COLLEGE OF HEALTH SCIENCES

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF BIOCHEMISTRY

INVESTIGATION ON THE EFFICACY OF CRUDE ROOT EXTRACT OF *CUCUMIS FICIFOLIUS A., RICH* ON SELECTIVE BIOCHEMICAL AND HISTOPATHOLOGICAL PARAMETERS IN HIGH-FAT-FRUCTOSE DIET-STREPTOZOTOCIN INDUCED TYPE-2 DIABETIC ALBINO WISTAR RATS

BY: YESHAMBEL AYELE MERA (BSc)

A THESIS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, SCHOOL OF GRADUATE STUDIES, ADDIS ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL BIOCHEMISTRY

SEPTEMBER, 2020

ADDIS ABABA, ETHIOPIA

DECLARATION

Addis Ababa University

School of Graduate Studies

This is to declare that the thesis prepared by Yeshambel Ayele, entitled **"Investigation on the Efficacy of Crude Root Extract of *Cucumis ficifolius A., Rich* on Selected Biochemical and Histopathological Parameters in High-Fat-Fructose Diet-Streptozotocin-Induced Type-2 Diabetic Albino Wistar Rats"** and submitted in partial fulfillment of the requirements for the Degree of "Master of Science in Medical Biochemistry" fulfills the regulations of the university and meets the accepted standards concerning to originality and quality. This thesis is Yeshambel Ayele's original work and has not been presented for a degree in any other university, and that all sources of material used for the thesis have been properly acknowledged.

Investigator: Yeshambel Ayele Mera (BSc, AAU) Signature: _____ Date: _____

E-mail: yayele613@gmail.com

Approved and Signed by the Examining Committee:

Examiner: Solomon Mequanente (PhD, Assistant Professor of Pharmacology, AAU)

Signature: _____ Date: _____

Advisor:

Daniel Seifu (PhD, Associate Professor of Biochemistry, AAU)

Signature: _____ Date: _____

Co-advisors:

Menakath Menon (PhD, Associate Professor of Biochemistry, AAU)

Signature: _____ Date: _____

Frehiwot Teka (MSc, Researcher, EPHI) Signature: _____Date: _____

Wondwossen Ergete (MD, Associate Professor of Pathology, AAU)

Signature: _____ Date: _____

Zeleke Geto (MSc, Associate Researcher, EPHI) Signature: _____ Date: _____

Chair of Department or Graduate Program Coordinator:

Maria Teklemariam (PhD)_____ Signature: _____ Date: _____

ACKNOWLEDGMENTS

Above all, I would like to express my indescribable gratitude to my Almighty GOD and His Mother Saint Virgin Mary for the wisdom, capability and strength that has been bestowed upon me during this thesis accomplishment, for saving me from many awful events particularly during the past 6-years, and indeed throughout my life.

My deepest gratitude goes to Jimma University and Addis Ababa University for sponsoring and educating my MSc program, respectively. My deepest gratitude forwards to Department of Biochemistry, School of Medicine, College of Health Sciences of Addis Ababa University and staffs for the supreme and countless contribution in various aspects throughout my academic and research success. My special thanks go to my instructors who delivered their unrestricted academic knowledge for my success in the field of Biochemistry.

It is my pleasure to gratefully and sincerely thank my advisors: Dr. Daniel Seifu, Dr. Menon Menakath, Mrs. Frehiwot Teka, Dr. Wondwossen Ergete, and Mr. Zeleke Geto for their unreserved guidance, advice and comment throughout this thesis accomplishment.

Then, my warmest thanks go to EPHI, TMMRD for positivity in funding this research project, and all members of TMMRD who paid lots of contribution in most challenges that had come up before me throughout the actual experimental period. Also, other divisions of EPHI such as National Experimental Animals Breeding Center; Department of National References Laboratory for Clinical Chemistry; Department of Security and staffs; Department of Finance and Purchase, and EFMHACA (Ethiopian Food, Medicine and Healthcare Administration and Control Authority) are thankful for their relevant support for the attainment of this paper.

My respectful thank goes to the Department of Pathology, Tikur Anbesa Specialized Hospital and the staffs for their contribution in the processing of histo-pathological data of this paper. Afterward, my gratitude is to Addis Ababa Abattoirs Enterprise and Loyal Supermarket PLC for their unreserved positivity in procurement of raw materials used for preparation of high fat diet.

I would like to express my heartfelt thank to Dr. Maria Teklemariam (Head, Department of Biochemistry) who helped me in reviewing my thesis and other administrative aspects, and Mr. Dereje Getachew (PhD candidate) in revising my thesis and giving advice. I also thanks to Dr. Getachew Addis (Botanist; from EPHI) in authenticating the experimental plant. My gratitude goes to Professor Tefera Belachew and Mr. Masresha Getnet (Biostatistician) (from Jimma University) who shared their knowledge in selection of proper statistical analysis method. I am beholden to my colleagues from Department of Biomedical Sciences of Jimma University and others for their advices and help in different aspects. Finally, my inexpressible gratitude is to my families for their countless contribution in my success.

TABLE OF CONTENTS

CONTENTS _____ Page no.

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

ADA……American Diabetes Association
ADP……Adenosine Diphosphate
AgRP…….. Agouti-Related Protein
Akt……...Protein Kinase B
ALP……Alkaline Phosphatase
ALT……Alanine Aminotransferase
AMP……Adenosine Monophosphate
AMPK…….AMP-activated Kinase
AST……. Aspartate Aminotransferase
ATP……..Adenosine TriPhosphate
C. ficifolius…Cucumis ficifolius A., Rich
CART……Cocaine–Amphetamine-Related Transcript
CoA……..Coenzyme-A
DAG…….Diacylglycerol
DMSO……..Dimethyl Sulfoxide
DPP-4……...Dipeptidyl Peptidase-4
EPHI……Ethiopian Public Health Institute
FA…….Fatty Acid
FAO……..Food and Agriculture Organization
FAS…….....Fatty Acid Synthase
FFA…….....Free Fatty Acid
FOXO (1/3/4)…Fork-head box subgroup O (isoform 1, 3 & 4)
GDM……..Gestational Diabetes Mellitus
GIT…….Gastrointestinal Tract
GLP-1…….Glucagon-Like Peptide-1
GLUT-2/4/5…Glucose Transporter- type 2 or 4 or 5
HDL-C……High-Density Lipoprotein-Cholesterol
HFD……High-Fat Diet
HFFD……..High-Fat-Fructose Diet
HMG-CoA.....3-Hydroxy-3-methylglutaryl-CoA
Hr……Hour
IDF……International Diabetes Federation
InsR…….Insulin Receptor
IR…….Insulin Resistance
IRS-1/2…….....Insulin Receptor Substrate Subunit-1 or -2
LDL-C……. Low-Density Lipoprotein-Cholesterol
LPL……..Lipoprotein Lipase
MDANOVA…Mixed Design ANOVA
MNSI……Mononuclear Spotty Infiltrates
NAD^+/H……Nicotinamide Adenine Dinucleotide non-reduced/reduced
$NADP^+$/H…..Nicotinamide Adenine Dinucleotide Phosphate-non-reduced/reduced
NPD……..Normal Pellet Diet
NPY……..Neuropeptide Y
OGTT……...Oral Glucose Tolerance Test
PEPCK……..Phosphoenol-Pyruvate Carboxykinase
PI3K……..Phosphatidylinositol-3-Kinase
POMC……...Proopiomelanocortin
PPAR-α/γ.....Peroxisome Proliferator-Activated Receptor-Alpha/gamma
PTP1B…Protein Tyrosine Phosphatase 1 Beta-subunit
RMANOVA…Repeated Measure ANOVA
ROS……...Reactive Oxygen Species
SD……..Standard Deviation
SREBP-1c…..Sterol Regulatory Element-Binding Protein-1c
STT……....Short-term Treatment Test
STZ……....Streptozotocin
T1DM or T2DM……Type One or Type Two Diabetes Mellitus
TC……..Total Cholesterol
TG……..Triglyceride
TMMRD…....Traditional and Modern Medicine Research Directorate
VLDL-C…….Very-Low Density Lipoprotein-Cholesterol

ABSTRACT

Background: Diabetes mellitus, mainly T2DM (type-2 diabetes mellitus), has resulted in large-scale and exponentially increasing burdens in health and socioeconomic aspects of various populations worldwide. The plant *Cucumis ficifolius A., Rich* root parts have been used for treatment of diabetes by traditional medical practitioners of Ethiopia.

Objective: This study was aimed to validate the efficacy of the crude 80% methanol extract of roots of ***C. ficifolius*** on blood glucose, pancreatic islets of Langerhans β-cells, lipid profile, body weight, and liver and kidney parameters on induced type 2 diabetic rats.

Method: The crude 80% methanol extract of roots *of C. ficifolius* was prepared using maceration technique. Phytochemical screening (qualitative) and acute toxicity of the crude extract were determined. T2DM rat model was developed by feeding "High-Fat-Fructose Diet" followed by induction of single 30mg/Kg dose Streptozotocin. Total of 6 groups (n=7) of albino wistar rats: three control groups (normal control, diabetic negative control and diabetic positive control (metformin 100mg/kg) groups) and three experimental groups (diabetic+ crude *C. ficifolius* root extract at 100, 200 & 400mg/kg doses) were used. The effect of crude root extract on blood glucose, body weight, fasting serum lipid profile, histo-pathology of pancreatic islets of Langerhans; liver and kidney biochemical and histo-pathological parameters were determined. Data analysis was done by SPSS version-24 using ANOVAs. The p-value <0.05 was considered as statistically significant.

Result: From crude 80% methanol extract of roots of *C. ficifolius* terpenoids, flavonoids, saponins, glycosides, phenols, alkaloids and steroids were present. There was no rat death and toxic sign at 2000mg/Kg acute test. Against diabetic negative control group, those groups treated with crude plant extract showed dose dependent: a) blood glucose reduction which was moderate in STT, continuing in progressive tests and significant ($p<0.05$) in 3-weeks long treatment; b) admirable glucose tolerance in OGTT, and c) notable increase in mass of pancreatic islets of Langerhans. Diabetic groups treated with plant extracts for 3-weeks displayed a dose dependent: significant reduction of fasting serum TC, LDL-C, TG, ALP, ALT and AST; b) moderate decrease in urea and fasting body weight; c) increase in HDL-C (significant) and creatinine (moderate), and d) slight improvement in liver and kidney histopathology. Treatment of plant extract at higher dose revealed better effect in pancreatic islets of Langerhans, HDL-C and creatinine than metformin 100mg/Kg treated group, and fairly comparable effect in rest of parameters.

Conclusion: These findings scientifically confirmed that the crude extract of *C. ficifolius* root has gotten antidiabetic activity in T2DM rat model. Molecular mechanisms of action and active compounds isolation of this extract, fractionation, and trial on human system are recommended.

Key words: *C. ficifolius*, traditional medicine, anti-diabetic activity, type-2 diabetes mellitus

1. INTRODUCTION

1.1. Definition and Classification of Diabetes Mellitus

The terms "Diabetes" and “Mellitus” are derived from Greek word: “Diabetes” denotes “a passer through, a siphon” whereas the “Mellitus” denotes “sweet”. It is because of the excessive amounts of urine produced by diabetics attracted bees and flies. Following this, the traditional practice of diagnosing diabetes mellitus was by noticing whether ants are attracted to a person's urine or not in ancient Chinese, and by tasting the urine themselves in Europe in medieval age (Patlak, 2002). Diabetes mellitus is a disease of complex and heterogeneous disturbances in carbohydrate, fat and protein metabolism, characterized mainly by chronic hyperglycemia ensuing from defects in insulin secretion, insulin sensitivity, or both (Kaur &Valecha, 2014; IDF, 2019). Diabetes mellitus is one of the foremost and the fastest growing global health and socioeconomic disasters of the 21st century (IDF, 2019).

The use of seriously controlled diagnostic and classification criteria of diabetes mellitus is the most important element for appropriate management of diabetes mellitus. Particularly, classification of type-one and and type-2 diabetes mellitus is somewhat difficult because age and keto-acidosis have recently been discovered as shared characteristics (Cernea & Cahn, 2016; ADA, 2019a). Thus, an improved classification based on patho-physiological mechanisms of the disease/biological markers (i.e. insulin resistance (IR), beta-cell dysfunction, autoimmunity) is more appropriate than traditional criteria alone. These possibly assist in better clinical decision and offer the opportunity of an optimized therapeutic strategy (Cernea & Cahn, 2016). Thus, based on the above criteria diabetes mellitus can be classified into four general categories (ADA, 2019a).

Type One Diabetes Mellitus (T1DM)

Type 1 diabetes mellitus (formerly referred as Insulin-Dependent Diabetes Mellitus, or Juvenile Diabetes) is chronic hyperglycemia characterized by loss of the insulin producing beta-cells of islets of Langerhans in the pancreas, leading to absolute insulin deficiency. It can be immune-mediated due to cellular-mediated autoimmune destruction of the pancreatic beta-cells or idiopathic (with no known etiologies). T1DM accounts for approximately 5–10% of those with diabetes (Ta, 2014). T1DM usually manifests in childhood or adolescence, and the patients require exogenous insulin (Jain *et al.*, 2016; ADA, 2019a).

Type Two Diabetes Mellitus (T2DM)

Type 2 diabetes mellitus (previously named as "Non–Insulin-Dependent Diabetes" or "Adult-Onset Diabetes) is chronic hyperglycemia due to a progressive loss of insulin secretion (relative insulin deficiency) on the background of IR. T2DM is the most prevalent category accounting for 90–95% of all diabetes (ADA, 2019a). Unlike T1DM, T2DM usually does not require insulin until insulin secretion has markedly decreased after years of disease (Jain *et al.,* 2016). It is frequently associated with metabolic syndrome (Ta, 2014).

Gestational Diabetes Mellitus (GDM)

GDM generally refers to milder hyperglycemia and lesser degree of glucose intolerance diagnosed in pregnant women who have never had diabetes before. It occurs in the second or third trimester of pregnancy, and may disappear (usually) or improve after delivery (Agarwal MM., 2016). GDM arises from IR because of hormone production by the placenta. Other risk factors for GDM include extreme weight gain during pregnancy, aging and a family history of diabetes (IDF, 2019). GDM can affect 1% - 20% of all pregnancies depending on the diagnostic criteria used or the population studied (Agarwal MM., 2016). If left untreated, it may precede the development of T2DM as it resembles T2DM in several respects, involving a combination of relative insulin deficiency and IR (Jain *et al.,* 2016).

Specific Types of Diabetes Mellitus

Specific types of diabetes mellitus are associated with specific causes. It involve monogenic diabetes syndromes due to specific gene defect (such as neonatal diabetes (before 6 months) and maturity-onset diabetes of the young (MODY-classically before age 25 years)), diseases of the exocrine pancreas (cystic fibrosis-related diabetes), post-transplantation (new onset) diabetes, infection related diabetes and drug or chemical-induced diabetes (such as glucocorticoid use) and many others (ADA, 2019a).

1.2. Etiology of Type 2 Diabetes Mellitus

In case of T2DM, genetic factors (associated with impaired insulin secretion and insulin action), environmental factors (such as obesity, over eating, physical inactivity, stress, heavy alcohol consumption, smoking and aging), gene-environment interaction, metabolic syndrome, family history of T2DM, and history of GDM are important determinants of IR and β-cell dysfunction (Zheng *et al.,* 2018). Beta-cell dysfunction is an impaired insulin secretion associated with a decrease in glucose responsiveness. IR is a condition in which insulin in the body does not exert

sufficient action proportional to its blood concentration in peripheral target tissues such as adipose, muscle, and liver (Kohei, 2010).

Genetic factor is one cause for IR and T2DM. Approximately 100 genes/genetic loci are involved in T2DM. Most of the genes identified by genome-wide association studies confer a small risk of diabetes with the genes (Nathan, 2015). Since the development of T2DM is clearly associated with a family history of diabetes, the genetic abnormalities in the molecules related to the regulatory system of glucose metabolism are typical factors. Some of the candidate genes responsible for process of insulin secretion and insulin action are glucokinase genes, mitochondrial genes, and insulin receptor genes (Pyke, 1979). Moreover, the genetic causes of IR are not only polymorphisms of genes that directly affect insulin signals but also polymorphisms of thrifty genes (diabetes-sensitive genes; such as the β-3 adrenergic receptor gene and the uncoupling protein (UCP) gene) associated with visceral obesity (Kohei, 2010).

Environmental factors, particularly excess weight and physical inactivity, are the major causes of IR. The sedentary life, accompanied by a decrease in muscle mass and associated decrease in insulin receptors (InsR), contributes for obesity. The alterations in dietary energy sources which involve (a) increase in consumption of low-quality fats (e.g., trans-fat and saturated-fat) and low quality carbohydrates (with high glycemic-index such as simple sugars), and (b) the decrease in intake of unsaturated fats, starch and dietary fibers are important causes of obesity (Kohei, 2010). Long-lasting excessive energy intake than expenditure is the most common factor of ectopic lipid deposition in the liver and skeletal muscle. It results in saturation of fat storage capacity of adipose tissues, and extra energy would be transported to other tissues (liver and skeletal muscle) where they accumulate as ectopic fat (Shulman, 2014).

In order to clearly understand the health risk of IR, appreciation of the metabolic role and mechanism of action of insulin in normal physiology is very important, discussed as follows.

1.2.1. Metabolic Role and Mechanisms of Action (Signaling Pathway) of Insulin in Normal Physiology and in Diabetes Mellitus (T2DM)

InsR/PI3K/Akt (Insulin Receptor/Phosphatidyl Inositol-3-Kinase/ Protein Kinase-B) pathway is a major mechanism in the development of IR because any alterations of protein expression in this stream probably influence insulin sensitivity. The insulin response begins when insulin binds to its receptor in cell membranes which leads to receptor auto-phosphorylation. Consequently, IRS-1/2 (insulin receptor substrate-1/2; a post-signaling intracellular protein) is phosphorylated at a tyrosine residue (activated) and function as a docking site for the recruitment and activation of

PI3K. PI3K, in turn, activate/phosphorylate/ Akt (Barbosa *et al.,* 2014). Akt is an effector molecule for all of insulin-induced regulation of carbohydrate (Barbosa *et al.,* 2014), protein (James *et al.,* 2017) and lipid metabolism (Vergès, 2010).

Insulin regulate carbohydrate metabolism to maintain normoglycemia. In skeletal muscle and adipose tissue, Akt up regulates the expression levels of PIP5K2α (phosphatidylinositol-5-phosphate-4-kinase type-2α) which result the translocation of GLUT-4 (glucose transporter type-4) into the membrane to initiate glucose uptake in to cytoplasm (Liu T., Yu B., *et al.,* 2016). In the liver, Akt increase the activation of glycogen synthase kinase-3and glycogen synthase, promote glucose storing as glycogen. Moreover, Akt inactivate FOXO (Fork-head box subgroup O), leading to decreased FOXO translocation to the nucleus and subsequent decrease in transcription of gluconeogenic enzymes, inhibit gluconeogenesis. Oppositely, IR leads to decreased glucose uptake, activation of gluconeogenesis and glycogenolysis, which all contribute for hyperglycemia (Barbosa *et al.,* 2014; Shulman, 2014).

Insulin controls protein metabolism. Activation of Akt (a) inhibits FOXO1/3/4 (isoform 1, 3 & 4) translocation and transcription of critical mediators of ubiquitin-proteasome- and autophagy-lysosome systems to decrease protein degradation, and (b) activates mTORC1 (mammalian target of rapamycin complex-1) which promotes downstream protein synthesis pathways. Conversely, in T2DM, IR causes protein degradation and muscle wasting (James *et al.,* 2017).

Insulin regulate lipid and lipoprotein metabolism to control dyslipidemia. Insulin via its effector molecule-Akt: (a) activates adipogenesis related genes such as membrane bound LPL (lipoprotein lipase) in circulation to promote TGs (triglycerides/triacyl glycerol) clearance from circulation and other enzymes important for *de novo* FA (fatty acid) and TGs synthesis and storage in adipose tissues; (b) inhibit adipolysis genes such as hormone sensitive lipase (HSL) in adipocytes to block lipolysis/TGs degradation and FA oxidation genes (Moodley *et al.,* 2015).

Insulin up regulates synthesis and secretion of intestinal apo-lipoprotein A subtype-4 (ApoA-4) which is important for chylomicron secretion, and so promotes absorption of dietary lipids for storage (Van Stee *et al.,* 2018). Insulin inhibits the assembly and secretion of VLDL-C (very low-density lipoprotein- cholesterol) particles by: (a) reducing its precursor-free fatty acids; (b) increasing degradation of Apo-lipoprotein B-100 (ApoB-100) and reducing the expression of microsomal transfer proteins (important for VLDL-C assembly) in the liver (Martín-Timón*et al.,* 2018). Insulin increase LDL-C B/E receptor expression and activity, promote the clearance of LDL-C (low density lipoprotein-cholesterol). Insulin also acts on HDL-C (high density lipoprotein-cholesterol) metabolism by activating LCAT (lecithin–cholesterol acyl transferase)

and hepatic lipase activities. The enzyme LCAT found attached to HDL-C particle and help esterification of cholesterol in HDL during HDL-C maturation. In the liver, hepatic lipase hydrolyzes TGs associated with HDL-C and LDL-C. This prevents abnormal elevation of circulating total cholesterol and TG levels (Vergès, 2010). However, impairment of this insulin signaling pathway in T2DM results dyslipidemia (Vergès, 2010; Moodley *et al.*, 2015; Martín-Timón*et al.*, 2018).

1.2.2. Mechanism of High-Fat-Fructose Diet (HFFD) on the Development of IR and T2DM

1.2.2.1. Mechanism of High-Fat Diet (HFD) on Induction of IR and T2DM

There are many mechanisms by which excess fat intake causes IR syndrome in muscle, liver and adipose tissue and ultimately T2DM development. Some of them are through induction of obesity, increasing the production of RBP-4(Retinol Binding Protein-4), induction of ectopic fat accumulation, increasing the level of circulating and tissue FFA (free fatty acids), and alteration of gut micro-biota (Samuel & Shulman, 2012;Zhang X., Zhao Y., *et al.*, 2012; El-Sayed *et al.*, 2013; Shulman, 2014).

HFD can induce obesity. Following entrance into adipocyte, the FAs are esterified by coenzyme A (CoA) into acyl-CoAs which bind glycerol backbone, forming triacylglycerol. This neutral lipid would be stored in adipocytes, resulting obesity (Samuel & Shulman, 2012).

HFD can induce systemic and hepatic IR by triggering the production of RBP-4. RBP-4 is fat derived peptide produces from adipose tissue (mainly in visceral) and liver, and acts as marker of intra-abdominal fat mass and predictor of diabetes and cardiovascular disease progression. HFD fed rats showed increased blood glucose and RBP-4. Increased levels of RBP-4 in adipocytes attenuate GLUT-4 expression and suppress adipocytes glucose intake. High RBP-4 in hepatocyte induces the expression of the gluconeogenic enzyme PEPCK (phosphoenol pyruvate carboxy kinase). Also, RBP-4 suppresses the activation of IRS-1 and PI3K (El-Sayed *et al.*, 2013).

HFD can induce ectopic fat, FFA deposition and associated IR. Excessive energy intake than expenditure is the most common factor. Long-term HFD can result in saturation of fat storage capacity of adipose tissues and consecutive increase in FFA level in the circulation. This high circulating level of FFA promote the greater flow and spread of FFA to other ectopic tissues (such as liver and skeletal muscle) where they accumulate as ectopic TG and increase the concentration of tissue FFA, leads to muscle and liver IR (Shulman, 2014).

Accumulation of ectopic lipid in skeletal muscle and liver encourages pathways that impair insulin signaling and causes for decreased glucose uptake and glycogen synthesis by muscle and liver, respectively (Samuel & Shulman, 2016). The ectopic lipid-induced muscle IR precedes liver IR. The diminished uptake of glucose by muscle causes for increased insulin independent flow of circulating glucose in to liver. In liver, glucose would be used as substrate for *de novo* lipogenesis, and consecutive hepatic IR (Samuel & Shulman, 2016).

HFD is also responsible for disturbance of gut micro-biota, leading to decreased bacterial communities which are responsible for production of short-chain fatty acids (SCFA). SCFA are important energy sources for intestinal epithelial cells; improve gut barrier function either by facilitating tight junction assembly and promoting cell differentiation or by up-regulating proglucagon gene expression in intestinal L-cells. Therefore, SCFA improve intestinal barrier function and prevent inflammation by blocking entry of exogenous antigen or endo-toxin into the blood. Thus, HFD causes for decrease in SCFA producing gut micro-biota and then increase in the levels of endo-toxins in circulatory systems (endo-toxemia) that initiates inflammation, obesity, IR and T2DM (Zhang X., Zhao Y., *et al*., 2012).

1.2.2.2. Mechanism of High-Fructose Diet on Development of T2DM

The chronic high intake of dietary fructose is strongly correlated with increased adiposity, hyperlipidemia, fatty liver, oxidative stress, inflammation, IR and T2DM (Dekker *et al.*, 2010; Simopoulos, 2013). The rapid metabolism, lipogenic, appetite stimulating and gut micro-biota altering properties of fructose are the important mechanisms for its risk on development of IR and T2DM (Dekker *et al.*, 2010; Ochoa *et al.*, 2015; Wong *et al.*, 2016).

The rapid metabolism of fructose (bypass the first rate-limiting step in glycolysis pathway) contributes for saturation of energy demand of the body and accumulation of excess acetyl-CoA which allows the shift of acetyl-CoA to *de novo* lipogenesis. A lipogenic property of fructose is because it cannot be used as primary energy source for body cells, unlike glucose (Wong *et al.*, 2016). Fructose could disturb gut micro-biota and leads to T2DM (Dekker *et al.*, 2010). Generally, these three properties of fructose are shared with HFD in resulting T2DM.

The most important and unique feature of fructose, unlike HFD, is its ability to increase appetite through peripheral and central mechanisms. Fructose promote food intake through peripheral endocrine mechanism by inhibiting satiety hormones expression, and so induce a positive energy balance and associated metabolic disorders. Oral and gastric/luminal load of fructose, contrary to glucose intake, brings weak release in circulating satiety hormones such as insulin, leptin,

glucagon-like peptide-1 (GLP-1; from L-cells), peptide tyrosine tyrosine (PYY) and 5-HT (5-hydroxytryptamine/serotonin; from entero-endocrine cells), and attenuates postprandial suppression of ghrelin (activate gherlin). Insulin, PYY and leptin are activator of reward neuron POMC/CART (proopiomelanocortin/cocaine–amphetamine-related transcript). Contrariwise, gherlin is an activator of appetite neuron NPY/AgRP (neuropeptide Y/agouti-related protein, an inhibitor of POMC/CART) by binding to its growth hormone secretagogue receptor (GHSR), and stimulate feeding. The fructose-induced weak release of 5-HT or GLP-1 causes for subsequent decrease in expression of 5-HT receptor and GLP-1 receptor in vagal afferents, and so prevent 5HT and GLP-1-induced secretion of insulin (Ochoa *et al.*, 2015).

The reasons for such features of fructose are limited expression of GLUT-5 (fructose transporter) in intestine and pancreatic beta-cells than GLUT-2, slower rate of absorption and partial intestinal metabolism in the intestine (Ochoa *et al.*, 2015).

Moreover, fructose can stimulate appetite by its central effect. There are evidences that not only intracerebroventricular fructose but also circulating fructose from oral (Shu *et al.*, 2006) and gastric load (Page *et al.*, 2013) is capable to promote neuronal appetite signal. This effect is because of its potent cellular inorganic phosphate scavenging ability. In fructose metabolism, the absence of feedback regulation of fructokinase causes continuous phosphorylation of fructose to fructose-1-phosphate and result in transient depletion of cellular inorganic phosphate (Pi). This leads to decreased availability of inorganic phosphate for ATP producing downstream and other pathways in the cells, creates a decreased ATP/AMP (adenosine triphosphate/adenosine monophosphate) ratio which induces activation of AMPK (AMP-activated Kinase, the cell sensor of AMP/ATP ratio) (Abdelmalek *et al.*, 2012). AMPK inhibit acyl-CoA carboxylase and consecutively decrease in malonyl-CoA level which is an important suppressor of appetite by inhibiting NPY/AgRP and activating POMC/CART (Dekker *et al.*, 2010; Ochoa *et al.*, 2015). Thus, high fructose diets have added effect if taken in combination with HFD to develop T2DM.

1.2.2.3. Mechanism of Excess Fatty Acids on Development of IR and T2DM

Free fatty acids (FFA) cause excessive production of DAG (diacylglycerol) and ceramides in tissues and induce peripheral and systemic IR. The increased FFA entrance to cells burdens the mitochondrial beta-oxidation and compromises ectopic storage as TGs. Increased FFA β-oxidation leads to increased NADH (nicotinamide adenine dineuclotide-reduced (-hydrohenated)) and ROS (Reactive Oxygen Species) production and associated mitochondrial death. In turn, decreased mitochondrial function leads to reduced rates of mitochondrial FA-oxidation. The increased influx of FFA to cells together with decreased FA-oxidation causes for accumulation of

cytoplasmic long-chain-CoA which increasingly esterified to DAG. However, the limited capacity of ectopic tissues to store neutral lipids causes for decreased rates of DAG incorporation into TG, raised accumulation of DAG in hepatocytes and myocytes (Shulman, 2014). Also, FA esterify with sphingosine to form ceramides (Samuel & Shulman, 2012).

In skeletal muscle, DAG results in the activation of members of the PKC-θ (protein kinase C family subunit-theta) which phosphorylate IRS-1/2 in serine residue /inactivated/ and activate nuclear factor kappa-B (NFκB) pathway and stimulate inflammation. Cytokines activate IκK-β (IκB (Inhibitor of Kappa light polypeptide gene enhancer in B-cells) Kinase-β) and JK (Janus kinase) both of which phosphorylate IRS-1 at serine residue and inhibit insulin signaling (Barbosa *et al.,* 2014). Also, ceramides accumulation impairs the activation of Akt in a mechanism dependent on PKC-θ (Barbosa *et al.,* 2014). Muscle IR causes for decreased translocation of GLUT-4 and uptake of plasma glucose, result hyperglycemia (Shulman, 2014).

FA over activates glucose-FA cycle and so have direct effect in increasing hyperglycemia and hyper-insulinemia. High FA flux in to myocyte leads to an increase in FA-oxidation in muscle, results in an increase in the ratio of intra-mitochondrial acetyl-CoA/CoA and NADH/NAD^+ (Nicotinamide Adenine Dinucleotide reduced/non-reduced). Higher level of NADH inactivates pyruvate dehydrogenase (PDH), causes for decline in glucose oxidation. Furthermore, excess acetyl-CoAs generate high concentration of intracellular citrates which inhibit phosphofructokinase-I (PFK-I), leads to succeeding increase in intracellular glucose-6-phosphate concentrations. Glucose-6-phosphate inhibits hexokinase activity, result in increased intracellular glucose concentrations and decreased glucose uptake by muscle from circulation. This assists the development of hyperglycemia and increased secretion of insulin (Randle *et al.,* 1963). Correspondingly, it cause for decreased access of insulin to muscle, leading to pre-receptor insulin signaling defect and hyper-insulinemia (Castro *et al.,* 2014).

Furthermore, DAG accumulation in hepatocytes causes for activation of PKC-ε (protein kinase C family subunit-epsilon) which binds to and inhibits the InsR (tyrosine kinase), leads hepatic IR. This increase hepatic glucose production and plasma glucose level (Shulman, 2014).

In addition, the excessive FA-oxidation in pancreatic islets of Langerhans contributes for: (a) increased ROS production and associated beta-cells apoptosis, and (b) increased accumulation of long-chain acyl-CoA which inhibits the insulin secretory function by opening β-cell potassium (K^+)-sensitive ATP channels (Tangvarasittichai, 2015).

1.2.3. Mechanism of Streptozotocin on Induction of Diabetes Mellitus

Streptozotocin (2-deoxy-2-[3-methyl-3-nitrosourea] 1-D-glucopyranose) is diabetogenic agent that induces stable diabetes in animal models by damaging pancreatic β-cells. STZ (Streptozotocin) toxic action involves its selective uptake into β cells. The 2-deoxy glucose moiety of STZ (structural analogue of glucose) allows its selective uptake into β cells via GLUT-2 (Eleazu *et al.,* 2013).

The reason why STZ selectively targets pancreatic cells, largely beta-cells, other than other tissues/ cell types is because their cell membrane is capable of highly expressing glucose transporter-2 (GLUT-2), a selective transporter of STZ (Pang *et al.,* 1994). Moreover, from all pancreatic cells, the beta-cell selective properties of STZ could be evidenced by its less persistent and less intact nature than non-β pancreatic endocrine cells (α- and δ-cell) and extra-pancreatic parenchyma after STZ challenge (Zhang Y., Zhang Y., *et al.*, 2012).

The possible molecular mechanisms of STZ β-cell toxicity are through carbamoylation and alkylation of DNA/cellular components, release of nitric oxide (NO), generation of free radicals and inhibition of O-GlcNAcase (O-linked glucosamine N-acetylase). Alkylation of DNA by STZ-derived carbonium ion (CH_3^+) cause extreme DNA damage and over activation of NAD^+-dependent poly ADP ribose synthetase (PARP, DNA repair nuclear enzyme), leading to extreme depletion of NAD^+ and ATP stores that causes pancreatic Islets of Langerhans beta-cell death. Free radicals nitric oxide ($NO^{\bullet-}$), superoxide ($O2^{\bullet-}$), hydroxide ($OH^{\bullet-}$), peroxynitrite ($ONOO^{\bullet-}$) generated during decomposition and metabolism of STZ diminishes the activities of mitochondrial enzymes (e.g., DNA replication and repair enzymes). This impairs mitochondria system and result in DNA damage, extreme depletion of energy (ATP) levels of cells, beta-cells destruction. Covalent modification and inhibition of OGlcNAcase (anti-hyperglycosylation post-translational modification enzyme), result in irreversibly glycosylated harmful proteins and beta-cells damage. These properties of STZ are responsible for necrosis of pancreatic β-cells and induction of experimental diabetes mellitus in laboratory animal models (Goud *et al.,* 2015).

1.3. Pathophysiology and Complications of Diabetes Mellitus

Pathophysiology of T2DM

Both IR and β-cell dysfunction occur early in the pathogenesis of T2DM, and they contribute more or less mutually to the development of pathophysiological conditions. Although IR often occurs with T2DM, it is inadequate for the T2DM development. Instead, the β-cells dysfunction is important event. It is because in early stage of IR, β-cells increase the secretory function to compensate and control hyperglycemia (Weyer *et al.,* 1999). However, IR often sets the stage for T2DM by placing a high demand on the insulin-producing beta-cells and progressively compromising beta-cells function (Kohei, 2010).

IR state not only impair carbohydrate metabolism but also protein and lipid metabolisms (Castro *et al*., 2014). These properties of IR cause for gluco-lipo-toxicity and associated apoptosis of pancreatic beta-cells, resulting beta-cells dysfunction (Kohei, 2010). In turn, β-cell dysfunction results in reduced insulin release, which is insufficient for maintaining normal glucose levels. Thus, the impairment of feedback loop between insulin secretion and insulin action is important for pathogenesis of T2DM (Zheng *et al.,* 2018).

Recently, the concept of pathophysiology of T2DM has arrived to advance from "triumvirate" (muscle, liver and pancreatic islets of Langerhans beta-cells) to the "Omnious octet" principle. The eight acknowledged pathophysiological abnormalities in development and maintenance of T2DM are: decreased peripheral glucose uptake, augmented hepatic glucose production, increased lipolysis with accumulation of intermediary lipid metabolites (like FFA), progressive deterioration of compensatory insulin secretion, concomitant inappropriate release of glucagon, "incretin defect" (inadequate gastrointestinal tract (GIT) incretin response to meal ingestion facilitate impaired insulin and excessive glucagon secretion), hypothalamic IR (with an elevated sympathetic drive, impair the ability of circulating insulin to suppress glucose production) and enhanced renal tubular glucose reabsorption capacity (Cersosimo *et al.,* 2018).

Complications of Diabetes Mellitus

All types of diabetes mellitus can result complications in many parts of the body, ensuing recurrent hospitalizations and increase the overall risk of premature mortality. Basically, untreated diabetes cause acute and chronic complications. Acute complications include hypoglycemia, hyperglycemic crises (keto-acidosis & hyperosmolar coma) or death (WHO, 2016)**.** Chronic hyperglycemia of diabetes is linked to long-term damage. The general and major categories of chronic complications involve: (a) micro-vascular complications such as retinopathy,

nephropathy, peripheral and autonomic neuropathies and lower extremity disease, and (b) macro-vascular complications such as cerebro-vascular disease/stroke, coronary artery disease and peripheral vascular disease due to atherosclerosis of large vessels (Baynest, 2015).

Complications of T2DM are very common because of its asymptomatic development. In an observational study of 28 countries in Asia, Africa, South America and Europe, about 50% and 27% of T2DM patients presented micro-vascular and macro-vascular complications, respectively (Zheng *et al.,* 2018). Most T2DM patients die from cardiovascular complications and end stage renal disease (Baynest, 2015). Moreover, T2DM increase the risks of disorders in hepatic (e.g., fatty liver disease) and musculoskeletal systems (Zheng *et al.*, 2018).

1.4. Management of Diabetes Mellitus

Early diagnosis and intervention are the two principal management strategies. The longer a person lives with undiagnosed and untreated diabetes, the worse their health outcomes. Diabetes outcomes can be reduced by having series of cost-effective interventions such as control of blood glucose via a combination of diet, physical activity and medication; control of blood pressure and lipids to reduce cardiovascular risk and other complications, and regular screening for damage to the eye, kidney and feet to facilitate early treatment (WHO, 2016).

The abnormalities underlying T2DM are reversible by having lifestyle modifications, like reducing dietary energy intake and having regular physical exercise. Normalization of both hepatic insulin sensitivity and beta-cell function in T2DM can be achieved by dietary energy restriction alone, which is associated with reduced TGs stores on those tissues (Lim *et al.,* 2011).

The major classes of modern drugs for diabetes management are described in Table-1.

Table-1: Modern Drugs for Management of Diabetes Mellitus and Their Modes of Action

Drug name	Mechanism of actions	Remark
1. Metformin	Mainly, it inhibits mitochondrial respiratory chain complex –I (NADH coenzyme Q oxido-reductase) which leads to increased AMP that directly inhibit adenylate cyclase and fructose-1,6-bisphosphatase and activate AMPK. Suppresses mitochondrial glycerol-phosphate dehydrogenase which affects transport of NADH from the cytoplasm into mitochondrion and leads to decreased conversion of lactate and glycerol to glucose. Improves gut microbiota by boosting beneficial microbial community profile and activating mucosal AMPK which retain gut integrity. Arouses production of GLP-1 in intestine and its receptor in pancreas	Pearson & Sakamoto, 2013; Hur & Lee, 2015
	Decrease hepatic glucose production. Improves insulin sensitivity by enhancing the expression and activity of InsR (tyrosine kinase). Lowers plasma lipid levels through a PPAR-α (Peroxisome Proliferator-Activated Receptor- alpha subunit) pathway, and reduces food intake by its incretin-like actions, induce modest weight loss	Chaudhury *et al.*, 2017
	DPP-4 (dipeptidyl peptidase-4) inhibitory, antioxidant, anti-hyperlipidemic, cardio-protective and anti-inflammatory properties	Suman *et al.*, 2016a
	First-line medication for the treatment of T2DM	ADA, 2019b
2. Sulfonylureas and meglitinides	Insulin secretagogues: increase insulin secretion by blocking ATP-sensitive K^+ channels located in the membrane of pancreatic beta-cells, and cell membrane depolarization to increase cytoplasmic Ca^{2+} levels which again increase insulin secretion.	Marín-Peñalver *et al.*, 2016
	Sulfonylureas limit gluconeogenesis in the liver, and decrease lipolysis and reduce clearance of insulin in the liver. Sulfonylureas are second line treatment options for T2DM.	Chaudhury *et al.*, 2017
3. SGLT2 Inhibitors	Promote glucosuria by blocking glucose reabsorption in proximal tubule.	Chaudhury *et al.*, 2017
4. DPP-4 inhibitor	Prevent degradation of GLP-1 by DPP-4.	
5. Glucosidase & amylase-inhibitors	Delay the digestion of starch in the small intestines to alleviate postprandial hyperglycemia.	
6.Thiazolidine diones	PPAR- γ (-gamma subunit) agonist to facilitate glucose uptake in peripheral tissues and increases insulin sensitivity.	
7. Insulin	Activates InsR and downstream signaling in multiple sensitive tissues. It can be used in the treatment all types of diabetes	(Marín-Peñalver *et al.*, 2016
8.GLP-1 Recep-tor Agonist	Augments insulin secretion and decrease secretion of glucagon.	
Limitations/side effects of market drugs	Metformin cause mild gastrointestinal fluxes; vitamin B12 and folic acid deficiency, and reduction in its efficiency as diabetes progresses.	Chaudhury *et al.*, 2017; ADA, 2019b
	Sulfonylureas, meglitinides & insulin: risk of hypoglycemia & weight gain. Glucosidase & amylase-inhibitors mainly have gastrointestinal risk (flatulence, diarrhea & abdominal pain); rare hypoglycemia.	Marín-Peñalver *et al.*, 2016; ADA, 2019b
	GLP-1 receptor agonist (risk of thyroid C-cell tumors); thiazolidinediones (risk of congestive heart failure & weight gain); SGLT2 inhibitors (canagliflozin-amputation & bone fracture) are under FAD black box.	

