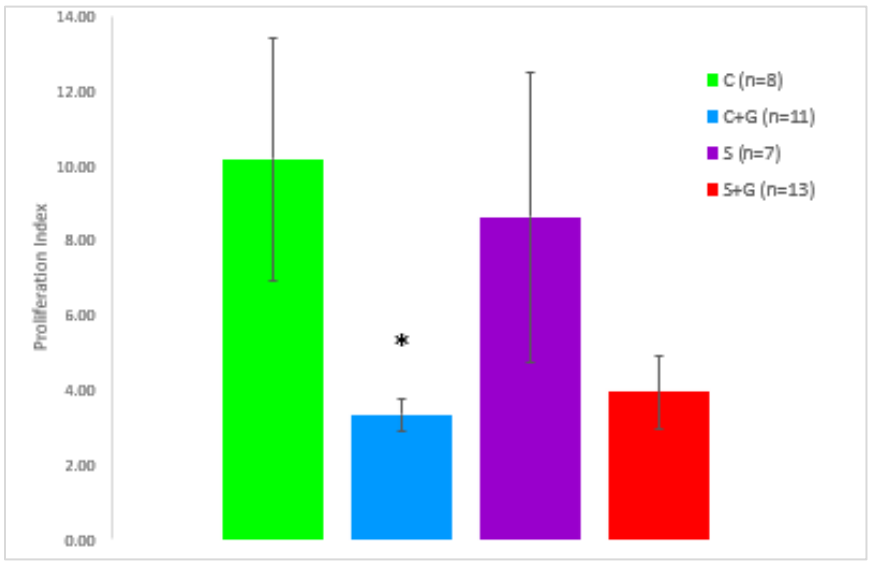


Figure 11. T cell proliferative index obtained on day 30 from the spleens of MLDSTZ and STZ-free C57BL/6J mice treated with aqueous GKE from seven to 13 weeks of age. T cell proliferation was induced by Con A stimulation during a 72-hour culture period and optical density was measured by an Alamar Blue assay. Proliferation index was obtained by dividing optical densities measured in ConA-stimulated by non-stimulated cells. Data presented as average ± SEM,*$p<0.05$ compared to control mice. The treatment groups' legend described in Figure 3.

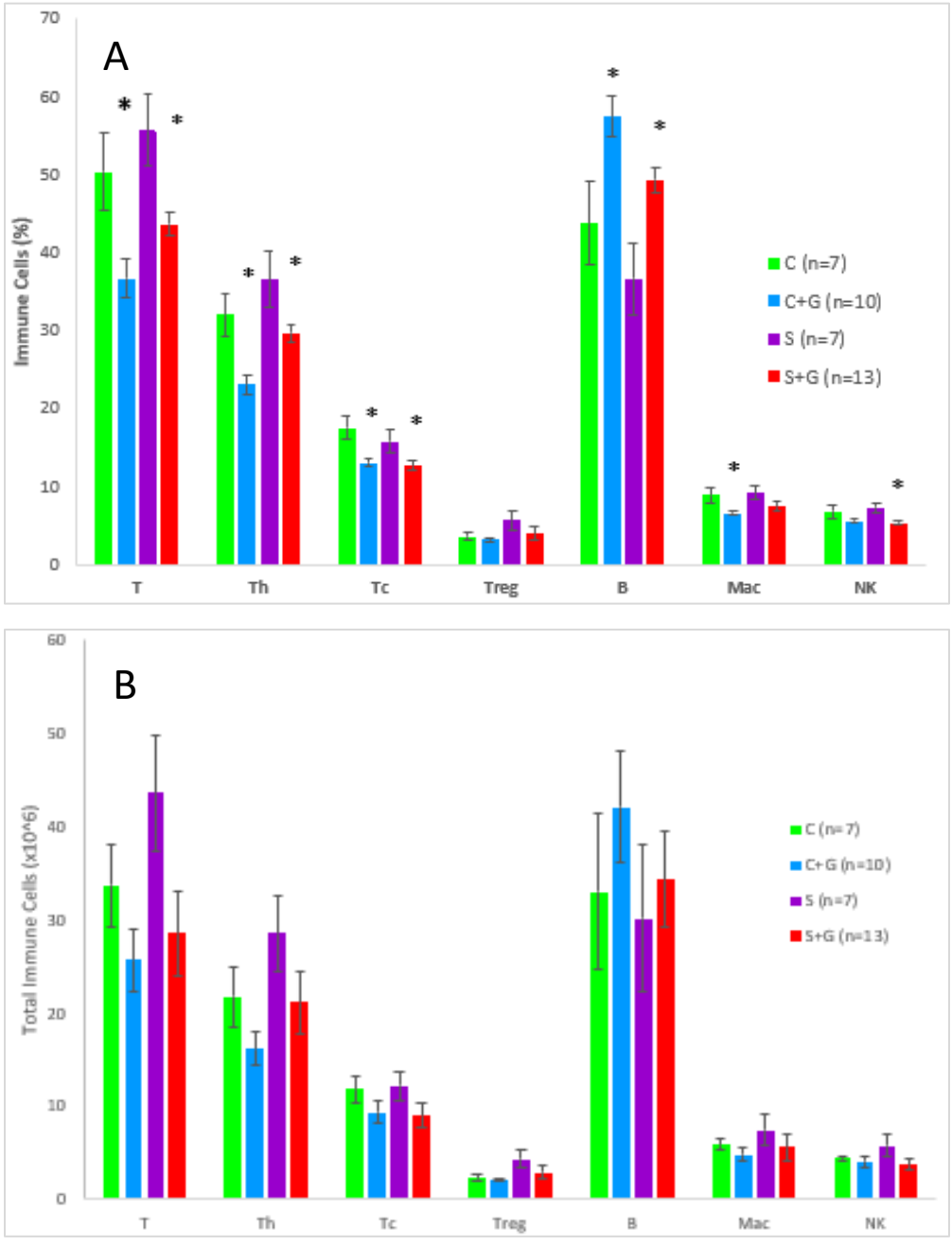


Figure 12. Immunophenotyping of splenocytes obtained on day 30 from MLDSTZ and STZ-free C57BL/6J mice treated with aqueous GKE from seven to 13 weeks of age. Immunophenotypes presented as percentages (A) and total cell counts (B). Data presented as average ± SEM, *$p<0.05$ compared to own, respective control group of mice for each particular cell type. The treatment groups' legend described in Figure 3.

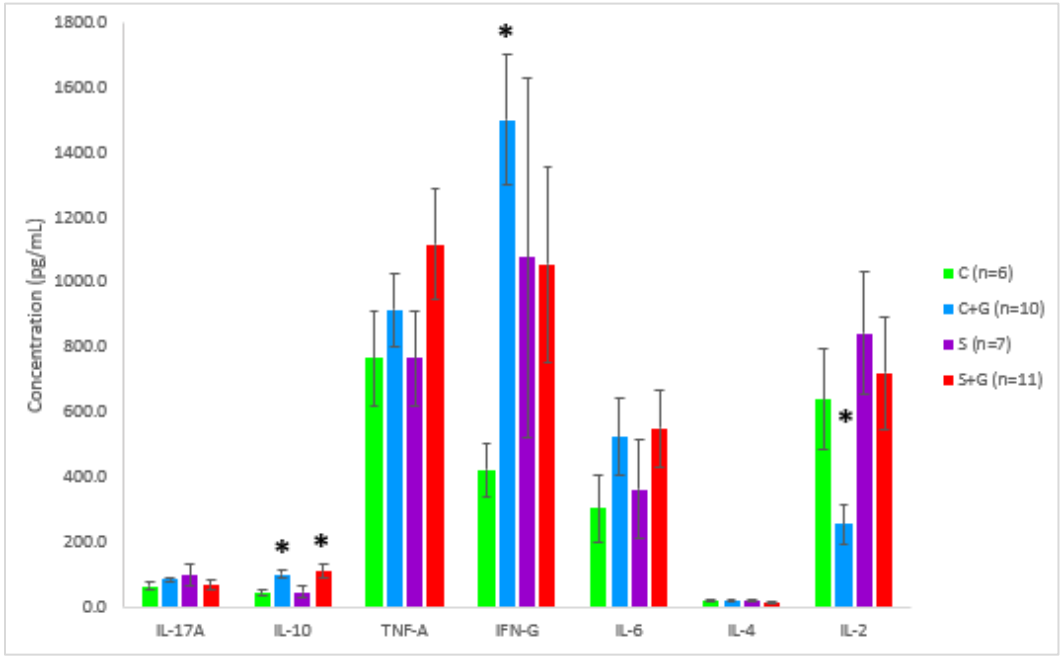


Figure 13. Cytokine profiles of splenocytes obtained on day 30 from MLDSTZ and STZ-free C57BL/6J mice treated with aqueous GKE from seven to 13 weeks of age. Data presented as average ± SEM, *p<0.05 compared to own control group of mice. The treatment groups' legend described in Figure 3.

In Vitro Effects of Ethanol GKE on T Cell Proliferation

Since aqueous GKE did not affect T cell proliferation *in vitro*, while influencing cytokine secretion, we wanted to study whether ethanol GKE would exhibit similar or different effects on T cells cultures. C57BL/6J splenocytes were stimulated with Con A (3 ug/mL) and GKE was added ranging in concentrations from 2 ug/mL to 1 mg/mL. As ethanol GKE stock was prepared in DMSO, and higher concentrations of DMSO are known to induce suppression of T cell proliferation (Cetkovic-Cvrlje, Olson, Schindler, & Gong, 2015), selected control cells, that did not receive GKE, were cultured with an equivalent amount of DMSO present in the higher GKE concentrations of 250 ug/mL, 500 ug/mL, and 1 mg/mL. Proliferation of splenocytes cultured with GKE at 250 ug/mL was significantly lower compared to its control DMSO equivalent. In contrast, T cell proliferation was significantly increased in splenocytes treated with GKE at 1

mg/mL compared to the DMSO equivalent of 1 mg/mL, whereas 500 ug/mL GKE did not affect T cell proliferation at all (Figure 14). Lower concentrations of GKE had no effect on T cell function.

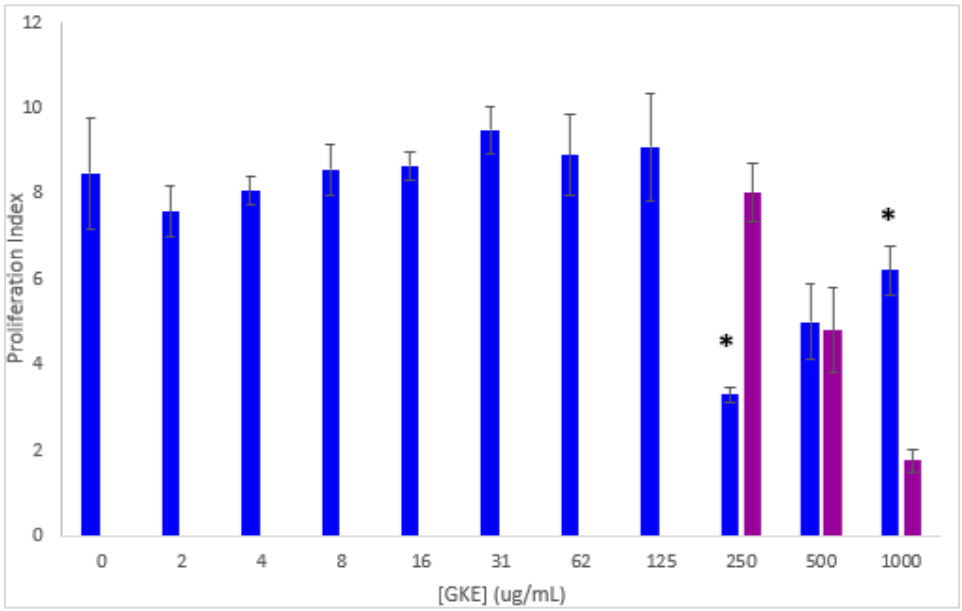


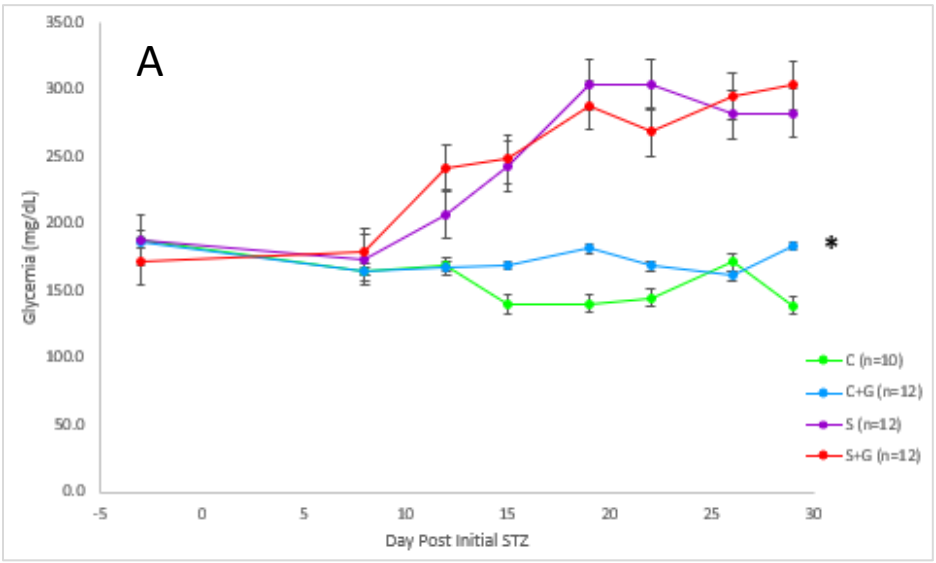
Figure 14. *In vitro* proliferation indices of stimulated splenocytes from C57BL/6J mice cultured with a serial dilution of GKE (blue bars). Select stimulated splenocytes received concentrations of DMSO that were equivalent to the DMSO content in the three highest GKE concentrations (purple bars). Data presented as average ± SEM, n=3, * $p < 0.05$. indicates significant difference compared to respective DMSO control.

Effects of *In Vivo* Ethanol GKE Treatment on Glycemia and Diabetes Incidence in MLDSTZ Model for T1D

A new set of experiments were performed in order to study effects of ethanol GKE on the diabetes development and glycemia. Seven-week-old male C57BL/6J mice were treated with ethanol GKE (100 mg/kg) in their drinking water until 13 weeks of age. Since GKE stock was prepared in DMSO, control mice received water enriched with 1.8% DMSO, the same DMSO concentration present in the GKE treatment water. At eight weeks of age, mice were intraperitoneally injected with multiple, low-doses (40 mg/kg) of streptozotocin (MLDSTZ) for five days. Body weight and glucose levels were measured the third day before STZ injections

and continued biweekly eight days after injections, until the experimental endpoint of 30 days after the first STZ injection (13 weeks of age). Glycemia and body weight measurements were also done in mice that were treated with ethanol GKE but did not receive STZ injections (STZ-free mice). GKE had no effect on glycemia levels nor body weight in MLDSTZ mice compared to their controls (Figure 15A & 15B). Treatment with ethanol GKE did not delay disease occurrence nor reduce incidence of disease. Diabetes occurred on an average of 19.8 ± 1.5 days for GKE treated mice and 19.4 ± 1.9 days for control mice. By the experimental endpoint, 92% of ethanol GKE-treated mice were diabetic and 83% of control mice were diabetic (Figure 15C).

Interestingly, STZ-free, non-diabetic mice treated with ethanol GKE had significantly increased glycemia levels compared to their controls ($p = 0.0017$), whereas GKE had no effect on body weight in these mice (Figure 15A and 15B).



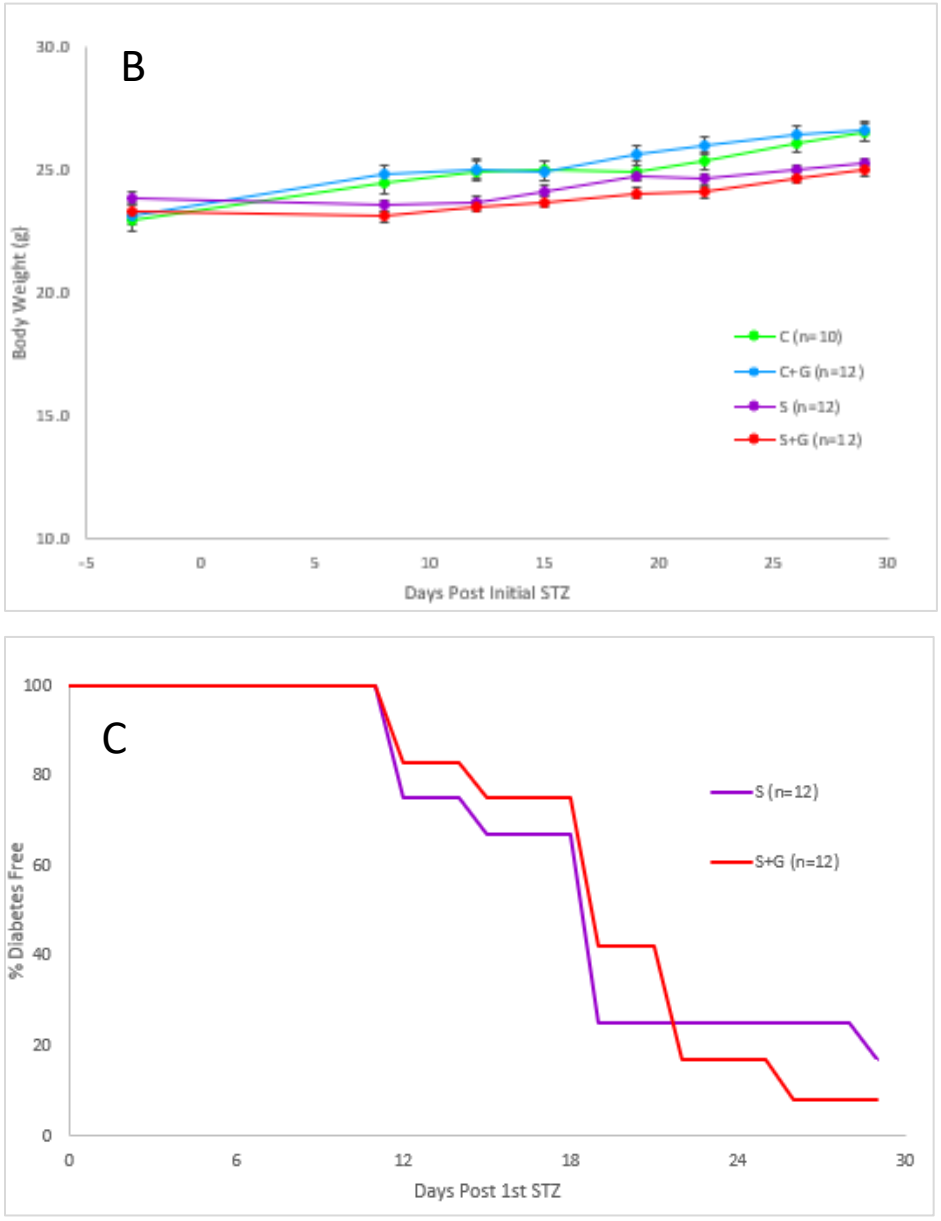


Figure 15. Average glycemia (A), average body weight (B), and diabetes incidence (C) of male MLDSTZ C57BL/6J mice and non-injected mice treated with ethanol GKE for six weeks. (A) Glycemia measurements were done beginning on the eighth day post the initial STZ injection up until 30 days after the initial STZ injection. Diabetes was defined as having two consecutive measurements of 250 mg/dL or greater. Data presented as average ± SEM, *$p<0.05$ compared to control mice. (B) Body weights measurements started three days before the initial STZ injection and lasted until the experimental endpoint. Data presented as average ± SEM. (C) Diabetes incidence presented as the percentage of diabetes-free mice. Diabetes was defined as having two consecutive measurements of glycemia of 250 mg/dL or greater. C and C+G groups were not graphed, as they stayed 100% diabetes-free throughout the experiment; The treatment groups' legend described in Figure 3, with a notion that G here represents ethanol GKE treatment.

Effects of Ethanol GKE on Immune Parameters During T1D Development

Mice were sacrificed at two time points – on days 11 and 30 post the initial STZ injection to evaluate ethanol GKE's effects on immune parameters such as: cell counts and viability, T cell proliferation (presented as proliferation index), T cell populations, and cytokine profiles. The following data are obtained from mice treated with ethanol GKE and controls on day 11. There were no significant effects on cell counts, cell viability (Figure 16A and 16B), or T cell proliferation (Figure 17) in mice treated with ethanol GKE compared to their controls. Immunophenotyping, presented as a percentage for a particular cell type, showed significant decreases in T, Th, Tc, and NK cells ($p= 0.006$, $p= 0.03$, $p= 0.04$, and $p= 0.01$, respectively), and a significant increase in B cells ($p= 0.001$) in GKE treated mice compared to their controls (Figure 18A). Immunophenotyping, presented in terms of total cell counts, exhibited significant decreases in T, Th, and NK cells ($p= 0.01$, $p= 0.01$, and $p= 0.04$ respectively), and trends of decrease in Tc, Tr, and MAC cells (Figure 18B) in mice treated with GKE compared to control mice. There were significant increases in IL-17A, IL-10, TNFα, and IL-6 ($p= 0.01$, $p= 0.001$, $p= 0.01$ and $p= 0.01$, respectively), and a significant decrease in IL-2 secretion ($p= 0.005$), obtained from the spleens of ethanol GKE-treated mice compared to controls; there was also a trend of increase in IFNγ in GKE-treated group of mice (Figure 19).

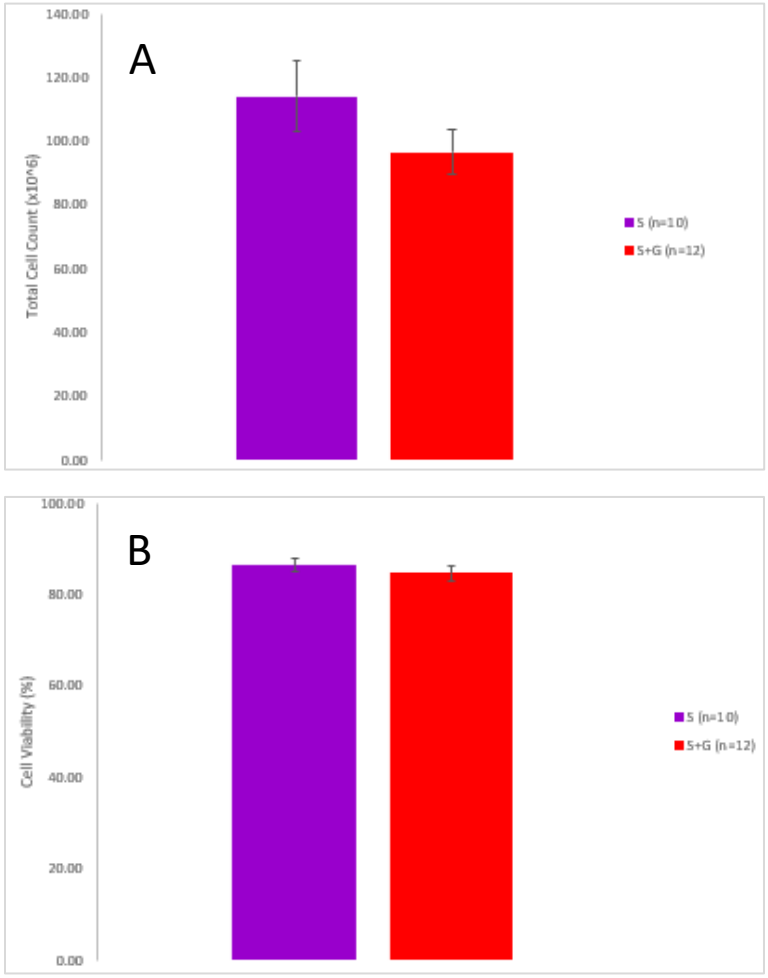


Figure 16. Total splenocyte counts (A) and cell viability (B) obtained on day 11 from MLDSTZ C57BL/6J mice treated with ethanol GKE. (A) Cell counts, and viability were measured using the Trypan blue exclusion method. Data presented as average ± SEM. (B) Cell viability was calculated by dividing alive cells by total cells x 100. Data presented as average ± SEM. The treatment groups' legend described in Figure 14.