1.5. Traditional Medicine and Medicinal Plants in Management of Diabetes Mellitus

1.5.1. Traditional Medicine

Traditional medicine is the sum total of knowledge, skills and practices based on theories, beliefs and experiences indigenous to different cultures that used to maintain health and to prevent, diagnose, improve or treat physical and mental illnesses (WHO, 2010). Approximately 70-80% of the world, 80% of developing countries (like Asia and Africa) (WHO, 2010) and about 80% of Ethiopia populations (Yadav, 2013) relied on traditional medicine.

Traditional medicines have prolific contributions for modern medicine. Natural products (as standardized extract or pure compound) provide boundless opportunities for new drug discoveries. It is because of their unique and supreme diversity of chemical structure and biological activities (Yuan *et al.,* 2016). Plant derived therapies take the foremost part, proved by the fact that many pharmaceuticals commonly used today are structurally derived from plants (Ota & Ulrih, 2017).

Many plant preparations have been scientifically investigated and confirmed as efficient against diabetes mellitus (Piero *et al.,* 2012). For instance, the development of Metformin is traced to the traditional use of the plant *Galega officinalis L.* (Goat's rue) (*Fabaceae*), which is rich in Guanidine and its isoprenyl derivative-Galegine, to treat diabetes (Ota & Ulrih, 2017).

Thus, traditional systems of medicine and medicinal plant research have become topics of global interest and importance. The WHO (World Health Organization) encouraged researchers to examine any beneficial clinical results the traditional medicines produced (WHO, 2010). Also, the government of Ethiopia supports and encourages traditional medicine through its policies despite sustainable use of traditional medicine and their integration with modern medical practice has been limited (Kassaye *et al.,* 2006).

1.5.2. Bioactive Phytochemicals with Anti-diabetic Properties and Their Mechanisms of Action

Phytochemicals identified from medicinal plants have promising opportunity for the development of new types of therapeutics for diabetes mellitus. The most prevalent bioactive phytochemical classes present naturally in many plants and known to possess potent anti-hyperglycemic activity are flavonoids, terpenoids, saponins, alkaloids, glycosides, steroids, polysaccharides, tannins, peptides, amines, peptidoglycans, guanidine and glycopeptides (Mentreddy, 2007; Semwal *et al.,* 2007; Gaikwad *et al.,* 2014; Bharti *et al.,* 2018).

The possible mechanisms of action of natural products from different plants against diabetes mellitus are: delaying and inhibition of food digestion and absorption in GIT (e.g., carbohydrate metabolizing enzymes); improvement of gut micro-biota; regulation of hepatic glucose metabolism (by restraining glycogenolysis and gluconeogenesis; augmenting hepatic glucose storage (glycogenesis)); increasing peripheral utilization of glucose, glycolysis and Krebs cycle; activation of the PPAR and AMPK; up regulation of lipolysis/beta-oxidation, inhibition of lipogenesis and cholesterol synthesis; inhibition of DPP-4 (Bharti *et al.*, 2018); enhancement of insulin secretion, pancreatic beta-cell proliferation and differentiation; activation of insulin signaling pathways, expression and translocation of GLUT-4; inhibition of pathways that impair insulin signaling (e.g., PTP1B (Protein Tyrosine Phosphatase 1-Beta subunit) activity & Nuclear Factor kappa B) (Governa *et al.*, 2018), anti-inflammatory, anti-apoptosis and anti-oxidative stress (Farzaei *et al.*, 2017). It is difficult to make definite suggestion about the main mechanism of action of a specific phytochemical class. It is, in part, because each phytochemical class has diverse mechanisms of action (Bharti *et al.*, 2018).

1.5.3. Medicinal Plants for the Management of Diabetes Mellitus

Plant based and traditional medicines are the basis in search of cure for diabetes. Some of plants that are most frequently used to help manage blood glucose include: *Momordica charantia (*Bitter melon*), Coccinia grandis (*Ivy gourd*), Trigonella foenum graecum (fenugreek), Gymnema sylvestre, Opuntia spp.* (nopal), Ginseng species (*panax ginseng* and *quinquefolius*), *Artemisia dracunculus* (Russian tarragon), *Cinnamomum cassia* (Cinnamon), *Plantago ovata*, *Zingiber officinale Roscoe* (Ginger) and *Allium sativum* (Garlic) (Ota & Ulrih, 2017).

On the other hand, anti-diabetic plant species that are enlisted in WHO monographs and supported by clinical data, or described in pharmacopoeias and in traditional systems of medicine comprise: *Ocimum tenuiflorum L., folium; Trigonella foenum-graecum L., semen; Allium cepa L., bulbus; Azadirachta indica A. Juss., folium; Momordica charantia L., fructus; Panax ginseng C.A. Meyer, radix; Panax quinquefolius L., radix, and Rehmannia glutinosa (Gaertn.) DC., radix.* The first two are supported with clinical data (Governa *et al.*, 2018).

In Ethiopia, as per the review done by TMMRD (Traditional and Modern Medicine Research Directorate) of EPHI (Ethiopian Public Health Institute), about 105 plant species were reported for claim of anti-diabetic activity. Among these plants *Moringa stenoptela, Allium sativum, Caylusea abyssinica, Ajuga remota, Calpurnia aurea, and Psidium guajava* were the most frequently indicated medicinal plant species. The root part is the second most frequently employed part next to leaf in the anti-diabetic herbal preparations (Meresa *et al*, 2017).

1.5.4. Anti-diabetic Plants in the Family *Cucurbitaceae*

The family *Cucurbitaceae*is the plant family in which the experimental plant of this study, *C. ficifolius* (*Cucumis ficifolius A. Rich*), belongs to. The family *Cucurbitaceae* consists of about 130 genera and 800 species **(**Rajasree *et al.*, 2016). Plants under the family *Cucurbitaceae* have many medicinal and nutritional benefits. It is one of many plant families confirmed to have anti-diabetic activity. The various phytochemicals extracted from the plants are reported to have anti-diabetic, antioxidant, anti-hyperlipidemic, anti-inflammatory activities, and against many other diseases (Dhiman *et al.*, 2012; Saboo *et al*., 2013; Rajasree *et al*., 2016).

The major phytochemicals present in *Cucurbitaceae* family are: glycosides, terpenoids, flavonoids, saponins, tannins, steroids, carotenoids, resins, and most commonly the terpenoid substance called cucurbitacins-give bitter property. Also, they contain carbohydrates, alkaloids and rich in proteins with many biological roles (Sood *et al.,* 2012; Rajasree *et al.,* 2016).

The plant species that are classified under the same family (e.g., *Cucurbitaceae*) and the same genus (e.g., *Cucumis*) have many shared phenotypic and genotypic properties. They, most probably, have communal phytochemicals with shared medicinal and nutritional values **(**Telford *et al*., 2011; Saboo *et al*., 2013; Rajasree *et al.,* 2016). Based on this general conception, the plant species in this family *Cucurbitaceae* and more closely in the genus *Cucumis* are described as **"sister species"** of *C. ficifolius* in this study.

Thus, purposively, their antidiabetic values are described here. From the many plants of the family *Cucurbitaceae* that have shown anti-diabetic activity, the most extensively studied are summarized in Table-2as follows.

Table-2: Summary of Anti-diabetic Activities of Plant Species under the Family *Cucurbitaceae* and Genus *Cucumis*

Plant Species	Anti-diabetic value and mechanism of action	Phytochemicals	Remarks
1. *Momordica charantia Linn Fructus* (Bitter melon/-gourd)	Antihyperglycemic activities: inhibition of glucose reabsorption in guts, suppression of gluconeogenic enzymes, increase of peripheral glucose utilization, insulin secretion and preservation of islets of Langerhans β-cells and their functions	Triterpenoids, polypeptide-p, vicine (alkaloid) and glycosides	Chang *et al*., 2013; Sarkar *et al*., 1996; Ota & Ulrih, 2017
	Ameliorate IR & dyslipidemia: by boosting the activity of AMPK, and diminishing expression of PEPCK & FA synthesis in high-fat fed mice	-	Joseph&Jini , 2013; Shih *et al*., 2014
	Increasing glucose uptake through stimulation of GLUT-4 translocation and increasing the phosphorylation of AMPK and IRS-1	Cucurbitane-type triterpenoids	Cheng *et al*., 2008; Ma *et al*., 2010

	Activate AMPK & stimulate GLUT-4 translocation, increase insulin sensitivity & FA-oxidation	Triterpenoids(momordicoside S & T, Karaviloside XI)	Tan *et al.*, 2008
	Activates PPARs and up regulates expression of the acyl-CoA oxidase gene (FA-oxidation)	-	Chao&Huang, 2003
	Insulin secretion in beta-cells and glycogen synthesis	Vicine, glycosides, polypeptide-p (plant insulin), & saponins	Khanna*et al.*, 1981; Sarkar *et al.*, 1996;Joseph & Jini, 2013
	Antioxidant properties: prevents oxidative damage in diabetic nephropathy in rats	-	Teoh *et al.*, 2010
	Capsules or tablets in brand name "Gourdin", "Karela" & "Glucobetic" is commonly marketed in the USA, Canada, UK, India & many Asian		Rahman *et al.*, 2015
2. *Coccinia grandis L. Voigt* (*Coccinia indica,*Ivy gourd)	Ayurvedic practice as an antidiabetic drug: insulin mimetic, insulin secreting and gluconeogenesis inhibitory effects	-	Ota & Ulrih, 2017
	Decrease glucose-6-phosphatase and lactate dehydrogenase level, and improve LPL activity, as per trial done on diabetic patients	-	Hossain *et al.*,1992; Kamble *et al.*, 1998
	Anti-obesity and anti-hyperlipidemic effect: fruit part in obese female rats	-	Ahmed & Manoj, 2012
3. *Cucurbita ficifolia* (Fig-leaf gourd)	Anti-hyperglycemic, antioxidant and anti-hyperlipidemic effect, and improve pancreatic β cells function.	Flavonoids, phenol, alkaloids, rich vegetal glutathione peroxidase &superoxide dismutase	Bayat *et al.*, 2014
	Fruit-significantly decreased blood glucose, plasma TC and TG levels.	-	Xia & Wang, 2007
	Anti-hyperglycemic effect (fruit extract) in STZ- induced rats	Higher D-Chiro-Inositol	Xia & Wang, 2006
	improved function of β cells, and the number of beta-cells per islets of Langerhans	-	Acosta-Patino *et al.*, 2001
	Antioxidant: by improving GSH redox state and increasing glutathione pool. decreased glucose, and plasma and pancreas lipid peroxidation, malondialdehyde level	Phenols	Liu Y, Jin H.,*et al.*, 2006; Diaz-Flores *et al.*, 2012
4. *Cucurbita pepo Linn* (Pumpkin)	Anti-hyperglycemic, anti-hyperlipidemic & antioxidant activities, Repair and restoration of pancreatic tissue. Fruit powder is responsible.	Rich phenolic compounds/flavonoids & polysaccharides	Sedigheh *et al.*, 2011
5. *Trichosanthes cucumerina* (Long tomato*)*	Anti-inflammatory & anti-diabetic activities by the root tubers & seeds, respectively	-	Adebooye, 2008
	Anti-hyperglycemic effect-Fruit pulp extract	Rich triterpenoids, saponins, flavonoids, carotenoids, phenolic acids, ascorbic acids.	Kolte *et al.*, 1997
6. *Citrullus colocynthis* (Bitter apple)	Anti-hyperglycemic effect: fruit part	carbohydrate,saponins, glycoside,steroid&resin	Verma *et al.*, 2015
	Promote insulin secretion & glycogen synthesis	Glycosides	Bharti *et al.*, 2018

	Root-significant reduction in serum level of glucose, creatinine, urea and protein.	-	Agarwal V., SharmaAK., *et al.*, 2012
Genus Cucumis			
7. *Cucumis sativus Linn* (Cucumber)	Potential anti-diabetic, lipid lowering and antioxidant activity Different parts: fruit, leave, and seed parts	alkaloids,flavonoids, tannins, cucurbitacins, terpene, phenol,vitexin, saponins, cucumerin-A &B, glycosides,orientin	Mukherjee *et al.*, 2013; Saidu *et al.*, 2014
	Regeneration of damaged pancreatic islets of Langerhans cells, stimulate glucose and calcium uptake, and anti-oxidant activity	Flavonoisa(flavonoid), tanins & ascorbic acids	Mukherjee *et al.*, 2013
	Lipid lowering activity	Pectin	Sudheesh & Vijayalakshmi, 1999
	Hypo-cholesterolemic effect	Steroids	Sood *et al.*, 2012
	Antioxidant	Tannins	
	Cardio-protective	Cardiac glycosides	(Abou-Zaid *et al.*, 2001)
8. *Cucumis melo* (musk melo/ Cantaloupe)	Anti-diabetic, anti-inflammatory, free radical scavenging and hepato-protective activities.	Flavonoids, terpenoids, glycolipids, ascorbic acid, β-carotenes, FAs, carbohydrates, amino acids, phospholipids minerals, &volatile one	Vishwakarma *et al.*, 2017
	Reduce serum lipids and hyperglycemia	Rich in polyphenols and ascorbic acids	Parmar & Kar, 2008
	Antioxidant and anti-inflammatory properties: Fruit extract.	Oxykine **(**rich in vegetal superoxide dismutase)	Vouldoukis *et al*, 2004; Naito *et al.*, 2005
	Anti-obesity and anti-hyperlipidemic activity: fruit peel extracts	-	(Bidkar *et al.*, 2012).
9. *Cucumis metuliferus*	Anti-hyperglycemic activity- fruit pulp extract	Alkaloids, saponins, tannins flavonoids, cardiac glycosides, steroids & cucurbitacin	Jimam *et al.*, 2010; Usman *et al.*, 2015
	Anti-hyperglycemic activity- fruit pulp	Glycosides	Gotep, 2011
10. *Cucumis prophetarum Linn*	Anti-diabetic, antioxidant & anti-inflammatory: by inhibiting alpha-amylase and alpha-glucosidase and reducing DPPH (2, 2-Diphenyl-1-picrylhydrazyl), superoxide radical and chelation of metal. Fruit part is responsible	Glycosides, saponins, sterols, carbohydrates, reducing sugars & amino acids.	Gawli & Lakshmidevi, 2015
11.Cucumis callosus	Anti-hyperglycemic: ethanol extract of fruit	Tannin, flavonoid, alkaloid, carbohydrate, glycosides & saponins	Verma *et al.*, 2015

1.5.5. The Experimental Plant *C. ficifolius A., Rich* (*Cucurbitaceae*) and Its Medicinal Values

The experimental plant species named as *Cucumis ficifolius A., Rich (C. ficifolius)* is grouped under class Angiosperm, family *Cucurbitaceae* and genus *Cucumis* (Garg *et al.*, 2007). The plant *C. ficifolius* is perennial usually prostrate herb stems up to 1meter long, hairy with coarse in the presence or absence of aculeate hairs and also with finer intermixed spreading hairs, and basal stem older, thickened with light/whitish colored somewhat scaly fissured bark, arising from a thickened root stock (see Figure-1 below). The habitation involves grassland, wooded grassland, acacia woodland, rocky slopes, secondary vegetation and cultivated places (Gelana, 2011). The plant found in altitude ranging from 1070–2700 meters above sea level. It is found in Africa largely in Ethiopia, Uganda, Kenya, Rwanda, and Tanzania (ITHAKA, 2016).

In Ethiopia, it is distributed in different parts of regions such as in Shoa, Gonder, Welega, Tigray, Gamo-Gofa, Gojam, Fiche, Sidama, Harerge, Somali and Afar. It has got different vernacular names in different languages: Yemidir-embuay=Amharic; Surupha= Sidamagna (Chekole *et al*., 2015); Lominbita/ Ramboramb =Tigrigna (Araya *et al*., 2015); Hidiihooloo/ Faca'aa/Hanchote= Oromiffa (Lulekal *et al.,* 2008; Bussmann *et al.,* 2011), and Anun demerit=Somaligna (Meragiaw & Asfaw, 2014).

Leaf blade in the presence or absence of ovate in outline, weakly cordate or sub-truncate at the base, somewhat sinuate toothed, very rough hairy above and beneath, 20–71 mm (milimeter) long, 20–74mm broad, 0.8–1.2 times as long as broad, palmately 3–5 lobed. Lobes are rather rounded above, somewhat narrowed below, with the central lobe largest, sometimes itself in the presence or absence of 3 lobulate, 20–53mm long, 0.7–0.9 times as long as the whole blade, narrowed at its base to 0.3–0.9 of its breadth at its broadest. Petiole is 10–35mm long, hairy like the stems. **Male flowers** solitary on 3–12mm long pedicels; Receptacle tube 2.5–4mm long; 1.5–2mm long fusiform lobes ; Corolla has yellow color, 4–7mm long and 2–3.5mm broad lobes and united below. **Female flowers** on 6–12mm long stalks; ovary ellipsoid, 5–7mm long, 3–3.5mm across, covered with forward pointing hyaline bristles; Receptacle tube 3–4mm long; 1.5–2mm long, filiform subulate lobes, and5–9mm long and 2.5–5mm broad corolla lobes. **Fruit** on a 7–25mm long stalk, bluntly ellipsoid, 23–50(–88) mm long, 12–30(-63) mm across, beset with rather closely scattered broadly conical low pustules each ending in a broad blunt bristle derived from the hairs of the ovary, dark green with 10 longitudinal pale green or grey green longitudinal stripes, ripening deep yellow. **Seeds** are ellipsoid, 5×2.5×1.2mm. **Roots** are taperoots growing deep in to soil, may be with lateral branches (Gelana, 2011; ITHAKA, 2016).

Figure-1: Morphology of the Plant *C. ficifolius A., Rich* (Photograph Taken during Plant Collection in the Field)

Medicinal values

There is no scientific study done about medicinal role of *C. ficifolius*, worldwide. This may be because this plant is distributed in limited areas, largely in Africa. In Ethiopia, it is widely distributed in most places. As a result, the people use this plant for treatment of many ailments which is confirmed by different ethno-botanical studies (discussed below). Moreover, the local traditional medical practitioners in Ethiopia (e.g., in Amhara region) recommends root part of *C. ficifolius* for treatment of diabetes mellitus (based on personal communication data source). They powder the root part, mix with honey and give three spoons per day for diabetic patients.

As per the ethno-botanical studies done in Amhara region in Ethiopia, the roots of *C. ficifolius* is used against bloody diarrhea (crush; mix with milk, and then sniff, drink and fumigate concoction), stomach ache & anthrax (peel, chew then swallow juice, or crush and drink with water). Fruit help to heal wound, and shoot help to expel ear mites (Chekole *et al*, 2015). In another related study, the roots of *C. ficifolius* are used for treatment of meningitis (root powder mixed

with honey taken orally until cure), rabies (crush fresh root together with water, leave it to ferment for 3-days, mix with honey and take orally early morning before breakfast till cure), stomach-ache (chewing fresh root and swallowing the juice), wound (tying fresh root around the injury until cure), amoebic dysentery (chewing fresh root and swallowing the juice before breakfast for 3-days). Wart can be treated by topical application of leaf and root powder of this plant (Teklehaymanot, 2009).

According to ethno-botanical study in Tigray region, roots of *C. ficifolius* are chewed for eye disease, jaundice, tuberculosis, snake bite, stomach ache following delivery and tooth ache. Roots and leaves are used for eczema (ground powder, mixed with honey and dressed on affected area). Leaves are used for treatment of anthrax, and fruits to treat ear infection, asthma and tetanus (Araya *et al*., 2015).

In another study, roots of *C. ficifolius* are used for toothache (by chewing part with the diseased tooth); for joint pain (crush, filter and drink the fluid); for abdominal pain (mix root of the plant with bark of *Croton across tachyus*, dry the paste, mix it with butter and drink it or chew the product and drink the fluid); for snake, scorpion and black spider bite (grind root, mix with honey and eat). Leaf is used for anthrax and tonsillitis; fruits for wound healing, and whole part is used for jaundice (Teklay *et al.*, 2013).

The ethno-botanical studies done in Oromiya region, Ethiopia showed the root and fruit parts of *C. ficifolius* are used for lung disease (crush the fresh form and drink) and gonorrhea (crush the fresh or dried form and drink). The root parts is used against jaundice (crush fresh /dried form, boil and drink), rabies (crush the fresh/dried form, boil and drink) (Lulekal *et al.*, 2008) and sudden illness (e.g. collapses or sudden stomach problems) (crush and drink with coffee). Fruits are used for wound (Bussmann *et al.*, 2011).

The fruit is used as an abortifacient for women, as an emetic, treatment of syphilis, stomach ache for children (in small doses with honey), meningitis, epistaxis, jaundice, anthrax, eczema, and also the roots is remedy for malaria (Teklehaymanot & Giday, 2007). As per the ethno-botanical studies done in Ethiopia, whole part of this plant also has antimalarial activity (Mesfin *et al*., 2012; Meragiaw & Asfaw, 2014). In Kenya, roots are used as remedy for malaria (Bioversity International – FAO, 2014). Moreover, *C. ficifolius* has antimicrobial activity. The hydro-methanol, acetone and ethylacetate leaves extracts has antibacterial activity against *Pseudomonas aeruginosa, Staphylococcus aureus and Shigella boydii* (Gelana, 2011).

Besides, *C. ficifolius* root *has* benefit on preparation of beverages, to make local honey-wine more powerful/alcoholic (Gelana, 2011). In addition to medicinal values for human, *C. ficifolius* also

has veterinary use. The fruit can be used as a medicine for fowls, as a vermifuge for horses by the Hausa people in Nigeria, and hurry expulsion of the placenta for cows in Ethiopia. This is used as grazing for all stocks in Kenya, Somalia, Sudan, Uganda, Mauritania and Senegal, and recommended for good milk production (Burkill, 1985; Gelana, 2011).

Generally, the medicinal values of *C. ficifolius* are summarized in Table-3 below.

Table-3: Summary of Traditional Medicinal Importance of *C. ficifolius*

Part used	**Medicinal value against**	**Remarks**
Root	Stomach ache, bloody diarrhea, & anthrax	Chekole *et al,* 2015.
	Stomach-ache, meningitis, wound, amoebic dysentery	Teklehaymanot, 2009
	Stomach ache following delivery, tooth ache, eye disease, jaundice, tuberculosis, snake bite,	Araya *et al*., 2015
	Abdominal pain, joint pain, toothache, snake, scorpion and black spider bite	Teklay *et al.,* 2013
	Jaundice, rabies	Lulekal *et al.,* 2008
	Sudden illness, e.g. when someone collapses	Bussmann *et al.,* 2011
	Malaria	Teklehaymanot & Giday, 2007; Bioversity International-FAO, 2014
	Beaverage: honey-wine, to make alcohol more powerful	Gelana, 2011
Root + Leaf	Wart	Teklehaymanot, 2009
	Eczema	Araya *et al*., 2015
Root + fruit	Lung disease & gonorrhea	Lulekal *et al.,* 2008
Leaves	Anthrax	Araya *et al*., 2015;
	Anthrax, tonsillitis	Teklay *et al.,* 2013
	Antimicrobial (antibacterial)	Gelana, 2011
Shoot	Expel ear mites	Chekole *et al, 2015*
Fruits	Wound healing	Bussmann *et al.,* 2011; Chekole *et al, 2015*
	Ear infection, asthma & tetanus	Araya *et al*., 2015
	Abortifacient for women, emetic, syphilis, stomach ache for children, meningitis, epistaxis, jaundice, eczema	Teklehaymanot & Giday, 2007
Whole part	Jaundice	Teklay *et al.,* 2013
	Antimalarial	Mesfin *et al*., 2012; Meragiaw & Asfaw, 2014
Veterinary uses:	Medicine for chickens& vermifuge for horses (Fruit); For hurry expulsion of the placenta for cows; Grazing for all stock, &advised for good milk production	Burkill, 1985; Gelana, 2011

In general, if *C. ficifolius* have these much medicinal roles other than diabetes, the anti-diabetic properties told by local traditional medical practitioners (personal communication data source) could be true. Also, most sister species of *C. ficifolius* (found in the same family *Cucurbitaceae* and genus *Cucumis*) have anti-diabetic properties, inspiring evidences.

1.6. Statement of the Problems

Diabetes mellitus, largely T2DM, is one of the leading and fastest growing health and socioeconomic disasters of the 21st century in the world, Africa and Ethiopia with no country immune from it (IDF, 2019). What makes T2DM annoying & most disastrous is, it chiefly affects productive manpower (adults) who are the pillar of whole aspect of the world and a specific country (IDF, 2019). How broad the gaps are discussed in detail as follows.

Very high health burden of diabetes mellitus

The human health catastrophe (illness and mortality) of diabetes is very high and rapidly increasing from year to year in the world with no country immune from it. WHO global report, diabetes cases increased from 108 million (4.7%) of 18+ ages in 1980 to422 million (8.5%) in 2014, and caused 3.7 million deaths in 2012 (WHO, 2016). As per IDF (International Diabetes Federation) report, the global diabetic cases in 20-79 age increased from 151 million in year 2000 to 463 million (i.e., 9.3% prev. (prevalence); 1/11 adults is diabetic; 50.1% undiagnosed) in 2019, and expected to be 700.2 million (10.9% prev.; 51% rate) in 2045. There were about 4.2 million mortality (1death/8 seconds; 11.3% of all-cause mortality) in 2019 (IDF, 2019).

In Africa region, WHO stated, diabetes caused illness of about 4 million (3.1%) adults (18+ ages) in 1980 and 25 million (7.1%) in 2014 (WHO, 2016). There were about 19.4 million (3.9%; 59.7% undiagnosed- highest of IDF regions) diabetic adults 20-79 age in 2019, and will be 47.1 million (4.4%; 143% rate) in 2045. It caused 366,200 deaths in 2019 (IDF, 2019).

In Ethiopia, the health risk of diabetes is increasing rapidly. There is limited national prevalence study. As per hospital based studies, the incidence of diabetes admission from all medical admissions was increased 1.9% (in 1970) to 9.5% (in 1999), and estimated cases of 0.8million in 2000 (Feleke & Enquselassie, 2005). According to the national community-based survey among people of 15-69 age, the prevalence of diabetes including those on medication was 3.2% (84.4% newly diagnosed) by the year 2015 (Gebreyes *et al.*, 2018). As per IDF report, in Ethiopia, there were about1.7 million cases (3.2%; ranked 4th in Africa in number of cases; 68.2% undiagnosed) and 23,157 deaths in 2019. Ethiopia will be one of top 10 countries of the world in number of adults 20-79 age with impaired glucose tolerance (14.7 million) in 2045 (IDF, 2019).

Large scale economic burden of diabetes mellitus

The economic burden of diabetes, chiefly T2DM, is distressing the whole world by increasing both direct and indirect cost (IDF, 2019).The average global absolute (direct and indirect) economic loss of diabetes mellitus in adults (20-79 age) in 2015 was 1.31 trillion USD(i.e.,1.8%

of global gross domestic product, GDP) (Bommer C., Heesemann E., *et al* 2017), and will be 2.2 trillion USD (2.2% GDP) by 2030 (Bommer C., Sagalova V., *et al* 2018). Also, the direct global loss of diabetes in 20-79 age has been rapidly growing from USD 232 billion in 2007 to 760.3 billion (10% global health costs) in 2019, and will be 845 billion in 2045 (IDF, 2019).

In Africa, diabetes care expenditure in 20-79 age was increased from USD 3.3 billion in 2017 (IDF, 2017) to 9.5 billion in 2019, and will be 17.4 billion in 2045 (IDF, 2019). In Ethiopia, diabetes related expenditure in 20-79 years was 126 million USD in 2017 (IDF, 2017), and increased to 192.54 million USD in 2019 (IDF, 2019). Table-4 precisely summarizes it.

Table-4: The Health and Economic Burden of Diabetes Mellitus in past, present and future

1	**Very high health burden of Diabetes in adults (18^{+} age for WHO; 20-79 age for IDF)**			
	Numbers (#) of Diabetic Cases in millions (% prevalence)	**Undiagnosed**	**Deaths**	**Remarks**
Globally	108 (4.7%) (in 1980)►422 (8.5%) in 2014	-	3.7mil. in 2012	WHO, 2016
	151 (in 2000)► 463 (i.e., 9.3%; 1/11 adults is diabetic) (in 2019)► will be 700.2 (10.9%; 51% rate) in 2045	50.1%	4.2mil.(1 death/8 sec; 11.3% of all mortality) in 2019	IDF, 2019
Africa	4(3.1%)(in 1980)►25(7.1%) in 2014	-	-	WHO, 2016
	19.4 (3.9%) (in 2019)►will be 47.1 (4.4%; 143% rate) in 2045.	59.7%	366,200 (6.8% of all mortality)	IDF, 2019
Ethiopia	Hospital based studies: of all medical admissions about 1.9% (in 1970) ►9.5% (in 1999), and then►0.8million (in 2000).	-	7% of all deaths >55 ages in medical wards	Feleke & Enquselassie ,2005
	National CB Survey: among 15-69 age=3.2%	84.4%	-	Gebreyes *et al.*, 2018
	1.7(3.2%; ranked 4th in Africa in # of cases	68.2%	23,157	IDF, 2019
2	**Large-scale economic loss (USD) by Diabetes Mellitus** (adults 20-79 age)			
Globally	Absolute cost=1.31 trillion USD (i.e., 1.8% of global GDP; indirect cost =34.7%) in 2015; ►Will be 2.2 trillion (i.e., 2.2% GDP) by 2030.			Bommer *et al*, 2017 ;2018
	Direct cost (billions):232 (in 2007)►727 (in 2017)►760.3 (in 2019) ►will be845 in 2045			IDF,2017; 2019
Africa	Direct cost (bil.): 3.3 (in 2017)► 9.5 (in 2019), and ►will be 17.4 (in 2045)			
Ethiopia	Direct cost (millions) among 20-79 age:126 (in 2017)►192.54 (in 2019)			

What enables diabetes this much disastrous and problematic?

Complicated features of diabetes largely T2DM such as heterogeneity in etiology, asymptomatic development, and higher cost of hospitalization are primary factors making its prevention and management difficult (Ta, 2014; WHO, 2016; IDF, 2019). Abnormal living standard of world population such as increment of urbanization, physical inactivity, over/unhealthy nutrition and obesity are key challenges ofT2DM (WHO, 2016). The presence of inadequate quality of care like insufficient diabetes education, low prioritization of diabetes screening and scarcity of resources worsen the complexity of diabetes prevention and management, primarily for low and middle-income countries including Ethiopia (Nigatu, 2012; WHO, 2016). As a result, the greatest increase

in rates has been recognized in these countries than developed ones, and accounted for 79.4% of diabetic and 84.3% of undiagnosed cases of world in 2019 (IDF, 2019).

Moreover, though there are many types of modern drugs for treatment of diabetes, their limited efficacy to cure, side effects, low access, higher cost and complex regimen is another challenge, cause poor adherence to medications (Tangvarasittichai, 2015; Edelman *et al.*, 2017).

In order to escape all the above challenges, most of people in the world (75%) and Ethiopia (80%) preferred to relay on traditional medicine (WHO, 2010; Yadav, 2013). Of course, traditional medicinal plant-based treatments have their own benefits: easily accessible, relatively cheaper, generally safe (natural) and even some of them are very effective with minimal side effect (Piero *et al.*, 2012; Ota & Ulrih, 2017). However, unlike modern drugs, it has its own limitations; mainly associated with toxicity and efficacy (not all are efficient as modern). The toxicity of herbal preparations could arise from inherent toxicity of target plant, dose-related, irregular use, higher chance of adulteration and undesired reaction among poly-herbal preparations. Also, the presence of inadequate registered practitioners in the population causes for increased acquisition of herbs from unknown sources (Abdel-Aziz *et al*, 2016).

Although the plant *C. ficifolius* root has been reported as candidate for treatment of diabetes by traditional medical practitioners in Ethiopia, there is no any scientific report proving this and its safety. Thus, the target plant *C. ficifolius* is in the borderline of these debates: either it has good efficacy with low side effects as that of market drugs, or has good efficacy but with toxic effects, or has no efficacy with or without toxic effects.

These were some of the gaps that magnify why doing this research project was needed and how much essential. Thus, the scientific validation of *C. ficifolius* root anti-diabetic property is very important and inspiring to reduce the health and socioeconomic misfortune of T2DM.

1.7. Significance of the Study

This research helps to scientifically validate the traditional use of *C. ficifolius* root against diabetes mellitus. That means, it provides clue about an alternative treatment opportunity of *C. ficifolius* root to fight diabetes mellitus. Accordingly, it has its own value in filling the above gaps.

There is no scientifically published data available about the anti-diabetic activity and other medicinal values of *C. ficifolius.* This study, for the first time, scientifically announces *C. ficifolius* (specifically root part) and its anti-diabetic importance. As a result, it can be used as an alternate based medicine and enables further research activity.

In Ethiopia, there is limited experience in the scientific confirmation of traditional medicinal plants for treatment of diabetes, though incorporated in policy. More specifically, the TMMRD of EPHI is the only one, at national level, in taking the responsibility of scientific approval of traditional medicine to modern medicine for sustainable use. Thus, this research paper may add its own value on encouraging such aspects.

2. OBJECTIVES

2.1. General Objective

🕮 To investigate the efficacy of crude 80% methanol extract of roots of the plant *C. ficifolius* on blood glucose, β-cells in pancreatic islets of Langerhans, lipid profile, body weight, liver and kidney biochemical and histo-pathological parameters on high fat-fructose diet-Streptozotocin-induced diabetic albino wistar rats.

2.2. Specific Objectives

- To identify the phytochemical constituents (qualitative) of the crude 80%-methanol extract of *C. ficifolius* roots.
- To assess the acute oral toxicity of crude extract of *C. ficifolius* root in apparently healthy albino wistar rats.
- To examine the effect of crude extract of *C. ficifolius* roots on blood glucose in diabetic rats using STT (Short-term Treatment Test), OGTT (Oral Glucose Tolerance Test), measurement of fasting blood glucose level progressively(weekly) and at end of 3-consecutive weeks treatment.
- To appraise the effect of crude extract of *C. ficifolius* roots on β-cells of pancreatic islets of Langerhans using pancreatic tissue histology in diabetic rats.
- To determine the effect of crude extract of *C. ficifolius* roots on lipid profile and body weight in diabetic rats.
- To explore the effects of crude extract of *C. ficifolius* roots on fasting serum biochemical and histological parameters of liver and kidney in diabetic rats

3. MATERIALS AND METHODS

3.1. Materials Used

The materials such as reagents, drug, chemicals, solvents, foods and instruments that were used in this study are summarized and presented in the following Table-5.

Table-5: Materials Used for the Conduct of This Study

Drugs, reagents/chemicals, instruments, solvents and foods		**Manufacturer**	
		Company	**Country**
Drug	Metformin Hydrochloride 500mg extended release Glucophage tablet	Merck Sante s.a.s, 2 rue du pressoir vert, 4400 Semoy	France
Reagents/ chemicals, solvents	Mercuric(II) chloride 99.5% AR (powder)	Riedel-de Haen	Germany
	Hydrochloric acid (solution)	Sigma Aldrich Cheme	Germany
	Lead acetate trihydrate cryst extra pure (powder)	Merck	France
	Methanol solution	F. Undutsch GmbH	Germany
	Chloroform AR (Trichloromethane), Sulfuric acid (H_2SO_4) (solution)	PS-PARK Scientific Ltd	UK (United Kingdom)
	Potassium iodide AR (powder)	Fischer scientific UK Ltd.	UK
	Sodium phosphate dibasic (powder)	SIGMA ALDRICH Inc.	USA
	Sodium phosphate monobasic (anhydrous 99%)	SIGMA chemical.co.	USA
	Dimethyl Sulfoxide solution	SIGMA chemical company	USA.
	Sodium citrate (trisodium salt: dihydrate) 99.9% ACS R	SIGMA chemical Co.	USA
	Streptozocin (powder); Citric acid anhydrous,	SIGMA ALDRICH. Co.	USA
	Ferric chloride ($FeCl_3$) AR (60% w/v solution)	BDH chemicals Ltd Poole	England
	Acetic acid glacial for synthesis,	Central drug ho(P) Ltd, New Delhi	India
	Sodium hydroxide pellets AR (98.0-100.5%); Bismuth subnitrate AR (powder) Diethyl ether AR (solution)	Chemicals UDYOG-121001	India
	Formalin (formaldehyde) (37-41% solution)	Oxford lab chem Pvt. Ltd.	India
	Sodium chloride 99.5% (powder)	Nice chemicals Pvt. Ltd.	India
	Kits for glucose, lipid profile (total cholesterol, triglycerides, low-density lipoprotein-cholesterol & high-density lipoprotein-cholesterol), kidney function (urea & creatinine), and liver function (alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase) tests	Roche Diagnostics GmbH	Germany
	Kits for tissues processing		

	Deionized water	Ethiopian Food, Medicine and Health-care Administration and Control Authority, Addis Ababa	Ethiopia
Foods	Normal pellet diet	National Experimental Animals Breeding Center, EPHI, Addis Ababa	Ethiopia
	D-Fructose 99% AR /D-Levulose, fruit sugar/	Loba chemie Pvt Ltd.	India
	RBD palm olein vegetable oil	MOI FOODS SDN BHD	Malaysia
	Cattle tallow	Addis Ababa City Abattoirs Enterprise, Addis Ababa	Ethiopia
	Pure peanut butter; Be Provolone cheese; Instant Yeast (Instant Kuru Ekmek Mayasi); Sodium chloride table salt.	Loyal Shopping Center (SOLAST.PL.C), Addis Ababa	Ethiopia
Plant	*C. ficifolius A., Rich* root	-	Ethiopia
Instruments			
Basic instruments	Water bath/KOTTERMANN/		Germany
	DS-500 orbital shaker		USA
	Whatman filter paper (no. 1 &42)	Whatman International Ltd Maidstone	England
	Rota-vapor	Buchi labortechnik AG	Switzerland
	Prodigy Auto-code Glucometer machine; No Coding blood glucose Test Strips	Ok Biotech Co.Ltd	Taiwan
	COBAS 6000/c501auto-analyzer	Roche diagnostics GmbH	Germany
	Ultra low temperature (ULT)freezer (model: DW-86L486)	Haier medical & laboratory products CO., Ltd	China
Others	Pocket pH meter, digital and analytical balances, animal cages (plastic), desiccator, tissue cassettes, oral gavage (16 gage), syringe (1ml & 5ml), serum separator test tube with gel, test tubes, neck tubes, pipettes, micropipettes, flasks (round bottom & conical), cylinder, funnels, beakers; disposable gloves, gauze and scissors		

3.2. Methods Applied

3.2.1. Study Design

Experimental study design, specifically completely randomized controlled experimental design was used to perform this research. The reason for why randomization, precisely complete randomization was used is because it better reduces bias than other designs. Statistical comparison among experimental groups only, without having any reference/control group, might reduce quality of experimental output and leads to false conclusion. However, having control groups and making statistical comparison between control groups and experimental groups could increase the data quality (i.e., help to precisely determine the effects of a specific experimental group on different dependent variables) and scientific acceptability of research work. Thus, incorporation of different control groups in this study was found as vital, and so used controlled experimental design.

3.2.2. Laboratory Setting and Study Period

This experimental study was conducted in different laboratory settings at EPHI and Tikur Anbesa Specialized Hospital, Addis Ababa, Ethiopia from December 2016 to May 2018 G.C.

3.2.3. Ethical Approval

The study was conducted after receiving ethical approval letter with "Reference number: SOM/DRERC/BCHM089/2009E.C" from the Departmental Research and Ethics Review Committee, Department of Biochemistry, College of Health Sciences, Addis Ababa University. Moreover, the study had gotten ethical approval from EPHI, TMMRD under the project "Research on Claimed and Selected Medicinal Plants against Diabetes Mellitus and Asthma Diseases". Efforts were made to minimize animal suffering during experimental protocols.

The methods of this paper have two general phases and three arms: (i) preparation phase (with two arms: (a) preparation of plant for experimentation and (b) preparation of animals for experimentation), and (ii) actual experimentation phase.

3.2.4. Preparation of the Plant Extract for Experimentation

3.2.4.1. Plant Material Collection, Extraction and Yield Calculation

The roots of *C. ficifolius* was collected on March 2017 from field: Bebeks Kebele, Fogera Woreda, South Gonder Zone, Amhara Region, Ethiopia (i.e., northwest Ethiopia, approximately 618 kilometers far from capital city, Addis Ababa). The plant was authenticated by a Botanist Dr. Getachew Addis (from EPHI), and a voucher specimen (number YA-01) was deposited at EPHI, TMMRD Herbarium for future reference.

The reason why the root part of *C. ficifolius A. Rich* choosen for experimentation was because the traditional medical practitioners in Ethiopia recommended its use as better than other parts of the plant (fruit, stem and leaf) for treatment of diabetes (based on personal communication data source). In addition, as different ethno-botanical studies stated above (in the literature part-entitled as "medicinal role of *C. ficifolius A. Rich*, including Table-3), the root part has superior medicinal roles for a number of ailments than other parts. Therefore, these increased the expectations that the root part might have better active ingredients against diabetes mellitus.

The roots of *C. ficifolius* were washed using tap water to remove dust materials and dried under the shade. Then, they were crushed separately to coarse powder, to increase the surface area for proper mixing with solvent, using a clean porcelain mortar and pestle.

The crude 80% methanol extract was prepared by maceration technique. Plant materials (coarsely powdered) was soaked in a stoppered flask with 80% methanol solvent and allowed to stand at room temperature for a period of 3-days with frequent agitation using mini orbital shaker until the soluble matter has dissolved (Handa *et al.*, 2008). The process intended to soften and break the plant's cell wall to release the soluble phytochemicals. After 3-days, the mixture was first filtered using gauze and filtrate was passed through Whatman's Number-1 filter paper.

For better estimation of percentage yield of the extract, the residue was re-macerated three times or until we get completely discolored filtrate. The resultant filtrate was pooled together and then evaporated using Rota-vapor set at 40 ºC to remove the solvent. Then, the concentrate was dried in to powder using open air under the shade which is better to preserve active phytochemicals than oven (Azwanida, 2015).

The percentage (%) yield of crude extract was computed using the following formula.

$$\%\ \text{yield of extract} = \frac{\text{Actual mass (dried extract weight (in gram))}}{\text{Theoretical mass (dried raw roots weight (in gram))}} * 100\%$$

Finally, the dried extract was stored in desiccator throughout experimentation.

3.2.4.2. Phytochemical Screening of Crude 80% Methanol Extracts of C. ficifolius Root

The qualitative phytochemical investigations of crude 80% methanol extract of roots of the plant *C. ficifolius* was carried out as per standard procedures with slight modifications (Ekpo *et al.*, 2013; Mir *et al.*, 2013; Pandey & Tripathi, 2014; Gul *et al.*, 2017).

It should be noted that all solutions required for phytochemical screening were prepared from their respective reagents as per standard procedures. Powdered chemicals were weighed using analytical balance, and deionized water was used as solvent. Moreover, for more accurate identification/screening of specific phytochemical class, each of all qualitative phytochemical tests was repeated three times.

Procedures and principles:

Test for Phenols (Ferric chloride test)

To 0.25g (gram) of 80% methanol-extract of *C. ficifolius* root, about 4 drops of 5% neutral ferric chloride solution was added. It was observed for appearance of a greenish precipitate/blue-black color/ which is an indicator of the presence of phenols (Mir *et al.*, 2013).

Test for Terpenoids (Salkowski's Test)

About 0.25g of 80% methanol-extract was added in 2ml of chloroform, heated on water bath for 5-7 minutes and filtered. Then, to the filtrate, about 3ml (milliliter) concentrated sulfuric acid solution was carefully added and allowed to stand to form a layer. It was observed for development of reddish brown coloration of the interface (above sulfuric acid and below chloroform) as an indicator of existence of terpenoids (Mir *et al.*, 2013).

Test for Saponins (foam test)

To 0.5g of 80% methanol-extract of *C. ficifolius*, about 5ml of distilled water was added in a test tube. Then, the solution was shaken vigorously for 5minutes and observed for stable persistent foam. It was watched for the formation of foam and its persistence for at least 10 minutes which is an indicator for saponins (Pandey & Tripathi, 2014; Gul *et al.*, 2017).

Test for Tannins

About 0.25g of 80% methanol-extract was heated for 5-7 minutes in 10ml of distilled H_2O in a test tube, and afterward filtered. About 6 drops of 0.1% ferric chloride solution was added to 5ml

filtrate, and then observed for the formation of brown, greenish or blue-black color as indicator of tannins (Mir *et al.,* 2013).

Test for Flavonoids

Sodium hydroxide/Alkaline Reagent/ Test: About 2ml of 2.0% NaOH solution was mixed with 0.25g of 80% methanol-extract, and observed for production of concentrated yellow color. A change in color from yellow to colorless on addition of 2 drops of diluted 1% HCl (hydrochloric acid) was considered as positive for flavonoids (Gul *et al.,* 2017).

Lead acetate Test**:** About 0.25g of80% methanol *C. ficifolius* extract was treated with 4 drops of 2% lead acetate solution. Development of pale-yellow color precipitate confirmed the presence of flavonoids (Pandey & Tripathi, 2014).

Test for Glycosides (Keller-kiliani Test)

To 0.25g of 80% methanol-extract, about 2ml of glacial acetic acid containing one drop of 2 % ferric chloride solution was added. This was treated with 1ml of concentrated sulfuric acid. The development of brown/blue-green/ ring at the interface indicated the presence of a deoxy-sugar characteristic of steroidal glycosides (Gul *et al.,* 2017).

Test for Steroids (Liebermann's Test)

About 2ml of chloroform and concentrated sulfuric acid were added with the 5ml 80% methanol-extract of *C. ficifolius*. The development of a red color in the lower chloroform layer showed the presence of steroids (Gul *et al.,* 2017).

Test for Alkaloids

About 0.5g of 80% methanol-extract was diluted in 10ml 1% HCl, stirred in water bath for 5 minutes and filtered. This was divided into two portions. About 1ml of Mayer's reagent was added to one portion and Dragendorff's reagent to the other. The formation of a cream/pall yellow/buff colored precipitate (with Mayer's reagent) or reddish brown/orange/ precipitate (with Dragendorff's reagent) revealed the presence of alkaloids (Ekpo *et al.,* 2013).

NB: For further clarity, please see annex-1a.

3.2.4.3. Testing for Acute Toxicity of Crude Extract of C. ficifolius Root

Acute oral toxicity test of crude 80% methanol extract of *C. ficifolius* root was done using the main test because there was no available estimate of its lethality. The main test was performed starting at dose of 175, 550 and 2000mg/10ml/Kg (i.e., a dose progression factor of 3.2 was used) as described by Organization for Economic Cooperation and Development (OECD) guideline 425 (OECD-425, 2008).

A total of seven apparently healthy, nulliparous and non-pregnant female albino wistar adult rats, 9-10 weeks old and weighing 200-210 grams were used. All animals were fasted overnight (food but not water) prior to dosing. The dose for a specific rat was calculated according to the fasting body weight. After the extract has been orally administered, food was withheld for a further 4 hour (Hr). First, one animal was given a dose of 175mg/Kg extract orally and followed for 48Hr for any sign of toxicity (frequently followed for the first 30 min after dosing and periodically, with special attention given during the first 4Hr, for the remaining 48Hr). The behavioral changes closely observed for were: hyperactivity, convulsions, salivation, diarrhea, lethargy, sleep and coma (OECD-425, 2008).

After the survival of first animal confirmed, one additional animal was given 550mg/Kg dose and followed for 48 hours for any sign of toxicity. After the survival of second animal confirmed, third animal was given 2000mg/Kg and followed for 48Hr for any sign of toxicity. After survival of the third animal, additional four animals were given similar 2000mg/Kg dose, sequentially. Note: all animals were: (a) housed separately in cages and observed individually; (b) frequently followed for the first 30 min after dosing, periodically during the first 48Hr (with close followup during the first 4Hr), and then daily for a total of 14-days for any change in general behavior and mortality (OECD-425, 2008; Bisht & Bhattacharya, 2013).

Note that the dose of extract was weighed using analytical balance, and about 1% DMSO (Dimethyl Sulfoxide) in deionized water was used as solvent.

3.2.5. Recruitment of Animals for Experimentation

3.2.5.1. Procuration of Experimental Animals

Apparently healthy albino wistar rats of either sex (i.e., female for acute toxicity test, and male for the remaining experiments) were procured from National Experimental Animals Breeding Center, EPHI, Ethiopia, for experimentation. The animals were housed in plastic cages under standard conditions, maintained on a 12:12 hour light-dark cycle at ambient room temperature (22 ± 2^{O}C), and have free access to food (pellet diet) and water being ad labium up to the date of experimentation. The animals were acclimatized to the laboratory condition for seven days before being subjected to the current experimental protocol (Umer *et al.*, 2013). The animals were handled according to the guideline for the care and use of experimental animals (Garber *et al.*, 2011).

3.2.5.2. Sample Size Determination and Sampling Technique

There is no doubt that the power analysis method is the most favored and scientific approach in sample size computation. However, applying this formula in this current study was impossible because of difficulty to obtain data about two basic requirements (the standard deviation and the effect size) of power analysis formula, not readily available in previous literatures that have similar nature with this current experimental study and difficulty to estimate in pilot study. Thus, using resource equation method was an exclusive choice. Of course, this method is recommended and suitable in exploratory animal researches (Charan & Kantharia, 2013; Arifin & Zahiruddin, 2017), for instance in this current study. Numerous researchers reflected six animals per group are adequate sample size though its scientific and statistical basis is limited. The formula is as follows (Arifin & Zahiruddin, 2017).

$$n = \left(\frac{DF}{K}\right) + 1 \quad \text{..... (Equation-1)}$$

Where; n=sample size per group; DF=degree of freedom ranging from 10 (minimum) to 20(maximum); k=numbers of study groups=6. In this approach to avoid false significant values associated with small sample size, maximum DF was used, to have maximum sample size of resource equation. Thus, the sample size per group would be 4, which is considered as adequate.

$$n = \left(\frac{20}{6}\right) + 1 = 4.3 \approx 4$$ (rounded down to keep "DF" within range).

Furthermore, in order to reduce false significance value /type-II error/ arising from small sample sizes and deaths during experimentation, expected 40% attrition of the above computed sample size was used (Arifin & Zahiruddin, 2017).

$$\text{Corrected sample size for MEx} = \frac{\text{Sample size(n)}}{1-[\%\ \text{attrition}/100]} \ldots\ldots\ldots\ldots\ldots\ldots \text{(Equation-2)}$$

$$= \frac{4}{1-[40\%\ \text{attrition}/100]} = 6.7 \approx 7$$

Hence, the corrected sample size used for main experiment (MEx) was n=7 rats per group (i.e., total of 42 rats for six groups).

Moreover, to avoid animal loss during STZ-induction (either due to death from hyperglycemia or reversal from true hyperglycemia) for five groups designed to be diabetic by HFFD-STZ induction (i.e., the sixth group was designed to be nondiabetic and fed on normal pellet diet (NPD)), 10% attrition of sample size of main experiment n=7 was considered to determine the sample size required for HFFD feeding. Therefore, the corrected sample size for HFFD feeding rats was computed as:

$$\text{Corrected sample size for HFFD} = \frac{\text{Sample size(n)}}{1-[\%\ \text{attrition}/100]} \ldots\ldots\ldots\ldots\ldots \text{(Equation-3)}$$

$$= \frac{7}{1-[10\%\ \text{attrition}/100])} = 7.8 \approx 8$$

Thus, the corrected sample size used for HFFD feeding was n=7 rats per group (i.e., total of 40 rats for five groups designed to be diabetic).

Also, about 7 rats for pilot STZ dose determination following HFFD feeding, and extra 7 rats for acute toxicity test were used. Thus, total of 61 rats were used for whole experimentation.

The simple random sampling (lottery method) technique was used to categorize experimental rats in to different groups. Maximum efforts were made to reduce outcome variation arising from differences in baseline characteristics such as sex, age, weight, health and reproductive status among rats. For acute toxicity test apparently healthy, nulliparous and non-pregnant female albino wistar rats with narrow range in age (9-10 weeks) and weight (200-210 grams) were used. For the rest of experiment, apparently healthy male albino wistar rats with narrow range in age 10-12 weeks and weight 210-252 grams (i.e., ±21g) were used. Note that in this simple random sampling procedure, another individual (other than investigator) who is not familiar with the objective of experiment was invited to pick one rat at a time by lottery method and place in to each of already prepared unlabeled clean plastic cages.

The sampling technique and sample sizes allocation is summarized well in Figure-2 below.

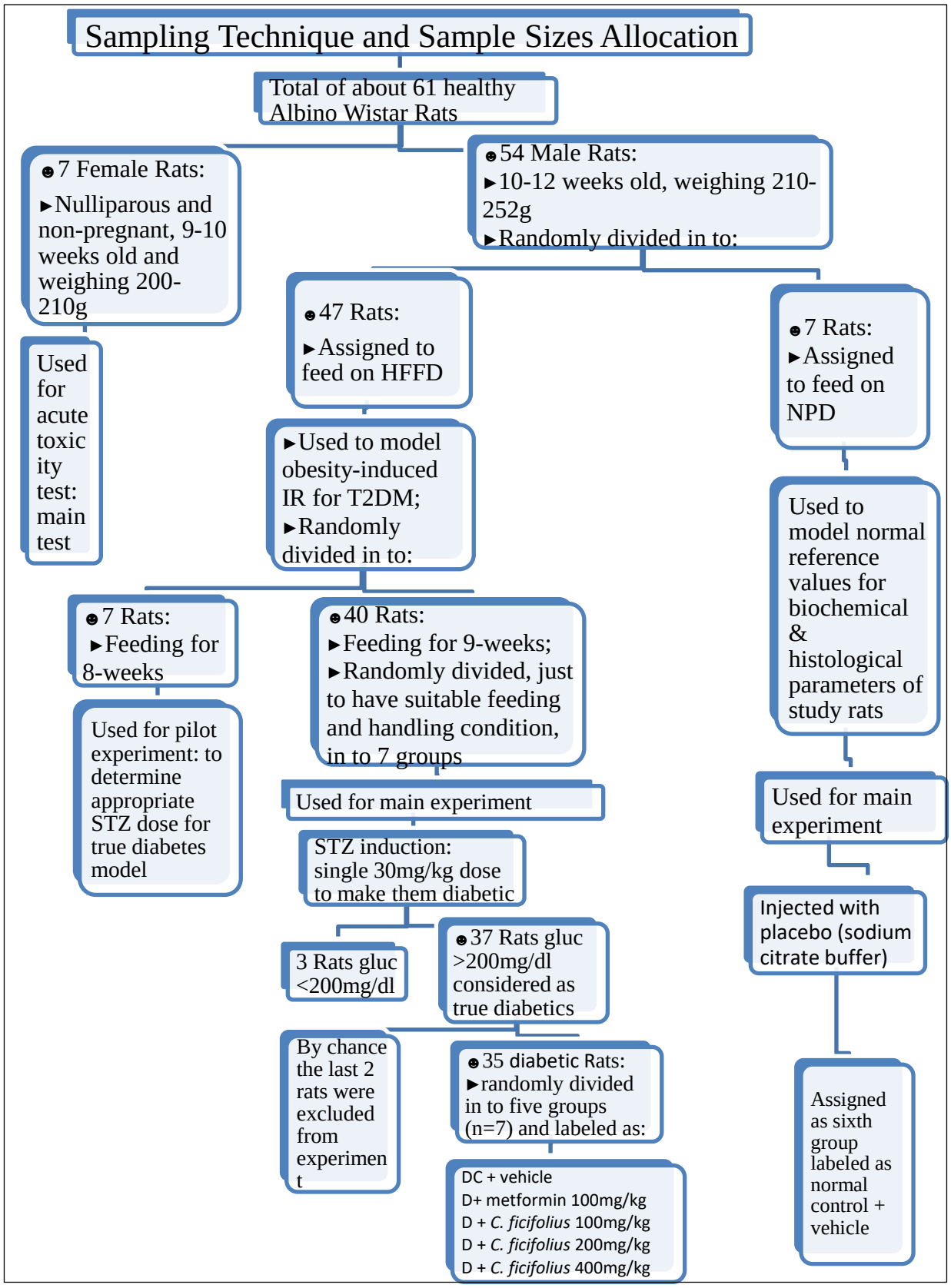


Figure-2: Framework for Sampling Technique and Sample Sizes Allocation, and May be for others in Section-3.2.5

3.2.5.3. Induction of T2DM Animal Model-Principle and Procedure

The T2DM model used for this study was developed by using HFD-10% fructose-single low (30mg/Kg) dose STZ. The reasons for why this model was selected are discussed as follows.

HFFD-single low dose STZ is the best of all models to induce true T2DM. High-fat-high-sugar feeding for 8 weeks combined with low-dose STZ (25mg/Kg) was reported to result in a rat model better representative of human T2DM (Xiang *et al.,* 2010). High-fat-sugar diet-single low dose STZ model was found as better than normal diet-low dose STZ model for efficient induction of T2DM. Compared with normal diet-repeated low dose STZ model, the High-fat-sugar diet (20% sucrose, 12% lard oil, 5% milk powder, 2% egg and 61% normal fodder) fed for 8-weeks followed by single 35mg/Kg dose STZ model showed significant increase in expression of inflammatory proteins and decrease in expression of insulin signaling molecules (such as InsR, Akt, PIP5K2α (phosphatidylinositol-5-phosphate-4-kinase type-2α) and GLUT-2), important for development of IR (Zhuo *et al.,* 2018).

Moreover, a combination of HFFD, rather than either of them alone, are better for development of long-term metabolic disorders such as glucose intolerance, pre-and post-prandial hyper-insulinaemia (IR), dyslipidemia and maintenance of fasting hyperglycemia/T2DM (Lozano *et al.,* 2016).

In general, HFFD cause IR and low dose of STZ partially impair pancreas islets of Langerhans β-cells function (insufficient insulin secretion) and result in relative insulin deficiency in the background of IR, the real T2DM (Zhuo *et al.,* 2018). These are some of evidences for suitability of HFFD-single low dose STZ for induction of real T2DM animal model in the present study.

3.2.5.3.1. Preparation of HFFD

The main purpose of HFFD feeding was to model diet-induced insulin resistant state, a prerequisite for the development of T2DM.

High (10%) fructose solution preparation: About 10% (w/v) of fructose solution was prepared from commercial D-fructose (Loba chemie Pvt. Ltd.). This solution was prepared on daily basis to avoid its contamination from microbial growth such as bacteria which uses carbohydrate as their energy source.

High-fat diet Preparation: High-fat diet was prepared manually because it was not commercially available. HFD was prepared based on standard procedure with few modifications (Srinivasan *et al.,* 2005; Maheswari *et al.,* 2015).

Step-1: Collection of ingredients for HFD: the normal diet of experimental rats, processed pure cattle tallow, RBD palm olein vegetable oil, peanut butter, dry burger (Be provolone) cheese, instant yeast powder, sodium chloride table salt were important ingredients collected for HFD preparation. The coarsely powdered normal chow/diet of experimental rats was procured from National Experimental Animals Breeding Center, EPHI. Processed pure tallow (raw cattle tallow and other remnants was passed through steam engine at temperature of 127^{o}C for 2Hr to grind and differentiate non-fat from fat components; then transferred to tank to which about 0.6% sodium chloride salt and 20-30 liter of water was added and boiled for 1½Hr to further purify and avoid any non-fat components; allowed to settle for 16Hr, yielding pure liquid tallow at top of tank) was bought from Addis Ababa Abattoir Enterprise, Addis Ababa. RBD palm olein vegetable oil, peanut butter, dry burger (Be provolone) cheese, instant yeast powder and sodium chloride table salt were bought from Loyal Shopping Center (SOLAST. Plc), Addis Ababa.

Step-2: Preparation of food components suitable for mixing: The coarsely powdered normal chow/diet of experimental rats was sieved to avoid any large and unwanted components. The solid tallow (at room temperature) was exposed to sunlight followed by large mortar and pestle to convert it in to semi liquid form which is suitable for mixing. Also, the dry burger (Be provolone) cheese was powdered in to mixable form using large mortar and pestle. Sodium table salt was dissolved in little amount of water to make it more mixable and easily distributable throughout the mix.

Step-3: Calculation of HFD proportion: the HFD proportion was computed based on standards for HFD preparation. Standard HFD proportion involves (for 1Kg): NPD-365grams (g), cholesterol-10g, lard-310g, casein-250g, vitamin and mineral mix-60g, di-methionine-3g, sodium chloride salt-1g and yeast powder-1g (Srinivasan *et al.*, 2005; Maheswari *et al.*, 2015).

In the present study, the manually prepared HFD proportion involved (for 1Kg HFD): about 350 grams(g)-normal chow/pellet diet/ of experimental rats, 290.5g-tallow, 124.5g- RBD Palm olein vegetable oil, 58.25g-peanut butter, 174.75g-dry burger (Be provolone) cheese, 1g-yeast powder and 1g-sodium chloride table salt (see Table-6 below). Note that digital balance was used to weigh each of the ingredients.

The approximated representation of these ingredients for standard HFD was as follows. Cattle tallow and palm olein vegetable oil was used as a representative of fat and cholesterol. The dry burger (Be provolone) cheese was purposively selected as one of the richest sources of casein protein. Peanut butter was used as rich source of di-methionine and proteins, and also vitamins, minerals and fats. The NPD of experimental rats was expected to contain all food components

with large proportion of carbohydrates. Thus, it was used mainly as representative of carbohydrate in addition to other components. It should be noted that not only the amount and type of fat but also the grams of carbohydrate in each formulation is important for true obesogenic/diabetogenic HFD. A true "Western Diet" would have high-fat and high-carbohydrate (Heydemann, 2016). Yeast was used for food fermentation, and sodium chloride table salt for food seasoning.

RBD Palm olein vegetable oil was selected purposively other than other market food oils. Because this oil is one of the leading food oils that are richest in saturated fats. The more saturated fatty acids are obesogenic and risky for health. It is because they are inefficiently used for energy production, and are therefore more readily stored (Heydemann, 2016).

Step-4: Mixing of all ingredients suitable for feeding: All ingredients were mixed thoroughly until each of all components get finely distributed and become doughy (soft, sticky and elastic). NB: no extra water was required and added for the preparation, the moisture content present the ingredients was enough. Then, the doughy mixture was prepared as bolus (round mass) suitable for feeding. Note that this HFD was prepared on two days interval to avoid its contamination because of its moisture content.

Table-6: Summary of Composition of Manually Prepared HFFD

No.	Representative ingredients	Specific composition	Proportion in grams (%)
1	Semi-powdered NPD	Estimated: ≈70%-*Zea maize*, ≈10%-inedible remnant of slaughtered cattle (blood/bone/others) & fish bone, ≈20%-sisal & castor bean	350g (35%)
2	Pure Tallow of Cattle	-	290.5 (29.05)
3	RBD Palm olein vegetable oil	Fat-90% (SFA-41.7%, MUSFA-36.7%, PUFA-9.3%, trans fat-0.3%)	124.5 (12.45)
4	Be provolone Cheese	-	174.75 (17.47)
5	Peanut butter	Fat-50%, carbohydrate-20%, protein-25%, vitamin & minerals-5%	58.25 (5.825)
6	Yeast powder	-	1 (0.1)
7	Salt	-	1 (0.1)
	Overall HFD		**1000g (100%)**
8	**D-Fructose**		**10% (w/v)**

3.2.5.3.2. Feeding the Animals with HFFD

Apparently healthy male albino wistar adult (10-12 weeks old with weight of 210-252 grams (mean weight 229 grams)) rats were purposively used for HFFD and NPD feeding. The reasons for why male than female and adult than young are because these rat models are best representative of human adult onset T2DM, justified as follows.

Male rodents are more susceptible to hyperglycemia from HFFD. This is due to adipocyte baseline. Females gained as much weight but did not suffer from pathogenic inflammation (Heydemann, 2016). Moreover, estradiol has protective effect on the ability of apolipoprotein-C3 to inhibit LPL activity and promote ectopic fat storage in premenopausal women (Camporez *et al.,* 2013). Rodents also show a substantial gender difference in STZ sensitivity. Male rats tend to be more susceptible to STZ-induced diabetes. This decreased sensitivity experienced by females may be attributed to estradiol's ability to protect pancreatic β-cells from apoptosis induced by oxidative stress (Le May *et al.,* 2006).

Adult rodents are more susceptible to fat induced T2DM. Young rodents, like young people, have the capacity to increase beta-cell mass. The older rodents (aged >1 year) and elder people (aged >30 years) do not seem to have this capacity (Skovsø, 2014). As a result, the use of male and adult wistar rats in this study was found to be appropriate.

From total of about 56 apparently healthy male albino wistar rats, about 47 rats were allowed to feed HFFD. HFFD feeing was done in two rounds: round one-for pilot experiment that began two weeks early from start of feeding animals for the main experiment and round two-for main experiment. For pilot experiment/appropriate STZ-dose determination/, about 7 rats were fed the prepared HFD and 10% fructose solution as a substitute of drinking water ad libitum for 8-weeks. For the main experiment, about 40 (forty) rats were fed the prepared HFD together with 10% fructose solution as a substitute of drinking water ad libitum for 9-consecutive weeks. Adjacently, the remaining 7 apparently healthy rats were fed the NPD and tape water ad libitum for 9 weeks to be used as one group named as normal control group in the main experiment.

Recording of diet-induced changes (body weight and blood glucose): This helped to assess whether the HFFD by itself induced hyperglycemia or not. NB: the body weights of all rats were measured soon before start of HFFD and NPD feeding. HFFD fed rats showed an increase in fasting blood glucose level and significant increase in body weight as compared to NPD fed rats. However, the increased level of glucose was not quite enough for decision of true hyperglycemia. In other words, HFFD feeding for 9-consecutive weeks alone was not enough to induce T2DM. Thus, HFFD fed rats were further exposed for low single-dose STZ to have true T2DM model (Zhuo *et al.,* 2018).

3.2.5.3.3. Streptozotocin Induction Principles and Procedure

The purpose of low dose STZ was to cause partial destruction of pancreas islets of Langerhans beta-cells which together with HFFD could help to develop true T2DM model.

A) Preparation of STZ solution: STZ solution was prepared by dissolving it in cold and freshly prepared sodium citrate buffer solution with 0.1M (Molarity) and pH 4.5 (prepared by mixing 0.1M 30ml sodium citrate and 0.1M 26ml citric acid, and then pH was adjusted using pocket pH meter). For each STZ-induction phase, the STZ solution was freshly prepared and immediately administered.

B) STZ-administration principle and procedure: The rats were allowed to fast for 12Hr before STZ administration. STZ dose was calculated based on fasting body weight. To avoid individual difference and to be more accurate/unbiased/, the STZ dose was specific for each of all rats, i.e., by making concentration fixed and adjusting/calculating/ the volume for each rat (volume/individual rat weight). The specific amount of STZ was injected intraperitoneally using insulin syringe at site of third quadrant (left lower part in ventral region; with 45^{o} degree angle inclination) of abdomen (Wilson & Islam, 2012; Suman *et al*., 2016b). Soon after STZ injection, 10% fructose solution was interrupted to avoid hyperosmolar coma and risk. To prevent hypoglycemic condition, which may arise from sickness-related none-feeding state, about 3% glucose solution was given for two days ad libitum.

C) Screening for hyperglycemic state/confirmation of true diabetes: The level of fasting blood glucose was determined by drawing blood from tail vein (after warming up the tail to make veins visible) using Prodigy Auto-code Glucometer machine and glucose-oxidase-peroxide reactive Test Strips. Note that the baseline (before STZ induction) fasting blood glucose was measured. The level of fasting blood glucose was assessed periodically, at day-3 and day-7, to confirm the induction and maintenance of diabetes mellitus. Finally, after 7^{th} day from STZ injection, the rats that maintained the fasting blood glucose ≥200 mg/dl at both day-3 and day-7 were considered as true diabetics and selected for the target pharmacological study. Whereas, rats that showed fluctuating hyperglycemia (<200mg/dl) in either of the two measurement periods were excluded from the experiment (Chaudhari *et al.,* 2013).

D) Appropriate STZ-dose determination (Pilot experiment): Appropriate STZ-dose was determined by pilot experiment using seven rats that fed on HFD-10% fructose for 8-consecutive weeks. The pilot experiment involved 45, 40, 35, 30, 25, 20mg/3.5ml/Kg. Note that the level of fasting glucose was measured, using Prodigy Auto-code Glucometer machine and glucose-oxidase-peroxide reactive Test Strips, at 3^{rd} and 7th day after STZ-induction to confirm persistent hyperglycemia(>200mg/dl). Dose selection trial started by inducing 45mg/3.5ml/Kg STZ for one rat, but this rat died at 4^{th} day after STZ induction. Second trial was done at dose 40mg/3.5ml/Kg for one rat and 20mg/3.5ml/Kg dose for another rat. However, rat taking 40mg/3.5ml/Kg died at

6th day and rat taking 20mg/3.5ml/Kg did not develop true hyperglycemia (<200mg/dl). Third trial was done at 25, 30 and 35mg/3.5ml/Kg doses, single rat for 25 and 35mg/Kg doses and two rats for 30mg/Kg dose. These doses appeared with good news; all the three rats showed persistent true hyperglycemia (>200mg/dl) with no death up to 7th day. However, the doses of 35 and 25mg/Kg were not trustable because of their tendency to cause death from hyperglycemia and reversal from true hyperglycemia, respectively, as the duration of experimentation get increased, and so excluded. Thus, the dose of 30mg/Kg was declared as optimum STZ dose to induce diabetes model and used for main experiment.

E) Induction of STZ for main experiment:

All 40 rats that previously fed HFFD for 9-weeks were injected with single low-30mg/Kg dose STZ, from which 37 rats were successfully true hyperglycemic (≥200mg/dl). These rats were allowed to continue feeding HFD and tape water ad libitum, instead of 10% fructose which was interrupted, until the end of experiment.

Furthermore, the 7 apparently healthy rats were injected with placebo (vehicle sodium citrate buffer-solvent for STZ), and allowed to continue feeding previous NPD until the end of experiment. For clarity of induction of T2DM-model using HFFD-STZ, please see annex-1b.

3.2.5.4. Dose Selection and Study Groups Allocation

From about 37 rats that developed true hyperglycemia, about 35 of them were randomly (by lottery method) grouped in to 5 groups (n=7), and then randomly allocated to specific doses. The last two rats remained were excluded. The seven apparently healthy rats were assigned as sixth group named normal control group.

Plant extract dose selection was done based on oral acute toxicity study of the crude extract. Therefore, the three doses 400, 200 and 100mg/Kg used for actual experiment were obtained by calculating the 1/5th, 1/10th and 1/20th of maximum dose 2000mg/Kg, respectively. Metformin dose was determined by extrapolating adult human dose 1000mg/day (using extrapolation factor=6.2). The test drugs (plant extract and metformin) were dissolved by using 1% DMSO solution (in deionized water). Normal control and diabetic negative control groups were allowed to receive vehicle (1% DMSO solution); one diabetic group received standard drug-metformin-100mg/10ml/Kg/day, and the remaining three groups were allowed to take the plant *C. ficifolius* root crude extract at dose 100, 200 and 400mg/10ml/Kg/day. Note that analytical balance was used to weigh the doses of test drugs.

🕮 Study groups:

- Normal control + vehicle (1% DMSO solution) 10ml/Kg/day=(NC)
- Diabetic control + vehicle (1% DMSO) 10ml/Kg/day=Diabetic Negative Control=(DC)
- Diabetic + metformin 100mg/10ml/Kg/day=Diabetic Positive Control=(D + Metf. 100)
- Diabetic + crude *C. ficifolius A., Rich* 100mg/10ml/Kg/day= (D + C.f 100)
- Diabetic + crude *C. ficifolius A., Rich* 200mg/10ml/Kg/day= (D + C.f 200)
- Diabetic + crude *C. ficifolius A., Rich* 400mg/10ml/Kg/day= (D + C.f 400)

In this paper, the term "study groups" stands for the above all six groups; the term "diabetic study groups" represent the five groups induced with diabetes excluding normal control group, and the term "experimental groups" stands for the last three groups treated with *C. ficifoliuas* at 100, 200 and 400mg/Kg doses.

The main purpose of inclusion of one apparently healthy/normal control group was for referencing the normal values of different biochemical and histo-pathological parameters. It is because of limitation of scientific literatures talking about the normal reference ranges of different biochemical parameters for experimental rats (including wistar rats). Most of the available literatures done on experimental animals (including rats) used the reference value of humans. Also, the reference intervals of different biochemical parameters of breeds of wistar rats present in EPHI National Experimental Animals Breeding Center were not characterized yet.

3.2.6. Actual Experimentation Principles and Procedures

This involves administration of test drugs, and samples collection and parameters determination. Samples collection and parameters determination was done in two general phases: phase one-during experimentation and phase two-at the end of experimentation (at sacrification day). The fasting hour for collection of all fasting samples/data was fixed, about 12Hr (from 8PM- 8AM).

3.2.6.1. Administration of Test Drugs

The test drugs (crude *C. ficifolius* root extracts and metformin) and vehicles for the assigned group were administered for one-day to determine their short-term treatment effects and for 3-consecutive weeks to determine their long-term treatment effects. A specified dose was administered once a day. The route of administration was oral by using oral gavage (16 gauge sized).

3.2.6.2. Samples Collected and Parameters Determined during the Experimentation

The samples collected during experimentation were blood and body weight. The effect of extract on blood glucose regulation during experiment was determined by STT, OGTT and progressive (weekly based) fasting blood glucose tests. The blood sample was drawn from tail veins of rats using Prodigy Auto-code Glucometer machine and glucose-oxidase-peroxide reactive Test Strips.

3.2.6.2.1. Determination of Glucose by OGTT

OGTT is diagnostic method of diabetes and one marker of IR or insulin deficiency. An OGTT is used clinically to diagnose impaired glucose tolerance and as a standardized test of carbohydrate metabolism. A prolonged elevation (>2Hr) in both plasma glucose and insulin constitutes impaired glucose tolerance and IR. Therefore, this test was aimed to determine glucose tolerance (Ernsberger & Koletsky, 2012).

In order to evaluate the effect of treatments/plant extract/ in alleviating glucose intolerance on diabetic rats, the OGTT was performed before and after treatment. The OGTT done before treatment (pre-treatment OGTT) was used as reference/baseline. The pre-treatment OGTT was done at day-9 after STZ induction (two days before start of extract administration) and OGTT after treatment (post-treatment OGTT) was done after 22-consecutive days extract/drug administration.

Procedure:

1) All of 6 groups were allowed to fast overnight (12Hr), and their baseline fasting glucose level (represented as "0Hr", i.e., just 30minutes before oral glucose administration) was measured using Prodigy Auto-code Glucometer machine and glucose-oxidase-peroxide reactive Test Strips at tail vein.

2) About 2g/8ml/Kg of glucose solution was orally administered using oral gavage (16 gauge sized) (Wang & Liao., 2012).

3) Blood glucose level was measured at 1^{st}, 2^{nd}, 4^{th} and 6^{th} Hr after oral glucose administration. At each measurement periods three readings of blood glucose were taken for each rats and the average of the three values was used for analysis.

3.2.6.2.2. Determination of Blood Glucose Using STT

In order to determine short-term effect of extract on blood glucose, short-term (one-day) treatment test was undertaken at first day of extract administration.

Procedure:

1) All six groups were fasted for 12Hr and baseline fasting blood glucose level (represented as "0Hr", i.e., just 30minutes before any extract/drug administration) was measured from tail vein by using glucometer.

2) Experimental groups/rats were allowed to take three different doses of extract (*C. ficifolius A. Rich* root 100, 200 & 400mg/Kg), and control groups take standard drug-metformin 100mg/Kg (diabetic positive control) and vehicle 1% DMSO solution (normal control and diabetic negative control groups)

3) The level of blood glucose was recorded at 1^{st}, 2^{nd}, 4^{th} and 6^{th} Hr after extract administration. At each measurement periods three readings of blood glucose were taken for each rat and the average of the three values was used for analysis.

3.2.6.2.3. Determination of Progressive (Weekly Based) Fasting Blood Glucose

This test was done throughout 3-weeks administration of test drugs. In order to assess the progressive effect of the extract/drug on blood glucose regulation, the fasting (12Hr) blood glucose was recorded at week-0/baseline (at day-1, specifically just 30minutes before start of any extract/drug administration), day-7 (week-1), day-15 (week-2) and day-25 (week-3). Fasting blood glucose collected at day-25 was soon before sacrification. At each measurement periods

three readings of blood glucose were taken for each rat and the average of the three values was used for analysis.

3.2.6.2.4. Determination of Progressive (Weekly Based) Body Weight Changes

In order to assess the progressive effect of the extract/drug on body weight regulation, the fasting (12Hr) body weight was weighed at week-0/baseline (at day-1, specifically, just 40minutes before start of any extract/drug administration), day-7, day-15 and day-25 using digital weighing balance. The body weight measured at day-25 was soon before sacrification.

3.2.6.3. Samples Collected and Parameters Determined at the End of Experimentation/at Sacrification Day/

At the end of the experiment, fasting blood sample and organs of pancreas, liver and kidney were taken. Fasting blood sample was used for analyses of biochemical parameters such as fasting serum glucose, serum lipid profiles (TC (total cholesterol), TG, LDL-C (low-density lipoprotein-cholesterol) & HDL-C (high-density lipoprotein-cholesterol), kidney function (serum urea & creatinine), and liver function (serum ALP (alkaline phosphatase), ALT (alanine aminotransferase) and AST (aspartate aminotransferase)) tests. These biochemical tests and histo-pathological examination were done for the purpose of assessment of effect of developed diabetes on altering these parameters and to evaluate usefulness of long-term (3-consecurtive weeks) treatment of test drugs (*C. ficifolius* root extract and metformin) on reversing these alterations.

Procedure for biochemical parameters:

1) **Blood sample collection:** All animals were fasted overnight (12Hr). The animals were anesthetized using diethyl ether, and then the blood samples were collected by cardiac puncture in to gel serum-separating tubes.

2) **Serum isolation:** The collected blood was left to form a clot at room temperature for 30 minutes, and then was centrifuged with 3500 revolution per minute for 10 minutes. The serum was transferred to neck tube using micropipette and stored in ice bag.

3) **Biochemical analysis:** This serum sample was directed for biochemical laboratory analysis. The serum glucose level was determined soon after centrifugation (before 8Hr from time of collection) to avoid its degradation by microbes. The remaining serum samples for determination of the rest biochemical parameters were stored in deep freezer (at -70^{o}C) until analysis. These biochemistry tests were done at National References Laboratory for Clinical Chemistry, EPHI by

professional and trained laboratory technologist, on COBAS 6000/c501 auto-analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Biochemical tests were done after daily quality control is done and become acceptable.

3.2.6.3.1. Determination of Fasting Serum Glucose Level

The concentration of glucose in blood is controlled within narrow limits by many hormones in normal physiology. However, a carbohydrate metabolism disorder named as diabetes mellitus is the most frequent cause of hyperglycemia. Measurement of fasting serum glucose is important in the diagnosis of diabetes mellitus (ADA, 2019a).

Thus, the purpose of this test was to evaluate the effect of diabetes mellitus on induction of hyperglycemia and successively to assess the efficacy of long-term treatment of test drugs (*C. ficifolius* root extract and metformin) on relieving these alterations.

Test Principle (Enzymatic reference method with hexokinase)

Hexokinase catalyzed the phosphorylation of glucose by ATP to form glucose-6-phosphate and ADP. The oxidation of glucose-6-phosphate by $NADP^+$ (Nicotinamide Adenine Dinucleotide Phosphate-non-reduced), under glucose-6-phosphate dehydrogenase (G6PDH), formed NADPH (NADP-reduced/hydrogenated). The concentration of the NADPH formed, which is directly proportional to the glucose concentration, can be determined by measuring the increase in absorbance at 340 nm (Roche Diagnostics Corporation (RDC)-Gluc2, 2017).

The reaction sequence is as follows.

D-glucose + ATP $\xrightarrow{\text{Hexokinase}}$ D-glucose-6-phosphate + ADP

D-glucose-6-phosphate + $NADP^+$ $\xrightarrow{\text{G6PDH}}$ D-6-phosphogluconate + NADPH + H^+

3.2.6.3.2. Determination of Fasting Serum Lipid Profile

LDL-C (bad cholesterol) is produced by the liver and carries cholesterol and other lipids from the liver to different areas of the body like muscles, tissues, organs and heart. The high levels of LDL indicate much more cholesterol in the blood stream than necessary and hence, increase the risk of heart disease. HDL (good cholesterol) is produced by the liver to carry cholesterol and other lipids from tissues back to the liver for degradation. High level of HDL-C is considered as a good indicator of a healthy heart. VLDL-C are formed by a combination of cholesterol and TGs, and so LDL-C level depend on TC and TG (Lakhne *et al*., 2015).

Therefore, the purpose of this test was to evaluate to effect of diabetes on induction of dyslipidemia and consecutively to assess the efficacy of long-term (3-consecutive weeks) treatment of test drugs (*C. ficifolius* root extract and metformin) on alleviating these alterations.