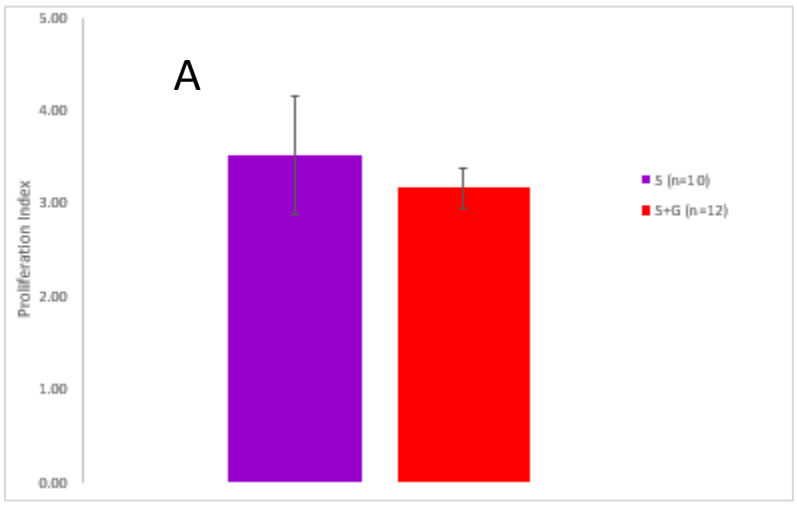
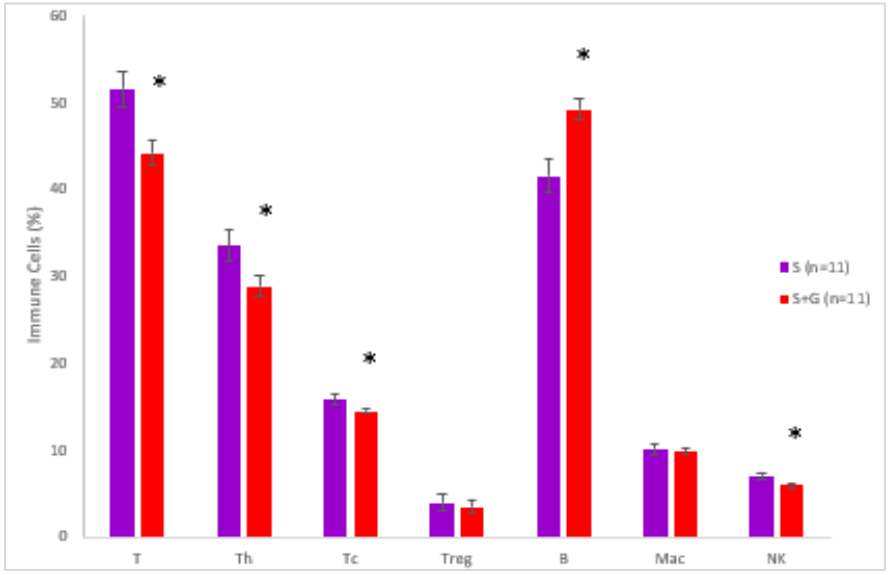


Figure 17. T cell proliferative index obtained on day 30 from the spleens of MLDSTZ C57BL/6J mice treated with ethanol GKE. T cell proliferation was induced by Con A stimulation during a 72-hour culture period and optical density was measured by an Alamar Blue assay. Proliferation index was obtained by dividing optical densities measured in ConA-stimulated by non-stimulated cells. Data presented as average ± SEM. The treatment groups' legend described in Figure 14.



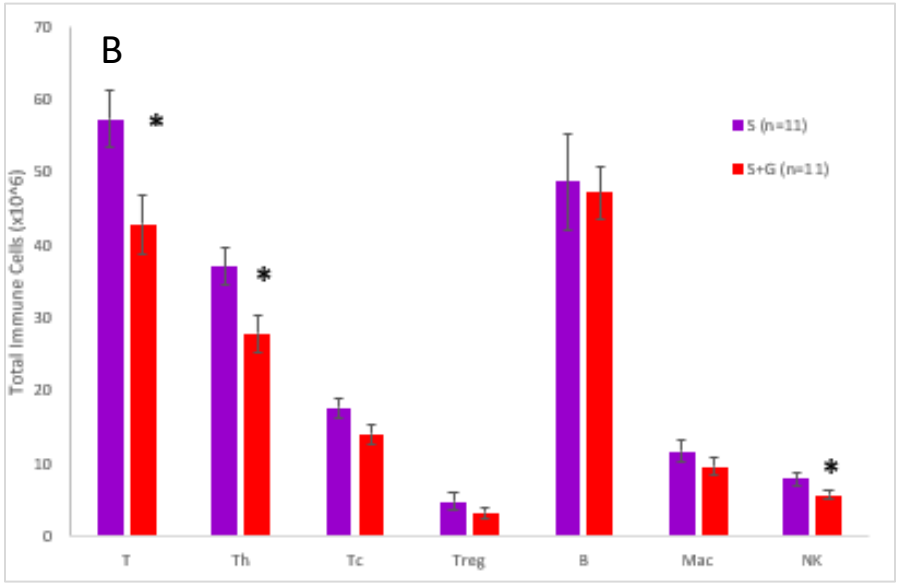


Figure 18. Immunophenotyping of splenocytes obtained on day 11 from MLDSTZ C57BL/6J mice treated with ethanol GKE. Immunophenotypes presented as percentages (A) and total cell counts (B). Data presented as average ± SEM, *p<0.05 compared to own, respective control group of mice for each particular cell type. The treatment groups' legend described in Figure 14.

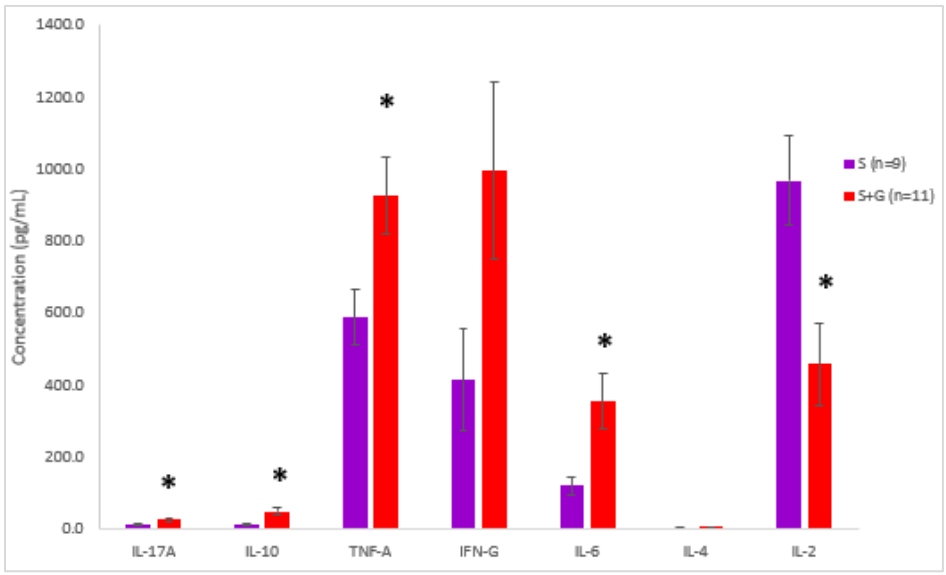


Figure 19. Cytokine profiles of splenocytes obtained on day 11 from MLDSTZ C57BL/6J mice treated with ethanol GKE. Data presented as average ± SEM, *p< 0.05 compared to control mice. The treatment groups' legend described in Figure 14.

The following data are from MLDSTZ mice treated with ethanol GKE and their controls on day 30. There were no significant effects observed on cell counts, cell viability, or T cell proliferation in mice treated with ethanol GKE compared to their controls (Figures 20A, 20B, and 21). Immunophenotyping, presented as a percentage, showed significant decreases in T, Th, Treg, B, and MAC cells ($p= 0.01$, $p=0.01$, $p= 0.02$, $p=0.008$, and $p= 0.03$, respectively) in mice treated with GKE compared to their controls (Figure 22A). There was also observed a trend of reduction in Tc in MLDSTZ mice treated with ethanol GKE compared to controls. Immunophenotyping in terms of total cell counts, showed significant decreases in T, Th, Tc, and Treg cells ($p= 0.008$, $p= 0.01$, $p= 0.02$, and $p= 0.01$, respectively) in GKE-treated mice compared to their controls (Figure 22B). There were trends of increase in TNFα, IFNγ, and IL-6, and a trend of reduction in IL-2 in ethanol GKE-treated MLDSTZ mice compared to controls (Figure 23).

The following data were obtained from STZ-free mice treated with ethanol GKE from seven to 13 weeks of age (equivalent to the day 30 timepoint). GKE significantly increased splenic cell counts ($p= 0.018$), while having no effect on cell viability, compared to control mice (Figure 20A and 20B). There was a trend of decrease in T cell proliferation in ethanol GKE-treated mice compared to controls (Figure 21). Immunophenotyping, presented as a percentage, showed significant decreases in all T and Tc cells ($p= 0.01$ and $p= 0.02$), and an increase in B cells ($p= 0.004$) in GKE-treated mice compared to their controls (Figure 22A). There was also a trend of decrease in NK and MAC cells in mice treated with GKE compared to controls. Immunophenotyping in terms of total cell counts showed a significant increase in B cells ($p= 0.002$), and trends of increase in T, Th, and Tc cells in ethanol GKE-treated mice compared to

their controls (Figure 22B). There were trends of increase in IL-17A, IL-10, TNFα, IFNγ, and IL-6, and a trend of reduction in IL-2 in STZ-free mice treated with ethanol GKE compared to controls (Figure 23).

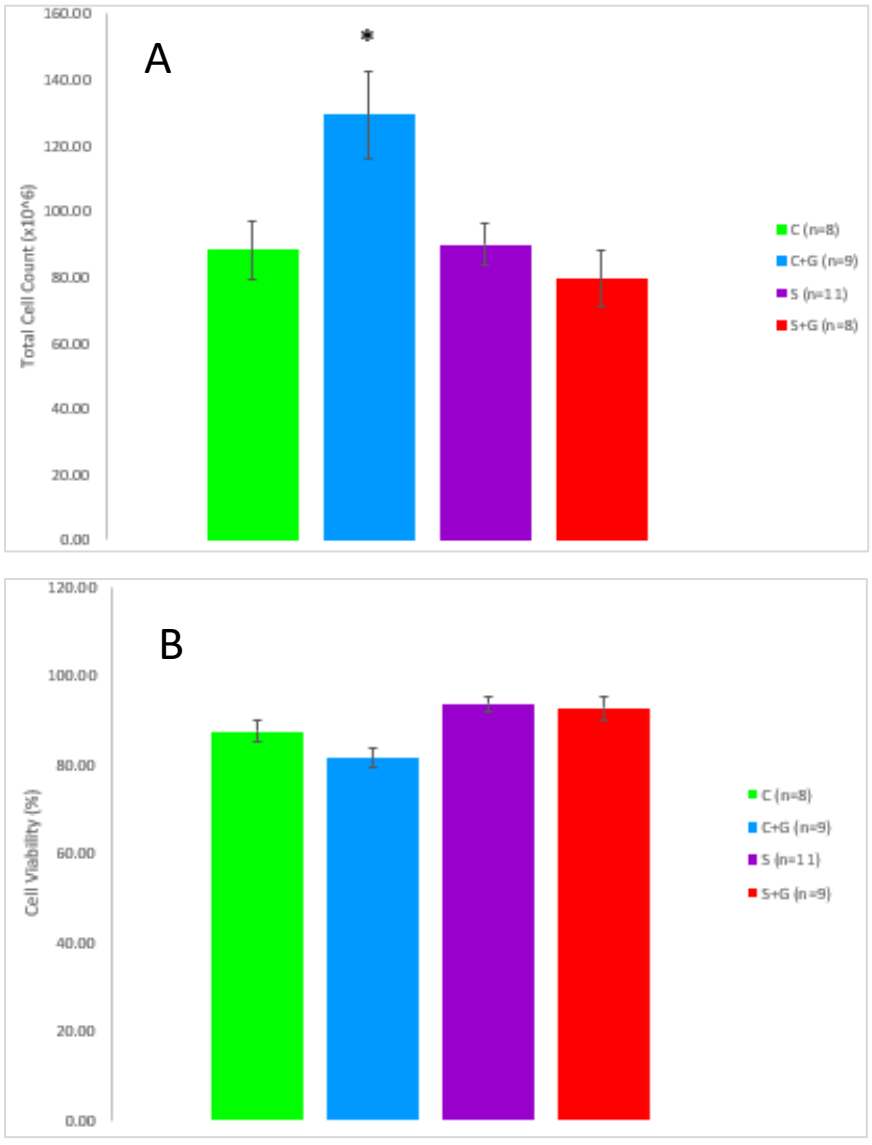


Figure 20. Total splenocyte counts (A) and cell viability (B) obtained on day 30 from MLDSTZ and STZ-free C57BL/6J mice treated with ethanol GKE from seven to 13 weeks of age. (A) Cell counts, and viability were measured using the Trypan blue exclusion method. Data presented as average ± SEM, *$p<0.05$ compared to control mice. (B) Cell viability was calculated by dividing alive cells by total cells x 100. Data presented as average ± SEM. The treatment groups' legend described in Figure 14.