Total Cholesterol Determination

Test principle (Enzymatic colorimetric method)

Cholesterol esterase cleaved cholesterol esters to yield free cholesterol and fatty acids. Cholesterol oxidase then catalyzed the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide helped the oxidative coupling of phenol and 4-aminoantipyrine to form a red quinone-imine dye. The color intensity of the dye formed is directly proportional to the cholesterol concentration, and was determined by measuring the increase in absorbance at 505nm (RDC-Chol2, 2017). The reaction sequence is as follows.

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{RCOOH}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinone-imine dye} + 4H_2O$$

Triglycerides Determination

Test principle (Enzymatic colorimetric test)

LPL catalyzed the rapid and complete hydrolysis of TGs to glycerol. Glycerol kinase with the help of Magnesium ion (Mg^{2+}) catalyzed the phosphorylation of glycerol by ATP to form glycerol-3-phosphate. Glycerol-3-phosphate oxidized by glycerol phosphate oxidase to form di-hydroxy-acetone phosphate and hydrogen peroxide. The H_2O_2 then reacted with 4-aminophenazone and 4-chlorophenol, under catalysis of peroxidase, to form a red dyestuff (Trinder end point reaction). The color intensity of the red dyestuff, which is directly proportional to the TG concentration, was measured photo-metrically at 505nm (RDC-TrigL, 2017). The reaction sequence is as follows.

$$\text{TGs} + 3H_2O \xrightarrow{\text{LPL}} \text{Glycerols} + 3\text{RCOOH}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol Kinase; } (Mg^{2+})} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{Glycerol phosphate oxidase}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{Peroxidase}} \text{4- (para-benzoquinone-monoimino) -phenazone} + 2H_2O + HCl$$

HDL-C Determination

Test principle (Homogeneous enzymatic colorimetric assay)

The cholesterol concentration of HDL-C was determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with Polyethylene Glycol (PEG) to the amino groups. PEG-cholesterol esterase cleaved cholesterol esters into free cholesterol and fatty acids. In the presence of oxygen, cholesterol oxidized by PEG-cholesterol oxidase to Δ^4-cholestenone (delta-4-cholestenone or cholest-4-en-3-one) and H_2O_2. Peroxidase catalyzed the interaction of hydrogen peroxide with HSDA (Sodium N- (2-Hydroxy-3-sulfopropyl)-3, 5-Dimethoxyaniline), 4-aminoantipyrine, hydrogen ion and water to form a purple blue pigment. The produced color intensity of the blue Quinone-imine dye, which is directly proportional to the HDL-C concentration, was determined by measuring the increase in absorbance at 600nm (RDC-HDLC, 2016).

The reaction sequence is as follows.

$$\text{HDL-Cholesterol esters} + H_2O \xrightarrow{\text{PEG-Cholesterol esterase}} \text{HDL-C} + \text{RCOOH}$$

$$\text{HDL-C} + O_2 \xrightarrow{\text{PEG-Cholesterol oxidase}} \Delta^4\text{-Cholestenone} + H_2O_2$$

$$2H_2O_2 + \text{4-Aminoantipyrine} + \text{HSDA} + H^+ + H_2O \xrightarrow{\text{Peroxidase}} \text{Purple blue pigment} + 5H_2O$$

LDL-C Determination

Low Density Lipoproteins (LDL) plays a key role in causing and influencing the progression of atherosclerosis, principally coronary sclerosis.

Test principle (Homogeneous enzymatic colorimetric assay)

The level of LDL-C was determined directly. Detergent was important for selective micellary solubilization of LDL-C. Cholesterol esters were broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, cholesterol becomes oxidized by cholesterol oxidase to Δ^4-cholestenone (delta-4-cholestenone or cholest-4-en-3-one) and hydrogen peroxide. Peroxidase catalyzed the reaction of hydrogen peroxide with 4-aminoantipyrine and HSDA (Sodium N-(2-hydroxy-3-sulfopropyl)-3, 5-dimethoxyaniline) to form a purple-blue dye. The color intensity of this dye is directly proportional to the cholesterol concentration and was measured photometrically at 600nm (RDC-LDLC, 2016).

The reaction sequence is as follows.

LDL-cholesterol ester + H_2O —**Detergent**, Cholesterol esterase, (Selective micellary solubilization)→ Cholesterol + Free fatty acid

LDL-cholesterol + O_2 —Cholesterol oxidase→ Δ^4-cholestenone + H_2O_2

$2H_2O_2$ + 4-aminoantipyrine + HSDA+ H^+ + H_2O —Peroxidase→ Purple blue dye + $5H_2O$

3.2.6.3.3. Determination of Fasting Serum Biochemical Parameters of Liver

The serum level of liver biochemical parameters ALT, AST and ALP were determined in order to evaluate the effect of diabetes and test drugs on liver function.

Serum ALT level determination

Test principle (Photometric; International Federation of Clinical Chemistry (IFCC) method)

ALT catalyzed the reaction between L-alanine and 2-oxoglutarate (α-ketoglutarate) to form pyruvate. The pyruvate gets reduced by NADH, under lactate dehydrogenase, to form L-lactate and NAD^+. The rate of the NADH oxidation is directly proportional to the catalytic ALT activity and was determined by measuring the decrease in absorbance at 340 nm (RDC-ALTL, 2018).

The reaction sequence is as follows.

L-Alanine + 2-Oxoglutarate —ALT→ Pyruvate + L-Glutamate

Pyruvate + NADH + H^+ —Lactate dehydrogenase→ L-Lactate + NAD^+

Serum AST level Determination

Test principle (Photometric; International Federation of Clinical Chemistry (IFCC) method)

AST catalyzed the transfer of an amino group between L-aspartate and 2-oxoglutarate (α-ketoglutarate) to form L-glutamate and oxaloacetate. Oxaloacetate reacted with NADH, under malate dehydrogenase, to form NAD^+. The rate of NADH oxidation, which is directly proportional to the catalytic AST activity, was determined by measuring the decrease in absorbance at 340 nm (RDC-ASTL, 2017). The reaction sequence is as follows.

L-Aspartate + 2-Oxoglutarate —AST→ Oxaloacetate + L-glutamate

Oxaloacetate + NADH + H^+ —Malate Dehydrogenase→ L-Malate + NAD^+

Serum ALP level Determination

Test principle (Colorimetric assay in accordance with a standardized method)

In the presence of magnesium and zinc ions, p-nitrophenyl phosphate is cleaved by ALP into phosphate and p-nitrophenol. The p-nitrophenol produced is directly proportional to the catalytic ALP activity, and was determined by measuring the increase in absorbance at 450 nm (RDC-ALP2, 2018).

p-Nitrophenyl phosphate + H2O $\xrightarrow{\text{ALP}}$ Phosphate + p-Nitrophenol

Where; p- = para- or 1,4-substituent or 4-nitro isomeric position

3.2.6.3.4. Determination of Fasting Serum Biochemical Parameters of Kidney

The serum level of kidney biochemical parameters urea and creatinine were determined in order to evaluate the effect of diabetes and test drugs on kidney function.

Serum Urea level determination

Test principle (Kinetic test/photometric method)

Urea could be hydrolyzed by urease to form ammonium and carbonate. Then, the 2-oxoglutarate reacted with ammonium in the presence of glutamate dehydrogenase and the coenzyme NADH to produce L-glutamate. In this reaction two moles of NADH was oxidized to NAD+ for each mole of urea hydrolyzed. The rate of decrease in the NADH concentration, which is directly proportional to the urea concentration in the specimen, was measured photometrically at 340nm (RDC-UreaL, 2017). The reaction sequence is as follows.

$Urea + 2H_2O \xrightarrow{Urease} 2NH_4^+ + CO_3^{2-}$

$NH_4^+ + 2\text{-Oxoglutarate} + NADH \xrightarrow{\text{Glutamate dehydrogenase}} \text{L-Glutamate} + NAD^+ + H_2O$

Serum Creatinine Determination

Test principle (Enzymatic colorimetric method)

Creatininase catalyzed the conversion of creatinine to creatine which in turn produced sarcosine by creatinase. Sarcosine-oxidase canalized the oxidation of sarcosine to produce glycine, formaldehyde (HCHO) and hydrogen peroxide. Finally, peroxidase catalyzed the reaction of hydrogen peroxide with 4-aminophenazone and HTIB for the formation of a quinone-imine-chromogen. The color intensity of the quinone imine chromogen, which is directly proportional

to the creatinine concentration in the reaction mixture, was measured photometrically at 546nm (RDC-Crep2, 2017).

The reaction sequence is as follows.

Creatinine + H_2O $\xrightarrow{\text{Creatininase}}$ Creatine

Creatine + H_2O $\xrightarrow{\text{Creatinase}}$ Sarcosine + Urea

Sarcosine + O_2 + H_2O $\xrightarrow{\text{Sarcosine oxidase}}$ Glycine + formaldehyde (HCHO) + H_2O_2

H_2O_2 + 4-Aminophenazone + HTIB $\xrightarrow{\text{Peroxidase}}$ Quinone-imine-chromogen + H_2O + HI

Where: HTIB =2, 4, 6-**tri**iodo-3-**h**ydroxy**b**enzoic acid; HI=hydrogen iodide

3.2.6.3.5. Histopathology Examination (Pancreas, Liver and Kidney)

The procedure for histopathology Screening was done as per the standard (Ross & Pawlina, 2011; Mescher, 2016) with minor modification and stated as follows.

Step-1: Tissues preservative buffer solution preparation:

Neutral formalin buffer (10%) was used for tissues preservation. Buffer preparation per 1L: about 6.5g sodium phosphate di-basic (Na_2HPO_4) and 4g sodium phosphate mono-basic (NaH_2PO_4) were dissolved in 100ml 40% formalin, and then filled with double distilled H_2O (900ml) up to 1L volume reached.

Step-2: Tissues isolation from animals:

At day-25, soon after the fasting blood samples collected, two representative rats from each of 6 groups were selected randomly and sacrificed by cervical dislocation method.

The pancreas (more of the head and partly body regions- where relatively large proportions of pancreatic beta-cells are expected to locate than other region (Tsuchitani *et al.*, 2016), kidney (left one) and liver (smaller left lobes plus any other visible altered region) tissues were isolated from those representative rats (two samples per group for each of the three organs).

Step-3: Tissues fixation: The isolated tissues were immediately fixed in 10% neutral formalin buffer, each in separate container, and preserved/stored until further processing done.

Step-4: Tissues processing: From the representative tissues, two samples of pancreas, one sample of kidney, one sample of liver tissues per group were allowed for tissues processing (dehydration-cleaning- infiltration-embedding). Tissues were dehydrated by ethanol at increasing concentrations (70% then 95%) to avoid excessive distortion of the tissue. Ethanol is miscible

with water in all proportions so that the water in the specimen is progressively replaced by the alcohol, suitable for paraffin-wax infiltration. Next, the tissues were cleaned/washed by xylene solution to displace the ethanol in the tissues and to remove a substantial amount of fat from the tissue which otherwise presents a barrier to wax infiltration. Then after, the tissues were infiltrated with 100% melted paraffin wax, and then embedded, placed in a small mold with melted paraffin and allowed to harden.

Step-5: Blocking out and sectioning: Then embedded tissue was trimmed into an appropriately sized block which was then sectioned at 5um (micrometer) using slicing machine-a microtome. The resulting sections were then mounted on glass slides using mounting medium (acrylic resins) as an adhesive.

Step-6: Staining and mounting: The sections were stained with Hematoxylin and Eosin dyes to increase the distinguishablity of cells. Before staining the section, the embedding agent-paraffin was removed out again with xylene, and the slide was rehydrated through a series of solutions of descending alcohol concentration. The tissue on the slides was then stained with hematoxylin in water. Because the counter-stain, eosin, is more soluble in alcohol than in water, the specimen was again dehydrated through a series of alcohol solutions of ascending concentration and stained with eosin in alcohol. After staining, the specimen was then passed through xylene to a non-aqueous mounting medium and covered with a cover slip to obtain a permanent preparation. These activities were done by pathology technologist at Laboratory of Pathology, Tikur Anbesa Specialized Hospital.

Step-7: Microscopic examination of tissue slides

The prepared tissue slides of pancreas, liver and kidney of study groups were examined under compound light microscope for histo-pathological changes. These activities were done by senior pathologist, Dr. Wondwossen Ergete at Department of Pathology of Tikur Anbesa Specialized Hospital, Addis Ababa University. The histopathological changes among study groups were determined through deep observation (examination) by interchanging the powers of objective lens (i.e., at 4x, 10x, 20x and 40x objectives). Computer's pointer arrow, counting and just an observation was used to measure the size, number and degree, respectively, of a particular change. Then, the pictures/images at both 20x and 40x objective lens (or 200x and 400x magnification powers, respectively) were taken for each representative samples of all study groups by using computer-based shooting camera attached to the microscope. Finally, the pictures at 200x magnification power were found to best describe the changes, and so used for actual comparison.

In the main, the figure-3 below briefly summarized the sample collection and parameters determination section of this study.

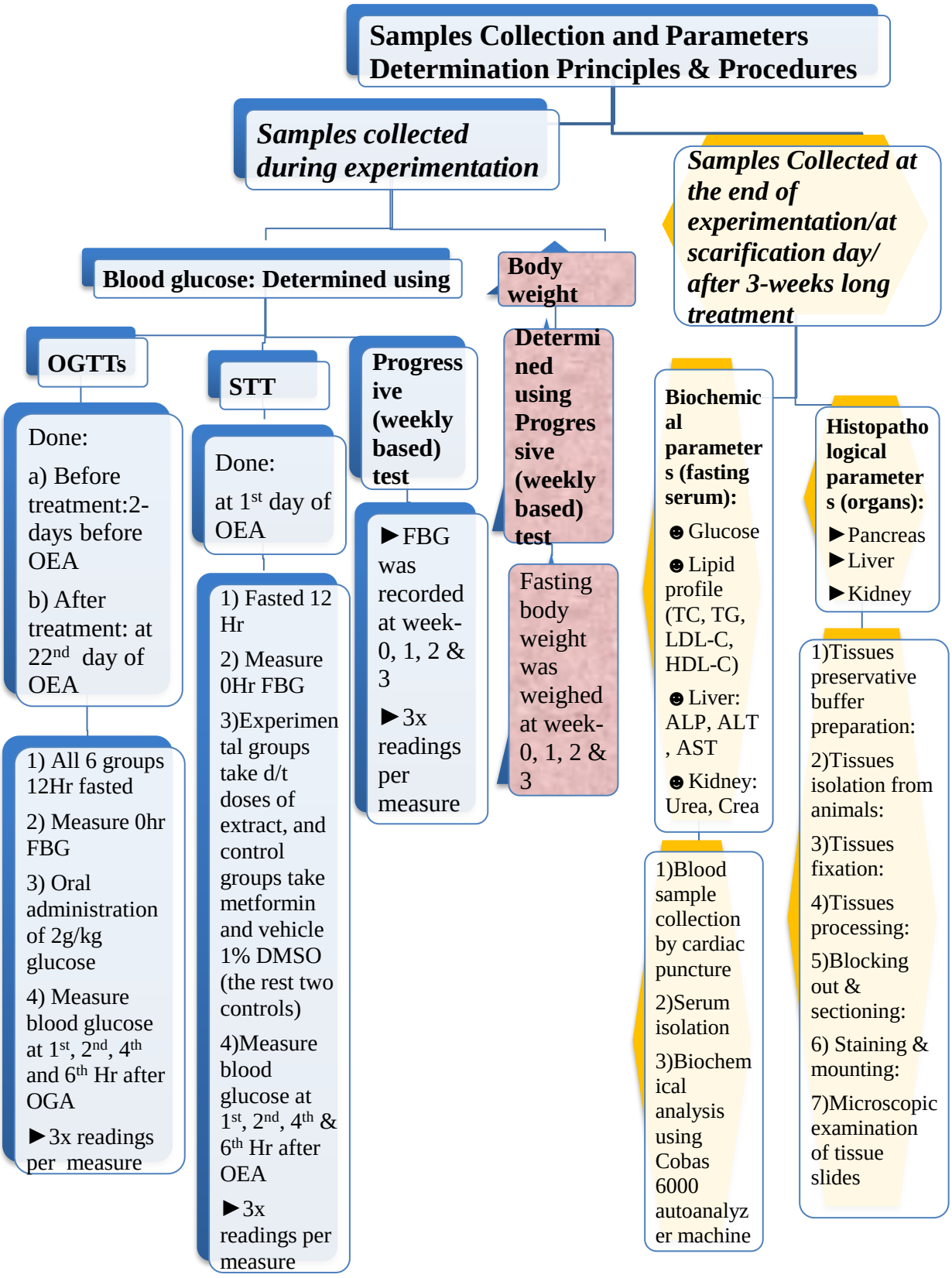


Figure-3: Summarized Framework for Samples Collection and Parameters Determination

Keys: OEA-oral extracts administration; OGA-oral glucose administration; FBG-fasting blood glucose

3.2.7. Statistical Analysis and Data Presentation

Data were analyzed using SPSS (Statistical Package for the Social Sciences) version-24. The central tendency and spread of data for blood glucose, lipid profile (TG, TC, HDL-C, LDL-C), liver enzymes (ALT, AST, ALP), kidney parameters (Urea, creatinine), and body weight were determined using mean and standard deviation (mean±SD). For data that hold more than two between subject factors with single measure of dependent variable, differences among study groups were analyzed using one-way independent ANOVA (Analysis of Variance) followed by post Hoc Tukey's HSD to compare all possible pairs of means. The parameters involve lipid profiles, liver enzymes, kidney parameters and fasting serum glucose which all were collected once at scarification day and determined using auto-analyzer machine (COBAS 6000/c501).

On the other hand, for data having more than two between-subject-factors (study groups) with two or more repeated measures (with-in-subject factors) of dependent variable, an advanced Mixed Design (split plot) ANOVA (MDANOVA) was used to determine the interaction effect and main effect. For multiple pairwise comparisons of main effects of study groups (between-subject-factors) and measurement periods (with-in-subject factors), post hoc Tukey's HSD and Bonferroni were used, respectively. The parameters include blood glucose measured in STT and OGTT (repeatedly measured at 0Hr, 1^{st}, 2^{nd}, $4^{th}$and 6^{th} Hr), and progressive fasting blood glucose and body weight (repeatedly measured at week-0, 1, 2 and 3 of test drugs administration periods). The simple main effects of between-subject factors (study groups) were analyzed by one-way-independent ANOVA followed by post hoc Tukey's HSD for multiple pairwise comparisons. The simple main effects of within-subject-factors (measurement periods) were analyzed by one-way Repeated Measure ANOVA (RMANOVA) followed by post hoc Bonferroni for multiple pairwise comparisons.

For purposive comparison of some parameters, such as fasting serum urea and creatinine levels, of normal control group against the remaining diabetic groups, one-way ANOVA followed by post hoc Dunnett's-t (2-sided) test was used. In all analyses stated above, the p-values < 0.05 were considered as statistically significant.

Furthermore, for insignificant differences in ANOVA among groups in some parameters such as body weight and serum creatinine, the percentage value difference was computed to precisely show the differences among groups. All the analyzed data were organized and presented using tables and line graphs.

In addition, for histo-pathological data (pancreas, liver and kidney) of all study groups, the microscopic observations of images were used to compare histo-pathological differences among groups. The analyzed data were presented as images with 200x magnification powers.

3.2.8. Data Quality Assurance

In order to assure data quality, maximum efforts were applied to minimize personal, instrumental and statistical errors. The investigator tried to acquire sufficient knowledge and experience, both practical and theoretical, by taking training from experienced bodies and reading numerous articles before and throughout the experimentation. Some of technical experiences developed were on animals handling and feeding, diabetes (STZ) induction, extract administration, blood drawing, animal scarification and organ collection, and other related experiences to assure the experimentation data quality. The procedures used in this study were developed after extensive review of quality and updated literatures, and also were strictly controlled. To avoid the metabolic effect of circadian rhythm and length of fasting hour on variation of blood biochemistry, particularly for measurement of fasting biochemical parameters, the fasting hours and time interval were fixed (12Hr; from 8PM-8AM,) and decidedly controlled. Blood chemistry tests and tissues processing were done by responsible trained professionals.

The biochemical parameters were analyzed at National Reference Laboratory for Clinical Chemistry which obtained accreditation certificate from ENAO (Ethiopian National Accreditation Office) for complete implementation of International Organization for Standardization-ISO-15189 medical laboratories standard. Internal quality control was done and acceptable according to the laboratory protocol before research sample were analyzed. For blood glucose tests done by using Prodigy Auto-code Glucometer machine and glucose-oxidase-peroxide reactive Test Strips, three samples/drawings per single measurement period were taken to guarantee the value more accurate. Also, appropriate statistical software (updated) and methods were used to analyze the data.

Generally, the study methods applied could be precisely summarized using this framework (Figure-4).

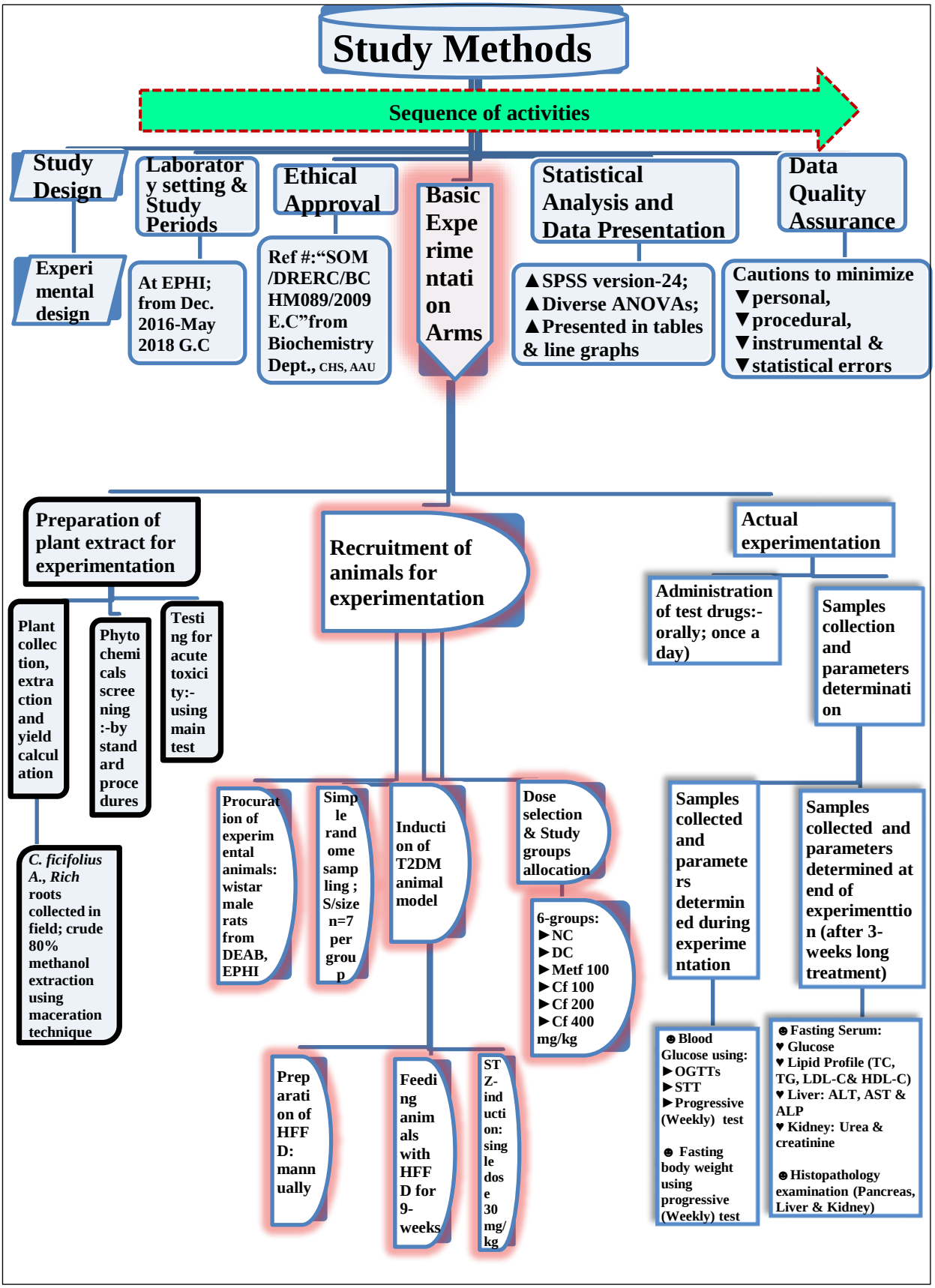


Figure-4: Summarized Framework for Study Methods Applied

4. RESULTS

4.1. Phytochemicals Screened in Crude 80% Methanol Extract of Root of *C. ficifolius*

According to qualitative phytochemical screening, the crude 80% methanol extract of roots of *C. ficifolius* contain relatively greater amounts of flavonoids and terpenoids followed by mild saponins, glycosides, alkaloids, phenols and steroids. However, tannins were absent (Table-7).

Table-7: Qualitative Phytochemical Separations of Crude 80% Methanol Extract of Root of *C. ficifolius*

No.	Phytochemicals classes	Observation	Qualitative detection level
1	Phenols	Blue black color	++
2	Terpenoids	Reddish brown coloration	+++
3	Saponins	About 1.8cm height foam; persisted for> 3Hr	++
4	Tannins	Indefinable	-
5	Flavonoids	Yellow color & pale yellow precipitate	+++
6	Glycosides	Brown/blue green/ ring	++
7	Steroids	Red ring	+
8	Alkaloids	Pale yellow & reddish orange color	+

Key: "+++"= high concentration; "++"= medium concentration; "+"= fair concentration; "-" absent

Also, the percentage yield of crude 80% extract of root part of *C. ficifolius* was 10.8%.

$$\%\ \text{yield of extract} = \frac{\text{Actual mass (dried extract weight (in gram))}}{\text{Theoretical mass (dried raw roots weight (in gram))}} * 100\%$$

$$= \frac{81.163\ \text{gram}}{750\ \text{gram}} * 100\% = 10.822\% \approx 10.8\%.$$

4.2. Acute Toxicity of Crude 80% Methanol Extract of Root of *C. ficifolius*

There was no death (all five rats that took 2,000mg/Kg survived) and no toxic signs could be recorded at 2,000mg/Kg dose. The behavioral changes such as hyperactivity, ataxia, tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma were not observed.

4.3. The Effect of Crude *C. ficifolius* Root Extract on Blood Glucose in Diabetic Rats

4.3.1. The Short-term Treatment Effect of Crude *C. ficifolius* Root Extract on Blood Glucose in Diabetic Rats

According to mixed design ANOVA (MDANOVA) output ("Greenhouse-Geisser" sphercity correction), there was interactive effect ($p<0.0000001$) between study groups (between-subject factor) and measurement periods (with-in-subject factor) on blood glucose level.

The main effect of diabetic study groups (MDANOVA) on blood glucose level indicated negligible difference ($p=0.915$) in blood glucose among them. The simple main effect (one-way ANOVA) of study groups on blood glucose level for each measurement periods (1^{st}, 2^{nd}, 4^{th}& 6^{th} Hr) showed non-significant difference among diabetic study groups in all measurement periods. However, the difference in blood glucose among diabetic study groups increased from 1^{st} Hr to 6^{th} Hr measurement period. Also, metformin 100mg/Kg treated group revealed greater discount in blood glucose followed by *C. ficifolius* root extract 400, 200 & 100mg/Kg treated groups as compared to diabetic negative control group in both main and simple main effects (Figure-5).

On the other hand, the main effect of measurement periods (MDANOVA) indicated remarkable difference ($P<0.000001$) in glucose reading among measurement periods. The multiple pairwise comparisons (MDANOVA, post-hoc Bonferroni) among all pairs, except 0Hr-1^{st}Hr pair) of measurement periods were substantial ($p<0.05$). It should be noted that the term "0Hr"-stated here and also in the following sections represents "the baseline fasting blood glucose level measured before (just 30minutes before) extract/drug administration". Moreover, the simple main effect of measurement periods (one-way RMANOVA) on blood glucose level for each diabetic study groups showed remarkable difference among measurement periods in all diabetic study groups. The multiple pairwise comparisons (one-way RMANOVA, post-hoc Bonferroni) among all pairs, except 0Hr-1^{st} Hr pair, of measurement periods were substantial ($p<0.05$) for each of all study groups. In addition, in simple main effect, the powers of significances were greater for metformin 100mg/Kg treated group followed by *C. ficifolius* root extract 400, 200 &100mg/Kg treated and diabetic negative control group.

As displayed in profile plot (Figure-5) below, experimental and positive control groups showed relatively greater reductions in blood glucose level as compared to diabetic negative control group throughout measurement periods (1^{st}, 2^{nd}, 4^{th}& 6^{th} Hr). Among experimental and positive control

groups, metformin 100mg/Kg treated group showed better discount in blood glucose followed by *C. ficifolius* root extract 400, 200 & 100mg/Kg treated groups throughout measurement periods.

Based on repeated measure comparison (self comparison of each study groups), as shown in profile plot (Figure-5) and relative percentage glucose reduction at each measurement periods (though result not stated here), metformin 100mg/Kg treated group exhibited highest reduction at 4th Hr followed by 6th and 2nd Hr. In contrast, *C. ficifolius* root extract treated groups (of all doses) showed greatest decrease in glucose level at 4th Hr followed by 2nd and 6th Hr. Though the data were not stated here, weekly measurement of blood glucose at 1st, 2nd, 4th& 6th Hr after extract administration (rats were deprived of food for 4Hr before extracts administration) showed similar result. Note that the mean fasting blood glucose levels for normal control group (that was not displayed in the graph below (Figure-5) to have a better and distinct comparison graph for the rest groups) at 0Hr, 1st, 2nd, 4th and 6thHr were 83.06±4.80, 84.47±4.50, 82.80±4.07, 80.74±3.76 and 79.53±4.63 (all in mg/dl), respectively.

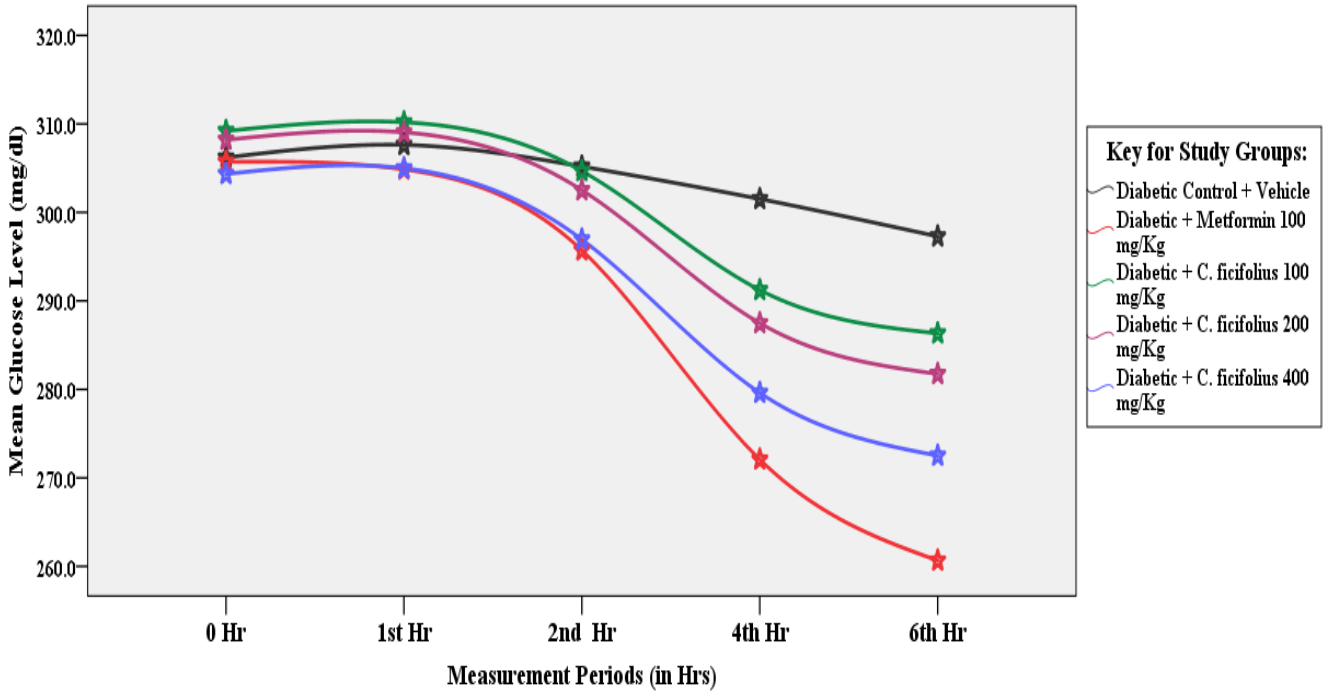


Figure-5: Short-term Treatment Effect of Crude Extract of *C. ficifolius* Root and Metformin in Diabetic Rats.

Where; Hr=hour; Sample size (N) for each of all groups was seven, N=7. "0Hr"-represents "the baseline fasting blood glucose level measured before (just 30minutes before) extract/drug administration".

4.3.2. The Effect of Crude *C. ficifolius* Root Extract on Glucose Tolerance (OGTT) in Diabetic Rats

Note: In this sub-topic the contextual definition of some terms is as follows. "**Reversal**" means from the total of orally loaded glucose almost all are utilized /metabolized/ by the body at specific time, or only small /statistically insignificant/ amount of glucose remained to be metabolized. "**Non-reversal"** means from the orally loaded glucose there are large amount of glucose remaining to be metabolized (non-metabolized) at specific time. "**Significant non-reversal"** means from the orally loaded glucose the amount of non-metabolized blood glucose is statistically significant. "**Insignificant non-reversal**" is analogous to the term "**reversal**".

As shown in Figure-6 below, MDANOVA output ("Greenhouse-Geisser" sphercity correction) for pre-treatment OGTT indicated the strongly insignificant interaction effect. Also, the main effect of between-subject factor/study groups/ was strongly insignificant. But, the main effect of with-in-subject factors/measurement periods/ was significant.

The MDANOVA ("Greenhouse-Geisser" sphercity correction) for post-3-weeks treatment OGTT revealed that the interaction effect of diabetic study groups/between-subject factors/ and measurement periods /with-in-subject factor/ was significant ($p<0.0000001$).

As per simple main effect of diabetic study groups (one-way ANOVA followed by post hoc Tukey's HSD), all experimental and positive control groups showed remarkable ($p<0.05$) drop in blood glucose against diabetic negative control group for each of all measurement periods (0Hr, 1^{st}, 2^{nd}, 4^{th}& 6^{th} Hr). It should be noted that the term "0Hr" stated here and also in the following sections represents "the baseline fasting blood glucose level measured before (just 30minutes before) oral glucose administration for OGTT". For all *C. ficifolius* root extract 100, 200 and 400mg/Kg treated groups, the powers of significances were increased from measurement period 0Hr ($p=0.024$, 0.016 and 0.004, respectively) to 6^{th} Hr (0.000098, 0.000064 and 0.000008, respectively) against diabetic negative control. Whereas, for metformin 100mg/Kg treated group, the powers of significances were increased from measurement period 0hr ($p=0.000483$) to 4^{th} Hr ($p=0.0000003$), and then declined at 6^{th} Hr ($p=0.00000086$). Among experimental and positive control groups, metformin 100mg/Kg treated group showed better reduction in glucose level followed by *C. ficifolius* root extract 400, 200 and 100mg/Kg at all measurement periods. However, the differences among them were negligible ($p>0.05$). Moreover, the extent of differences among experimental and positive control groups had gotten larger as we go from 0Hr to 4^{th} Hr, and then, declined at 6^{th} Hr (see Figure-6). Also, the main effect (MDANOVA) of diabetic study groups on blood glucose level, in OGTT done after 3-consecutive weeks treatment,

was remarkably (p=0.000013) different. According to multiple comparisons (MDANOVA, post hoc Tukey's-HSD) of main effects among diabetic study groups, all experimental and positive control groups presented significant discount ($p \leq 0.002$) in blood glucose against diabetic negative control group. Among experimental and positive control groups, metformin 100mg/Kg treated group showed better discount in blood glucose followed by *C. ficifolius* root extract 400, 200 and 100mg/Kg, however, the differences among them were minimal ($p > 0.05$). In other word, for diabetic study groups, these main effects were equivalent to the simple main effects and strengthened it (see Figure-6 below).

Pairwise comparisons of simple main effect of measurement periods (RMANOVA, post hoc Bonferroni) for diabetic negative control showed significant difference (non-reversal) at 1^{st}, 2^{nd}, 4^{th}, 6^{th} Hr against 0Hr measurement period. Metformin 100mg/Kg treated group presented significant non-reversal in pairs of 0Hr-1^{st}Hr and 0Hr-2^{nd} Hr, and insignificant non-reversal (there is reversal) in pairs of 0Hr-4^{th} & 0Hr-6^{th} Hr. All of *C. ficifolius* root extract treated (i.e., 100, 200 & 400mg/Kg doses) groups revealed significant non-reversals in pairs of 0Hr-1^{st}, 0Hr-2^{nd} and 0Hr-4^{th} Hr, and insignificant non-reversal in pair of 0Hr-6^{th} Hr. Generally, metformin 100mg/Kg treated group exhibited better reversal/reduction of the loaded glucose level followed by *C. ficifolius* root extract 400, 200, 100mg/Kg and diabetic negative control at each of all measurement points. The main effect analysis (MDANOVA, "Greenhouse-Geisser" sphercity correction) of measurement periods showed significant effect on blood glucose level ($p < 0.0000001$). According to pairwise multiple comparisons (MDANOVA, post hoc Bonferroni correction) the non-reversals of blood glucose levels were significant among all pairs of measurement periods. The strongly significant ($p \leq 0.004$)) non-reversal in pairs of 0Hr-1^{st}, -2^{nd}, -4^{th} and -6^{th} Hr with the power of significances decreasing from 0Hr-1^{st} Hr pair to 0Hr-6^{th} Hr pair.

On the other hand, simple main effect (RMANOVA, post hoc Bonferroni) of measurement periods in normal control group showed significant non-reversal in pairs of 0Hr-1^{st}, and strongly insignificant non-reversal in pairs of 0Hr-2^{nd}, -4^{th}& -6^{th} Hr.

Moreover, analysis of main effect of diabetic study groups on blood glucose using combined OGTT (meaning, using both pre- and post- treatment OGTTs at a time) for each measurement periods was important. This analysis was computed by considering the five diabetic study groups as five between-subject factors and taking two similar measurement periods from pre- and post-treatment OGTTs as two with-in-subjects. For instance, the measurement periods 0Hr from pre-treatment OGTT and 0Hr from post-treatment OGTT were considered as two with-in-subject factors. That means the analysis was 5x2 MDANOVA design, and multiple pairwise comparisons

were computed by post hoc-Tukey's HSD. It is because this analysis probably ignored the issue of baseline differences in glucose tolerance among diabetic study groups observed from pre-treatment OGTT and so best reflected the effect of treatments on improvement of glucose tolerance. Therefore, based on this analysis, all experimental and positive control groups showed non-remarkable glucose reduction (P>0.05) as compared to diabetic negative control at 0Hr and 1st Hr measurement periods. Metformin 100mg/Kg treated group presented significant glucose reduction at 2nd, 4th and 6th Hr (p=0.03, 0.004, 0.006, respectively). Also, *C. ficifolius* root extract at 400mg/Kg dose (but not at dose 100 and 200mg/Kg) treated group exhibited substantial reduction of glucose at 4th and 6th Hr (p=0.024 and 0.02, respectively). Regarding to the parallel comparisons, the statistical power of glucose reduction was increased as we go from 0Hr to 6th Hr for each of *C. ficifolius* root extract treated groups and from 0Hr-4th Hr for metformin 100mg/Kg treated group as compared to diabetic negative control group. However, in metformin 100mg/Kg treated group, the statistical power was decreased (instead of increasing) from 4th Hr (p=0.004) to 6th Hr (p=0.006). Generally, metformin 100mg/Kg treated group revealed better glucose reduction followed by *C. ficifolius* root extract 400, 200 and 100mg/Kg treated groups, however, differences among them were insignificant.

The difference in glucose between glucose levels in pre- and post-treatment OGTT was computed by subtracting glucose level in pre-treatment OGTT from glucose level in post-treatment OGTT following formula:

$$\mathrm{D/ce\ Gluc\ at\ Hr(i))} = \text{"GlucBfT OGTTat Hr (i)"} - \text{"GlucAfT OGTT at Hr (i)"}$$

Where: Hr(i)= specific hour at which glucose level measured during OGTT, and include 0Hr, 1st, 2nd, 4th and 6th Hr.

D/ce Gluc = Difference in blood Glucose at Hr (i)

GlucAfTOGTT = blood Glucose levels in After-Treatment OGTT.

GlucBfTOGTT = blood Glucose levels in Before-Treatment OGTT.

This data helped to clearly show the effect of 3-weeks-long treatment of extract/drug on glucose tolerance as compared to pre-treatment OGTT.

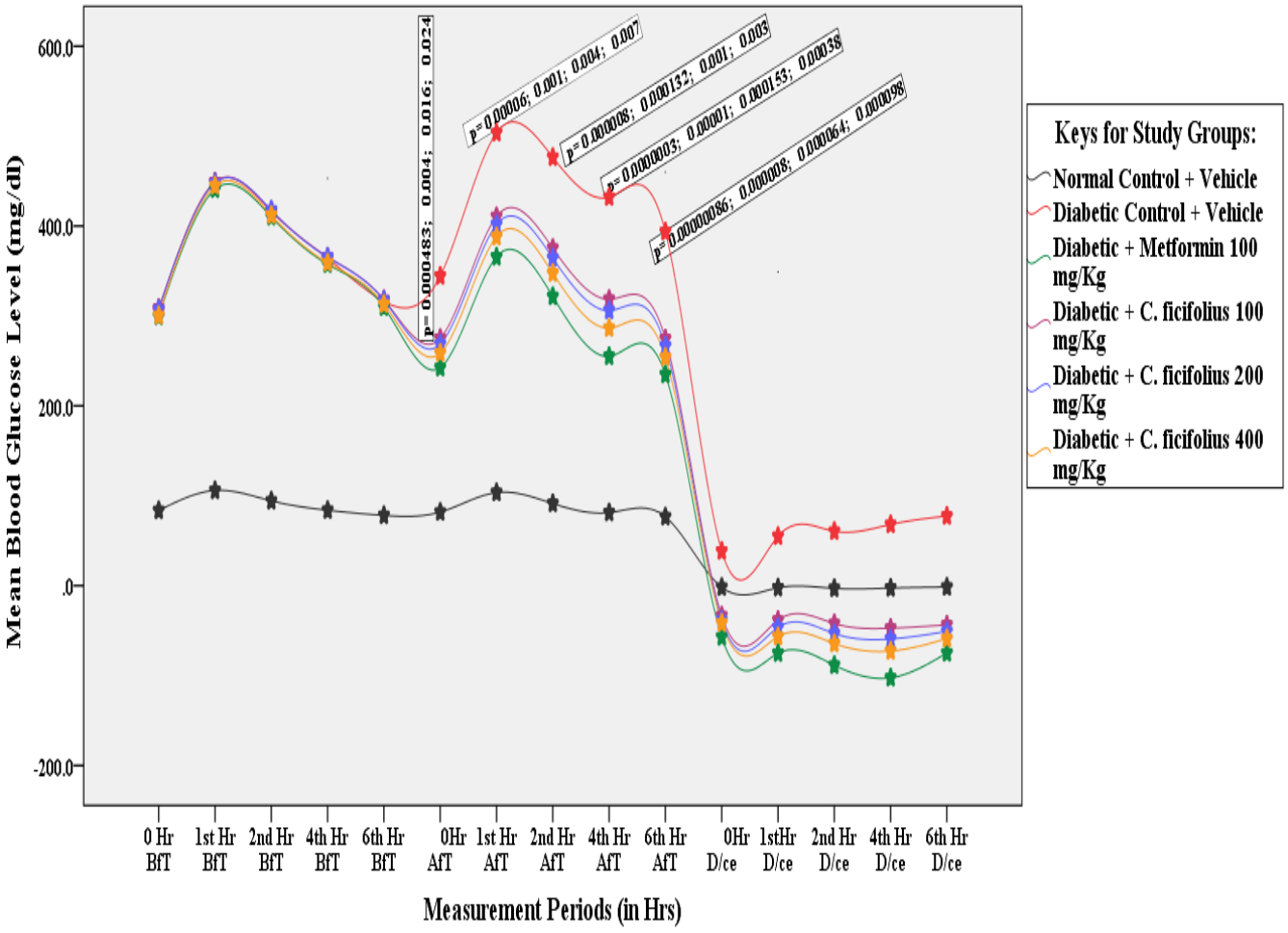


Figure-6: **Effect of Crude *C. ficifolius* Root Extract and Metformin on Glucose Tolerance Using OGTT done before-and after-3-Consecutive Weeks Treatment**

Key: p=the statistical p-value and the respective stated p-values (numbers) separated in "semicolon (;)" are the statistical values for metformin 100mg/Kg treated, *C. ficifolius* root extract 400, 200 and 100mg/Kg treated groups, respectively, at a specific measurement period for post-3-weeks treatment. All p-values were against diabetic negative control group. The p-value for OGTT after treatment was from one-way ANOVA, post hoc Tukey's HSD. Hr=hour, 0Hr= baseline hour; BfT=before any treatment; AfT=after 3-weeks treatment; D/ce=difference in blood glucose between OGTT done "before- (pre-)" and "after (post-)"-treatment. In before-treatment OGTT, the sample sizes were similar for all groups, n=7. However, in post-treatment OGTT, the sample size for diabetic negative control and *C. ficifolius* root extract 200mg/Kg treated groups were n=6 and for the remaining groups n=7. It is because one rat from Cf 200mg/Kg treated group was died at day-14 and one rat of diabetic negative control group was died at day-19.

4.3.3. The Progressive Effect of Crude *C. ficifolius* Root Extract on Fasting Blood Glucose in Diabetic Rats throughout 3-Weeks Treatment

This result purposively helped to assess & confirm the progressive effect of target drugs, and to evaluate the effective treatment duration required for specific test drug to induce sufficient anti-hyperglycemic effect.

The mixed design ANOVA (using "Greenhouse-Geisser" sphercity correction) interaction effect of diabetic study groups and measurement periods was strongly significant (p-value< 0.000001).

Analysis of simple main effect (one-way ANOVA) of treatment /study/ groups demonstrated significant differences in blood glucose among diabetic study groups after 2-weeks-long treatment (p=.047) and after 3-weeks-long treatment (p=.000472), but was negligible at 1-week-long treatment. Multiple pairwise comparisons (post hoc Tukey's HSD) among diabetic study groups after 2-consecutive weeks treatment revealed that only metformin 100mg/Kg treated group had significantly dropped (p=0.036) fasting blood glucose level against diabetic negative control. Pairwise comparisons (post hoc Tukey's HSD) among diabetic study groups at the end of 3-weeks-long treatment indicated that all experimental and positive control groups (metformin, *C. ficifolius* root extract 100, 200 and 400mg/Kg) showed substantial decrease (p= 0.000285, 0.016, 0.011 and 0.002, respectively) in their fasting blood glucose level as compared to diabetic negative control. Additionally, metformin 100mg/Kg treated group showed greater reduction in glucose level followed by *C. ficifolius* root extract 400, 200 & 100mg/Kg treated groups at week-1, 2 & 3 measurement periods, yet the differences among them were trivial (p>0.05). On the other hand, the main effect (MDANOVA) of diabetic study groups was not significant (p-value= 0.218) (see Figure-7 below).

Repeated measure ANOVA of simple main effect of treatment durations/measurement periods/ for diabetic negative control group showed that there was time wise and significant elevation of fasting blood glucose, and multiple comparisons (post hoc Bonferroni correction) were significant ($p \leq 0.018$) among all treatment durations. On the other hand, in all experimental and positive control groups (RMANOVA), there were time wise and significant reductions of blood glucose level. Pairwise comparisons (RMANOVA, post hoc Bonferroni correction) among treatment durations for metformin 100mg/Kg treated group showed significant reduction in fasting blood glucose level (p<0.002) among all pairs of treatment durations (week-0 (baseline) versus week-1, week-2 and week-3, and also other pairs). Pairwise comparisons among treatment durations for *C. ficifolius* root extract 100, 200 and 400mg/Kg doses revealed remarkable (p<0.05) drop in fasting blood glucose level among all pairs of treatment durations. In addition, the main effect of

treatment duration (MDANOVA) was significant (p-value< 0.000001). The pairwise comparisons (MDANOVA, post hoc Bonferroni) were also significant in all pairs of treatment durations, which truly represented simple main effect. Note that the mean fasting blood glucose levels for normal control group (that was not displayed in the graph below (Figure-7) to have a better and distinct comparison graph for the rest groups) at week-0, 1, 2 and 3 were83.06±4.80, 84.00±4.73, 82.37±3.95, 81.34±5.28, respectively.

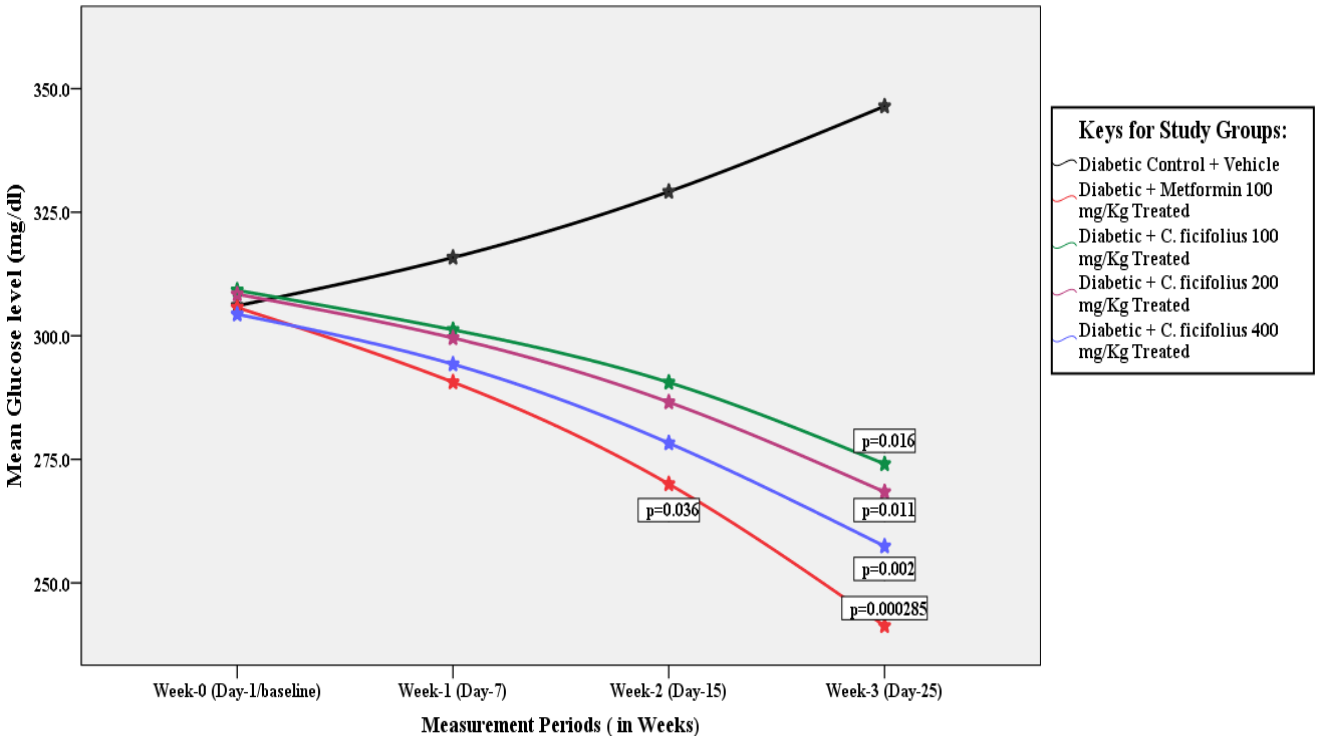


Figure-7: The Progressive Effect of Crude *C. ficifolius* Root Extract on Fasting Blood Glucose in Diabetic Rats throughout 3-Weeks Treatment.

Key: p-value was against diabetic negative control group using one-way independent ANOVA followed by Post hoc Tukey's HSD. From *C. ficifolius* root extract 200mg/Kg treated group one rat was died at day-14, and also from untreated diabetic negative control one rat was died at day-19. Week-0 (day-1) represents the baseline fasting blood glucose level measured before (just 30minutes before) any extract/drug administration. Week-1 (day-7), week-2 (day-15) and week-3 (day-25) fasting blood glucose level measured before the usual daily extract/drug administration.

4.3.4. The Effect of Crude *C. ficifolius* Root Extract on Fasting Serum Glucose in Diabetic Rats at the End of Long-Term (3-Weeks) Treatment

Based on comparison of differences in fasting serum blood glucose levels among diabetic study groups (one-way independent ANOVA, followed by post hoc Tukey's HSD), those groups treated with *C. ficifolius* root extract 100, 200 & 400mg/Kg, and metformin 100mg/Kg for 3-consecutive weeks showed strongly significant decrease (p-values=0.014, 0.009, 0.002, and 0.000251, respectively) in glucose against untreated diabetic negative control group. Metformin 100mg/Kg treated group showed greater reduction in glucose level followed by *C. ficifolius* root extract 400, 200 & 100mg/Kg treated groups, yet the differences among them were not remarkable (p>0.05) (see Table-8).

Table-8: The Effect of Crude Extract of Root of *C. ficifolius* on Fasting Serum Glucose after 3-Weeks Treatment

Study Groups	**N**	**Fasting Serum Glucose (mg/dl)**			
		Mean±SD	*p-value*	95% Confidence Interval for mean difference	
				Lower bound	Upper bound
NC +vehicle	7	83.70±5.04	-	`-	-
DC+ vehicle	6	348.35±46.40	-	-	-
D + Metf. 100	7	243.47±32.50	***.000251***	43.76	165.99
D + C.f 100	7	275.96±38.36	***.014***	11.28	133.51
D + C.f 200	6	269.17±37.06	***.009***	15.76	142.61
D + C.f 400	7	259.46±34.17	***.002***	27.78	150.01

Key: The p-value was against diabetic negative control group using one-way independent ANOVA followed by Post hoc Tukey's HSD.

4.4. The Effect of Crude *C. ficifolius* Root Extract on Histopathology of Pancreas Islets of Langerhans Cells of Diabetic Rats after 3-Weeks Treatment

As shown in figure-8, the size and structure of pancreatic islets of Langerhans cells of diabetic negative control, and experimental and positive control groups were small; irregular shaped and relatively shrank/atrophic/ as compared to normal control group. Moreover, compared to nondiabetic normal control group, the degree of destruction of pancreas islets of Langerhans cells in diabetic model groups was comparatively partial destruction/ not completely destructed/. Metformin and *C. ficifolius* root extract (100, 200 & 400mg/Kg doses) treated groups had greater islets of Langerhans cells mass as compared to diabetic negative control (i.e., diabetic negative control group developed extra atrophic change).The *C. ficifolius* root extract treated groups possessed larger islets of Langerhans cells mass at 400mg/Kg followed by 200 & 100mg/Kg in reference to diabetic negative control groups. Metformin 100mg/Kg treated group had larger pancreatic islets of Langerhans cells mass as compared to *C. ficifolius* root extract 200 & 100mg/Kg treated groups. Interestingly, *C. ficifolius* root extract 400mg/Kg treated group showed markedly increased islets of Langerhans cells mass and also it was greater than metformin 100mg/Kg treated group.

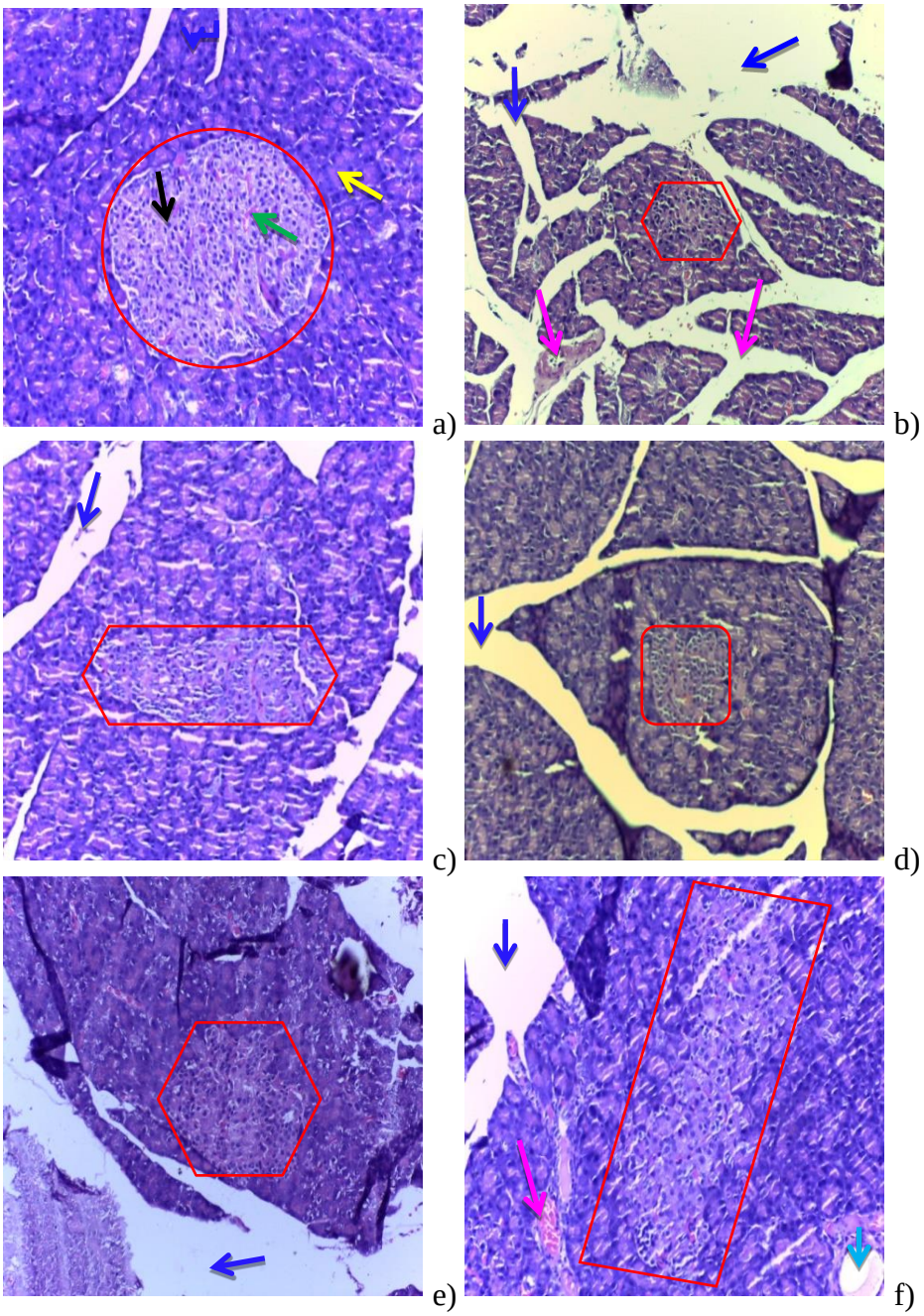


Figure-8: **Histopathology of Pancreatic Islets of Langerhans Cells in Negative Controls and Treated (Metformin and *C. ficifolius* Root Crude Extract) Groups of Diabetic Rats**

H&E stained. a) Normal control/apparently healthy/ group (200x); b) diabetic negative control group(200x); c) Metf. (Metformin) 100mg/Kg treated group (200x); d) C.f100mg/Kg treated group (200x); e) C.f 200mg/Kg treated group (200x), and f) C.f 400mg/Kg treated group (200x). Where: C.f =*C. ficifolius* crude 80% methanol roots extract. Arrows key: islets of Langerhans cells (encircled in red); Exocrine (acinar/ductal) cells (yellow arrow), black dots with in islets are nucleus of islets of Langerhans cells (black arrow); red dots and/or aggregates within islets of Langerhans cells are RBCs and/or blood vessels (green arrow); blood vessels (pink arrow), ducts (light blue arrow); large white spaces/mostly cracks (deep blue arrow).

4.5. The Effect of Crude *C. ficifolius* Root Extract on Lipid Profile and Body Weight in Diabetic Rats after 3-Weeks-Long Treatment

4.5.1. The Effect of Crude *C. ficifolius* Root Extract on Lipid Profile in Diabetic Rats after 3-Weeks-Long Treatment

As per one-way independent ANOVA analysis, metformin (100mg/Kg dose) and *C. ficifolius* root extract(at all three 100, 200 & 400mg/Kg doses) treated groups showed significant (p-value <0.05) reduction in fasting serum TC, LDL-C and TG level, and significant increment in HDL-C (good cholesterol) level as compared to diabetic negative control group (see Table-9). Metformin 100mg/Kg treated group exhibited greatest reduction in LDL-C followed by TC, TG, and better increase in HDL-C. On the other hand, the *C. ficifolius* root extract 100, 200 and 400mg/Kg treated groups revealed greater drop in TG level followed by TC and LDL-C level, and supreme augmentation in HDL-C level. The crude *C. ficifolius* root extract at higher dose (400mg/Kg) treated group had nearly comparable reduction in TG level as that of metformin 100mg/Kg treated groups. As compared to metformin 100mg/Kg treated groups, *C. ficifolius* root extract400 and 200mg/Kg treated groups exhibited relatively better increase in HDL-C level, but the differences between them were not substantial ($p > 0.05$). On the other hand, metformin 100mg/Kg treated group revealed greater reduction in LDL-C and total cholesterol level as compared to all *C. ficifolius* root extract treated groups, yet the differences between them were not weighty (p-value > 0.05). Generally, experimental and positive control groups possessed better improvement in lipid profiles than diabetic negative control group.

Table-9: **The Effect of Crude 80% Methanol Extract of Root of *C. ficifolius* on Lipid Profile in Diabetic Rats after Long-term (3-weeks) Treatment**

Groups & Dose (mg/Kg)	**Fasting Serum Lipid profiles (all mean values expressed as mg/dl)**							
	Total Cholesterol		**LDL-C**		**TG**		**HDL-C**	
	Mean±SD	*p-value*	Mean±SD	*p-value*	Mean±SD	*p-value*	Mean±SD	*p-value*
NC+vehicle	75.44±4.37	-	12.40±1.23	-	136.17±18.47	-	51.84±2.18	-
DC+ vehicle	252.15±50.47	-	143.43±32.79	-	365.75±56.12	-	48.63±12.41	-
D + Metf. 100	164.89±20.95	*.000491*	76.17±18.86	*.000285*	253.17±28.70	*.001*	66.14±7.94	*.011*
D + C.f 100	193.29±32.34	*.026*	103.31±25.85	*.046*	287.50±46.96	*.022*	65.10±8.56	*.019*
D + C.f 200	187.48±31.74	*.016*	97.57±24.37	*.023*	277.78±43.54	*.011*	68.90±8.48	***.004***
D + C.f 400	179.01±25.4	*.004*	90.80±19.16	*.005*	267.07±37.55	*.003*	73.06±6.68	***.000298***

Key: The p-value was against diabetic negative control group using one-way-ANOVA followed by Post hoc Tukey's HSD. Total number of samples was n=6 for diabetic negative control and C.f200mg/Kg treated groups and n=7 for the remaining. Data of normal control is just for referencing purpose.

4.5.2. The Progressive Effect of Crude *C. ficifolius* Root Extract on Body Weight in Diabetic Rats throughout 3-Weeks-Long Treatment

According to MDANOVA output (Greenhouse-Geisser sphercity correction), interaction effect between measurement periods and study groups in affecting body weight was insignificant ($p>0.05$). Main effect (MDANOVA) of diabetic study groups was strongly insignificant ($p= 0.967$), whereas, main effect of measurement periods was significant ($p<0.0000001$). Based on one-way ANOVA of simple main effect of diabetic study groups, experimental and positive control groups showed weight reduction, but less significant ($p>0.05$), as compared to diabetic negative controls, in each of all measurement periods. The degree of difference in weight between diabetic negative control group and experimental and positive control groups at a particular measurement period (week) increased when we go from week-0 to week-3 (i.e., the weight difference at week-3 was greater than the difference at week-2 followed by week-1 and week-0). Moreover, among experimental and positive control groups, metformin 100mg/Kg treated group showed greater weight reduction followed by *C. ficifolius* 400, 200 and 100mg/Kg treated groups at each measurement periods (week-1, -2 and -3) (see Table-10).

Moreover, simple main effect of measurement periods (RMANOVA) on weight change was significant ($p<0.05$) for each of all diabetic groups (diabetic negative control, metformin 100mg/Kg, *C. ficifolius* root extract 100, 200 and 400mg/Kg treated groups). Multiple pairwise comparisons of the simple main effect of measurement periods (RMANOVA, post hoc Bonferroni) showed that weight reductions were significant ($p<0.0001$) among measurement periods for each of all diabetic groups. Specifically, when we look the output in pairwise comparison of measurement period (week)-1, -2 and -3 against week-0, treatment week-1 had lower power of significance followed by week-2 and week-3 for each of all diabetic groups. In these pairs of comparison, each of all treatment weeks-1, -2 and -3 presented the lowest weight reduction for diabetic negative control group followed by for *C. ficifolius* root extract 100, 200 and 400mg/Kg, and metformin 100mg/Kg treated groups.

Based on percentage weight change, all diabetic groups (diabetic negative control, metformin 100mg/Kg, *C. ficifolius* root extract 100, 200 and 400mg/Kg treated groups) exhibited weight reduction as the treatment duration increase. The experimental and positive control groups showed better cumulative weight reduction (i.e., metformin 100mg/kg, *C. ficifolius* root extract 400, 200 and 100mg/Kg treated groups reduced body weight by -9.4%, -8.84%, -8.36% and -8.09%, respectively) as compared to diabetic negative control group (-7.60%) after 3-consecutive weeks treatment (see Table-10). This data revealed that among experimental and positive control groups, metformin 100mg/Kg treated group showed superior weight reduction followed by *C. ficifolius* root extract 400, 200 and 100mg/Kg.

The percentage (%) cumulative body weight change by a particular (x) study group (G) (%CBWG(x)) at specific (i) week (w (i)) was calculated by using the following formula:

$$\%\text{CBWCG(x) at w(i)} = \left(\frac{[\text{mean weight of G(x) at w(i)}] - [\text{mean weight of G(x) at w(0)}]}{\text{mean weight of G(x) at w(0)}}\right) * 100$$

Where: w (i) = week-1, 2 & 3 w (0) = week-0(baseline at day-1)

G(x) =normal control, diabetic negative control, metformin 100mg/Kg treated, *C. ficifolius* root extract 100, 200 &400mg/Kg treated groups

Table-10: The Progressive Effect of Treatment of *C. ficifolius* root extract and Metformin on Body Weight in Diabetic Rats

Measurement periods	Study groups and dose (mg/Kg/10ml)	N	Fasting body weight changes	
			Mean±SD (grams)	Percentage %CBWCG(x) at w(i)
Before Treatment (week-0)	NC + Vehicle	7	299.00±11.17	
	DC + Vehicle	7	306.00±17.68	
	D + Metf. 100	7	303.86±16.00	
	D + C.f 100	7	300.43±16.83	
	D + C.f 200	7	300.00±17.38	
	D + C.f 400	7	303.29±19.05	
After 1-week treatment (Week-1)	NC + Vehicle	7	304.86±11.52	1.96%
	DC + Vehicle	7	299.71±18.04	-2.06
	D + Metf. 100	7	296.29±16.55	-2.50
	D + C.f 100	7	293.86±15.88	-2.18
	D + C.f 200	7	293.29±16.93	-2.24
	D + C.f 400	7	296.29±19.08	-2.32
Week-2	NC + Vehicle	7	310.86±11.31	3.97
	DC + Vehicle	7	293.00±18.57	-4.27
	D + Metf. 100	7	287.86±16.86	-5.28
	D + C.f 100	7	286.86±17.47	-4.54
	D + C.f 200	6	286.33±19.97	-4.75
	D + C.f 400	7	288.43±19.82	-4.92
Week-3	NC + Vehicle	7	318.00±12.40	6.35
	DC + Vehicle	6	283.00±19.87	-7.60
	D +Metf. 100	7	275.29±14.50	-9.4
	D + C.f 100	7	276.14±16.53	-8.09
	D + C.f 200	6	275.50±19.53	-8.36
	D + C.f 400	7	276.43±16.63	-8.84

Key: In percentage data the "-" sign represents weight reduction, whereas no sign represents weight increase.

4.6. The Effect of Crude *C. ficifolius* Root Extract on Liver Parameters in Diabetic Rats after 3-Weeks-Long Treatment

4.6.1. The Effect of Crude *C. ficifolius* Root Extract on Liver Biochemical Parameters in Diabetic Rats

As shown in Table-11, compared to normal control group (one-way ANOVA, post hoc Dunnet t-test two-sided analysis), diabetic negative control group exhibited strongly altered liver parameters (highly increased fasting serum ALP followed by ALT and AST levels (for all $p<0.0001$)). On the other hand, as compared to diabetic negative control group (one-way ANOVA, post hoc Tukey's HSD), experimental (*C. ficifolius* root extract 200 & 400mg/Kg treated) and positive control (metformin 100mg/Kg treated) groups showed significant ($p<0.05$) decrease in fasting serum ALP, ALT and AST levels. Exceptionally, *C. ficifolius* root extract 100mg/Kg showed nearly significant reduction ($p=0.067$) in AST level. Moreover, metformin 100mg/Kg treated group showed extra reduction in fasting serum ALP, ALT and AST levels than *C. ficifolius* root extract treated groups. However, the difference among them was inconsequential ($p>0.05$).

Table-11: The Effect of Crude *C. ficifolius* Root on Liver Biochemical Parameters of T2DM Rats after 3-Consecutive Weeks Treatment

Groups & Dose (mg/Kg)	**Fasting Serum Liver parameters (all mean values expressed in U/L)**					
	ALP		**ALT**		**AST**	
	Mean±SD	*p-value*	Mean±SD	*p-value*	Mean±SD	*p-value*
NC+vehicle	148.64±13.24	-	100.07±7.07	-	154.61±10.31	-
DC+ vehicle	516.58±80.77	-	204.31±24.56	-	243.23±31.78	-
D + Metf. 100	362.57±41.52	*.000225*	157.50±12.27	*.000425*	196.66±15.56	*.006*
D + C.f 100	420.89±51.71	*.030*	174.91±19.40	*.040*	208.91±22.89	*.067*
D + C.f 200	406.13±49.51	*.013*	170.47±16.21	*.018*	205.37±20.19	*.045*
D + C.f 400	390.43±46.90	*.003*	163.26±13.55	*.002*	200.67±18.70	*.014*

Key: p-value was against diabetic negative control group using one-way ANOVA followed by Post hoc Tukey's HSD. Sample size n=6 for DC and C.f 200mg/Kg treated groups and n=7 for the rest.

4.6.2. The Effect of Crude *C. ficifolius* Root Extract on Liver Histo-pathological Parameters in Diabetic Rats

As revealed in Figure-9, unlike normal control/apparently healthy/ group, diabetes-induced groups showed mild pathological changes. Changes in diabetic negative control group include intra-cytoplasmic fat; observable kupffer cells development and mononuclear spotty infiltrates (MNSI). The first three changes were relatively pronounced than experimental and positive control groups, whereas, MNSI (though slight) was specific to diabetic negative control. Metformin 100mg/Kg treated group revealed normal hepatocyte with few intracellular fat droplets and negligible kupffer cells. In *C. ficifolius* root extract 100mg/Kg treated group the pathological changes were comparable to diabetic negative control with slight improvement. These include intracellular fat droplets (with few macro-vesicular fat vacuoles) and visible kupffer cells. The *C. ficifolius* root extract 200mg/Kg treated group possessed normal hepatocyte with few kupffer cells. The *C. ficifolius* root extract 400mg/Kg treated group had diffused small sized intracellular fat droplets but nearly normal smooth hepatocyte cells architecture. Generally, although there were no marked difference among diabetic study groups, those *C. ficifolius* root extract 100, 200 and 400mg/Kg treated and metformin 100mg/Kg treated groups showed relatively better improvement in those minor pathological changes as compared to diabetic negative control. Metformin 100mg/Kg treated group showed better reversal of minor pathological changes followed by *C. ficifolius* root extract 400, 200 & 100mg/Kg treated groups showed dose dependent improvement.

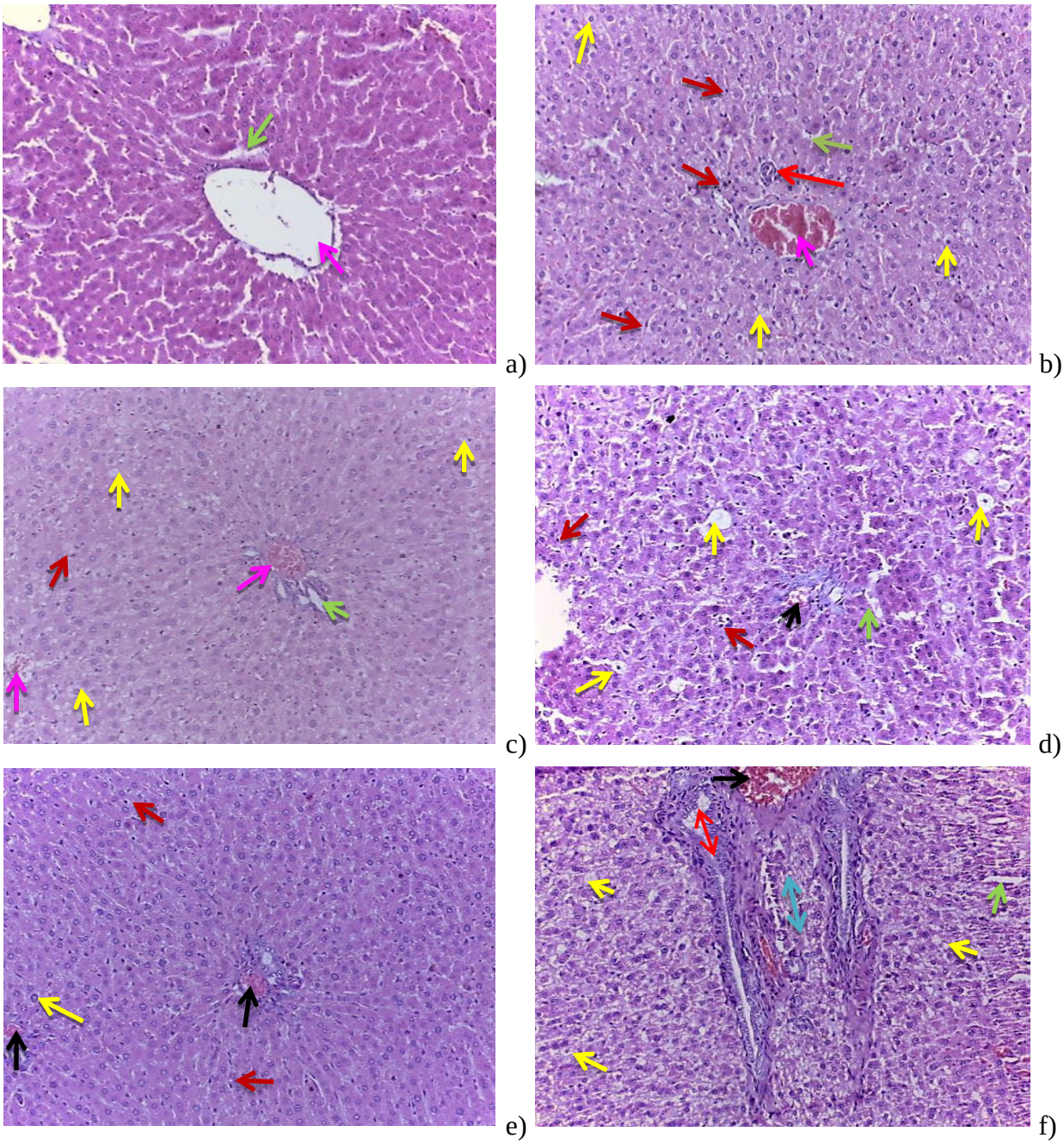


Figure-9: Liver Histopathology in Negative Controls and Treated (Metformin and *C. ficifolius* Root Crude Extract) Groups of Diabetic Rats

H&E-stained: a) Normal control group (200x); b) Diabetic negative control group(200x); c) Metformin 100mg/Kg treated group (200x); d) C.f 100mg/Kg treated group(200x); e) C.f 200mg/Kg treated group(200x), and f) C.f 400mg/Kg treated group (200x). Where: C.f =*C. ficifolius* crude 80% methanol roots extract. Arrows key: normal hepatocytes (green arrow), sinusoidal cells (light green arrow), central vein (purple arrow, in NC-diluted type), portal triad (black arrows), MNSI (red arrow), kuppfer cells (dark red arrows), intracellular fat droplets/intra-cytoplasmic fat (yellow arrow), bile duct (red double arrow) and large cord (light blue double arrow).

4.7. The Effect of Crude *C. ficifolius* Extract Root on Kidney Parameters in Diabetic Rats after 3-Weeks-Long Treatment

4.7.1. The Effect of Crude *C. ficifolius* Root Extract on Kidney Biochemical Parameters in Diabetic Rats

Based on purposive comparison one-way ANOVA followed by Dunnett-t (2-sided) analysis, against normal control group, the fasting serum level of urea were significantly ($p<0.05$) increased in diabetic negative control and *C. ficifolius* root extract 100mg/Kg treated groups, but not significant in metformin 100mg/Kg, *C. ficifolius* root extract 200 & 400mg/Kg treated groups. However, the increased urea level in diabetic negative control was moderately high, near to the upper bound of normal reference range (see Table-12).

As per one-way ANOVA followed by post hoc Tukey's HSD analysis among diabetes induced groups, metformin 100mg/Kg treated group showed notable (p-value <0.05) fall in fasting serum urea level against diabetic negative control group. Compared with diabetic negative control group, *C. ficifolius* root extract treated groups showed dose dependent reduction in serum urea level, but insignificant (p-value >0.05) with statistical trend towards significance at 400mg/Kg dose (see Table-12). In general, metformin 100mg/Kg treated group showed greater drop in fasting serum urea level followed by *C. ficifolius* root extract 400, 200 & 100mg/Kg treated group, however, differences among them were negligible ($p>0.05$).

Based on purposive comparison one-way ANOVA followed by Dunnett-t (2 sided) analysis, as compared to normal control group, the fasting serum level of creatinine were significantly lower in all diabetes induced groups. The power of significance was higher in diabetic negative control followed by *C. ficifolius* root extract 100mg/Kg, *C. ficifolius* 200mg/Kg, metformin 100mg/Kg and *C. ficifolius* 400mg/Kg treated groups. That means diabetic negative control group revealed lower level of fasting serum creatinine than that of normal control group (see Table-12).

In one-way ANOVA analysis among diabetic study groups, experimental and positive control groups showed insignificant increase ($P>0.05$) in fasting serum level of creatinine as compared to diabetic negative control group. Specifically, *C. ficifolius* 400mg/Kg treated group exhibited greater increase in fasting serum creatinine followed by metformin 100mg/Kg, *C. ficifolius* root extract 200 and 100mg/Kg treated groups, yet the difference among them were trivial. Also, the percentage creatinine data displayed similar order of increase (Table-12).

Generally, although the experimental and positive control groups showed increase in fasting serum creatinine level against diabetic negative control group, still the values were significantly lower as compared to normal control group.

As per urea: creatinine ratio, diabetic negative control showed greater value as compared to normal control group. As compared to diabetic negative control group, experimental and positive control groups showed lower urea: creatinine ratio. Specifically, metformin 100mg/Kg treated group showed lower ratio followed by *C. ficifolius* root extract 400, 200 and 100mg/Kg.

Table-12: **The Effect of Crude Extract of Root of *C. ficifolius* on Kidney Biochemical Parameters of T2DM Rats after 3-Consecutive Weeks Treatment**

Groups & Dose (mg/Kg)	Kidney biochemical parameters							
	Urea			**Creatinine**				**Urea/creatinine ratio**
	Mean±SD	***p***	$p^{a)}$	Mean±SD	***p***	$p^{a)}$	% Increase	
NC+vehicle	42.06±3.02	-		.3714±.0255	-		-	113.30
DC+ vehicle	59.75±10.83	-	*.000355*[a]	.2850±.0378	-	*.000026*[a]	-	210.38
D +Metf. 100	46.04±6.75	***.025***	*.743*[a]	.3186±.0261	*.271*	*.007*[a]	**11.79**	145.74
D + C.f 100	54.50±7.92	*.734*	*.010*[a]	.3014±.0313	*.852*	*.00031*[a]	5.75	182.01
D + C.f 200	51.88±7.16	*.407*	*.069*[a]	.3133±.0273	*.471*	*.004*[a]	9.93	166.92
D + C.f 400	48.99±5.09	*.114*	*.259*[a]	.3286±.0241	*.087*	*.036*[a]	**13.27**	149.90

Key*:* The p-values were against diabetic negative control group using one-way ANOVA, post hoc Tukey's HSD. The p-values with "[a)]" sign ($p^{a)}$) represent comparison against normal control group using one-way ANOVA followed by post hoc Dunnett-t test (2-sided). Sample size n=6 for DC and C.f 200mg/Kg treated groups and n=7 for the rest.