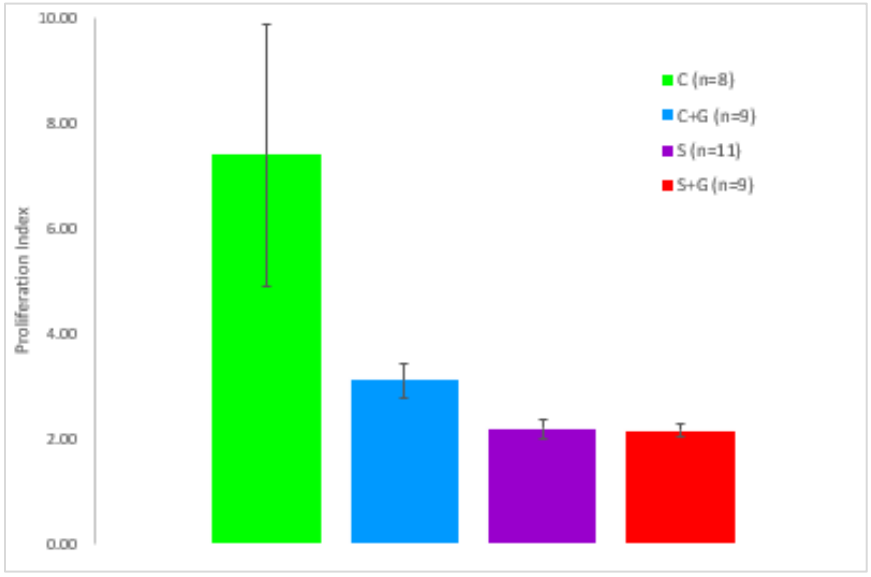
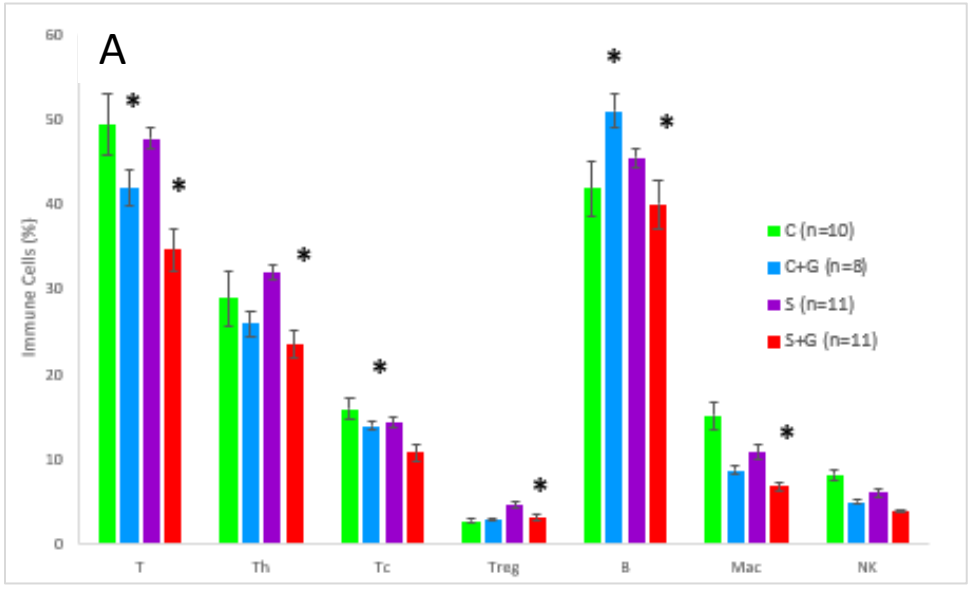


Figure 21. T cell proliferative index obtained on day 30 from the spleens of MLDSTZ and STZ-free C57BL/6J mice treated with ethanol GKE from seven to 13 weeks of age. T cell proliferation was induced by Con A stimulation during a 72-hour culture period and optical density was measured by an Alamar Blue assay. Proliferation index was obtained by dividing optical densities measured in ConA-stimulated by non-stimulated cells. Data presented as average ± SEM. The treatment groups' legend described in Figure 14.



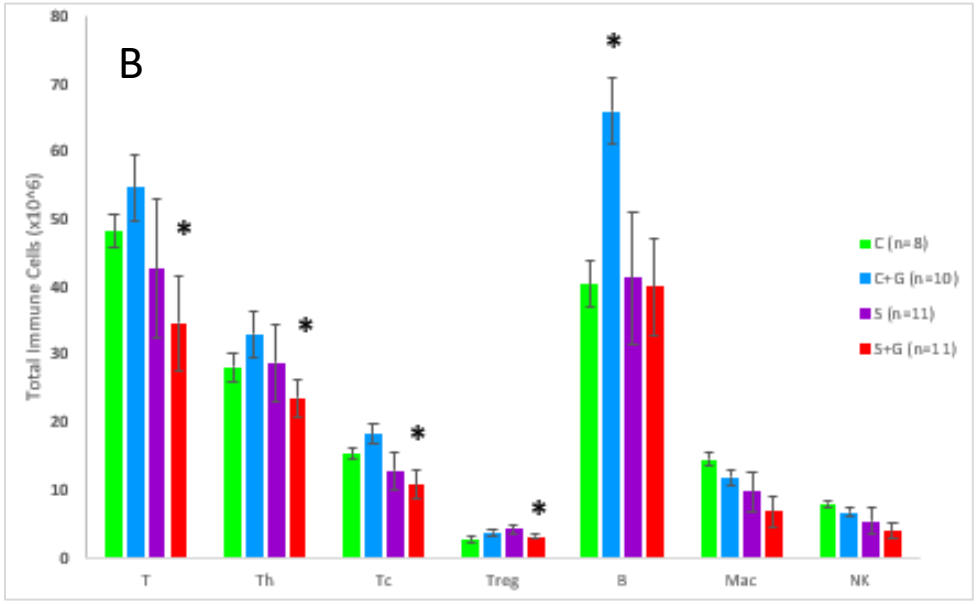


Figure 22. Immunophenotyping of splenocytes obtained on day 30 from MLDSTZ and STZ-free C57BL/6J mice treated with ethanol GKE from seven to 13 weeks of age. Immunophenotypes presented as percentages (A) and total cell counts (B). Data presented as average ± SEM, *p<0.05 compared to own, respective control group of mice for each particular cell type. The treatment groups' legend described in Figure 14.

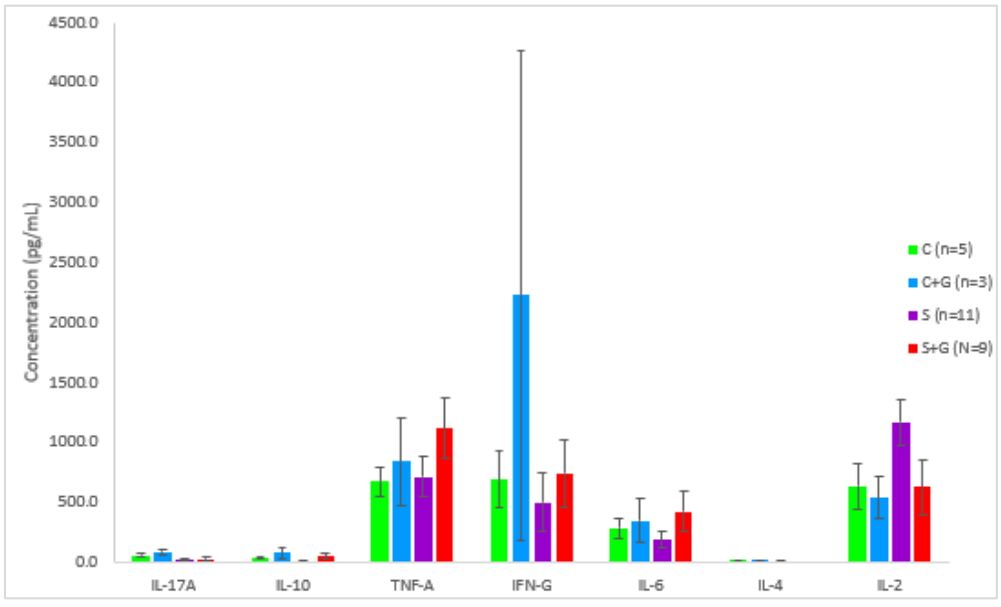
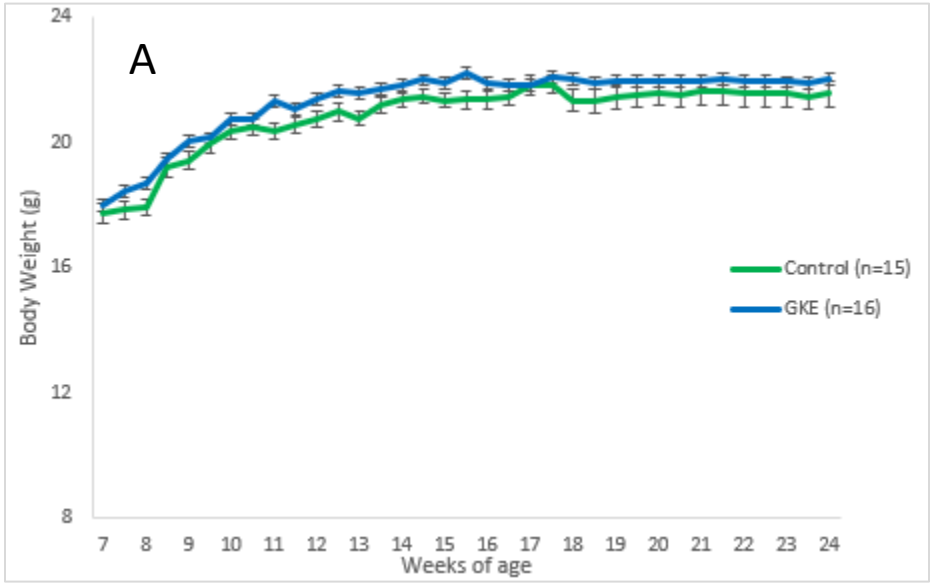
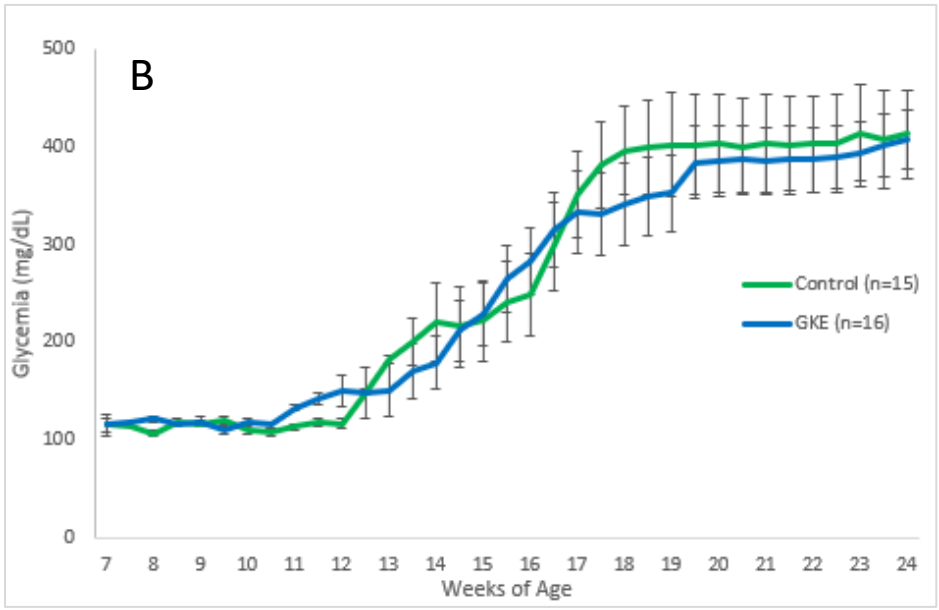


Figure 23. Cytokine profiles of splenocytes obtained on day 30 from MLDSTZ and STZ-free C57BL/6J mice treated with ethanol GKE from seven to 13 weeks of age. Data presented as average ± SEM. The treatment groups' legend described in Figure 14.

Effects of *In Vivo* Ethanol GKE Treatment on Glycemia and Diabetes Incidence in NOD Mouse Model

Since hypoglycemic effect, as well as a reduction of disease incidence have not been observed in chemically-induced MLDSTZ model of T1D, we questioned whether GKE treatment would exhibit an effect on these parameters in NOD/LtJ females, a mouse model that spontaneously develops T1D. Four-week-old female NOD/LtJ mice were treated with ethanol GKE (100 mg/kg) in their drinking water until the experimental endpoint of 24 weeks of age. Control mice received water enriched with 1.8% DMSO to have the same DMSO concentration in their drinking water as the GKE treatment group. Whereas no difference in efficacy in reduction of hyperglycemia and diabetes incidence was observed between the aqueous and ethanol GKE treatments, the ethanol GKE extract was chosen, as potentially more potent extract, for studying antidiabetic GKE effects in NOD mice. The initial body weight and glycemia measurements were done at four weeks of age. Biweekly body weight and glycemia measurements started at seven and lasted until 24 weeks of age. Diabetes was defined as having two consecutive measurements of 250 mg/dL or greater; mice were euthanized as they became diabetic. Treatment with ethanol GKE had no effect on body weight or glycemia levels (Figures 24A and 24B). In addition, GKE had no effect on disease incidence nor disease prevalence (Figure 24C). Disease developed on an average of 16.6 ± 0.9 weeks in GKE-treated mice and 15.3 ± 0.5 weeks for control mice. By the experimental endpoint of 24 weeks of age, 94% of mice treated with GKE were diabetic in comparison to 73% controls.


A
Body Weight (g)
24
20
16
12
8
7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Weeks of age
Control (n=15)
GKE (n=16)


B
Glycemia (mg/dL)
500
400
300
200
100
0
7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Weeks of Age
Control (n=15)
GKE (n=16)

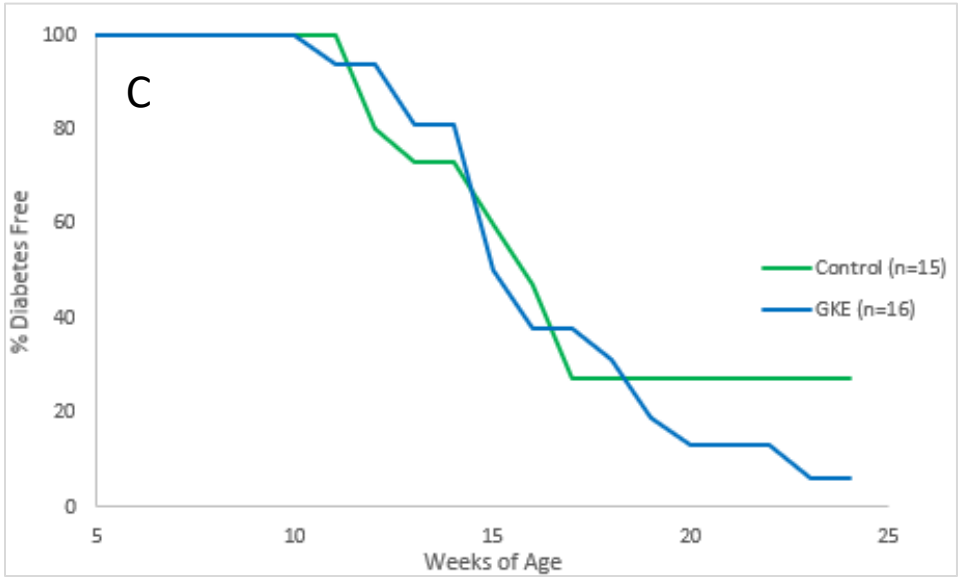
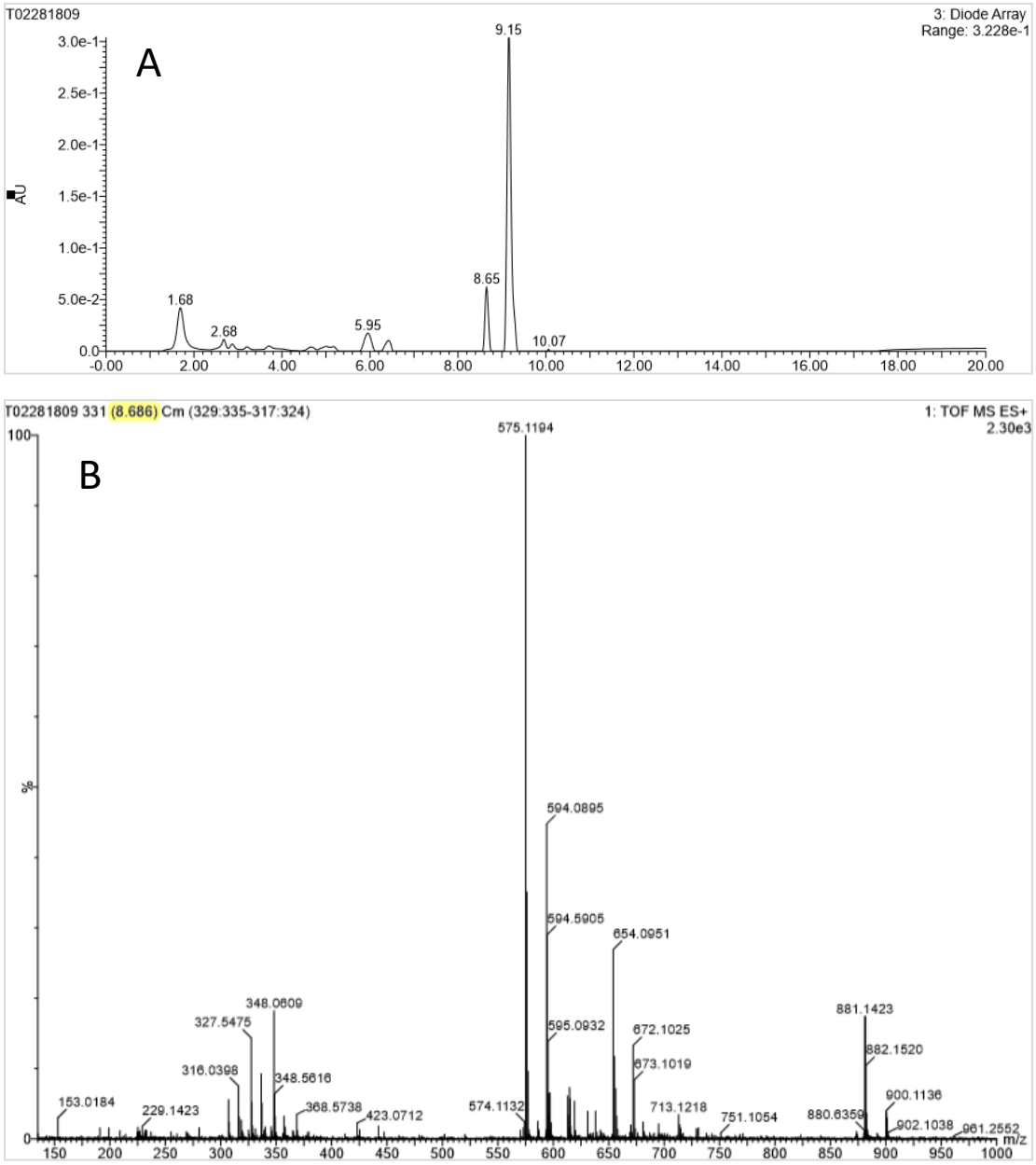


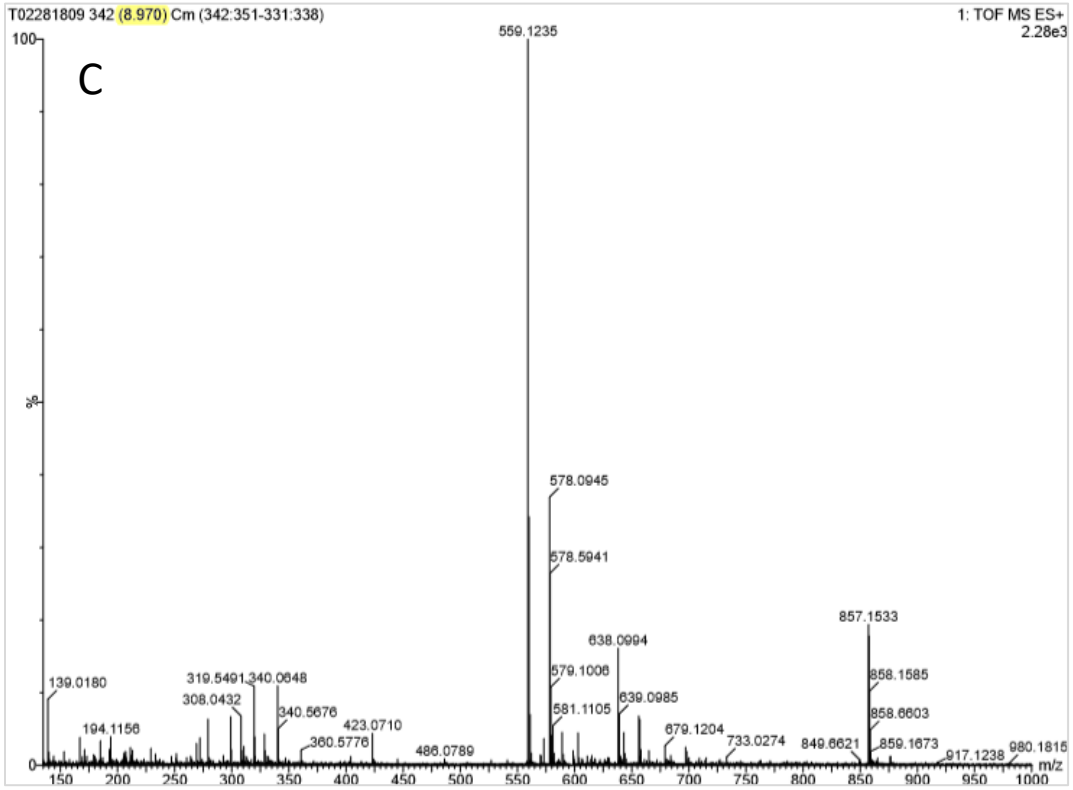
Figure 24. Average body weight (A), average glycemia (B), and diabetes incidence (C) of female NOD/LtJ mice treated with ethanol GKE for five months. (A) Body weight measurements continued biweekly from 7 weeks of age until the experimental endpoint at 24 weeks of age. Data presented as average ± SEM. (B) Glycemia measurements continued biweekly from 7 weeks of age until the experimental endpoint at 24 weeks of age. Data presented as average ± SEM. (C) Diabetes incidence presented as the percentage of diabetes free mice. Diabetes was defined as two consecutive glycemia measurements of 250 mg/dL or greater.

LC-MS Analysis of GKE

As the performed extraction method was not the primary method done in previous studies (Adaramoye, 2012; Ayepola et al., 2013; Iwu et al., 1990; Tchieme et al., 2016), samples of aqueous and ethanol GKE were sent to the University of Iowa for LC-MS analysis to confirm whether Kolaviron, the active biflavonoid complex, was present in our extracts. In addition, we wanted to know whether there would be differences between aqueous and ethanol GKE samples. The following compounds present in aqueous GKE were confirmed to be *Garcinia* biflavonoid 2 (Figure 25B), *Garcinia* biflavonoid 1 (Figure 25C), kolaflavonone (Figure 25D), and binarigenin (Figure 25E). The same four compounds were also present in ethanol GKE (Figure 26). Thus, within both GKE extract types *Garcinia* biflavonoid 1, *Garcinia* biflavonoid 2, kolaflavonone,

and binarigenin were present. The fifth flavonoid, previously found in Kolaviron (Ayepola et al., 2013), kolaflavone, was not present in either aqueous or ethanol GKE.

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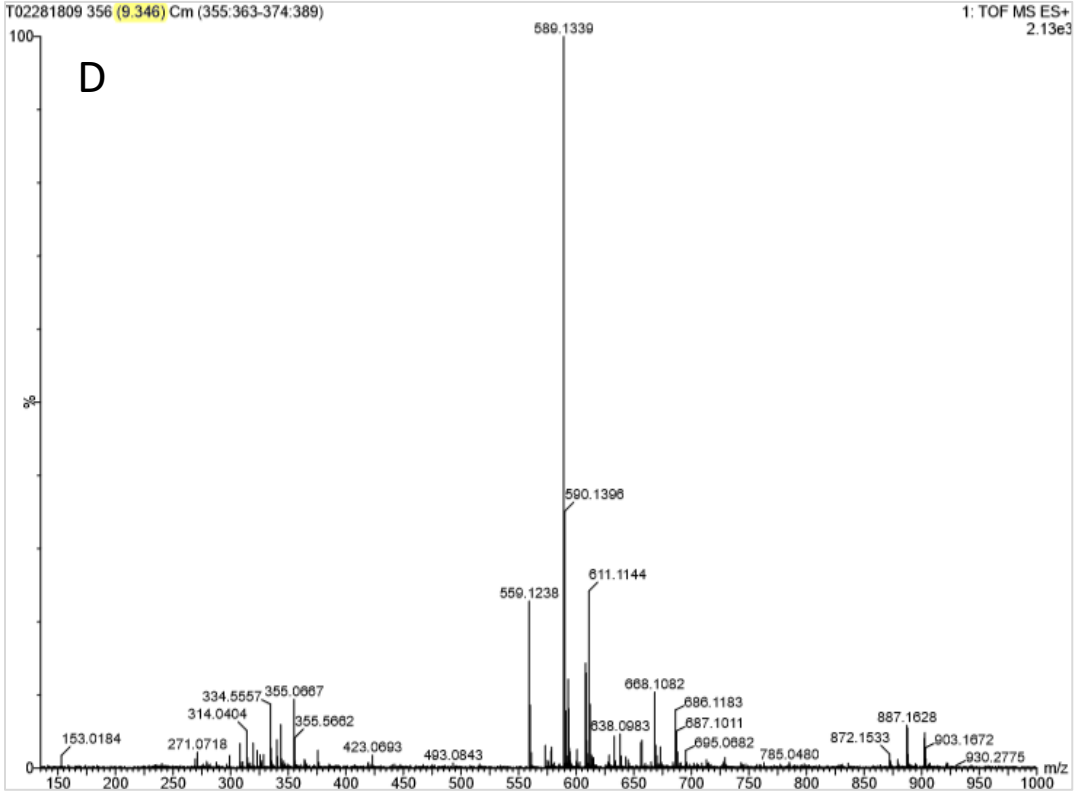
1: TOF MS ES+
2.28e3
C
559.1235
578.0945
578.5941
857.1533
638.0994
579.1006
858.1585
639.0985
581.1105
858.6603
679.1204
733.0274
849.6621
859.1673
917.1238
980.1815
139.0180
319.5491
340.0648
308.0432
340.5676
194.1156
423.0710
360.5776
486.0789
%
m/z


T02281809 356 (9.346) Cm (355:363-374:389)
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2.13e3
D
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590.1396
611.1144
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668.1082
686.1183
687.1011
695.0682
638.0983
887.1628
872.1533
903.1672
930.2775
785.0480
334.5557
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314.0404
355.5662
153.0184
271.0718
423.0693
493.0843
%
m/z