The percentage (%) increase (↑) in creatinine of a particular experimental and positive control group was computed using the following formula:

$$\% \uparrow \text{Creatinine} = \left(\frac{\text{Creatinine of EPCG} - \text{Creatinine of DNCG}}{\text{Creatinine level of DNCG}}\right) * 100$$

Where: EPCG= Experimental and positive control groups

DNCG= Diabetic negative control group

4.7.2. The Effect of Crude *C. ficifolius* Root Extract on Kidney Histo-pathological Parameter in Diabetic Rats

As shown in Figure-10, the Hematoxylin- & Eosin-stained histo-pathological data of kidney demonstrated that all diabetic groups showed essentially normal histologic appearance like that of normal control group. Generally, there was no marked histo-pathological difference between diabetic negative control and normal control, and also between diabetic negative control group, and experimental and positive control groups.

However, if we look more strictly, there were slight alterations in diabetic negative control and *C. ficifolius* root extract 100mg/Kg treated groups. These two groups had slightly enlarged and observable proximal tubules than normal control and remaining groups. At this point, what should be taken in to mind is that these changes are subjective, not scientifically measured. In the remaining groups such as metformin 100mg/Kg treated, *C. ficifolius* root extract 200 and 400mg/Kg treated groups there were comparative normal proximal convoluted tubules number and size (subjectively).

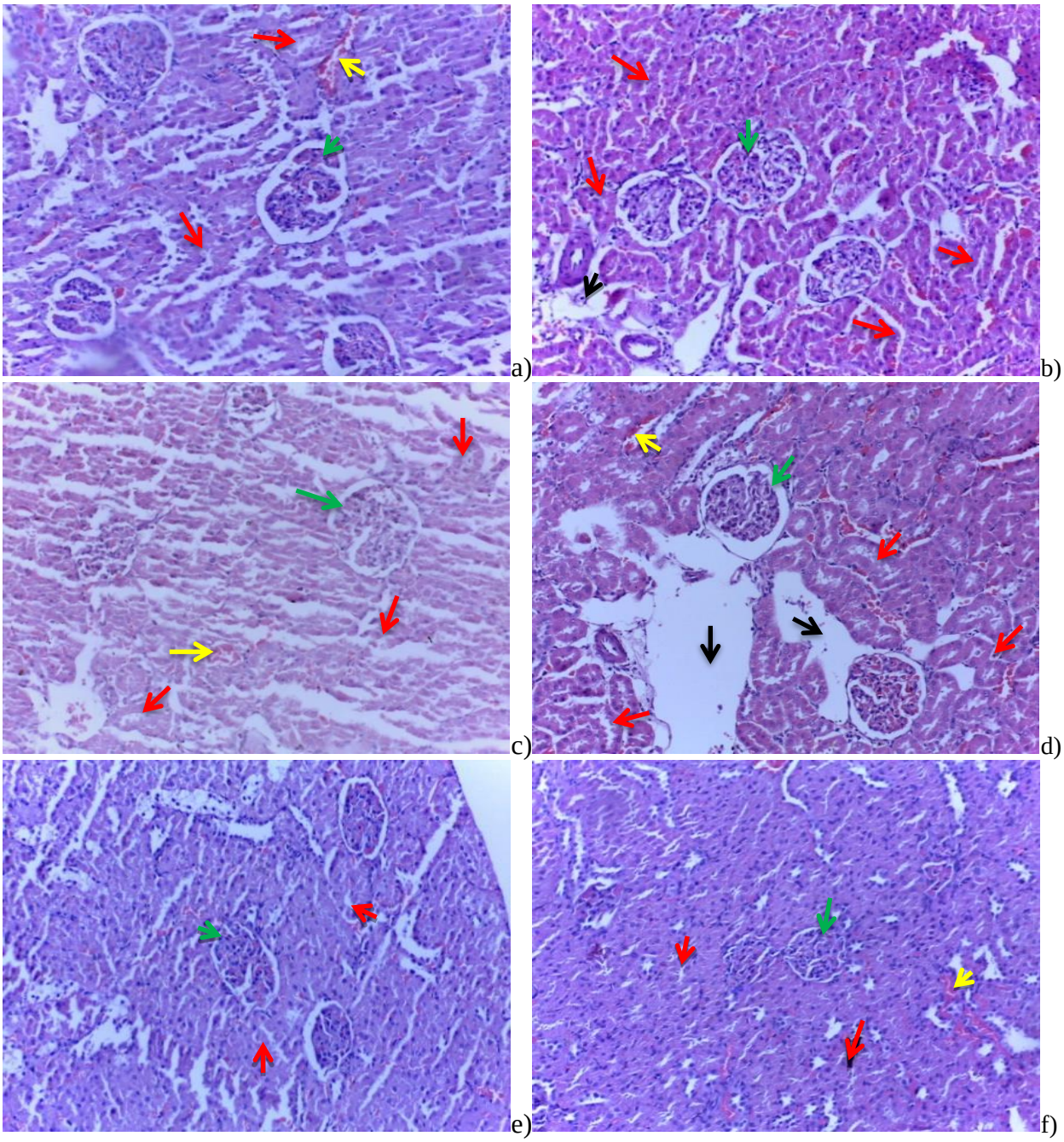


Figure-10: Kidney Histopathology in Negative Controls and Treated (Metformin and *C. ficifolius* root extract) Groups of Diabetic Rats

(H&E stained, and all with 200x magnification): a) Normal control group; b) diabetic negative control group; c) Metformin 100mg/Kg treated group; d) Cf 100mg/Kg treated group; e) Cf 200mg/Kg treated group, and f) Cf 400mg/Kg treated group. Where: Cf=*C. ficifolius* crude 80% methanol roots extract. Arrows key: Proximal tubules (red arrows), glomerulus (green arrows), blood vessels/aggregate of red blood cells (yellow arrow) and artifacts (black arrow).

5. DISCUSSIONS

5.1. Phytochemicals of Crude 80% Methanol Extract of Root of *C. ficifolius*

The effective anti-diabetic preparation is from the anti-diabetic plants with the maximum number of active compounds targeting many proteins that are involved in multiple and therapeutically relevant pathways related to diabetes (Fayaz *et al*., 2014). Therefore, the presence of flavonoids, terpenoids, saponins, glycosides, alkaloids, phenols and steroids in crude extract of *C. ficifolius* root is very interesting and providing evidence about the anti-diabetic role of crude extract of *C. ficifolius* root. It is because all of these phytochemicals are commonly present in its family *Cucurbitaceae* (Sood *et al.*, 2012; Rajasree *et al.*, 2016), and have anti-diabetic properties through various mechanisms of action, discussed in the following paragraphs.

It is difficult to make a definite suggestion about the main mechanism of action of a specific phytochemical class. It is, in part, because each of these phytochemical classes has diverse mechanisms of action (Bharti *et al.*, 2018). According to the study incorporating many active compounds, majority of active compounds of alkaloids delay carbohydrate digestion and absorption; glycosides promote insulin secretion and glycogen synthesis; saponins increase regeneration of pancreatic beta-cells and insulin secretion; flavonoids scavenge free radicals, promote insulin secretion and insulin-mimetic; polyphenols affect carbohydrate digestion and absorption and insulin secretion (Bharti *et al.*, 2018). In another study, alkaloids were found to promote basal glucose uptake and reduce oxidative damage; glycosides increase hepatic glycolysis and lower hepatic gluconeogenesis; flavonoids diminish glucose absorption and lipid metabolism in diabetic states (Gaikwad *et al.*, 2014).

Terpenoid compounds have several anti-diabetic actions mainly by modulating carbohydrate metabolism (inhibiting digestion at GIT, gluconeogenesis and glycogenolysis) and by preventing the development of IR and improving insulin signaling through inhibiting PTP1B. PTP1B is key negative regulator of insulin signaling pathway by dephosphorylating the InsR-β (beta-subunit). They also have antioxidant, anti-glycation, and anti-hyperlipidemic activities. Triterpenes are also promising agents in the prevention of diabetic complications due to its strong antioxidant and AGE inhibitory properties. The cucurbitane, ursane, oleanane and lupane type triterpenoids are well recognized for such effects (Ramírez-Espinosa *et al.*, 2011; Nazaruk & Borzym, 2015).

Flavonoids have potential anti-diabetic and anti-obesity/lipid-lowering, anti-atherogenic, antioxidant, and anti-inflammatory effects by activating or inhibiting different enzymes,

cytokines, metabolites, transcription factors, genes and intracellular signaling pathways such as AMPK, Nuclear Factor kappa-B and insulin-signaling IRS-1/PI3K/Akt pathways (Kawser *et al.*, 2016). On the other hand, the anti-diabetic potential of flavonoids is mainly through their modulatory effects on glucose transporter by increasing expression and translocation of GLUT-4 by activating InsR/IRS-1/PI3K/Akt and AMPK pathways (Hajiaghaalipour *et al.,* 2015). Moreover, flavonoids could enhance GLUT-2 expression in pancreatic β-cells, insulin secretion, reduce apoptosis and promote proliferation of pancreatic β-cells. Thus, they are effective supplements for prevention and management of diabetes and its long-term complications (Hajiaghaalipour *et al.,* 2015; Vinayagam & Xu, 2015).

Polyphenolic compounds can prevent the development of long-term diabetes complications. Specifically, anthocyanins, flavonols (quercetin) and catechins are reported for these purposes (Bahadoran *et al.,* 2013). Polyphenols mainly flavonoids and phenolic acids are the most important and extensively used phytochemicals against obesity and weight management. Their mechanisms of action are through inhibition of adipogenesis-related genes and up regulation of fat oxidation-related genes (Hsu &Yen, 2007; Sun *et al*., 2016).

As a result, the secondary metabolites identified in crude *C. ficifolius* root extract in the current study could be promising evidence to talk about the anti-diabetic activity of this plant. The specific anti-diabetic values and mechanisms of action of these phytochemicals could be discussed in each subtopic below.

Moreover, the obtained 10.8% yield of crude 80% methanol extract of *C. ficifolius* root is a good yield. This yield is important in reducing the issue of accessibility for large scale use. Therefore, the good yield together with its abundance and widespread distribution in Ethiopia (Lulekal *et al.*, 2008; Meragiaw & Asfaw, 2014; Araya *et al*., 2015; Chekole *et al*., 2015) positively supports the practice of the medicinal, such as anti-diabetic, values of *C. ficifolius* root without difficulty.

5.2. Acute Toxicity of Crude 80% Methanol Extract of Root of *C. ficifolius*

Safety must be the first issue in drug development strategies for herbal medicines (Abdel-Aziz *et al,* 2016). The absence of death and even any observable toxic sign including behavioral changes in acute oral administration of dose of 2000mg/Kg indicated that this plant is relatively safe or the lethal dose is greater than 2000mg/Kg (Lethal Dose causing 50% mortality (LD_{50})> 2000mg/Kg). Moreover, the nonexistence of any toxic changes in liver and kidney histo-pathological data during the daily oral administration of *C. ficifolius* root extract at doses of 400, 200 and 100mg/Kg for 3-consecutive weeks (discussed in detail below) strengthened the safety of this plant extract. Generally, the observed relatively good safety profile of crude 80% methanol extract of root part of *C. ficifolius* is supporting the idea about the medicinal, including anti-diabetic, importance.

5.3. The Anti-hyperglycemic Effect of Crude *C. ficifolius* Root Extract

5.3.1. Anti-hyperglycemic Effect of Crude *C. ficifolius* Root Extract during STT

The remarkable interactive effects suggested that both factors (study groups and measurement periods) influenced the dependent variable-glucose level, and also are interdependent. That means the glucose lowering effects of specific study groups depended on measurement period, and the level of glucose at specific measurement period depended on type of study group.

According to the ANOVA result of both main and simple main effects of study groups, the insignificant difference in blood glucose level among diabetic study groups implied that short-term (single day) treatment of *C. ficifolius* root extract (100, 200 & 400mg/Kg) and metformin (100mg/Kg) have only moderate efficiency on lowering blood glucose level. The data expressed in profile plot (Figure-5) clearly confirmed that short-term treatment of *C. ficifolius* root extract 100, 200 & 400mg/Kg and metformin 100mg/Kg possess anti-hyperglycemic effect as compared to untreated diabetic negative control. Metformin allowed better net glucose utilization/better efficacy/ followed by *C. ficifolius* root extract 400, 200 & 100mg/Kg than diabetic negative control.

On the other hand, based on ANOVA result of both main and simple main effects of measurement periods, the significant differences in blood glucose levels among measurement periods implied that there were greater net glucose utilizations than productions by the body cells of each of all diabetic study groups. In case of diabetic negative control group, this difference might be associated with time dependent physiological metabolic difference (time wise net increase in glucose metabolism/utilization by body cells). In early stage T2DM, insulin signaling may not be completely blocked and allow insulin-dependent uptake of small amount of endogenous glucose by peripheral tissues (Weyer *et al.*, 1999).

Additionally, the time wise difference in effectiveness between metformin and *C. ficifolius* root extract proposed that they have distinct bioavailability and plasma peak time. The delayed effect seen in metformin 100mg/Kg treated group is probably due to the metformin brand used, metformin Denk 500mg Glucophage extended release (XR), Metformin Hydrochloride 500mg film coated tablets whose plasma peak time ranges from 4-8Hrs. On the other hand, *C. ficifolius* root extract treated groups showed relatively immediate effect than metformin 100mg/Kg treated group. As per rough look on the result of this study, peak effective time of *C. ficifolius* root extract

ranged from approximately 2-4Hrs. Thus, as compared to XR metformin, *C. ficifolius* root extract effect started relatively early. But direct studies are needed to determine the metabolism and bioavailability (pharmacokinetics and pharmacodynamics) of extract.

As a whole, crude 80% methanol extract of *C. ficifolius* roots part had short-term treatment anti-hyperglycemic effect, and its efficacy was relatively less than metformin.

The observed short-term anti-hyperglycemic effect of metformin is likely associated with its strong effect on inhibition of hepatic gluconeogenesis. The direct supply of metformin from gut to liver by portal vein and higher expression of its transporter (organic cation transporter (OCT1)) in liver are responsible for the potent and preferential effects of metformin in liver (Pearson & Sakamoto, 2013).

On the other hand, the observed short-term anti-hyperglycemic mechanisms of crude 80% methanol extract of *C. ficifolius* root are discussed in the following paragraphs.

The crude *C. ficifolius* root extract might have inhibitory effect on hepatic glucose production either by directly inhibiting specific enzymes or indirectly by activating AMPK. The presence of terpenoids, saponins and alkaloids in *C. ficifolius* root extract and the presence of sister species belonging to the same family *Cucurbitaceae* such as *Coccinia grandis* and *momordica charantia* with such mechanism of action are supporting evidences. *Coccinia grandis* extract was described to decrease glucose-6-phosphatase and lactate dehydrogenase level, and control hyperglycemia in diabetic patients (Hossain *et al.,* 1992; Kamble *et al.,* 1998). *M. charantia* was reported to boost the activity of the AMPK pathway and diminish expression of PEPCK in high-fat-fed mice (Shih *et al.,* 2014). The cucurbitane-type triterpenoids from *Momordica charantia* was responsible for increased phosphorylation of AMPK (Ma *et al.,* 2010). Terpenoids (e.g., oleanolic acid and hederagenin/Oleanane-type derivatives/) had been reported as the most active glycogen phosphorylase inhibitors (Luo *et al.,* 2008). Saponins (protopanaxadiol and protopanaxatriol) down regulated PEPCK and glucose-6-phosphatase (Deng *et al.,* 2017). Alkaloids (such as trigonelline, sotolon, gentianine and carpaine) were found to down regulate the activity of fructose-1, 6-bisphosphatase (Khosla *et al.,* 1995).

Another mechanism of short-term treatment effect of *C. ficifolius* root extract in reducing blood glucose might be by enhancing insulin-independent uptake of circulating glucose to peripheral tissues probably by promoting insulin-independent recruitment of GLUT-4 to cell membrane. The comparatively greater level of flavonoids presents in *C. ficifolius* root extract might be responsible this for effect. Flavonoids are well acknowledged to promote insulin-independent translocation of

GLUT-4 via PI3K/Akt and AMPK pathways, and consecutively allow glucose uptake by cells (Hajiaghaalipour *et al.*, 2015).

Moreover, the presence of alkaloids in *C. ficifolius* root extract might be supporting for its possible acute effect by up regulating glucose metabolism (glycolysis) with in cells of peripheral tissues, which in turn promote uptake of glucose from circulation. An alkaloid-berberine was acknowledged to augment the activity of hexokinase and phosphofructokinase, and so increased rates of glucose catabolism with in cells (Singh SS., Pandey SC., *et al*., 2003).

Consequently, the possible short-term anti-hyperglycemic mechanism of *C. ficifolius* root extract might be more likely by: (a) mainly inhibiting hepatic glucose production from gluconeogenesis and glycogenolysis; (b) enhancing insulin-independent uptake of glucose to peripheral tissues; (c) increasing rate of glucose metabolism within the cells which in turn may increase glucose uptake from circulation, and/or (d) combination of them. The presence of relatively high amount of terpenoids and flavonoids, and moderate amount of saponins and alkaloids in crude extract of *C. ficifolius* root could be responsible for this effect.

5.3.2 The Glucose Tolerance Improving Effect of Crude *C. ficifolius* Root Extract

In pre-treatment OGTT, the strongly insignificant main effect of diabetic study groups truly reflected that no difference among them in altering blood glucose. However, significant main and simple effect of measurement periods suggested that the given measurement time intervals were large enough for the body to significantly metabolize orally loaded glucose. This might be associated with natural physiological effect. Also, the insignificant interaction effect implied that two factors (study groups and measurement periods) have no communal influence on blood glucose level.

In post-treatment OGTT, the significant interaction effect suggested the influence of study groups and measurement periods on blood glucose reduction were interdependent. The significant value in both of main effects implied that each factor has remarkable effect on reducing blood glucose level.

According to results from simple main effect of diabetic study groups, the significant difference at 0Hr was baseline difference which indicated the cumulative effect of 3-weeks-long treatment on fasting blood glucose. In experimental and positive control groups, the extra difference (reduction in glucose level) seen at 1^{st}, 2^{nd}, 4^{th} and 6^{th} Hr in reference to baseline (0Hr) difference suggested that treatment of metformin and *C. ficifolius* root extract for 3-consecutive weeks

improved glucose intolerance as compared to diabetic negative control. The relatively narrow differences among groups at 6th Hr in reference to differences at 4th Hr were because body cells of experimental and positive control groups completely utilized exogenous (orally loaded) glucose and shift their source of glucose to endogenous source, becomes in steady state/very slow decline/ at 6th Hr. Also, metformin better alleviated glucose intolerance followed by *C. ficifolius* root extract 400, 200 & 100mg/Kg. Moreover, the main effect of diabetic study groups truly supported the simple main effect statement. Generally, both the simple main and main effects of diabetic study groups implied that metformin and *C. ficifolius* root extract recovered glucose intolerance in diabetic rats.

The result of simple main effect of measurement periods reflected that more than 6Hr for diabetic negative control, approximately 4Hr for metformin 100mg/Kg treated and less than 6Hr for *C. ficifolius* root extract (with low time required as the dose gets higher, 400mg/Kg) treated groups were required to metabolize nearly all amount of orally loaded glucose. This means diabetic negative control group developed poor glucose tolerance, whereas, treatment of metformin and *C. ficifolius* root extract improved glucose tolerance. Based on parallel comparison among the with-in-subject factors, treatment of metformin had a better insulin sensitizing /glucose tolerance improving/ effect followed by treatment of *C. ficifolius* root extract 400, 200 & 100mg/Kg in diabetic rats as compared to untreated diabetic negative control. However, the main effect result (i.e., more than 6Hr was needed to completely metabolize orally loaded blood glucose) did not truly reflect the simple main effect. This means, even though each of metformin 100mg/Kg and *C. ficifolius* root extract 100, 200 & 400mg/Kg treatments alone had blood glucose reversing/glucose intolerance alleviating/ effects their cumulative effect was minimal. Also, depending on outcome of simple main effect of measurement periods for normal control group, nearly 2Hr would be enough for apparently healthy/normal control group to utilize almost all of orally loaded glucose which was equivalent to the real situation.

On the other hand, based on the results from main effect of diabetic study groups on blood glucose using cumulative OGTT (from pre-and post-treatment OGTT), the increased statistical power of glucose reduction from 0Hr to 6th Hr suggested that treatment of metformin and *C. ficifolius* root extract possessed positive effect on alleviating glucose intolerance. An exceptionally, in metformin 100mg/Kg treated group, the decreased statistical power at 6th Hr as compared to 4th Hr implied that the effect of oral glucose in this group was eliminated in post-treatment OGTT and so the group started normal physiological activities/ reduction in blood glucose became slow/. This caused the cumulative glucose at 6th Hr to be greater, approached to glucose value of diabetic negative control group. On the other hand, diabetic negative control group had extra glucose (from

orally loaded) that was being eliminated even at 6th Hr (in post-treatment OGTT). Thus, the difference in blood glucose level between metformin 100mg/Kg treated and diabetic negative control groups was found to be lower at 6th Hr than at 4th Hr. Generally, the long-term (3-weeks) treatment of metformin 100mg/Kg showed better reversal of glucose intolerance followed by *C. ficifolius* root extract 400, 200 and 100mg/Kg in diabetic rats.

Largely, as per the result of post-treatment and cumulative OGTTs for both of study groups and measurement periods, the root extract of *C. ficifolius* and metformin had interesting effect in augmenting glucose intolerance/IR/ in T2DM rats. Also, the insulin sensitizing effect of *C. ficifolius* root extract was roughly comparable to standard drug metformin.

The possible reason for increased severity of glucose intolerance in diabetic negative control could be because T2DM is known to induce gluco-lipo-toxicity which leads to oxidative stress, beta-cells dysfunction and further impairment of insulin signaling pathways (Tangvarasittichai, 2015). Conversely, the sufficient level of glucose transporter proteins, functioning insulin signaling pathways and upgraded peripheral glucose metabolism are important requirements for improvement of glucose tolerance/insulin sensitivity (Prentki & Nolan, 2006; Eddouks *et al.*, 2014). Thus, these might be the apparent reasons for the observed improvement of glucose tolerance by treatment of crude *C. ficifolius* root extract and metformin.

Metformin is well documented to relieve systemic and hepatic IR. Metformin promote AMP-induced mitochondrial β-oxidation (which protects ceramide and DAG-induced inhibition of IRS-1 and Akt) and peripheral glucose uptake, and inhibit hepatic glucose production (Hur & Lee, 2015; Zabielski *et al.*, 2018). It also enhances the gene expression and activity of InsR (Chaudhury *et al.*, 2017).

Different phytochemicals have been shown to improve insulin sensitivity through mechanism of stimulating insulin-signaling pathways including: increasing the expression levels of InsR-α (alpha-subunit), IRS-1, PI3K; tyrosine-induced phosphorylation of IRS; inhibition of PTP1B; the phosphorylation of AMPK/acyl-CoA carboxylase and MAPKs (mitogen activated protein kinases); activation of PPAR-γ which increase glucose uptake; enhancement of expression and translocation of the GLUT-4 and GLUT-2; augmentation of glycolysis and glycogenesis, and down regulation of glucogenesis and lipolysis (Eddouks *et al.*, 2014). Therefore, the glucose intolerance alleviating properties of crude 80% methanol extract of roots of *C. ficifolius* might be by targeting at least some of these mechanisms.

The IR alleviating mechanisms of crude extract of *C. ficifolius* root part might be by activating insulin signaling pathways and inducing the gene expression and translocation of GLUT-4. The

presence of comparatively great level (qualitative) of flavonoids and terpenoids, and moderate level of saponins and glycosides is supporting evidence. The terpenoids, flavonoids and glycosides have been reported as the main phytochemicals in improving insulin sensitivity (Eddouks *et al.*, 2014). Interestingly, the sister plant species found in the same family *Cucurbitaceae* named as *M. charantia* was found to contain different types of triterpenoids such as cucurbitane-type, momordicoside S, momordicoside T and Karaviloside XI responsible for increased phosphorylation of AMPK and IRS-1, translocation of GLUT-4 and insulin sensitivity (Cheng *et al.*, 2008; Tan *et al.*, 2008; Ma *et al.*, 2010).

Flavonoids, as one of their main anti-diabetic mechanisms, found to have strong modulatory effects on glucose transporter by promoting the gene expression and translocation of GLUT-4 (Hajiaghaalipour *et al.*, 2015). A number of flavonoids were described to promote insulin independent GLUT-4 translocation by activating upstream insulin signaling (PI3K/Akt and AMPK) pathways including: activating AMPK (e.g., procyanidins, quercetin, epigallocatechin-3-gallate, naringin and anthocyanin), PI3K (epicatechin & epigallocatechin), Akt (epigallocatechin-3-gallate and kaempferitrin). Anthocyanin and catechin found to increase GLUT-4 expression. Also, flavonoids promote InsR phosphorylation (anthocyanin, catechin) and activate IRS-1 (naringin) which are important for insulin signaling (Hajiaghaalipour *et al.*, 2015).

Glycosides (Quercetin 3-O-glycosides) were reported to have potential role for prevention and treatment of IR in muscle cells by stimulating AMPK pathway and glucose uptake (Eid *et al.*, 2010). The saponin known as compound K (a metabolite of panaxadiol) was reported to promote the expression of InsR, IRS-1, PI3K, pAkt and GLUT-4 and enhanced insulin sensitivity in high-fat and low STZ-induced diabetic rat model (Jiang *et al.*, 2014).

Moreover, the mechanism of *C. ficifolius* root extract on improving glucose tolerance might be by protecting further impairment of insulin signaling by inhibiting different molecules, such as PTP1B, responsible for such effect**.** The relatively greater level (qualitative) of terpenoids present in *C. ficifolius* root extract might be suggestive for such effect. It is because diverse numbers of triterpenoids are acknowledged to have insulin sensitizing properties principally by inhibiting PTP1B. From triterpenoids, the oleanane-type, ursane-type and lupane-type classes were recognized as the most potent PTP1B inhibitors (Ramírez-Espinosa *et al.*, 2011; Nazaruk & Borzym, 2015).

Another mechanism of *C. ficifolius* root extract on improving glucose tolerance might be by increasing glucose uptake and promoting its storage as glycogen, and flavonoids and glycosides might be responsible phytochemicals. The flavonoid-quercetin was documented to stimulate the

gene expression of glucokinase, increased liver glucose uptake in diabetic mice. Glucokinase is a marker enzyme for excess glucose level (with high Km-Michaelis-Menten constant or substrate concentration) specific to pancreas and liver, and so promote insulin secretion in pancreas and storage of excess glucose as glycogen and fat in liver (Kobori *et al.,* 2009). Numerous glycosides from many plants were found to have anti-hyperglycemic effect mainly by boosting insulin secretion and glycogen synthesis which help glucose tolerance (Bharti *et al.,* 2018). More interestingly, glycosides from sister species of *C. ficifolius* named as *Momordica charantia* (momorcharaside A and B, momorcharin A and B, momordin, momordicine and charantin) and *Citrullus colocynthis* (citrullol, colocynthin, elaterin, elatericin B and colosynthetin) were reported for such effects (Bharti *et al.,* 2018). Also, *Cucurbita ficifolia (Cucurbitaceae)* was stated to significantly improve glucose tolerance in OGTT done after treatment of extract for 28-days (Xia & Wang, 2007).

In the main, the crude 80% methanol extract of root part of *C. ficifolius* had glucose toxicity/IR/ alleviating properties. The possible mechanisms might be by: (a) activating molecules in insulin signaling pathway, (b) inducing the gene expression and translocation (insulin-dependent and independent) of GLUT-4, (c) protecting insulin signaling from impairment by other factors such as PTP1B, and (d) increasing liver glucose uptake and promoting its storage as glycogen. The presence of phytochemicals like terpenoids, flavonoids, glycosides, saponins in *C. ficifolius* root extract and presence of sister species with insulin sensitizing properties could be good indicators and evidences of *C. ficifolius* root extract anti-diabetic effect by alleviating IR.

Here, it should be noted that there must be further research focusing on each specific above stated mechanisms of action to know the exactly involved mechanism for *C. ficifolius* root extract.

5.3.3. The Progressive Antihyperglycemic Effect of Crude *C. ficifolius* Root Extract throughout 3-Weeks Treatment

The significant interaction effect implied that the two factors (study groups and treatment durations) were interdependent on altering blood glucose level in diabetic rats. This means the effect of a particular study group on fasting blood glucose depended on duration for how long the specific treatment type was taken. On the other hand, the effect of specific treatment duration on blood glucose depended on types of treatment that have been taken.

As per the result from simple main effect of diabetic study groups, for metformin to bring significant reduction of hyperglycemia against diabetic negative control, it should be administered for at least two-weeks. Whereas, for *C. ficifolius* root extract (even at higher doses, 400mg/Kg) to result remarkable discount of hyperglycemia against diabetic negative control, it should be administered for greater than two but less than three consecutive weeks. On the other hand, the insignificant main effect among diabetic study groups suggested that test drugs (metformin and *C. ficifolius* root extract) should be administered for more than three consecutive weeks /were less efficient/ to have cumulative substantial decrease of hyperglycemia in the present T2DM diabetes model. Generally, based on the data from both simple main and main effect of diabetic study groups, both metformin and *C. ficifolius* root extract had glucose lowering effect with insignificant difference among them.

According to the result from both simple main effect and main effect of treatment durations, the significant difference in glucose levels among treatment durations suggested that the given time interval (one week) was enough for: (a) the current T2DM model to induce significant increase in glucose (hyperglycemic) in diabetic negative control group, and (b) for metformin and *C. ficifolius* root extract to bring remarkable reduction of fasting blood glucose (anti-hyperglycemic). This commended that *C. ficifolius* root extracts possessed stimulating efficacy in at least 1-week-long treatment.

Based on progressive results from comparisons among study groups and treatment durations, treatment of *C. ficifolius* root extract (dose dependently) for diabetic rats have interesting effect in reducing hyperglycemia with better effect as treatment duration increase. Thus, crude 80% methanol extract of root of *C. ficifolius* had promising effect in alleviating hyperglycemia, and its efficiency at higher dose is fairly comparable with the effects shown by the standard drug metformin.

5.3.4. The Effect of Crude Extract of *C. ficifolius* Root on Fasting Serum Glucose in Diabetic Rats at the End of Long-Term (3-Weeks) Treatment

According to long-term treatment results, treatment of metformin 100mg/Kg and *C. ficifolius* root extract (100, 200 & 400mg/Kg) possessed interesting anti-hyperglycemic effects in diabetic rats. Although metformin took a better efficacy than *C. ficifolius* root extract (at all three doses), the negligible difference among them suggested that the anti-hyperglycemic efficiency of extract was moderately comparable to standard drug-metformin. This result which was determined by laboratory kits is nearly similar to the result of the third week progressive test value that was determined by Prodigy Auto-code Glucometer machine and glucose-oxidase-peroxide reactive Test Strips. The slight difference might arise from difference in sensitivity of test method/machine.

In addition to the above proposed mechanisms, the presence of terpenoids, flavonoids, saponins and alkaloids in *C. ficifolius* root extract suggested that it might has other anti-hyperglycemic mechanisms, such as effect at GIT level. The mechanism of action might be by inhibiting carbohydrate digesting enzymes, improving gut micro-biota and promoting GLP-1 secretion. The sister plant species of *C. ficifolius* named as *M. charantia* was found to inhibit glucose reabsorption in guts (Chang *et al.,* 2013).

A number of alkaloids from various plants have been reported to delay carbohydrate digestion and absorption (Bharti *et al.,* 2018). Triterpenoids (1β, 2β, 3β, 19α-tetrahydroxy-12-en-28-oic acid and corosolic acid) and flavonoids (quercitrin and hyperoside) possessed strong α-glucosidase inhibitory activities (Liu X., Zhu L., *et al*., 2014). Flavonoids (flavonols and flavones) (Sales *et al.*, 2012) and terpenoids (oleanolic and ursolic acids) (Ali *et al.*, 2006) were found to inhibit α-amylase.

Phenolic compounds (from *Morinda citrifolia Linn.)* were reported to improve bacterial colonization of the gut and intestinal morphology by their prebiotic (e.g., flavonoids-quercetin and proanthocyanidin) and pathogenic microbes inhibitory (e.g., phenolics) actions (Inada *et al.*, 2017). Also, ginseng total saponins have been reported to increase glucagon-like peptide-1 and significantly reduced hyperglycemia in diabetic rats (Liu C., Hu MY., *et al*., 2014; Governa *et al.*, 2018).

5.4. The Effect of Crude *C. ficifolius* Root Extract on Pancreas Islets of Langerhans Cells after 3-Weeks Treatment

Histo-pathological alterations in T2DM include decreased islets of Langerhans cells mass (degeneration), irregular islets of Langerhans cells shape, vacuolization of islets of Langerhans cells and acinar cells (Nugent *et al.,* 2008). Therefore, unlike the normal control group, the greater atrophic change of pancreatic islets of Langerhans cells seen in the remaining five diabetic study groups were due to the degenerative effect of diabetes mellitus on islet of Langerhans cells, particularly beta-cells. Although firm evidence is required to make such inference, the observed relatively partial destruction of pancreas in diabetic model groups compared to nondiabetic normal control group suggested that the diabetes model developed is likely to be T2DM model rather than T1DM. Furthermore, the extra atrophic change of pancreatic islets of Langerhans cells in diabetic negative control group than the experimental and positive control groups suggested that the exacerbation of diabetes induced apoptosis of pancreatic islets of Langerhans cells. Conversely, treatment of *C. ficifolius* root extract and metformin reversed this effect. It is clear that diabetes-induced gluco-and lipo-toxicity could encourage oxidative stress and associated beta-cell apoptosis (Tangvarasittichai, 2015).

The larger/extra/ mass of pancreatic islets of Langerhans cells seen in experimental and positive control groups as compared to diabetic negative control group was probably linked to mass of beta-cells instead of alpha-cells. However, what should be taken in to mind here is that the fasting serum insulin level test and/or special pancreatic tissue stain should be done to exactly differentiate which islets of Langerhans cells type (beta-cells? or non-beta/alpha/-cells?) increased. In diabetes mellitus, the ideally expected condition is increased alpha cells mass rather than beta-cells, as a body's physiological compensatory mechanism against peripheral tissues glucose deprivation secondary to IR/deficiency. However, this response is detrimental than benefiting. The inappropriate increased alpha-cells represent an increased α-cell function and concomitant secretion of glucagon, increased glucogenesis, leading to hyperglycemia (Godoy-Matos, 2014). Thus, if we say the increased pancreatic islets of Langerhans cells mass observed in experimental and positive control groups was linked to alpha-cells rather than beta-cells, it implied treatment of *C. ficifolius* root extract (100, 200 & 400mg/Kg doses) and metformin 100mg/Kg had glucagon-secreting/hyperglycemia aggravating/ effect beyond that of diabetic negative control. However, the decreased fasting glucose level/relief from hyperglycemia/ seen in experimental and positive control groups as compared to diabetic negative control group disproved this concept. Evidentially, anti-hyperglycemic and β-cell function improving effect of

leaf extract of *Clerodendrum volubile P Beauv (Labiatae)* in T2DM rats have been presented by evenly distributed alpha-cells and increased β-cells mass (Erukainure *et al.,* 2018). Therefore, the observed increased pancreatic islet mass in experimental and positive control groups was associated with pancreatic beta-cell mass, not alpha cells. This means treatment of *C. ficifolius* root extract and metformin had favorable effects on beta-cell mass for their anti-hyperglycemic action.

Islets of Langerhans β–cell failure in T2DM possibly happens when Islets of Langerhans are incapable to sustain β-cell compensation for IR. The compensatory mechanisms are by increasing β-cell mass and enhancing β–cell function. Improving insulin gene expression and insulin secretion are important mechanisms for maintaining beta-cells function (Prentki & Nolan, 2006). On the other hand, the maintenance of mature β-cell population/mass/ is controlled by β-cell differentiation, trans-differentiation from other cell lines, beta-cells self-replication and regulation of apoptosis (Khadra & Schnell, 2015).

In this study, the mechanism of extension or maintenance of beta-cells mass observed in experimental and positive control groups was unlikely by increasing beta-cells proliferation because the model rats used were adults. Adult pancreatic beta-cells could be formed by self-duplication of mature cells rather than stem-cell differentiation which is the case of young rats (Dor *et al.,* 2004). Therefore, the possible mechanisms of maintenance and/or extension of beta-cells mass observed in experimental and positive control groups might be by reducing further apoptosis, increasing duplication of mature beta-cells and up regulating trans-differentiation of non-beta islets of Langerhans cells (alpha, delta & ductal) to beta-cells. Islet of Langerhans cells plasticity allows conversion of non-beta islets of Langerhans cells into beta-cells to replenish beta-cell mass and function (Khadra & Schnell, 2015; Da Silva, 2018).

The relative improvement in islets of Langerhans cells (beta-cells) in metformin 100mg/Kg treated group, as compared to diabetic negative control, was probably due to its anti-apoptotic effect than direct islets of Langerhans cells/beta-cells regenerative effect. It has been reported that metformin has no direct effect on increasing beta-cells mass (regeneration) and function (insulin secretion), instead, it protects pancreatic beta-cells dysfunction by defending their apoptosis (Van Stee *et al.*, 2018). Metformin could protect beta-cells by reducing T2DM-derived gluco-toxicity and lipo-toxicity, and associated ROS production, oxidative- and ER (endoplasmic reticulum)-stresses and inflammation which are known elements for beta-cells apoptosis (Zhou *et al.,* 2018).

On the other hand, the possible mechanism of action of *C. ficifolius* root extract on relative improvement of mass of beta-cells of pancreatic islets of Langerhans was either by protecting

beta-cells from apoptosis or by promoting regeneration of beta-cells via replication and trans-differentiation, or both. Therefore, the possible justifications for the relatively greater pancreatic islets of Langerhans cells (most likely beta-cells) mass observed in *C. ficifolius* root extract 400mg/Kg treated group than metformin 100mg/Kg treated group are either the extract had a better anti-apoptotic effect than metformin as dose increase, or had additional direct beta-cells regenerative effect (Khadra & Schnell, 2015; Da Silva, 2018).

On the other hand, this islet of Langerhans beta-cells mass improving effect of *C. ficifolius* root extract might help to augment insulin secretion and improve beta-cells function, and so alleviate hyperglycemia. This concept can be evidenced by the fact that the main cellular mechanism of traditional natural products for increasing the secretion of insulin are regeneration of pancreatic β-cell, inhibiting the apoptosis of pancreatic islets of Langerhans tissue, and enhancement of cellular signaling pathways like insulin promoter factor-1(Farzaei *et al.*, 2017).

The presence of phytochemicals such as flavonoids, saponins, glycosides, alkaloids and terpenoids, in crude 80% methanol extract of roots of *C. ficifolius* might be responsible for its beta-cell regenerative (by promoting beta-cells replication and differentiation) and anti-apoptotic (by owning anti-oxidant effect or directly inhibiting apoptosis signaling pathway) effect. It has been indicated that flavonoids (like silymarin, quercetin, genistein and epigallocatechin-3-gallate (EGCG)) and saponins (ginsenoside) protect pancreatic beta-cell apoptosis; vinca alkaloid (conophylline) encourage differentiation and replication of beta-cells, and glycoside (momordicin) increase pancreatic beta-cell replication (Oh, 2015). A number of active compounds of saponins extracted from different plants have been described to play anti-diabetic role mainly by increasing regeneration of pancreatic beta-cells and insulin secretion (Bharti *et al.*, 2018). Terpenoid-asiatic acid was presented to upsurge beta-cell replication and beta-cell pro-survival signaling in STZ-induced diabetic rats (Liu J., He T., *et al*., 2010).

Different sister species of *C. ficifolius* such as *Cucurbita pepo Linn* (Rauter *et al.*, 2010), *Cucumis sativus* (Mukherjee *et al.*, 2013), *Cucurbita ficifolia* (Acosta-Patino *et al.*, 2001) *and Momordica charantia* (Chang *et al.*, 2013) have been described to have beta-cell regenerative and anti-apoptotic effects. These properties might be shared by *C. ficifolius* root extract and strengthen the evidence of its effect on maintaining pancreas beta-cells mass.

Therefore, the positive effect of crude 80% methanol extract of *C. ficifolius* roots on Beta-cells of pancreatic islets of Langerhans might be one mechanism for its anti-diabetic effect.

5.5. The Effect of Crude *C. ficifolius* Root Extract on Lipid Profile and Body Weight in Diabetic Rats

5.5.1. The Effect of Crude *C. ficifolius* Root Extract on Lipid Profile after 3-Weeks-Long Treatment

In this study, the increased levels of LDL-C, VLDL-C and TC along with decreased HDL-C level noticed in all diabetic study groups as compared to normal control group implied that the current T2DM model started to alter lipid metabolism, ensued dyslipidemia. Diabetes mellitus is known to result abnormal lipid metabolism which is characterized by accumulation of plasma LDL-C, VLDL-C and TC as well as decreased HDL-C. Elevated levels of LDL-C, VLDL-C and TC are major risk factors for cardiovascular disease (CVD). Conversely, increased HDL-C reduces the risk of cardiovascular disease by transporting cholesterol from periphery to liver for clearance (Moodley *et al.,* 2015).