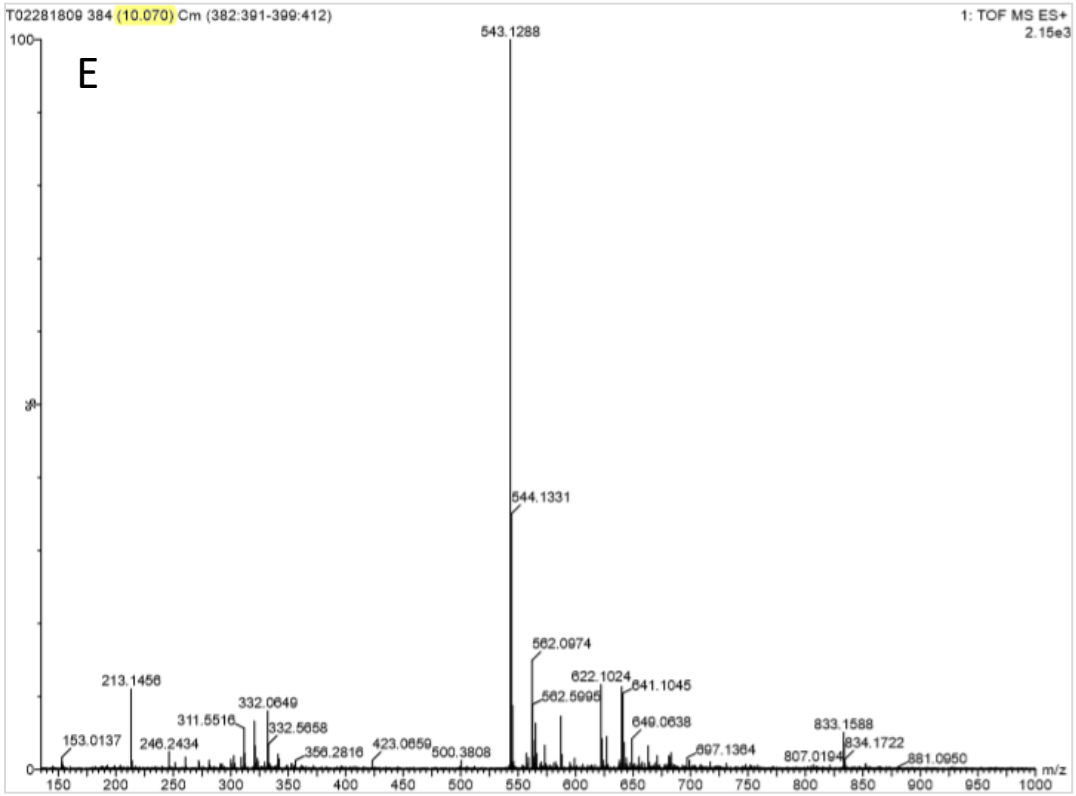
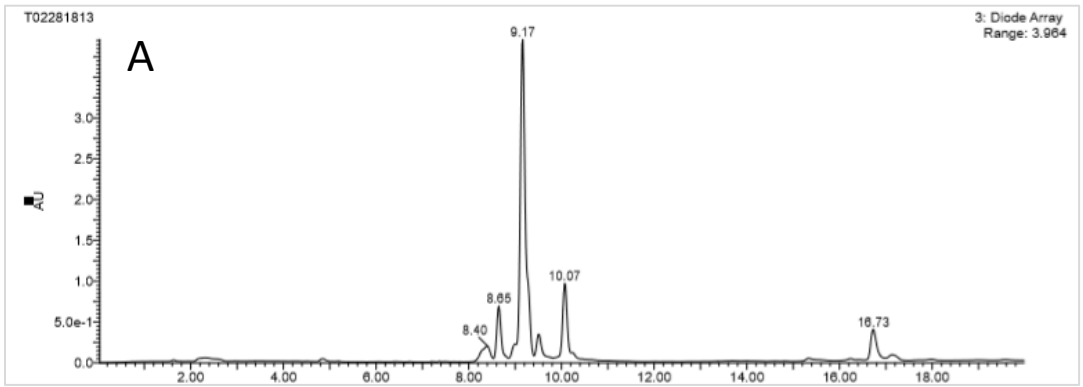
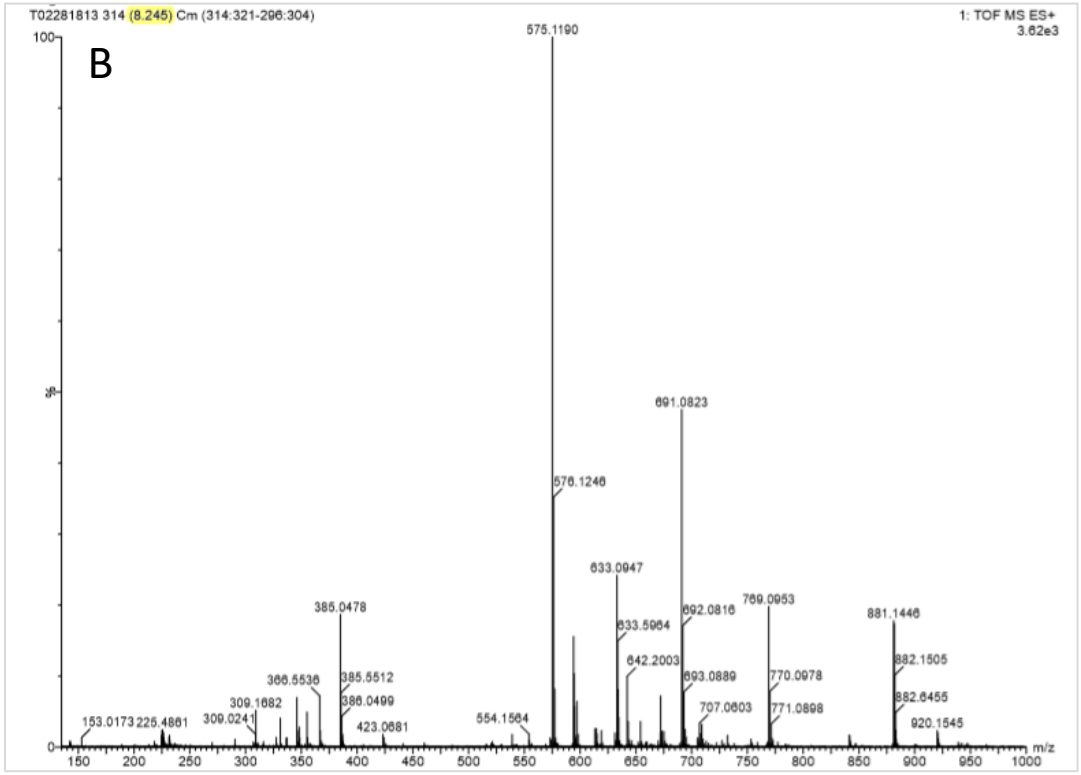


Figure 25. LC-MS profile of an aqueous GKE sample. The first chromatography plot indicates retention times (A), and the subsequent four mass spectrophotograms (B-E) are from highlighted retention times, the following (m/z) are of compounds with 100% abundance; (B) *Garcinia* biflavonoid 2 (m/z) 575.1194, (C) *Garcinia* biflavonoid 1 (m/z) 559.1235, (D) kolaflavonone (m/z) 589.1339, and (E) binarigenin (m/z) 543.1288.

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3.62e3
B
575.1190
691.0823
576.1246
633.0947
385.0478
769.0953
692.0816
881.1446
633.5964
642.2003
882.1505
366.5536
385.5512
693.0889
770.0978
309.1682
386.0499
707.0603
771.0898
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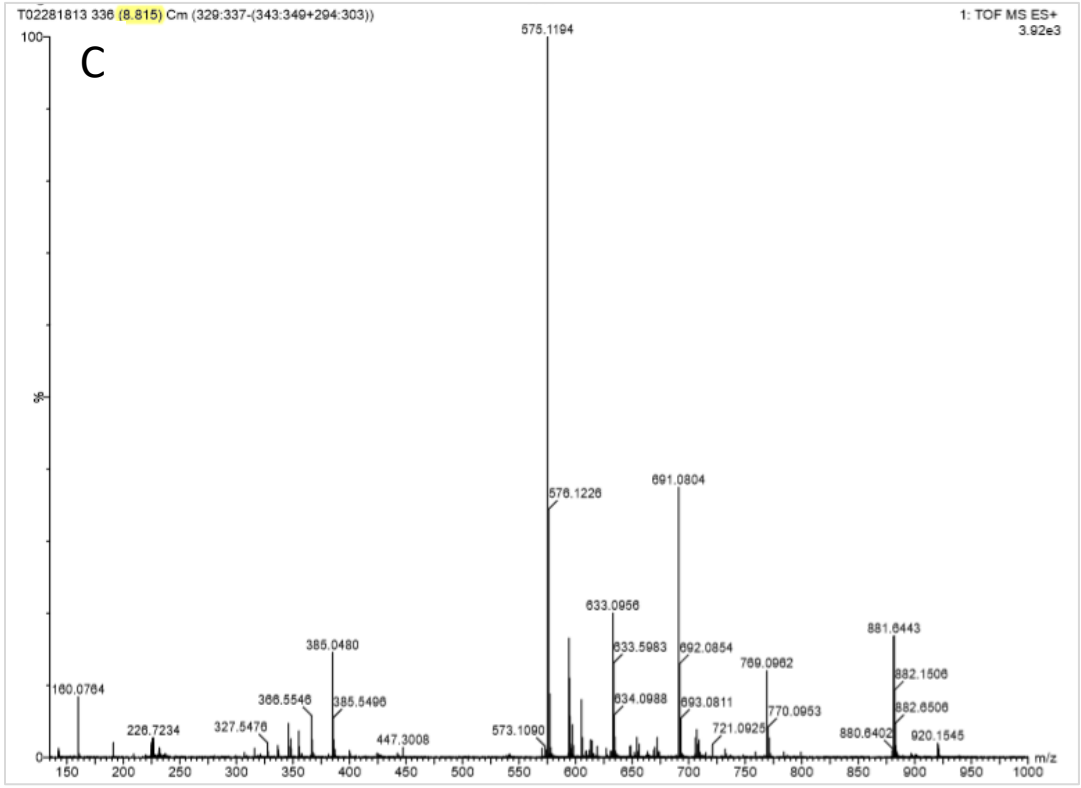
153.0173
225.4861
309.0241
423.0681
554.1564
920.1545
%
m/z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C
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691.0804
576.1226
633.0956
881.6443
385.0480
633.5983
692.0854
769.0962
882.1506
160.0764
366.5546
385.5496
634.0988
693.0811
770.0953
882.6506
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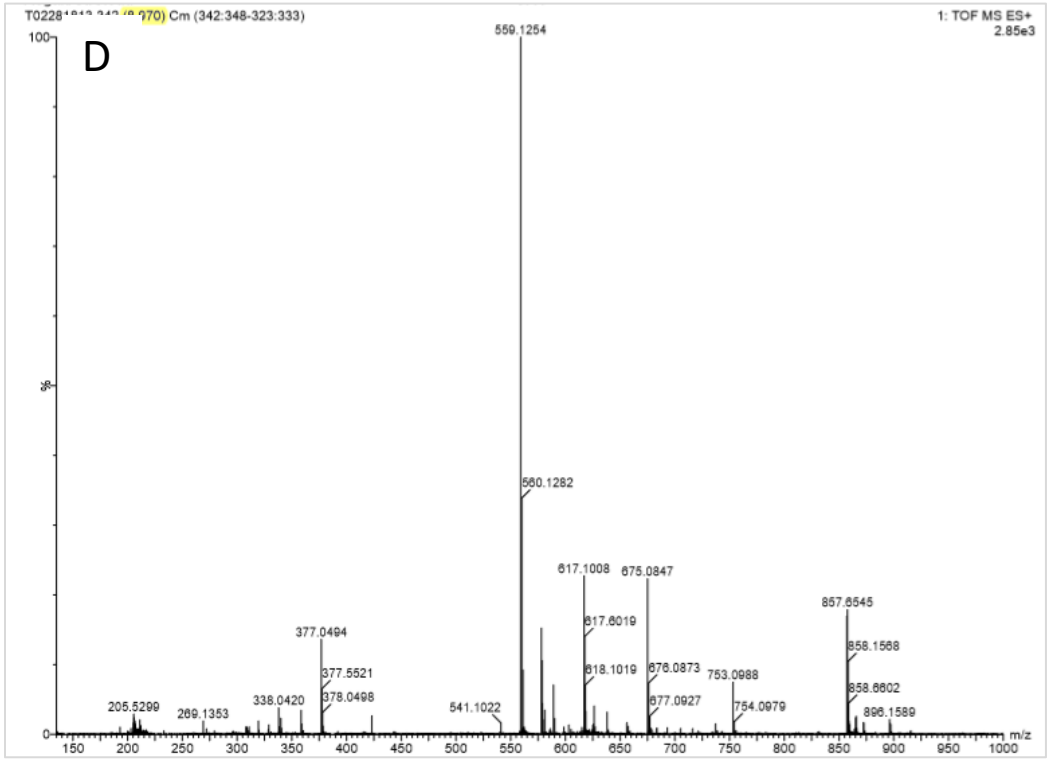
447.3008
573.1090
721.0925
880.6402
920.1545
%
m/z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2.85e3
559.1254
560.1282
617.1008
675.0847
857.6545
617.6019
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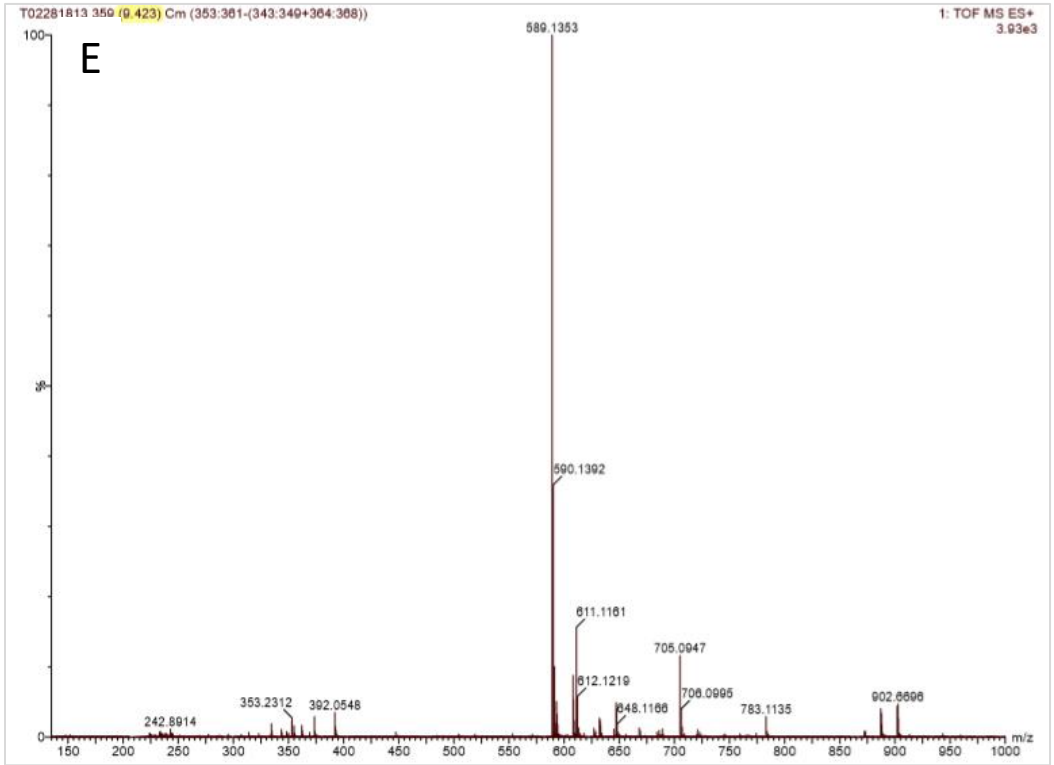
858.1568
377.5521
618.1019
676.0873
753.0988
858.6602
338.0420
378.0498
677.0927
754.0979
205.5299
269.1353
541.1022
896.1589
%
m/z


E
1: TOF MS ES+
3.93e3
589.1353
590.1392
611.1161
705.0947
612.1219
706.0995
353.2312
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783.1135
902.6696
242.8914
%
m/z

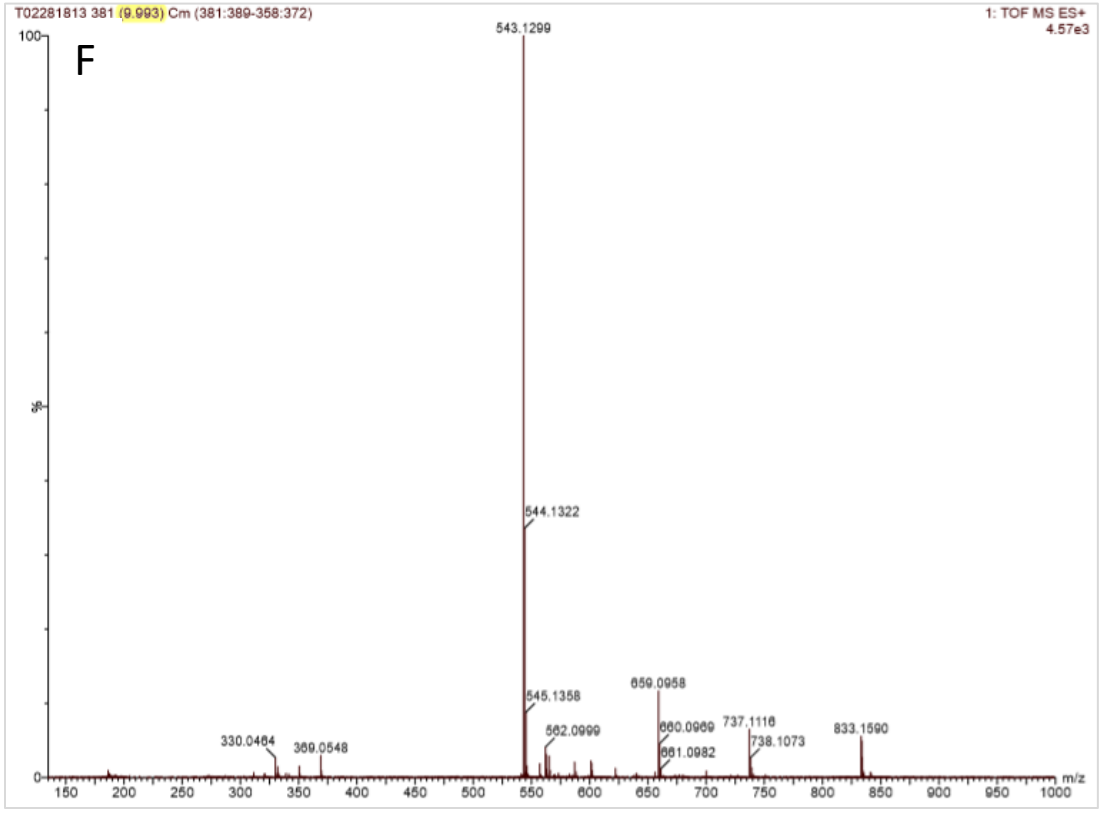

Figure 26. LC-MS profile of an ethanol GKE sample. The first chromatography plot indicates retention times (A), and the subsequent five mass spectrophotograms (B-F) are from highlighted retention times, the following (m/z) are of compounds with 100% abundance; (B) *Garcinia* biflavonoid 2 (m/z) 575.1190, (C) *Garcinia* biflavonoid 2 (m/z) 575.1194, (D) *Garcinia* biflavonoid 1 (m/z) 559.1254, (E) kolaflavonone (m/z) 589.1353, and (F) binarigenin (m/z) 543.1299.

Summary

Effects of GKE treatment on glycemia, body weight, diabetes incidence, and immune parameters are summarized here:

- **Glycemia:** Hypoglycemic effect of GKE was not observed regardless of GKE extraction type (aqueous or ethanol), GK seed source, frequency of freshly-prepared GKE treatment solution, or the experimental mouse T1D model studied (STZ-induced MLDSTZ C57BL/6J or spontaneous NOD/LtJ). STZ-free mice treated with ethanol GKE had increased glycemia compared to control.

- **Body weight:** There was no effect of different modalities of GKE treatment on body weight, tested in different types of experimental mouse models, besides an increase of body weights observed in MLDSTZ mice treated with aqueous GKE treatment.
- **Incidence of T1D:** There was no effect of different modalities of GKE treatment on the incidence and prevalence of T1D obtained in MLDSTZ or NOD models of T1D.
- **Immune parameters (day 11):** In a MLDSTZ model of T1D, there was
  - no effect on splenic cell counts and cell viability obtained by aqueous/ethanol GKE;
  - no effect/trend of decrease in T cell proliferation obtained by aqueous/ethanol GKE;
  - a decrease in the percentage of T cells and T cell subpopulations obtained by aqueous/ethanol GKE
  - an increase in TNFα, IFNγ, and IL-6 levels with a decrease of IL-2 obtained in splenocytes of mice treated with aqueous GKE; an increase of IL-17A, IL-10, TNFα, and IL-6, a trend of increase in IFNγ, and a decrease of IL-2 obtained in splenocytes of mice treated with ethanol GKE.
- **Immune parameters (day 30):** In a MLDSTZ model of T1D, there was
  - no effect on splenic cell counts and cell viability obtained by aqueous/ethanol GKE;
  - no effect on T cell proliferation obtained by aqueous/ethanol GKE
  - a decrease in the percentage of T cells and T cell subpopulations obtained by both GKE types

- an increase in IL-10, and a trend of increase in TNFα, and IL-6 levels with a trend of decrease in IL-2 obtained in splenocytes of mice treated with aqueous GKE; a trend of increase of TNFα, IFNγ and IL-6, with a trend of decrease in IL-2 obtained in splenocytes of mice treated with ethanol GKE.

Chapter V: Discussion

Effects of GKE Extracts on Glycemia, Body Weight and T1D Incidence

In initial experiments, mice were treated with aqueous GKE in their drinking water, as this was the most physiologically relevant route to humans' consumption of GK seeds. Contrary to the previous reports of GK-induced hypoglycemic effects (Adedara et al., 2015; Adaramoye 2012; Ayepola et al., 2013; Farahna et al., 2017; Iwu et al., 1990; Tchieme et al., 2016), the aqueous GKE treatment did not lower glycemia in diabetic mice in our study. However, in previous studies glucose measurements were obtained on fasted animals, which we did not use, as fasting is known to stress the experimental mice (Jensen, Kiersgaard, Sørensen, & Mikkelsen, 2013). To ensure that the STZ itself did not interfere with the potential effects of GKE on glycemia, the experimental group was formed in which STZ-free animals were treated by aqueous GKE; still, no hypoglycemic effects were observed in these mice.

The dose of 100 mg/kg GKE was chosen as it was the most commonly used efficient dose in previous studies. The hypoglycemic effects were observed in both diabetic and not diabetic animals by using this dosage (Adaramoye 2012; Ayepola et al., 2013; Farahna et al., 2017). Since no hypoglycemic effect was obtained by 100 mg/kg GKE, we assumed that a lower dose would not be efficient. In a 2017 study done by Farahna et al., diabetic rats treated with aqueous GKE at 50 mg/kg did exhibit a hypoglycemic effect, but it was not restored to control levels. A dose higher than 100 mg/kg was also not used because of the potential adverse health effects. A study done by Chinedu et al. described rats treated with aqueous GKE at 450 mg/kg and 900 mg/kg. Whereas hypoglycemic effects were achieved, an elevation of hepatic serum enzymes, indicating toxicity-related liver damage, was observed as well (Chinedu et al., 2013).

We also questioned whether differences in the experimental design, particularly freshness of GKE, treatment administration, and the extraction method influenced observed differences in glycemia results compared to previous studies. Since the *Garcinia kola* seeds were provided a year before the experimental execution, we wondered whether the freshness of the seeds impacted the presence of the active compound, kolaviron (KV). Thus, aqueous GKE samples were sent for LC-MS analysis, which confirmed the presence of four out of five flavonoids that constitute KV. Though our extracts did contain active flavonoid compounds, the fresh seeds were purchased, and a new experimental group of mice was treated with aqueous GKE prepared from these seeds. Again, a hypoglycemic effect was not observed.

Another factor taken into consideration was the frequency of freshly prepared GKE-enriched water, as in a few previous studies the GKE preparation was performed daily (Adedara et al., 2015; Farahna et al., 2017). Thus, a daily preparation and administration of GKE working solution was executed, instead of originally proposed every five days. Even this modification did not make a difference in observed glycemic levels of treated mice.

Furthermore, in previous studies, the main method of GKE administration was oral gavage (Table 1). Whereas this method ensures precise dosage, it is not as physiologically relevant as the method we chose in mimicking human consumption. In addition, when animals are treated orally (drinking or eating), an interaction with mucosal surfaces in the mouth can lead to more efficient absorption and transport as well as evasion of first pass metabolism, resulting in higher bioavaibility of the compound (Vandenberg, Welsons, vom Sall, Toutain, & Myers, 2014).

Unlike previous work, the GKE treatment in this study began before induction of T1D, ensuring its effects on the pancreas and glycemia level before chemically-induced damage of beta cells. In diabetic mice treated with aqueous GKE, significant increase in body weight was observed, suggesting that GKE can restore the weight lost caused by the progression of T1D. This weight gain is congruent with the previously published data on rats chronically treated with KV for six weeks (Ayepola et al., 2013), three weeks (Adaramoye, 2012) and 30 days (Farahna et al., 2017). Interestingly, in non-diabetic STZ-free mice treated with GKE, although not significant, there was observed a trend of decrease in body weight, which was also previously seen in non-diabetic rats (Ayepola, et al., 2013).