Conversely, among diabetic study groups, the diminished level of LDL-C, VLDL-C and TC together with increased HDL-C level in metformin 100mg/Kg treated and *C. ficifolius* root extract 100, 200 and 400mg/kg treated groups as compared to untreated diabetic negative control group suggested that treatment of both metformin and *C. ficifolius* root extract had anti-hyperlipidemic effect in T2DM rats. More specifically, treatment of extract had nearly comparable effect on TG level as that of metformin. However, treatment of metformin possessed relatively greater effect in improving LDL-C and TC level than *C. ficifolius* root extract. Conversely, treatment of *C. ficifolius* root extract resulted better effect in improving HDL-C level than metformin. These differences suggested that metformin and *C. ficifolius* root extract might have somewhat different mechanisms of action in regulating dyslipidemia. The possible mechanisms are discussed as follows.

Metformin's effect on lipid metabolism is more localized to the intestine than liver tissue (Zhou *et al.,* 2018). Metformin has serum lipid lowering effect by: (a) inhibit chylomicron assembly and its absorption in intestine through inhibiting gene expression of Apo-lipoprotein A subtype-4 (ApoA-4, important for chylomicron secretion); (b) inhibiting intestinal apical sodium-dependent bile acid transporter (ASBT) to prevent bile acids absorption (promote fecal excretion); (c) improving gut micro-biota which metabolize bile acids and (d) activating AMPK which in turn inhibit cholesterol (intestinal HMG-CoA reductase) and TG (ACC1 and FAS) synthesis and improve LDL-C-receptor recycling (Van Stee *et al.,* 2018). Intestinal deficiency of bile acids promotes uptake of plasma LDL-C particle to liver as a source of cholesterol for synthesis of

compensatory bile acids (Xu *et al*., 2015). AMPK-induced SREBP-1c inhibition leads to up regulation of fatty acid desaturase 1(FADS1) and FADS2 which results decreased arachidonic acid levels. Reduction of arachidonic acid level cause increased membrane fluidity, thereby increasing LDL-C receptor recycling and plasma LDL-C clearance (Sone *et al.*, 2013).

The effect of metformin on HDL-C could be by reducing its degradation. HDL-C levels are linked with TG and LDL-C levels (Zang C., Gao F., *et al*., 2015). Thus, in this study, the greater effect of metformin on LDL-C and total cholesterol levels than on TG and HDL-C could be by increasing clearance of plasma LDL-C and intestinal cholesterol, respectively (Van Stee *et al.,* 2018).

The greater effect of *C. ficifolius* root extract on HDL-C than on LDL-C and total cholesterol suggested that the main mechanism of this extract seems to be by increasing HDL-C production rather than by decreasing its catabolism/clearance. As long as there is high plasma TC and TG level, an increased catabolism of HDL-C is a must. It is because the main function of HDL-C is clearing cholesterol and TG from plasma and peripheral cells by transporting them to liver where they get degraded (Moodley *et al.,* 2015). Therefore, the possible mechanism of extract on high HDL-C level tends to be by increasing its synthesis and assembly (Vergès, 2010). Increased HDL-C production might, in part, help for increased TG and cholesterol clearance (Lakhne *et al*., 2015).

The presence of sister species of *C. ficifolius* such as *Coccinia grandis (*fruit part) (Ahmed & Manoj, 2012), *Cucumis melo* (rich in polyphenols and ascorbic acids) (Parmar & Kar, 2008; Bidkar *et al.,* 2012) and *Cucurbita pepo* (rich in flavonoids) (Sedigheh *et al.,* 2011) with significant increasing effect in HDL-C level, together with reducing effect in TC, TG and LDL-C levels, partially supports the current effect of extract on HDL-C level. Thus, the presence of flavonoids and other polyphenols in *C. ficifolius* root extract might be responsible for the suggested effects. However, the mechanisms of action remain to be determined.

The possible mechanisms of actions of *C. ficifolius* root extract on reduction of TG seems to be by (i) increasing its clearance through augmenting HDL-C-induced TG degradation, membrane LPL activity, FA-uptake and FA-oxidation, and (ii) by inhibiting TG synthesis. Membrane LPL promotes degradation of plasma TGs, from both endogenous (VLDL &LDL-C) and exogenous (chylomicron) sources (Moodley *et al.,* 2015). Saponins, named as ginsenosides, were recognized to: (a) increase the expression of LPL and its cofactor Apo C-III genes; (b) promote the transcription of proteins involved in fatty-acid uptake such as activating PPAR (Auwerx *et al.*, 1996), and (c) down regulate FAS (Chen F., Chen Y., *et al.,* 2012). Sister species of *C. ficifolius* known as *Coccinia grandis* had been reported to improve LPL activity (Kamble *et al.,* 1998). *Momordica charantia* was rcognized to activate PPARs and up regulate the gene expression of

the acyl-CoA oxidase which catalyzes the first step of peroxisomal beta-oxidation. The promotion of fatty acids-oxidation allows reduction of FA (precursor of TG synthesis) and so decreases TG level (Chao & Huang, 2003). *Momordica charantia* (Joseph & Jini, 2013) and *Cucurbita ficifolia* (Xia & Wang, 2007) were described to reduce TG and TC.

The total cholesterol reducing effect of crude extract of *C. ficifolius* root might be by increasing cholesterol clearance (secondary to high HDL-C) or by directly inhibiting cholesterol synthesis. The existence of flavonoids and steroids in the extract could be responsible phytochemicals. Flavonoid compounds were identified to inhibit cholesterol synthesis by suppressing HMG-CoA reductase (Huseini *et al.*, 2006). Steroids from *Cucumis sativus Linn*, which is a sister species of *C. ficifolius*, were reported for cholesterol decreasing effect (Sood *et al.*, 2012).

The LDL-C reducing mechanism of crude extract of *C. ficifolius* root could be by inhibiting LDL-C formation and by increasing its clearance by promoting LDL-C-receptor production. The flavonoids present in this extract could be the proposed evidence. Flavonoids were found to increase LDL receptor expression and suppress the secretion of Apo B-100 (important for assembly of VLDL-C) (Pal *et al*., 2003). Because VLDL-C synthesis depends on level of TGs and TC present, the decrease in these precursors may also reduce LDL-C formation from VLDL-C (Lakhne *et al.*, 2015).

5.5.2. The Progressive Effect of Crude *C. ficifolius* Root Extract on Body Weight throughout 3-Consecitive Weeks Treatment

The insignificant interaction effect implied that the dependency of one factor on another factor (i.e., particular measurement period on study groups, or vice versa) in affecting body weight was weak. The insignificant main and simple main effects among diabetic study groups suggested that treatment of a particular test drug (metformin 100mg/Kg or *C. ficifolius A Rich* 100, 200 or 400mg/Kg) to diabetic rats had minimal weight reducing effect as compared to non treated diabetic negative control group. However, the increased weight reducing effect of test drugs as the treatment duration increased proposed the advantage of having longer treatment durations. Moreover, treatment of metformin (standard) still had greater weight reducing effect followed by treatment of *C. ficifolius* root extract 400, 200 and 100mg/Kg in each of measurement periods (week-1, 2 and 3).

The remarkable main and simple main effects of measurement periods on weight reduction for all diabetic groups (control and experimental and positive control groups) implied that a given measurement period was enough for the disease-diabetes mellitus itself and test drugs (metformin

and *C. ficifolius* root extract) to result in weight loss in diabetic rats. By each measurement periods (week-1, 2 and 3), the extra increase in power of significance for experimental and positive control groups than for diabetic negative control confirmed the additional weight reducing effects of test drugs. In addition, metformin 100mg/Kg possessed better weight reducing effect followed by *C. ficifolius* root extract 400, 200 and 100mg/Kg. This means extract revealed dose dependent weight reducing effect, and its efficacy was relatively lower than metformin. The percentage result clearly confirmed the above explanations (e.g., the net percentage weight reduction for *C. ficifolius* root extract at higher dose-400mg/Kg was 1.24%, whereas, for metformin 100mg/Kg was1.8%).

Since obesity is one of main factors contributing for T2DM development (Kohei, 2010; Shulman, 2014; Samuel & Shulman, 2016; Furukawa *et al.*, 2017), treating obesity/hyperlipidemia is the best strategy for prevention and management of T2DM. In this study, the time-wise decrease (from week-1 to week-3) in body weight in diabetic negative control could be associated with the pronounced time-wise catabolic effect of diabetes mellitus on body lipids (Vergès, 2010), carbohydrates (Barbosa *et al.,* 2014) and proteins (James *et al.,* 2017).

The body weight reducing effect of *C. ficifolius* root extract could be more evidenced by its effect on decreasing biochemical lipid parameters (discussed above). It is because body compositions (body mass index and pattern of fat distribution) have strong positive correlation with body fat and blood lipids profile (Zamani *et al.,* 2012). This strengthens the dual effect of *C. ficifolius* root extract on body weight reduction and on improvement of dyslipidemia. The good anti-obesity effects could be reflected by reduction in body weight with simultaneous decrease in the levels of TG, TC and LDL-C and increase in HDL-C level (Patra *et al,* 2015).

The weight reductions by *C. ficifolius* root extract and metformin might more likely be associated with fat mass than lean mass. According to the previous study that incorporated different factors responsible for weight loss, from the total of weight loss composition the fat mass accounts for approximately three-fourth of weight loss and the remaining one-fourth for fat-free (lean) mass (Heymsfield *et al.,* 2014).

Metformin was found to induce modest weight loss in overweight and obese individuals at risk for diabetes by reducing food intake via GLP-1 mediated incretin-like actions and by increasing FA-oxidation through PPAR-α and AMPK activation (Hur & Lee, 2015; Chaudhury *et al.,* 2017).

The suggestive weight reducing mechanisms of crude extract of *C. ficifolius* roots involve stimulating lipolysis, inhibiting lipogenesis, delaying lipid digestion and suppressing appetite signals. The flavonoids, phenols, alkaloids, steriods and saponins present in *C. ficifolius* root extract might be responsible for such effects. Polyphenols mainly flavonoids (such as epicatechin,

catechin, rutin, quercetin and kaempferol) and phenolic acids (e.g., gentisic acid, p-hydroxybenzoic acid and the derivative chlorogenic acid) were acknowledged as the most important and extensively used phytochemicals against obesity and weight management. Their mechanism of actions involves up regulation of fat oxidation-related genes (such as hormone-sensitive lipase, carnitine palmitoyl transferase 1 (CPT-1), uncoupling protein-2 (UCP-2), PPAR-α and adiponectin protein levels) and inhibition of adipogenesis-related genes (such as PPAR-γ, C/EBP-α (CCAAT (Cytosine-Cytosine-Adenosine-Adenosine-Thymidine)/Enhancer-Binding Protein-alpha), SREBP-1c, FAS, tissue LPL and leptin levels) (Hsu & Yen, 2007; Sun *et al*., 2016). Also, alkaloids were reported to inhibit adipogenesis by reducing the expression levels of several adipocyte marker genes including PPAR-γ and C/EBP-α in adipocytes (Choi *et al.,* 2014).

In earlier different studies, saponin (like, chakasaponin-II) with NPY mRNA (messenger RNA) suppressive (Wang L., Yamasaki M., *et al*., 2011); phyto-sterols formulation (Cylaris) with appetite suppressant and anti-lipase (Oben *et al.,* 2006), and flavonoid (flavones-without glucose) with pancreatic lipase inhibitory (Ahn *et al.,* 2013) activities were reported. In this study, as per the rough observational assessment on food consumption difference among study groups (though not definitely quantified and not stated in result section), those metformin and crude *C. ficifolius* root extract (dose dependently) treated groups showed relatively lower food consumption. In rough food consumption assessment during experimentation, all diabetic study groups were allowed to take amount of food (300 grams of bolus) of food bolus per day. Then, how much uneaten food remained in each cages per day was observed. However, whether it was due to the test drug's direct appetites suppressant and lipid digestion inhibitory properties or due to indirect effect on food consumption by reducing diabetes-induced polyphagia remains to be determined.

Furthermore, the extract from sister species of *C. ficifolius* found in same family *Cucurbitaceae* such as *Gynostemma pentaphyllum, Cucumis melo* and *Coccinia grandis* were reported to reduce body weight. The fruit peel extract of *Cucumis melo* (Bidkar *et al.,* 2012) and fruit extract of *Coccinia grandis* (Ahmed & Manoj, 2012) were found to have body weight reducing activity in atherogenic cholesterol fed rat models. In human clinical trial, active compound-actiponin from *Gynostemma pentaphyllum* leaves extract was stated to activate AMPK which in turn down regulate adipogenesis-related genes (Park *et al.,* 2014). These effects might be shared by *C. ficifolius* root extract and strengthened its anti-weight effect.

Taken together, the significant anti-hyperlipidemic and the moderate weight-reducing effect of crude 80% methanol extract of roots of *C. ficifolius*, in part, explained its advantage for management of T2DM.

5.6. The Effect of Crude *C. ficifolius* Root Extract on Liver of Diabetic Rats after 3-Consecutive Weeks Treatment

5.6.1. The Effect of Crude *C. ficifolius* Root Extract on Liver Biochemical Parameters

Liver is the most important organ in regulation of carbohydrate and lipid metabolism (Rui, 2014). However, T2DM is known to deregulate liver metabolism and result in liver complications. More specifically, the ALT, AST, ALP and fatty liver (NAFLD, Non-Alcoholic Fatty Liver Disease) are independent risk factors and predictor for incident T2DM (Chen SCC., Tsai S.P., *et al*., 2017).

Therefore, in this study, the elevated levels of fasting serum ALT, AST and ALP observed in all diabetic study groups as compared to normal control group implied that the changes were caused by diabetes (the current T2DM model). Conversely, treatment of crude root extract of *C. ficifolius* 100, 200 &400mg/Kg and metformin 100mg/Kg significantly reversed diabetes-induced elevation of liver enzymes ALT, AST and ALP as compared to untreated diabetic negative control group. Treatment of metformin 100mg/Kg possessed a better efficacy followed by *C. ficifolius* root extract 400, 200 & 100mg/Kg, however, the negligible difference among them implied that crude *C. ficifolius* root extract had relatively comparable effect as that of metformin. In the main, treatments of crude 80% methanol extract of roots of *C. ficifolius* and metformin for diabetic rats had positive effect in alleviating T2DM-induced liver biochemical complications.

5.6.2. The Effect of Crude *C. ficifolius* Root Extract on Liver Histo-pathological Parameters

In reference to normal control group, the abnormal minor liver histo-pathological changes such as intracellular fatty degenerations (steatosis), inflammatory kupffer cell development and mononuclear spotty infiltrates seen in diabetic groups were associated with diabetes disease. This implied that the present diabetes model started to result liver complications (Tolman *et al.*, 2007; Lucchesi *et al.*, 2015).

The observed intracellular fat droplets in hepatocytes are indicators of development of fatty liver/simple steatosis, and caused by the existing T2DM model. Fatty liver is an independent predictor for incident T2DM (Chen SCC., Tsai S.P., *et al*., 2017). T2DM is known to cause peripheral tissues lipolysis which allows massive inflow of FFA to liver where accumulated as TG, leads to fatty liver. Non-alcoholic fatty liver disease could also progress to non-alcoholic steato-hepatitis/NASH/, cirrhosis and acute liver disease (Mohamed *et al.*, 2016). Intracellular fat

is an early sign of hepatocyte hypertrophy which in large scale may develop to liver hypertrophy and lead to liver dysfunction (Moodley *et al.,* 2015).

The increased numbers of kupffer cells (liver macrophages) observed in almost all diabetic groups (with different degree) and the minor MNSI (specific to diabetic negative control) indicated the onset of inflammation in the present diabetes model. T2DM could result liver damage by inducing oxidative stress and inflammation (Mohamed *et al.,* 2016). T2DM-induced fatty liver might be the cause for such inflammatory changes. It has been reported that fatty acid trigger mitochondrial production of ROS and activation of innate immunity, both of which are important factors for inflammation. Over activation of innate immunity, particularly the hepatic resident phagocytic cells (macrophages, known as Kupffer cells), is known to persuade inflammation (Williams *et al.*, 2012).

Conversely, treatment of crude extract of root of *C. ficifolius* 100, 200 & 400mg/Kg and metformin 100mg/Kg improved those diabetes-induced mild liver histo-pathological aberrations as compared to untreated diabetic negative control group. What should be underlined here is that the histo-pathological differences among untreated- and treated-diabetic groups were not large enough, and so comparisons were subjective. The greater improvement of those slight pathological changes in metformin than *C. ficifolius* root extract advocated the better efficacy of metformin against liver complications. On the other hand, the dose dependent improvement in pathological changes in extract treated groups, especially at doses of 400 and 200mg/Kg, indicated better hepato-protective potential of extract as dose increased. Thus, treatments of *C. ficifolius* root extract and metformin for diabetic rats have affirmative effect in alleviating T2DM-induced liver pathological complications.

On the other hand, the amelioration of those slight pathological changes and biochemical parameters in *C. ficifolius* root extract (dose dependently) and metformin 100mg/Kg treated groups as compared to diabetic negative control group, at least, suggested that treatment of *C. ficifolius* root extract and metformin have no extra hepatotoxic effect (not hepato-toxicants) during experimentation period. Hepato-toxicants are characterized by disturbing liver clinico-biochemical markers such as increasing liver enzymes (AST, ALT and ALP) and resulting liver pathological injuries (zonal necrosis, hepatitis, cholestasis, granuloma and vascular lesions) (Singh A., Bhat TK., *et al*., 2011). This statement supported that the biochemical and pathological changes seen in diabetic groups is most likely associated with T2DM rather than treatments' toxicity. However, chronic toxicity test should be done to make clear inference.

In general, the observed improvements in those slight histo-pathological alterations and liver biochemical parameters suggested that crude 80% methanol extract of *C. ficifolius* roots and metformin have hepato-protective effect against diabetes-induced complications.

The possible mechanism might be associated with the presence of phytochemicals with anti-hyperglycemic, anti-hyperlipidemic or direct antioxidant properties in crude extract of *C. ficifolius* root. The anti-hyperglycemic and anti-hyperlipidemic properties are preventive strategies against development of oxidative stress and inflammation (Tangvarasittichai, 2015). Moreover, the presence of relatively greater level of strong antioxidant phytochemicals, such as flavonoids, in crude extract proposed that this plant might have direct antioxidant effect to take part in prevention of oxidative damage of liver (Bharti *et al.*, 2018). However, the directly antioxidant activity of this extract remains should be determined, future work.

According to ethno-botanical study done in Ethiopia, root part of *C. ficifolius* was used against jaundice (crush the fresh /dried form, and then boil and drink) (Teklehaymanot & Giday, 2007; Lulekal *et al.*, 2008; Araya *et al*., 2015). This might, to some extent, support the beneficial effect of *C. ficifolius* to liver.

In addition, different sister plant species of *C. ficifolius* found in same genus *Cucumis* such as *Cucumis prophetarum* (Gawli & Lakshmidevi, 2015) and *Cucumis melo* (Vishwakarma *et al.*, 2017) have been reported for their hepato-protective effect. The strong anti-inflammatory and antioxidant effect, together with anti-hyperglycemic properties, of these plants were suggested mechanisms for such effects.

5.7. The Effect of Crude *C. ficifolius* Extract Root on Kidney of Diabetic Rats after 3-Consecutive Weeks Treatment

5.7.1. The Effect of Crude *C. ficifolius* Root Extract on Kidney Biochemical Parameters

Blood urea and creatinine levels are simple tests helpful to assess the renal function in diabetic patients who are poorly controlled (Bamanikar *et al.,* 2016). In this study, the increased serum urea level in diabetic study groups as compared to normal control group could be associated with T2DM induced alterations. It has been described that serum urea level has strong positive relationship with duration and severity of diabetes (i.e., as blood glucose level increased, the serum urea level also increased) (Shrestha *et al.,* 2008; Bamanikar *et al.,* 2016). In the present study, the observed concurrent increment of fasting serum urea and glucose level clearly supported this association.

The moderately high level of increase in fasting serum urea in diabetic study groups, including untreated diabetic negative control group which has relatively greater urea level than those treated diabetic groups but still near to the upper bound of normal reference range, implied that the effect of T2DM was not severe. As compared to creatinine, urea is more sensitive indicator of renal function, and so better helps the detection of early renal disease than creatinine (Higgins, 2016a). This evidence supported that the moderately increased urea level seen in all diabetic study groups reflected T2DM-induced early kidney alteration (Bamanikar *et al.,* 2016).

The possible mechanism by which T2DM resulted in elevation of serum urea level in diabetic study groups, largely in untreated diabetic negative control group could be: (a) by causing increased production of urea by liver (increased protein degradation from muscle tissue under normal liver function), and (b) by causing decreased clearance of urea by kidney (renal disease/failure). T2DM causes for greater protein catabolism which produces increased availability of precursor molecules for urea synthesis. This condition leads to increased urea synthesis and secretion by liver, which burdens the kidney function in clearing the urea (Shrestha *et al.,* 2008). On the other hand, diabetes-induced hyperglycemia is known to alter kidney structure such as glomeruli and proximal tubule, and so affect its glomerular filtration rate (GFR) (Teoh *et al.,* 2010; Pourghasem *et al.,* 2015).

Conversely, against diabetic negative control group, treatments of metformin 100mg/Kg and *C. ficifolius* root extract 100, 200 & 400mg/Kg for diabetic rats reversed serum urea level, and so had positive role in improving kidney function. Precisely, treatment of metformin 100mg/Kg had

comparatively greater efficacy followed by extract 400, 200 &100mg/Kg treatments. This implied that treatments protected the kidney from diabetes-induced abnormally increased urea synthesis and decreased urea clearance (Shrestha *et al.,* 2008; Pourghasem *et al.,* 2015).

The lower serum creatinine level observed in diabetic study groups (largely in untreated diabetic negative control group) as compared to normal control group could arises from low protein diet and diabetes-induced declined muscle mass (Bamanikar *et al.,* 2016; Thongprayoon *et al.,* 2016). On the other hand, neither increased renal clearance/glomerular filtration rate (renal over functioning) nor decreased creatinine synthesis by liver (failure of liver function) contributed for the lower creatinine effect. In order to say both or either of these factors have such roles, parallel dose dependent increase in urea clearance and consecutive low serum urea level would be expected, but did not. To be more evidential, decreased function of liver to synthesize creatinine could occur only in state of advanced liver disease (Thongprayoon *et al.,* 2016). The biochemical and histo-pathological result of liver discussed above, partially, could disprove the condition of advanced liver disease.

The lower creatinine levels in diabetic study groups than normal control group might be linked to low protein diet they fed on. In fact, diabetic negative control group was fed on HFD, whereas, normal control group was fed on NPD. Although the nutritional composition of prepared HFD was not analyzed scientifically, as per manual preparation, the proportion in gram of protein was lower in HFD than NPD. Diets containing low protein proportion are known to cause for decreased amino acid pool required for protein and creatinine synthesis, and cause for diminished muscle mass (Bamanikar *et al.,* 2016).

Additionally, the decreased serum creatinine levels in diabetic study groups than normal control could be due to T2DM-induced muscle wasting. It is because T2DM is known to cause increased protein degradation and consecutive skeletal muscle wasting (James *et al.,* 2017). Plasma creatinine (Kaplan *et al.,* 2003) and serum creatinine concentrations are directly proportional to skeletal muscle mass, and can be used as a surrogate marker of skeletal muscle mass (Patel *et al.,* 2013; Thongprayoon *et al.,* 2016). It is because the amount of creatine per unit of skeletal muscle mass and the break down rate of creatine in to creatinine are consistent (Kaplan *et al.,* 2003). As an evidence, blood creatinine and T2DM were found to be inversely correlated, and low serum creatinine could be a predictor of increased risk of T2DM (Harita *et al.,* 2009; Hjelmesæth *et al.,* 2010). As a result, HFD-induced low protein diet and T2DM-induced muscle wasting were the most likely mechanisms responsible for the observed low serum creatinine level in diabetic study groups than normal control group.

Accordingly, low serum creatinine level observed in diabetic study groups might reflect the incidence of IR in these groups. The possible justification is that skeletal muscle mass, which is the direct reflection of serum creatinine level, is directly proportional to the amount of InsR which is important for sufficient insulin signaling. The lower InsR causes for decreased amount of insulin binding to its receptors and leads to decreased insulin action, increased insulin accumulation in blood, reflected incidence of receptor level IR and T2DM (Castro *et al.*, 2014).

In comparisons among diabetic study groups, the increased serum creatinine level observed in diabetic study groups treated with metformin 100mg/Kg and *C. ficifolius* root extract 100, 200 & 400mg/Kg contrary to untreated diabetic negative control group implied that those treatments reversed T2DM-induced muscle wasting (i.e., improved muscle mass). Improved muscle mass is source of precursor molecules and a prerequisite for increased creatinine synthesis and secretion by liver (Nigam & Ch, 2017).

Neither decreased renal creatinine clearance (due to test drugs' (metformin and *C. ficifolius* root extract) tubular toxicity to kidney), nor improved liver function by test drugs took part for the above effect. In order to say both or either of these factors have such roles, parallel dose dependent increase in serum urea level beyond the level seen in diabetic negative control would be expected in these metformin and *C. ficifolius* root extract treated groups, but did not. In fact, drugs with toxic effect might lead to inhibition of proximal tubule creatinine clearance (Nigam & Ch, 2017). Additionally, the production of creatinine by liver strongly depends on the availability of precursor molecules (main source is muscle mass). That means, the improvement in functionality of liver (in this case, let's say characterized by synthesis of creatinine) could be realized if and only if there is no issue of amount of precursor molecules required for creatinine synthesis (Thongprayoon *et al.*, 2016).

Moreover, the differences in serum creatinine level among experimental and positive control groups were associated with differences in the ability of each test drugs on improvement of muscle mass. The crude *C. ficifolius* root extract at higher dose-400mg/Kg had greater efficacy on improving muscle mass followed by metformin 100mg/Kg, *C. ficifolius* root extract 200 & 100mg/Kg. That means, increasing the dose of extract could be more beneficial in augmenting muscle mass, even better than that of metformin.

Besides, the existing muscle mass boosting effect of metformin and crude extract of *C. ficifolius* root could have two implications. First, it reflected their advantage on improving insulin sensitivity/glucose tolerance. Increased skeletal muscle mass is directly proportional to the increased amount of InsR which causes for increased amount of insulin binding to its receptors

and leads to increased insulin action/improved insulin sensitivity/ (Castro *et al.,* 2014). This could be, partially, supported by OGTT result discussed above. Secondly, it suggests their body weight reducing effect (discussed above) was associated with decrease in fat mass (lipolysis) rather than decrease in muscle mass (proteolysis). Evidentially, the relation of serum creatinine level with body mass was reported to be specific to lean body mass rather than total body mass (Baxmann *et al.,* 2008; Vodičar *et al.,* 2018). Thus, decreased body weight by treatments did not imply the decrease in muscle mass.

When we interpreted biochemical parameters separately, creatinine level better reflected muscle mass rather than kidney function, whereas, urea level better reflected kidney function. However, the low specificity of urea and low sensitivity of creatinine to kidney function might limit us to completely make such inference (Higgins, 2016a). To avoid such argument, the serum "Urea: Creatinine ratio" would be relatively good to make conclusion about the effect of test drugs on kidney function (Higgins, 2016b).

Based on computed data, the greater level of "serum urea: creatinine ratio" in diabetic study groups (largely in diabetic negative control group) than normal control suggested that the present T2DM model altered kidney function to some extent. The decreased "Urea: Creatinine ratio" in experimental and positive control groups as compared to diabetic negative control group implied that treatments of metformin and *C. ficifolius* root extract (dose dependently) improved kidney function rather than having toxic effect. Moreover, the relatively greater reduction of level of "Urea: Creatinine ratio" in metformin 100mg/Kg treated group than *C. ficifolius* root extract treated groups indicated that metformin possessed a better kidney protective effect than *C. ficifolius* root extract. Therefore, based on combined effect of the two parameters expressed as "Urea: Creatinine ratio", crude 80% methanol extract of root part of *C. ficifolius* and metformin had beneficial effect on kidney function.

The logical mechanism for muscle mass increasing effect of treatment of *C. ficifolius* root extract and metformin in diabetic rats might be either by protecting protein degradation from T2DM, or by augmenting protein synthesis, or both. Metformin was found to promote muscle mass by indirectly reducing protein catabolism and promoting protein synthesis. Metformin-induced restoration of insulin sensitivity promotes dual influence of insulin on both glucose and muscle protein metabolism and stimulate protein synthesis. Mechanisms of metformin in reducing protein catabolism are: (a) by inhibition of gluconeogenesis which allows for decreased efflux of amino acids (gluconeogenesis precursor) from muscle and (b) by augmentation of glucose uptake by

peripheral tissues, which allows for increased glucose oxidation as fuel and reduced use of amino acids as energy substrate (Gore *et al.,* 2005).

On the other hand, the mechanism of action of *C. ficifolius* root extract on muscle mass might be similar to metformin. The positive effect of this extract on glucose tolerance in OGTT (discussed above) could be supporting evidence. In addition, the better efficacy of extract at higher (400mg/Kg) dose implied that this plant might have better effect in protecting protein catabolism or in promoting protein synthesis than the standard drug-metformin.

5.7.2. The Effect of Crude *C. ficifolius* Root Extract on Kidney Histo-pathological Parameter

As demonstrated in different studies, diabetes mellitus has been recognized to cause numerous pathological changes in kidney. Some of them are: glomerular and tubular basement membrane thickening, glomerular and tubular hypertrophy, mesangial and interstitial fibroblast expansion, nodular sclerosis and glomerulo-sclerosis; thickening of renal arterial walls, tubular atrophy, interstitial fibrosis (Pourghasem *et al.,* 2015) and altered proximal convoluted tubule (such as vacuolization, hyper-cellularity, muco-polysaccharide deposits) (Teoh *et al.,* 2010).

In the present study, the possible justification for no visible kidney pathological change among diabetic groups (including diabetic negative control) could be that the study duration was short and might be insufficient for the induced-T2DM model to result noticeable kidney complication. It has been reported that histological changes, including early changes such as glomerular basement membrane thickening, could not be detected soon (i.e., need time) after onset of diabetes (Pourghasem *et al*., 2015).

However, as per the subjective result, the slightly enlarged and observable proximal convoluted tubules seen in diabetic negative control and *C. ficifolius* root extract 100 mg/Kg treated groups might be related to pathological change (although it needs extra and firm evidence to confirm) caused by current T2DM model (Teoh *et al.,* 2010). The greater levels of abnormality in biochemical parameters parallel to those groups partly supported this idea. Renal structural alteration is characterized by early tubular and glomerular hypertrophy. It is because renal cortex particularly glomeruli and proximal convoluted tubules are involved in glucose homeostasis (filtration and reabsorption of blood glucose, respectively), and so more likely to be early affected by T2DM-induced hyperglycemia (Triplitt, 2012).

The possible mechanism of proximal tubule ballooning could be associated with T2DM-induced hyperglycemia. Hyperglycemia is attributed to cause increased non-enzymatic glycation,

synthesis and accumulation of different structural proteins and growth factors, and so leads to cellular ballooning (Pourghasem *et al.,* 2015). Hyperglycemia-associated activation of polyol pathway is important in the formation of fructose-6 phosphate and consecutive activation of hexosamine pathway which is important for increased synthesis of different structural compounds and growth factors (Nordquist & Palm, 2007).

On the contrary, when those slight histo-pathological changes are taken in to account, the improvement of those changes by treatment of metformin 100mg/Kg and *C. ficifolius* root extract 200 and 400mg/Kg for diabetic rats as compared to untreated control group implied that those treatments had relatively positive effect. Also, *C. ficifolius* root extract at 400 & 200mg/Kg ensured comparable effect as metformin 100mg/Kg.

In addition, these histo-pathological data might in part suggest that crude 80% methanol extract of roots of *C. ficifolius* root extract (100, 200, 400mg/Kg) and metformin 100mg/Kg per se were less likely to have toxic effect to the kidney during the experimentation period. This increases the medicinal value of *C. ficifolius* root extract against diabetes mellitus. However, to make a definite inference about the extract toxic effect, there should be adoption of longer durations of treatments, future direction.

Thus, although the existing renal data was insufficient to make definite statement, these slight histopathological and biochemical changes together implied that the induced diabetes model started to alter kidney function to some extent, and treatment of metformin and crude 80% methanol extract of *C. ficifolius* reversed these effects.

The possible mechanisms for such positive effect of *C. ficifolius* root extract and metformin on kidney could be by preventing hyperglycemia- and hyperlipidemia-induced oxidative stress and inflammation, both of which are important for development of diabetes complications (Tangvarasittichai, 2015). Metformin was found to have direct antioxidant and anti-inflammatory properties (Suman *et al.*, 2016a). The presence of comparatively higher level of flavonoid (a strong antioxidant) in crude extract of *C. ficifolius* root suggests it might have direct antioxidant effect. The sister species of *C. ficifolius* named as *Cucumis melo* (Naito *et al.,* 2005) and *Momordica charantia* (Teoh *et al.,* 2010) were acknowledged for their kidney-protective effects (prevent diabetic nephropathy) by their strong antioxidant properties.

6. CONCLUSIONS

The secondary metabolites qualitatively identified in crude 80% methanol extract of root part of *C. ficifolius A., Rich* were terpenoids, flavonoids, saponins, phenols, glycosides, alkaloids and steroids. The crude 80% methanol extract of *C. ficifolius* root had remarkable dose dependent anti-hyperglycemic, pancreatic islets of Langerhans β-cells protective and regenerative, anti-hyperlipidemic, body weight reducing, hepato-and nephro-protective, and muscle mass boosting effects in HFFD-single 30mg/Kg dose STZ-induced diabetic rats. The above stated phytochemicals might be the cause for such inducing effects. These activities are interlinked one another and important for effective management of diabetes mellitus (largely T2DM) and its complications. Precisely, the pancreatic beta-cells regenerating and protective effect, anti-hyperlipidemic, body weight reducing, muscle mass improving effects of this plant extract could be important mechanisms of action in reducing hyperglycemia in diabetic rats.

Moreover, from the available three test doses (100, 200 and 400mg/Kg) of crude 80% methanol extract of *C. ficifolius* root, the higher dose-400mg/Kg was found to be the better effective dose in all tested parameters. The extract had fairly comparable efficacy in most tested parameters and relatively greater efficacy in some parameters such as in pancreatic islets of Langerhans, HDL-C and creatinine levels as compared to the standard drug-metformin. Interestingly, the good safety profile, in addition to the existing efficacy, magnified its anti-diabetic values.

Overall, these findings scientifically confirmed that the crude 80% methanol extract of Ethiopian species of the plant *C. ficifolius A., Rich* root has gotten antidiabetic activity, as shown in the type-2 diabetic rat model.

STRENGTHS AND LIMITATIONS

Strengths

One of the strengths of this paper is its originality, and procedures were standardized. This most challenging and tedious work, but accomplished in an attractive and successful manner, in this study was development of HFFD-single low dose STZ induced rat model for T2DM. The commercial HFFD is not accessible in Ethiopia and many other developing countries. To solve this, here, we prepared HFFD manually by using easily available resources in our country. Lots of efforts were paid to assure accuracy of measured data, increased the truthfulness and acceptability of the finding.

This paper attempted to cover many parameters both biochemical and histological, most of which were added after approval of the proposal (beyond the scope/optimum requirement/of the proposal). The parameters added after approval of the proposal include OGTT, STT, measure of progressive (weekly) blood glucose and body weight, pancreas histopathology, liver and kidney biochemical and histopathology parameters. These helped to dilate and better confirm the anti-diabetic values of *C. ficifolius*.

Most importantly, this paper tried to discuss broadly each subtopic at molecular level by touching most of the likely mechanisms of actions of *C. ficifolius* on the basis of its qualitatively identified phytochemical classes. This impressively inspires any interested body to work on and assess each specific mechanism of action of the root extracts or other plant parts.

Limitations

Determinations of biochemical parameters were done using human kit instead of rat specific kit due to difficulty of obtaining it. The exact composition of HFD was not done due to trouble to make analysis.

RECOMMENDATIONS

- Devoting extra efforts on medicinal /anti-diabetic/ values of *C. ficifolius A., Rich* root is profitable. Further study accounting the above limitations would help to better confirm its anti-diabetic roles.
- Molecular mechanisms of action of crude extract of *C. ficifolius* root against different (non-) enzymatic pathways involved in T2DM development should be done.
- Quantitative phytochemical screening and active compounds isolation of crude root extract would be good to know which phytochemical classes and specific active compounds are dominant and responsible.
- The direct antioxidant and anti-inflammatory activities of the crude root extract would be more inclusive.
- Chronic toxicity test should be done to best confirm the safety of *C. ficifolius* root extracts with human systems.
- This result obtained with rat model should be tested clinically with humans and that can be useful in traditional healing of diabetes in Ethiopia.
- The anti-diabetic activities of different fraction may help to select the best extraction solvent with more anti-diabetic active compounds.
- Further study with respect to the origin of plant collected in Ethiopia including collections at different habituations, seasons and altitudes should be done.
- The anti-diabetic role of other structural parts such as stem, leaf, fruit & flower, in addition to root, should be examined.

REFERENCES

Abdel-Aziz, S.M., Aeron, A. and Kahil, T.A., 2016. Health benefits and possible risks of herbal medicine. *In Microbes in Food and Health*, pp. 97-116. Springer, Cham.

Abdelmalek, M.F., Lazo, M., Horska, A., Bonekamp, S., Lipkin, E.W., Balasubramanyam, A., Bantle, J.P., Johnson, R.J., Diehl, A.M., Clark, J.M. and Fatty Liver Subgroup of the Look AHEAD Research Group, 2012. Higher dietary fructose is associated with impaired hepatic adenosine triphosphate homeostasis in obese individuals with type 2 diabetes. *Hepatology*, 56(3), pp.952-960

Abou-Zaid, M.M., Lombardo, D.A., Kite, G.C., Grayer, R.J. and Veitch, N.C., 2001. Acylated flavone C-glycosides from *Cucumis sativus*. *Phytochemistry*, 58(1), pp.167-172.

Acosta-Patino, J.L., Jimenez-Balderas, E., Juarez-Oropeza, M.A. and Dıaz-Zagoya, J.C., 2001. Hypoglycemic action of *Cucurbita ficifolia* on Type 2 diabetic patients with moderately high blood glucose levels. *Journal of ethnopharmacology*, 77(1), pp.99-101.

ADA (American Diabetes Association), 2019a. 2. Classification and diagnosis of diabetes: standards of medical care in diabetes—2019. *Diabetes Care*, 42(Supplement 1), pp.S13-S28.

ADA (American Diabetes Association), 2019b. 9. Pharmacologic approaches to glycemic treatment: standards of medical care in diabetes—2019. *Diabetes Care*, 42(Supplement 1), pp.S90-S102.

Adebooye, O.C., 2008. Phyto-constituents and anti-oxidant activity of the pulp of snake tomato (*Trichosanthes cucumerina L.).African Journal of Traditional, Complementary and Alternative Medicines*, 5(2), pp.173-179)

Agarwal, M.M., 2016. Gestational diabetes mellitus: Screening with fasting plasma glucose. *World Journal of Diabetes*, 7(14), p.279.

Agarwal, V.I.P.I.N., Sharma, A.K., Upadhyay, A.N.S.H.U., Singh, G.O.P.E.N.D.R.A. and Gupta, R.A.J.I.V., 2012. Hypoglycemic effects of *Citrullus colocynthis* roots. *Acta Pol Pharm*, 69(1), pp.75-9.

Ahmed, S.K. and Manoj, J., 2012. Anti obesity activity of *Coccinia indica* in female rats fed with cafeteria and atherogenic diets. *Scholars research library*, 4(5), pp.1480-1485.

Ahn, J.H., Kim, E.S., Lee, C., Kim, S., Cho, S.H., Hwang, B.Y. and Lee, M.K., 2013. Chemical constituents from *Nelumbo nucifera* leaves and their anti-obesity effects. *Bioorganic & medicinal chemistry letters*, 23(12), pp.3604-3608.

Ali, H., Houghton, P.J. and Soumyanath, A., 2006. α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus*. *Journal of ethnopharmacology*, 107(3), pp.449-455.

Araya, S., Abera, B. and Giday, M., 2015. Study of plants traditionally used in public and animal health management in SehartiSamre District, Southern Tigray, Ethiopia. *Journal of ethnobiology and ethnomedicine*, 11(1), p.1.

Arifin W.N., Zahiruddin W.M., 2017. Sample size calculation in animal studies using resource equation approach. *Malaysian Journal of medical sciences*, 24(5), pp.101-105.