The extraction method we utilized (modified version of Ogunmoyole et al., 2012) used water as a solvent, whereas most of the previous studies used a method originally developed by Iwu (1984). This method involved more polar solvents, which yielded the active compound mixture (KV). Since no hypoglycemic effect was observed in aqueous GKE-treated mice, we wondered whether an ethanol-extracted GKE would do so. It was previously reported that an ethanolic extraction of GK fruits yielded more flavonoid content than an aqueous extraction, suggesting that an ethanol-based extraction would have more potent antidiabetic effects (Ogynmoyole et al., 2012). However, our results obtained using ethanol GKE did not confirm its hypoglycemic effect in diabetic or STZ-free mice. Hypoglycemic effects were previously observed in non-diabetic rats treated with KV at the same dose of 100 mg/kg (Ayepola et al., 2013; Farahna et al., 2017; Iwu et al. 1990). In contrast, non-diabetic STZ-free mice treated with ethanol GKE have significantly higher glycemia levels compared to control mice.

To our best knowledge, this study is the first study to investigate the effects of GKE on T1D incidence. Treatment with either aqueous or ethanol GKE had no effect on diabetes incidence and prevalence. GKE of either extraction type did not delay T1D development, nor reduced the number of diabetic animals.

Though no hypoglycemic or antidiabetic effects were seen in STZ-treated C57BL/6 mice, we questioned whether the STZ introduction, necessary to generate chemically-induced MLDSTZ model of T1D (Leiter & Schile, 2014), would interfere with the potential effects of GKE. Thus, later experiments were conducted on female NOD/LtJ mice, a strain that spontaneously develops T1D. (Leiter & Schile, 2014). Though there was no difference in glycemia when C57BL/6J mice were treated with ethanol GKE, we chose to treat NOD females with ethanol GKE, speculating about its higher potency (Ogynmoyole et al., 2012). The five-month-long ethanol GKE treatment of NOD females did not affect either glycemia levels or diabetes incidence. Based on the results of multiple experiments, performed with both aqueous and ethanol GKE it can be concluded that treatment with GKE does not reduce glycemia nor diabetes incidence in MLDSTZ and NOD models of T1D.

The lack of glycemic effects may be related to how the active compounds within GKE are absorbed and metabolized. The metabolism and absorption of flavonoids is not completely understood, and rates of both differ among the subclasses of flavonoids (Hollman, 2004). KV is a complex containing five flavonoids: GB-1, GB-2, kolaflavone, kolaflavonone, and binarigenin. The most active flavonoids in this complex, previously studied and found to exhibit hypoglycemic effects, are mainly GB-2, then GB-1 and kolaflavone (Adaramoye, 2012; Ayepola et al., 2013). The individual flavonoids are structurally similar; differences are observed

primarily within different side chains at the R3 and R4 position (Adedara et al. 2015; Ayepola et al. 2013).

In both of our aqueous and ethanol GKE samples, that were analyzed via LC-MS (positive ion ESI), GB-1, GB-2, kolaflavonone, and binarigenin were present, whereas kolaflavone was not present in either extraction type. Interestingly, the (m/z) values for each flavonoid present in the GKE samples were consistently an additional two atomic mass units higher than the (m/z) values previously published by Ayepola et al. (2013). This consistency suggests that the two ketones present in each flavonoid were protonated. Despite the confirmation of four flavonoids in the GKE extracts used in this study, three of which are most attributed to hypoglycemic effect, a reduction of blood glucose levels was not observed in this study, regardless of the type of GKE extract.

Effects of GKE Extracts on Immune Parameters

To our best knowledge, this study was the first study to extensively investigate the effects of GKE on the immune system. Immune parameters, such as T cell proliferation, splenic cell counts and viability, T cell subtypes and their cytokine profiles were studied at two different time points, on day 11 and 30, during the development of T1D in MLDSTZ mice treated with aqueous and ethanol GKE. Overall, no reduction in splenic cell numbers and viability was observed, suggesting that GKE extracts did not exhibit toxic effects on the immune system. The reduction in percentages of T cells and T cell subpopulations, with a trend of decreased T cell proliferation were observed. The cytokine profiles suggested a Th1 pro-inflammatory shift, with a significant elevation of Th-1-type cytokine levels, especially on day 11. Day 11 was chosen, as a representative day in the second week after the initial STZ injection, which is a critical period in

the development of T1D in the MLDSTZ model. T cells are the most active and mice are becoming diabetic in that period (Hobbs, 2011). Thus, the most prominent GKE-induced effects on T cell activity are expected to be observed during that period, with the lingering, declining effects on day 30 (Hobbs, 2011). Th-1-type cytokines were profoundly elevated at the beginning of disease development on day 11, whereas a non-significant trend in increase of Th1-cytokines remained on day 30, suggesting that a GKE-induced shift towards a pro-inflammatory response occurs early in T1D development. Even a significant increase of IL-10 was observed on day 30, indicating a compensatory increase of Th-2-type response. Interestingly, among both aqueous and ethanol GKE-treated mice, a decrease in IL-2 was observed. This finding supports the decreased proliferation index, as IL-2 is crucial for T cell activation and subsequent proliferation. However, IL-2 results do not corroborate GKE-induced shift to the Th1 response. Due to the increase in the pro-inflammatory cytokines, it would be expected to see a parallel increase in T cell proliferative capacity.

Based solely on immunophenotyping data, showing significant reduction in T cell population and T cell subtypes, one would expect to observe the signs of immunosuppression in GKE-treated mice, and a consequent prevention of T cell-mediated T1D. Though there are decreased relative and even absolute levels of T cells and Th/Tc subpopulations in GKE-treated mice, the profiling of their cytokines suggests GKE-induced skew towards pathogenic Th1-type response. Thus, it seems that GKE treatment, while decreasing T cells, makes them even more pathogenic, with a resulting unaltered T1D development.

A possible mechanism of action, not elucidated in our study, would be that GKE promotes NFκB expression, a key transcription factor involved in the production of numerous

pro-inflammatory cytokines and other inflammatory markers. Another plausible speculation would be an interaction of GKE with the aryl hydrocarbon receptor (Ahr). The Ahr is responsible for binding environmental pollutants that are metabolized primarily through the upregulation of cytochrome p450 (Julliard, Fechner, & Mezrich, 2014). Once the receptor is activated, it can promote the differentiation of Th17 or Tregs. It is of general thought that exogenous ligands (i.e. environmental pollutants) promote Th17 differentiation, whereas endogenous ligands promote Tregs (Julliard et al., 2014). Although flavonoids are considered as ligands for Ahr, which can act either agonistically or antagonistically (Amakura et al., 2008), the interaction of GKE and the Ahr remains to be elucidated. While the effects of KV or GKE on T cells have never been studied before, results from Ayepola et al. (2013) showed that treatment with KV exhibited anti-inflammatory properties in the lysate of rat liver through a reduction of IL-6 and TNFα levels. They proposed that KV suppresses NFκB expression (Ayepola et al., 2013) (Table 2). It is apparent that neither aqueous nor ethanol GKE exhibited anti-inflammatory effects in the MLDSTZ experimental model of T1D. However, GKE-induced shift towards a Th1 response, observed in our study, could be beneficial in other diseases, such as asthma or allergies in general, in which increased Th1 cytokines serve as a protective by decreasing pathogenic Th2 response. In a study done by Liu, Li, Zhang, Zhang, and Yang, the effects of a citrus fruit flavonoid, tangeretin, were investigated on ovalbumin-induced asthma in BALB/c mice. It was found that tangeretin decreased IL-4, IL-5 and IL-13 levels, while increasing IFNγ (Liu et al., 2017).

Our *in vitro* exploration of GKE effects on T cell function, using low (2-100 ug/mL)- to extremely high-concentrations (250-1000 ug/mL) GKE, showed that aqueous extract had no

effect on Con A-induced T cell proliferation. Even a slight indication of a trend of immunosuppression was not observed. However, aqueous GKE, added in those high concentrations of 500 and 1000 ug/mL, induced a significant increase in three pro-inflammatory cytokines, TNFα, IL-17 and IL-6, whereas just the highest concentration of 1000 ug/mL GKE even increased the level of anti-inflammatory cytokine IL-4. Previous work done by Abarikwu found that LPS-activated RAW264.7 murine macrophages treated with KV (~2.85 ug/mL to ~57 ug/mL) decreased IL-6 production, while KV had no effect on TNFα (Abarikwu, 2014). In contrast, a study done by Onasanwo et al. found that LPS-activated BV2 microglia cells treated with KV (~2.85 ug/mL to ~11.4 ug/mL) exhibited a decrease in both IL-6 and TNFα (Onasanwo, Velagapudi, El-Bakoush, & Olajide, 2016). This study also investigated the effect of KV on NFκB and found that KV inhibited the activation and binding to target DNA (Onasanwo et al., 2016). In our case, similar to the effects of GKE observed on cytokines *ex vivo*, it is likely that GKE does not inhibit NFκB activation, but rather enhances it, explaining the increases in pro-inflammatory cytokines.

Chapter VI: Conclusion

In conclusion, this study does not support previously observed hypoglycemic and anti-inflammatory effects of GKE. T1D incidence and prevalence, for the first time studied here, were not affected by either aqueous or ethanol GKE treatment. However, both types of GK extracts exhibited prominent effects on the immune system, inducing a reduction of T cells and T cell subpopulations, while skewing cytokine production towards pathogenic pro-inflammatory Th-1-type immune response. Whereas our data do not support GKE as a potential preventative treatment for T1D, they provide a strong evidence about GKE effects on T cells, suggesting GKE usage in other pathologies in which potentiation of pro-inflammatory Th-1 response would be beneficial.

Table 1. Summary of GKE's effects on glycemia and body weight in previous studies.

Reference	Extraction method	Experimental model	GK dose, treatment regimen, and duration	Administration method	Effects on glycemia and body weight (bw)
Iwu, et al (1990)	Iwu soxhlet method (Iwu, 1984)	Rabbits Alloxan single dose 150 mg/kg	100 mg/kg One-time administration of KV	IP injection	Hypoglycemic effect
Adaramoye, et al (2006)	Iwu soxhlet method (Iwu, 1984)	Male wistar rats STZ single dose 65 mg/kg	100 mg/kg Daily 14 days KV, Fraction 1, Fraction 2, and Fraction 3	Oral gavage	Hypoglycemic effect seen in KV, F1, F2 but not F3 Decreased body weight in diabetic rats (not F3)
Adaramoye (2012)	Iwu Soxhlet method (Iwu, 1984)	Male wistar rats STZ single dose 35 mg/kg	100 mg/kg Daily 3 weeks	Oral gavage	Increased body weight in diabetic treated rats Hypoglycemic effect
Ayepola, et al (2013)	Iwu Soxhlet method (Iwu, 1984)	Male wistar rats STZ single dose 50 mg/kg	100 mg/kg 5x a week 6 weeks	Gastric gavage	Hypoglycemic effect Improved weight loss in diabetic rats
Adedara, et al (2015)	No extraction done Stock solution of GK (100mg/mL) was prepared fresh every other day with corn oil	Male wistar rats STZ single dose 50 mg/kg	250 mg/kg 14 days	Orally* *not clearly defined	No effect on bw Hypoglycemic effect
Tchieme, et al (2016)	Modified Iwu Soxhlet method (Iwu, 1984)	Male and female wistar rats Alloxan single dose 200 mg/kg	50 mg/kg KV, gb1, gb2, crude ethanol extract, garcinoic acid	Oral gavage	Overall hypoglycemic effect, best improvement in gb2 and crude extract treatment groups
Farahna, et al. (2017)	No extraction done Stock solution of GK (25 mg/mL) was prepared daily in distilled water	Male wistar rats STZ single dose 60 mg/kg	100 mg/kg Daily 30 days	Oral gavage	Hypoglycemic effect Decreased bw in non diabetic rats increased bw in diabetic rats

Table 2. Summary of the effects of KV *in vitro* from previous studies.

Reference	Extraction method	KV (particular fraction used), dose and treatment duration	Cell and molecular targets studied	Results
Abarikwu (2013)	Iwu Soxhlet method (Iwu, 1984)	5, 10, 15, 25, 50, 100 uM ~2.85 ug/mL to 57 ug/mL 24 hours	mRNA expression and secretion of TNFα and IL-6 in RAW 264.7 macrophages	No effect on TNF-α Decreased IL-6
Onasanwo et al. (2016)	Iwu Soxhlet method (Iwu, 1984)	5-20 uM ~2,85 ug/mL to 11.4 ug/mL 24 hours	BV2 mouse microglia Cell viability TNFα and IL-6 secretion	No effect on cell viability Decreased TNFα and IL-6
Muhammed et al (2017)	Iwu Soxhlet method (Iwu, 1984)	5 ug/mL 48 hours	Human PBMCs Mitotic index TLR2 and VEGFC mRNA expression	Decreased mitotic index Down regulation of TLR2 and VEGFC
Okoko & Ere 2012	Not described	**GB-1 and GB-2 used 2, 4, 8, 16 uM (~1.14 ug/mL to 9.12 ug/mL) 72 hours	U937 macrophages Cell viability Cell activation TNF-α, IL-1, and IL-2 secretion	Increased cell viability Inhibition of activation Decreased TNF-α, IL-1 and IL-2 GB-2 more impactful than GB-1

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