Auwerx, J., Schoonjans, K., Fruchart, J.C. and Staels, B., 1996. Transcriptional control of triglyceride metabolism: fibrates and fatty acids change the expression of the LPL and apo C-III genes by activating the nuclear receptor PPAR. *Atherosclerosis*, 124, pp.S29-S37)

Azwanida, N.N., 2015. A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. *Medicinal & Aromatic Plants*.

Bahadoran, Z., Mirmiran, P. and Azizi, F., 2013. Dietary polyphenols as potential nutraceuticals in management of diabetes: a review. *Journal of diabetes & metabolic disorders*, 12(1), p.43.

Bamanikar, S.A., Bamanikar, A.A. and Arora, A., 2016. Study of Serum urea and Creatinine in Diabetic and nondiabetic patients in a tertiary teaching hospital. *The Journal of Medical Research*, 2(1), pp.12-15.

Barbosa-da-Silva, S., Bringhenti Sarmento, I., Bargut, L., Thereza, C., Souza-Mello, V., Barbosa Aguila, M. and Mandarim-de-Lacerda, C.A., 2014. Animal Models of Nutritional Induction of Type 2 Diabetes Mellitus. *International Journal of Morphology*, 32(1).

Baxmann, A.C., Ahmed, M.S., Marques, N.C., Menon, V.B., Pereira, A.B., Kirsztajn, G.M. and Heilberg, I.P., 2008. Influence of muscle mass and physical activity on serum and urinary creatinine and serum cystatin C. *Clinical Journal of the American Society of Nephrology*, 3(2), pp.348-354.

Bayat, A., Jamali, Z., Hajianfar, H. and Beni, M.H., 2014. Effects of *Cucurbita ficifolia* Intake on Type 2 Diabetes: Review of Current Evidences. *Shiraz E-Medical Journal*, 15(2).

Baynest, H.W., 2015. Classification, Pathophysiology, Diagnosis and Management of Diabetes Mellitus. *Journal of Diabetes & Metabolism*.

Bharti, S.K., Krishnan, S., Kumar, A. and Kumar, A., 2018. Antidiabetic phytoconstituents and their mode of action on metabolic pathways. *Therapeutic Advances in Endocrinology and metabolism*, 9(3), pp.81 100.

Bidkar, J.S., Ghanwat, D.D., Bhujbal, M.D. and Dama, G.Y., 2012. Anti-hyperlipidemic activity of *Cucumis melo* fruit peel extracts in high cholesterol diet induced hyperlipidemia in rats. *Journal of Complementary and Integrative Medicine*, 9(1)

Bioversity International-FAO, 2014. Economic importance of *cucurbitaceous* species found in the Kenyan Central Highlands. *Plant Genetic Resources Newsletter*, 10(121), pp.1-5. *http://www.bioversityinternational.org/fileadmin/PGR/tables/table2-132.htm*. [Accessed on January 6, 2016].

Bisht, R. and Bhattacharya, S., 2013. Effect of various extracts of *Desmodium gangeticum* on Streptozotocin-nicotinamide induced type-2 diabetes. *Asian Journal of Plant Science and Research*, *3*(3), pp.28-34.

Bommer, C., Heesemann, E., Sagalova, V., Manne-Goehler, J., Atun, R., Bärnighausen, T. and Vollmer, S., 2017. The global economic burden of diabetes in adults aged 20–79 years: a cost-of-illness study. *The lancet Diabetes & endocrinology*, 5(6), pp.423-430.

Bommer, C., Sagalova, V., Heesemann, E., Manne-Goehler, J., Atun, R., Bärnighausen, T., Davies, J. and Vollmer, S., 2018. Global economic burden of diabetes in adults: projections from 2015 to 2030. *Diabetes care*, 41(5), pp.963-970.

Burkill, H.M., 1985. *The Useful Plants of West Tropical Africa*. 2nd Edn, Vol.1. London: Kew, Royal Botanic Gardens.

Bussmann, R.W., Swartzinsky, P., Worede, A. and Evangelista, P., 2011. Plant use in Odo-Bulu and Demaro, Bale region, Ethiopia. *Journal of ethnobiology and ethnomedicine*, 7(1), p.1.

Camporez, J.P.G., Jornayvaz, F.R., Lee, H.Y., Kanda, S., Guigni, B.A., Kahn, M., Samuel, V.T., Carvalho, C.R., Petersen, K.F., Jurczak, M.J. and Shulman, G.I., 2013. Cellular mechanism by which estradiol protects female ovariectomized mice from high-fat diet-induced hepatic and muscle insulin resistance. *Endocrinology*, 154(3), pp.1021-1028.

Castro, A.V.B., Kolka, C.M., Kim, S.P. and Bergman, R.N., 2014. Obesity, insulin resistance and comorbidities? Mechanisms of association.*Arquivos Brasileiros de Endocrinologia & Metabologia*, 58(6), pp.600-609.

Cernea, S. and Cahn, A., 2016. Diabetes mellitus: in search of an improved classification and treatment algorithm. *Revista Romana de Medicina de Laborator*, 24(1), pp.9-20.

Cersosimo, E., Triplitt, C., Solis-Herrera, C., Mandarino, L.J. and DeFronzo, R.A., 2018. Pathogenesis of type 2 diabetes mellitus. In *Endotext [Internet]*. MDText. com, Inc..

Chang, C.L., Lin, Y., Bartolome, A.P., Chen, Y.C., Chiu, S.C. and Yang, W.C., 2013. Herbal therapies for type 2 diabetes mellitus: chemistry, biology, and potential application of selected plants and compounds. *Evidence-Based Complementary and Alternative Medicine*, 2013.

Chao, C.Y. and Huang, C.J., 2003. Bitter gourd (*Momordica charantia*) extract activates peroxisome proliferator-activated receptors and upregulates the expression of the acyl CoA oxidase gene in H4IIEC3 hepatoma cells. *Journal of biomedical science*, 10(6), pp.782-791.

Charan J., Kantharia N.D., 2013. How to calculate sample size in animal studies. *Journal of pharmacology and pharmacotherapeutics*, 4(4), pp.303-306.

Chaudhari, H.S., Bhandari, U. and Khanna, G., 2013. Embeliaribes extract reduces high fat diet and low dose streptozotocin-induced diabetic nephrotoxicity in rats. *EXCLI journal*, 12, p.858.

Chaudhury, A., Duvoor, C., Dendi, R., Sena, V., Kraleti, S., Chada, A., Ravilla, R., Marco, A., Shekhawat, N.S., Montales, M.T. and Kuriakose, K., 2017. Clinical review of antidiabetic drugs: implications for type 2 diabetes mellitus management. *Frontiers in endocrinology*, 8, p.6.

Chekole, G., Asfaw, Z. and Kelbessa, E., 2015. Ethnobotanical study of medicinal plants in the environs of Tara-gedam and Amba remnant forests of LiboKemkem District, northwest Ethiopia. *Journal of ethnobiology and ethnomedicine*, 11(1), p.1.

Chen, F., Chen, Y., Kang, X., Zhou, Z., Zhang, Z. and Liu, D., 2012. Anti-apoptotic function and mechanism of *Ginseng* saponins in Rattus pancreatic β-cells. *Biological and Pharmaceutical Bulletin*, 35(9), pp.1568-1573.

Chen, S.C.C., Tsai, S.P., Jhao, J.Y., Jiang, W.K., Tsao, C.K. and Chang, L.Y., 2017. Liver fat, hepatic enzymes, alkaline phosphatase and the risk of incident type 2 diabetes: a prospective study of 132,377 adults. *Scientific reports*, 7(1), p.4649.

Cheng, H.L., Huang, H.K., Chang, C.I., Tsai, C.P. and Chou, C.H., 2008. A cell-based screening identifies compounds from the stem of *Momordica charantia* that overcome insulin resistance and activate AMP-activated protein kinase. *Journal of Agricultural and Food Chemistry*, 56(16), pp.6835-6843

Choi, J.S., Kim, J.H., Ali, M.Y., Min, B.S., Kim, G.D. and Jung, H.A., 2014. *Coptis chinensis* alkaloids exert anti-adipogenic activity on 3T3-L1 adipocytes by downregulating C/EBP-α and PPAR-γ. *Fitoterapia*, 98, pp.199-208.

Da Silva Xavier, G., 2018. The cells of the islets of langerhans. *Journal of clinical medicine*, 7(3), p.54.

Dekker, M.J., Su, Q., Baker, C., Rutledge, A.C. and Adeli, K., 2010. Fructose: a highly lipogenic nutrient implicated in insulin resistance, hepatic steatosis, and the metabolic syndrome. *American Journal of Physiology-Endocrinology and Metabolism*, 299(5), pp.E685-E694.

Deng, J., Liu, Y., Duan, Z., Zhu, C., Hui, J., Mi, Y., Ma, P., Ma, X., Fan, D. and Yang, H., 2017. Protopanaxadiol and protopanaxatriol-type saponins ameliorate glucose and lipid metabolism in type 2 diabetes mellitus in high-fat diet/streptozocin-induced mice. *Frontiers in pharmacology*, 8, p.506.

Dhiman, K., Gupta, A., Sharma, D.K., Gill, N.S. and Goyal, A., 2012. A review on the medicinally important plants of the family *Cucurbitaceae*. *Asian Journal of Clinical Nutrition*, 4(1), pp.16-26.

Diaz-Flores, M., Angeles-Mejia, S., Baiza-Gutman, L.A., Medina-Navarro, R., Hernandez-Saavedra, D., Ortega-Camarillo, C., Roman-Ramos, R., Cruz, M. and Alarcon-Aguilar, F.J., 2012. Effect of an aqueous extract of *Cucurbita ficifoliaBouché* on the glutathione redox cycle in mice with STZ-induced diabetes. *Journal of ethnopharmacology*, 144(1), pp.101-108)

Dor, Y., Brown, J., Martinez, O.I. and Melton, D.A., 2004. Adult pancreatic β-cells are formed by self-duplication rather than stem-cell differentiation. *Nature*, 429(6987), p.41.

Eddouks, M., Bidi, A., El Bouhali, B., Hajji, L. and Zeggwagh, N.A., 2014. Antidiabetic plants improving insulin sensitivity. *Journal of Pharmacy and Pharmacology*, 66(9), pp.1197-1214.

Edelman, S.V. and Polonsky, W.H., 2017. Type 2 diabetes in the real world: the elusive nature of glycemic control. *Diabetes Care*, 40(11), pp.1425-1432.

Eid, H.M., Martineau, L.C., Saleem, A., Muhammad, A., Vallerand, D., Benhaddou-Andaloussi, A., Nistor, L., Afshar, A., Arnason, J.T. and Haddad, P.S., 2010. Stimulation of AMP-activated protein kinase and enhancement of basal glucose uptake in muscle cells by quercetin and quercetin glycosides, active principles of the antidiabetic medicinal plant *Vaccinium vitis-idaea*. *Molecular nutrition & food research*, 54(7), pp.991-1003.

Ekpo, I.A., Osuagwu, A.N., Agbor, R.B., Okpako, E.C. and Ekanem, B.E., 2013. Phytochemical composition of *Aframomum melegueta* and *Piper guineense* seeds. *Journal of Current Research in Science*, 1(1), p.24.

Eleazu, C.O., Eleazu, K.C., Chukwuma, S. and Essien, U.N., 2013. Review of the mechanism of cell death resulting from streptozotocin challenge in experimental animals, its practical use and potential risk to humans. *Journal of Diabetes & Metabolic Disorders*, 12(1), p.60.

El-Sayed, M.M., Ghareeb, D.A., Talat, H.A. and Sarhan, E.M., 2013. High fat diet induced insulin resistance and elevated retinol binding protein 4 in female rats; treatment and protection with *Berberis vulgaris* extract and vitamin A. *Pak J Pharm Sci,* 26(6), pp.1189-1195.

Ernsberger, P. and Koletsky, R.J., 2012. The glucose tolerance test as a laboratory tool with clinical implications. *Glucose Tolerance*, p.1.

Erukainure, O.L., Hafizur, R.M., Kabir, N., Choudhary, M.I., Atolani, O., Banerjee, P., Preissner, R., Chukwuma, C.I., Muhammad, A., Amonsou, E.O. and Islam, M., 2018. Suppressive effects of *Clerodendrum volubile P beauv.[labiatae]* methanolic extract and its fractions on type 2 diabetes and its complications. *Frontiers in pharmacology*, 9, p.8.

Farzaei, F., Morovati, M.R., Farjadmand, F. and Farzaei, M.H., 2017. A mechanistic review on medicinal plants used for diabetes mellitus in traditional Persian medicine. *Journal of evidence-based complementary & alternative medicine*, 22(4), pp.944-955.

Fayaz, S.M., Kumar, V.S.S. and Rajanikant, K.G., 2014. Finding needles in a haystack: application of network analysis and target enrichment studies for the identification of potential anti-diabetic phytochemicals. *PloS one,* 9(11), p.e112911.

Feleke, Y. and Enquselassie, F., 2005. An assessment of the health care system for diabetes in Addis Ababa, Ethiopia. *Ethiopian journal of health development*, 19(3), pp.203-210.

Gaikwad, S.B., Mohan, G.K. and Rani, M.S., 2014. Phytochemicals for diabetes management. *Pharmaceutical Crops*, 31(2), pp.261-6.

Garber, J.C., Barbee, R.W., Bielitzki, J.T., Clayton, L.A., Donovan, J.C., Hendriksen, C.F.M., Kohn, D.F., Lipman, N.S., Locke, P.A., Melcher, J. and Quimby, F.W., 2011. Guide for the care and use of laboratory animals. The National Academic Press, Washington DC, *8*, p.220.

Garg, N., Sidhu, A.S. and Cheema, D.S., 2007. Systematics of the genus *Cucumis*: a review of literature. *Haryana Journal of Horticulture Science, Ann Arbor,* 36(1-2), pp.192-197.

Gawli, K. and Lakshmidevi, N., 2015. Antidiabetic and antioxidant potency evaluation of different fractions obtained from *Cucumis prophetarum* fruit. *Pharmaceutical biology*, 53(5), pp.689-694.

Gebreyes, Y.F., Goshu, D.Y., Geletew, T.K., Argefa, T.G., Zemedu, T.G., Lemu, K.A., Waka, F.C., Mengesha, A.B., Degefu, F.S., Deghebo, A.D. and Wubie, H.T., 2018. Prevalence of high bloodpressure, hyperglycemia, dyslipidemia, metabolic syndrome and their determinants in Ethiopia: Evidences from the National NCDs STEPS Survey, 2015. *PloS one,* 13(5), p.e0194819.

Gelana, T., 2011. *Antimicrobial activity of solvent-extracts of Cucumis ficifolius and zehneria scabra on some test microorganisms.* Masters Thesis. School of Graduate Studies Faculty of Life Sciences, Addis Ababa University.

Godoy-Matos, A.F., 2014. The role of glucagon on type 2 diabetes at a glance. *Diabetology & metabolic syndrome,* 6(1), p.91.

Gore, D.C., Wolf, S.E., Sanford, A., Herndon, D.N. and Wolfe, R.R., 2005. Influence of metformin on glucose intolerance and muscle catabolism following severe burn injury. *Annals of surgery*, 241(2), p.334.

Gotep, J., 2011. Glycosides fraction extracted from fruit pulp of *Cucumis metuliferus E. Meyer* has antihyperglycemic effect in rats with alloxan-induced diabetes. *Journal of Natural Pharmaceuticals*, 2(2).

Goud, B.J., Dwarakanath, V. and Chikka, B.K., 2015. Streptozotocin-a diabetogenic agent in animal models. *International Journal of Pharmacy & Pharmaceutical Research*, 3(1), pp.253-269.

Governa, P., Baini, G., Borgonetti, V., Cettolin, G., Giachetti, D., Magnano, A., Miraldi, E. and Biagi, M., 2018. Phytotherapy in the management of diabetes: A review. *Molecules*, 23(1), p.105.

Gul, R., Jan, S.U., Faridullah, S., Sherani, S. and Jahan, N., 2017. Preliminary phytochemical screening, quantitative analysis of alkaloids, and antioxidant activity of crude plant extracts from *Ephedra intermedia* indigenous to Balochistan. *The Scientific World Journal.*

Hajiaghaalipour, F., Khalilpourfarshbafi, M. and Arya, A., 2015. Modulation of glucose transporter protein by dietary flavonoids in type 2 diabetes mellitus. *International journal of biological sciences*, 11(5), p.508.

Handa, S.S., Khanuja, S.P.S., Longo, G. Rakesh, D.D. and United Nations Industrial Development Organization, 2008. Extraction technologies for medicinal and aromatic plants. *Earth, Environmental and Marine Sciences and Technologies.*

Harita, N., Hayashi, T., Sato, K.K., Nakamura, Y., Yoneda, T., Endo, G. and Kambe, H., 2009. Lower serum creatinine is a new risk factor of type 2 diabetes: the Kansai healthcare study. *Diabetes care*, 32(3), pp.424-426.

Heydemann, A., 2016. An overview of murine high fat diet as a model for type 2 diabetes mellitus. *Journal of diabetes research*.

Heymsfield, S.B., Gonzalez, M.C., Shen, W., Redman, L. and Thomas, D., 2014. Weight loss composition is one-fourth fat-free mass: a critical review and critique of this widely cited rule. *Obesity Reviews*, 15(4), pp.310-321.

Higgins, C., 2016(a). Urea and the clinical value of measuring blood urea concentration.

Higgins, C., 2016(b). Urea and creatinine concentration, the urea: creatinine ratio. *Acute Care Testing Handbook*, pp.1-8.

Hjelmesæth, J., Røislien, J., Nordstrand, N., Hofsø, D., Hager, H. and Hartmann, A., 2010. Low serum creatinine is associated with type 2 diabetes in morbidly obese women and men: a cross-sectional study. *BMC endocrine disorders*, 10(1), p.6.

Hossain, M.Z., Shibib, B.A. and Rahman, R., 1992. Hypoglycemic effects of *Coccinia indica*: inhibition of key gluconeogenic enzyme, glucose-6-phosphatase. *Indian journal of experimental biology*, 30(5), pp.418-420.

Hsu, C.L. and Yen, G.C., 2007. Effects of flavonoids and phenolic acids on the inhibition of adipogenesis in 3T3-L1 adipocytes. *Journal of agricultural and food chemistry*, 55(21), pp.8404-8410.

Hur, K.Y. and Lee, M.S., 2015. New mechanisms of metformin action: Focusing on mitochondria and the gut. *Journal of diabetes investigation*, 6(6), pp.600-609.

Huseini, H.F., Larijani, B., Heshmat, R., Fakhrzadeh, H., Radjabipour, B., Toliat, T. and Raza, M., 2006. The efficacy of *Silybum marianum (L.)* Gaertn.(silymarin) in the treatment of type II diabetes: a randomized, double-blind, placebo-controlled, clinical trial. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, 20(12), pp.1036-1039.

IDF (International Diabetes Federation), 2017. *IDFDiabetes Atlas*. 8th ed.Brussels, Belgium: International Diabetes Federation.https://www.idf.org/e-library/epidemiology-research/diabetes-atlas/134-idf-diabetes-atlas-8th-edition.html

IDF (International Diabetes Federation), 2019. *IDFDiabetes Atlas*. 8th ed.Brussels, Belgium: International Diabetes Federation. https://www.diabetesatlas.org/upload/resources/2019/IDF_Atlas_9th_Edition_2019.pdf

Inada, A., Figueiredo, P., Santos-Eichler, R., Freitas, K., Hiane, P., Castro, A. and Guimarães, R., 2017. *Morinda citrifolia Linn,*(Noni) and its potential in obesity-related metabolic dysfunction. *Nutrients*, 9(6), p.540.

ITHAKA (Information Technology for Humanitarian Assistance, Cooperation and Action), 2016. JSTOR Global plants: *Cucumis ficifoius*. *https://plants.jstor.org/compilation/Cucumis.ficifolius*. [Accessed on November 13, 2016].

Jain, R., Jain, P. and Jain, P., 2016. A review on treatment and prevention of diabetes mellitus. *International Journal of Current Pharmaceutical Research*, 8(3).

James, H.A., O'Neill, B.T. and Nair, K.S., 2017. Insulin regulation of proteostasis and clinical implications. *Cell metabolism*, 26(2), pp.310-323.

Jiang, S., Ren, D., Li, J., Yuan, G., Li, H., Xu, G., Han, X., Du, P. and An, L., 2014. Effects of compound K on hyperglycemia and insulin resistance in rats with type 2 diabetes mellitus. *Fitoterapia*, 95, pp.58-64.

Jimam, N.S., Omale, S., Wannang, N.N. and Gotom, B., 2010. Evaluation of the Hypoglycemic Activity of *Cucumis metuliferus (Cucurbitaceae*) Fruit Pulp Extract in Normoglycemic AlloxanInduced Hyperglycemic Rats. *Journal of Young Pharmacists*, 2(4), pp.384-387.

Joseph, B. and Jini, D., 2013. Antidiabetic effects of *Momordica charantia* (bitter melon) and its medicinal potency. *Asian Pacific Journal of Tropical Disease*, 3(2), pp.93-102.

Kamble, S.M., Kamlakar, P.L., Vaidya, S. and Bambole, V.D., 1998. Influence of *Coccinia indica* on certain enzymes in glycolytic and lipolytic pathway in human diabetes. *Indian journal of medical sciences*, *52*(4), pp.143-146.

Kaplan, L.A., Kazmierczak, S.C. and Pesce, A.J., 2003. *Clinical chemistry: theory, analysis, correlation*. Mosby

Kassaye, K.D., Amberbir, A., Getachew, B. and Mussema, Y., 2006. A historical overview of traditional medicine practices and policy in Ethiopia. *Ethiopian Journal of Health Development*, 20(2), pp.127-134.

Kaur, M. and Valecha, V., 2014. Diabetes and antidiabetic herbal formulations: An alternative to Allopathy. *Int J Pharmacogn*, 1, pp.614-626.

Kawser Hossain, M., Abdal Dayem, A., Han, J., Yin, Y., Kim, K., Kumar Saha, S., Yang, G.M., Choi, H. and Cho, S.G., 2016. Molecular mechanisms of the anti-obesity and anti-diabetic properties of flavonoids. *International journal of molecular sciences*, 17(4), p.569.

Khadra, A. and Schnell, S., 2015. Development, growth and maintenance of β-cell mass: Models are also part of the story. *Molecular aspects of medicine*, 42, pp.78-90.

Khanna, P., Jain, S.C., Panagariya, A. and Dixit, V.P., 1981. Hypoglycemic activity of polypeptide-p from a plant source. *Journal of Natural Products*, 44(6), pp.648-655.

Khosla, P., Gupta, D.D. and Nagpal, R.K., 1995. Effect of Trigonella foenum graecum (Fenugreek) on serum lipids in normal and diabetic rats. *Indian Journal of Pharmacology*, 27(2), p.89.

Kobori, M., Masumoto, S., Akimoto, Y. and Takahashi, Y., 2009. Dietary quercetin alleviates diabetic symptoms and reduces streptozotocin-induced disturbance of hepatic gene expression in mice. *Molecular nutrition & food research*, 53(7), pp.859-868.

Kohei, K.A.K.U., 2010. Pathophysiology of type 2 diabetes and its treatment policy. *JMAJ*, 53(1), pp.41-46.

Kolte, R.M., Bisan, V.V., Jangde, C.R. and Bhalerao, A.A., 1997. Anti-inflammatory activity of root tubers of *Trichosanthes cucumerina (Linn)* in mouse's hind paw oedema induced by carrageenin. *Indian J Indigenous Med*, 18, pp.117-21.

Lakhne, R., Gupta, R. and Gupta, R.S., 2015. Active Phytoconstituents for Controlling Hypercholesterolemia: A Review. *International Journal of clinical endocrinology and metabolism*, pp.22-30.

Le May, C., Chu, K., Hu, M., Ortega, C.S., Simpson, E.R., Korach, K.S., Tsai, M.J. and Mauvais-Jarvis, F., 2006. Estrogens protect pancreatic β-cells from apoptosis and prevent insulin-deficient diabetes mellitus in mice. *Proceedings of the National Academy of Sciences*, 103(24), pp.9232-9237.

Lim, E.L., Hollingsworth, K.G., Aribisala, B.S., Chen, M.J., Mathers, J.C. and Taylor, R., 2011. Reversal of type 2 diabetes: normalisation of beta cell function in association with decreased pancreas and liver triacylglycerol. *Diabetologia*, 54(10), pp.2506-2514.

Liu, C., Hu, M.Y., Zhang, M., Li, F., Li, J., Zhang, J., Li, Y., Guo, H.F., Xu, P., Liu, L. and Liu, X.D., 2014. Association of GLP-1 secretion with anti-hyperlipidemic effect of ginsenosides in high-fat diet fed rats. *Metabolism*, 63(10), pp.1342-1351.

Liu, J., He, T., Lu, Q., Shang, J., Sun, H. and Zhang, L., 2010. Asiatic acid preserves beta cell mass and mitigates hyperglycemia in streptozocin-induced diabetic rats. *Diabetes/metabolism research and reviews*, 26(6), pp.448-454.

Liu, T., Yu, B., Kakino, M., Fujimoto, H., Ando, Y., Hakuno, F. and Takahashi, S.I., 2016. A novel IRS-1-associated protein, DGKζ regulates GLUT4 translocation in 3T3-L1 adipocytes. *Scientific reports*, 6, p.35438.

Liu, X., Zhu, L., Tan, J., Zhou, X., Xiao, L., Yang, X. and Wang, B., 2014. Glucosidase inhibitory activity and antioxidant activity of flavonoid compound and triterpenoid compound from *Agrimonia pilosa Ledeb*. *BMC complementary and alternative medicine*, 14(1), p.12.

Liu, Y., Jin, H., Xu, Z.Q., Nan, W.K., Wang, T. and Cheng, Y.Y., 2006. Effects of pumpkin polysaccharides on blood glucose and blood lipids in diabetic rats. *Zhongguo ying yong sheng li xue za zhi= Zhongguo yingyong shenglixue zazhi= Chinese journal of applied physiology*, 22(3), pp.358-361.

Lozano, I., Van der Werf, R., Bietiger, W., Seyfritz, E., Peronet, C., Pinget, M., Jeandidier, N., Maillard, E., Marchioni, E., Sigrist, S. and Dal, S., 2016. High-fructose and high-fat diet-induced disorders in rats: impact on diabetes risk, hepatic and vascular complications. *Nutrition & metabolism*, 13(1), p.15.

Lucchesi, A.N., Cassettari, L.L. and Spadella, C.T., 2015. Alloxan-induced diabetes causes morphological and ultrastructural changes in rat liver that resemble the natural history of chronic fatty liver disease in humans. *Journal of diabetes research*.

Lulekal, E., Kelbessa, E., Bekele, T. and Yineger, H., 2008.An ethnobotanical study of medicinal plants in ManaAngetu District, southeastern Ethiopia. *Journal of ethnobiology and Ethnomedicine*, 4(1), p.1.

Luo, J.G., Liu, J. and Kong, L.Y., 2008. New pentacyclic triterpenes from *Gypsophila oldhamiana* and their biological evaluation as glycogen phosphorylase inhibitors. *Chemistry & biodiversity*, 5(5), pp.751-757.

Ma, J., Whittaker, P., Keller, A.C., Mazzola, E.P., Pawar, R.S., White, K.D., Callahan, J.H., Kennelly, E.J., Krynitsky, A.J. and Rader, J.I., 2010. Cucurbitane-type triterpenoids from *Momordica charantia*. *Planta medica*, 76(15), pp.1758-1761.

Maheswari, R. and Vennarasi, M., 2015. Hypoglycemic antioxidant potential of herbal extract studied in high fat fed and low dose streptozotocin induced type 2 diabetic rats. *International Journal of Bioinformatics and Biomedical Engineering*, 1(2), pp.85-92.

Marín-Peñalver, J.J., Martín-Timón, I., Sevillano-Collantes, C. and del Cañizo-Gómez, F.J., 2016. Update on the treatment of type 2 diabetes mellitus. *World journal of diabetes*, 7(17), p.354.

Martín-Timón, I., Sevillano-Collantes, C., García-Domínguez, M., Marín-Peñalver, J.J., Ugalde-Abiega, B. and del Cañizo-Gómez, F.J., 2018. Update on the Management of Diabetic Dyslipidaemia. *DIABETES*.

Mentreddy, S.R., 2007. Medicinal plant species with potential antidiabetic properties. *Journal of the Science of Food and Agriculture*, 87(5), pp.743-750.

Meragiaw, M. and Asfaw, Z., 2014. Review of antimalarial, pesticidal and repellent plants in the Ethiopian traditional herbal medicine. *Res. Rev. J. Herbal. Sci*, 3(3), pp.21-45.

Meresa, A., Gemechu, W., Basha, H., Fekadu, N., Teka, F., Ashebir, R. and Tadele, A., 2017. Herbal medicines for the management of diabetic mellitus in Ethiopia and Eretria including their phytochemical constituents. *AJADD*, 5(01), pp.040-58.

Mescher, A.L., 2016. Junqueira's Basic Histology: Text and Atlas. 14th ed. McGraw Hill, NY.

Mesfin, A., Giday, M., Animut, A. and Teklehaymanot, T., 2012. Ethnobotanical study of antimalarial plants in Shinile District, Somali Region, Ethiopia, and in vivo evaluation of selected ones against Plasmodium berghei. *Journal of Ethnopharmacology*, 139(1), pp.221-227.

Mir, M.A., Sawhney, S.S. and Jassal, M.M.S., 2013. Qualitative and quantitative analysis of phytochemicals of *Taraxacum officinale*. *Wudpecker Journal of Pharmacy and Pharmocology*, 2(1), pp.1-5.

Mohamed, J., Nafizah, A.N., Zariyantey, A.H. and Budin, S.B., 2016. Mechanisms of diabetes-induced liver damage: the role of oxidative stress and inflammation. *Sultan Qaboos University Medical Journal*, 16(2), p.e132.nzkkz

Moodley, K., Joseph, K., Naidoo, Y., Islam, S. and Mackraj, I., 2015. Antioxidant, antidiabetic and hypolipidemic effects of *Tulbaghia violacea Harv*.(wild garlic) rhizome methanolic extract in a diabetic rat model. *BMC complementary and alternative medicine*, 15(1), p.408.

Mukherjee, P.K., Nema, N.K., Maity, N. and Sarkar, B.K., 2013. Phytochemical and therapeutic potential of cucumber. *Fitoterapia*, 84, pp.227-236.

Naito, Y., Akagiri, S., Uchiyama, K., Kokura, S., Yoshida, N., Hasegawa, G., Nakamura, N., Ichikawa, H., Toyokuni, S., Ijichi, T. and Yoshikawa, T., 2005. Reduction of diabetes-induced renal oxidative stress by a *Cantaloupe melon* extract/gliadin biopolymers, oxykine, in mice. *Biofactors*, 23(2), pp.85-95.

Nathan, D.M., 2015. Diabetes: advances in diagnosis and treatment. *Jama*, 314(10), pp.1052-1062.

Nazaruk, J. and Borzym-Kluczyk, M., 2015. The role of triterpenes in the management of diabetes mellitus and its complications. *Phytochemistry Reviews*, 14(4), pp.675-690.

Nigam, P.K. and Ch, A., 2017. Positive and negative false estimates of serum creatinine. *Interventional Cardiology*, 9(4), pp.163-167.

Nigatu, T., 2012. Epidemiology, complications and management of diabetes in Ethiopia: a systematic review. *Journal of diabetes*, 4(2), pp.174-180.

Nordquist, L. and Palm, F., 2007. Diabetes-induced alterations in renal medullary microcirculation and metabolism. *Current diabetes reviews*, 3(1), pp.53-65.

Nugent, D.A., Smith, D.M. and Jones, H.B., 2008. A review of islet of Langerhans degeneration in rodent models of type 2 diabetes. *Toxicologic pathology*, 36(4), pp.529-551.

Oben, J., Kuate, D., Agbor, G., Momo, C. and Talla, X., 2006. The use of a *Cissus quadrangularis* formulation in the management of weight loss and metabolic syndrome. *Lipids in Health and Disease*, 5(1), p.24.

Ochoa, M., Lalles, J.P., Malbert, C.H. and Val-Laillet, D., 2015. Dietary sugars: their detection by the gut–brain axis and their peripheral and central effects in health and diseases. *European journal of nutrition*, 54(1), pp.1-24.

OECD-425 (Organization for Economic Cooperation and Development guidline 425), 2008. For the testing of chemicals, Guidance document on acute oral toxicity. *Environmental Health and Safety Monograph Series on Testing and Assessment*, pp.1-27.

Oh, Y.S., 2015. Plant-derived compounds targeting pancreatic beta cells for the treatment of diabetes. *Evidence-Based Complementary and Alternative Medicine*, 2015.

Ota, A. and Ulrih, N.P., 2017. An overview of herbal products and secondary metabolites used for management of type two diabetes. *Frontiers in pharmacology*, 8, p.436.

Page, K.A., Chan, O., Arora, J., Belfort-DeAguiar, R., Dzuira, J., Roehmholdt, B., Cline, G.W., Naik, S., Sinha, R., Constable, R.T. and Sherwin, R.S., 2013. Effects of fructose vs glucose on regional cerebral blood flow in brain regions involved with appetite and reward pathways. *Jama*, 309(1), pp.63-70.

Pal, S., Ho, N., Santos, C., Dubois, P., Mamo, J., Croft, K. and Allister, E., 2003. Red wine polyphenolics increase LDL receptor expression and activity and suppress the secretion of ApoB100 from human HepG2 cells. *The Journal of nutrition*, 133(3), pp.700-706.

Pandey, A. and Tripathi, S., 2014. Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. *Journal of Pharmacognosy and Phytochemistry*, 2(5).

Pang, K., Mukonoweshuro, C. and Wong, G.G., 1994. Beta cells arise from glucose transporter type 2 (Glut2)-expressing epithelial cells of the developing rat pancreas. *Proceedings of the National Academy of Sciences*, 91(20), pp.9559-9563.

Park, S.H., Huh, T.L., Kim, S.Y., Oh, M.R., Tirupathi Pichiah, P.B., Chae, S.W. and Cha, Y.S., 2014. Antiobesity effect of *Gynostemma pentaphyllum* extract (actiponin): A randomized, double-blind, placebo-controlled trial. *Obesity*, 22(1), pp.63-71.

Parmar, H.S. and Kar, A., 2008. Possible amelioration of atherogenic diet induced dyslipidemia, hypothyroidism and hyperglycemia by the peel extracts of Mangifera indica, *Cucumis melo* and *Citrullus vulgaris* fruits in rats. *Biofactors*, 33(1), pp.13-24.

Patel, S.S., Molnar, M.Z., Tayek, J.A., Ix, J.H., Noori, N., Benner, D., Heymsfield, S., Kopple, J.D., Kovesdy, C.P. and Kalantar-Zadeh, K., 2013. Serum creatinine as a marker of muscle mass in chronic kidney disease: results of a cross-sectional study and review of literature. *Journal of cachexia, sarcopenia and muscle*, 4(1), pp.19-29.

Patlak, M., 2002. New weapons to combat an ancient disease: treating diabetes. *The FASEB Journal*, 16(14), pp.1853e-1853e.

Patra, S., Nithya, S., Srinithya, B. and Meenakshi, S.M., 2015. Review of medicinal plants for anti-obesity activity. *Translational Biomedicine*, 6(3).

Pearson, E.R. and Sakamoto, K., 2013. Molecular mechanism of action of metformin: old or new insights?. *Diabetologia*, 56(9), pp.1898-1906.

Piero, N.M., Njagi, M.J., Kibiti, M.C., Ngeranwa, J.J.N., Njagi, N.M., Njue, W.M. and Gathumbi, P.K., 2012. Herbal management of diabetes mellitus: A rapidly expanding research avenue.

Pourghasem, M., Shafi, H. and Babazadeh, Z., 2015. Histological changes of kidney in diabetic nephropathy. *Caspian journal of internal medicine*, 6(3), p.120.

Prentki, M. and Nolan, C.J., 2006. Islet β cell failure in type 2 diabetes. *The Journal of clinical investigation*, 116(7), pp.1802-1812.

Pyke, D.A., 1979. Diabetes: the genetic connections. *Diabetologia*, 17(6), pp.333-343.

Rahman, I.U., Khan, R.U., Rahman, K.U. and Bashir, M., 2015. Lower hypoglycemic but higher antiatherogenic effects of bitter melon than glibenclamide in type 2 diabetic patients. *Nutrition journal*, 14(1), p.13.

Rajasree, R.S., Sibi, P.I. and Femi Francis, H.W.,2016. Phytochemicals of *Cucurbitaceae* Family–A Review.

Ramírez-Espinosa, J.J., Rios, M.Y., López-Martínez, S., López-Vallejo, F., Medina-Franco, J.L., Paoli, P., Camici, G., Navarrete-Vázquez, G., Ortiz-Andrade, R. and Estrada-Soto, S., 2011. Antidiabetic activity of some pentacyclic acid triterpenoids, role of PTP–1B: In vitro, in silico, and in vivo approaches. *European journal of medicinal chemistry*, 46(6), pp.2243-2251.

Randle, P.J., Garland, P.B., Hales, C.N. and Newsholme, E.A., 1963. The glucose fatty-acid cycle its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *The Lancet*, 281(7285), pp.785-789.

Rauter, A.P., Martins, A., Borges, C., Mota-Filipe, H., Pinto, R., Sepodes, B. and Justino, J., 2010. Antihyperglycaemic and protective effects of flavonoids on streptozotocin–induced diabetic rats. *Phytotherapy Research*, 24(S2).

Roche Diagnostics Corporation (RDC), 2016. HDLC: Method sheet. Accessed on March, 2018. https://pim-eservices.roche.com/eLD_SF/nz/en/Documents/GetDocument?documentId=9f366996-7ad5-e511-739a-00215a9b3428

Roche Diagnostics Corporation, 2016. LDLC: Method sheet. Accessed on March, 2018. https://pim-eservices.roche.com/eLD_SF/nz/en/Documents/GetDocument?documentId=3464c6c1-7ed5-e511-739a-00215a9b3428

Roche Diagnostics Corporation, 2017. ASTL: Method sheet, updated in 2020. Accessed on March, 2018https://pim-eservices.roche.com/eLD_SF/nz/en/Documents/GetDocument?documentId=05891529-80c2-e811-2d93-00215a9b3428

Roche Diagnostics Corporation, 2017. CHOL2: Method sheet, updated in 2019. Accessed on March, 2018.https://pim-eservices.roche.com/eLD_SF/nz/en/Documents/GetDocument?documentId=1f1f3277-133a-e911-12b7-00215a9b3428

Roche Diagnostics Corporation, 2017. Creatinine (Crep2): Method sheet, updated in 2019. Accessed on March, 2018https://pim-eservices.roche.com/eLD_SF/nz/en/Documents/GetDocument?documentId=879aec73-cb49-e911-939f-00215a9b3428

Roche Diagnostics Corporation, 2017. Gluc2: Method sheet. Accessed on March, 2018https://pim-eservices.roche.com/eLD_SF/nz/en/Documents/GetDocument?documentId= 5a37fc54-fb3f-e711-acb2-00215a9b3428

Roche Diagnostics Corporation, 2017. TRIGL: Method sheet. Accessed on March, 2018https://pim-eservices.roche.com/eLD_SF/nz/en/Documents/GetDocument?documentId= 1aabd09a-50c4-e711-b48d-00215a9b3428

Roche Diagnostics Corporation, 2017. Urea (Ureal): Method sheet, updated in 2019. Accessed on March, 2018https://pim-eservices.roche.com/eLD_SF/nz/en/Documents/GetDocument?documentId=13f6cb5e-7944-ea11-fc90-005056a71a5d

Roche Diagnostics Corporation, 2018. ALP2: Method sheet, updated in 2020. Accessed on March, 2018https://pim-eservices.roche.com/eLD_SF/nz/en/Documents/GetDocument?documentId=8535677d-242b-e911-a49d-00215a9b3428

Roche Diagnostics Corporation, 2018. ALTL: Method sheet, updated in 2020. Accessed on March, 2018https://pim-eservices.roche.com/eLD_SF/nz/en/Documents/GetDocument?documentId=134bd1a6-77c2-e811-2d93-00215a9b3428

Ross, M.H. and Pawlina, W., 2011. Histology: a Text and Atlas: With correlated cell and molecular biology. 6th ed. Lippincott Williams & Wilkins.

Rui, L., 2014. Energy Metabolism in the Liver. Comprehensive Physiology, 4(1), p.177.

Saboo, S.S., Thorat, P.K., Tapadiya, G.G. and Khadabadi, S.S., 2013. Ancient and recent medicinal uses of *Cucurbitaceae* family. *International Journal of Therapeutic Applications*, 9, pp.11-19.

Saidu, A.N., Oibiokpa, F.I. and Olukotun, I.O., 2014. Phytochemical screening and hypoglycemic effect of methanolic fruit pulp extract of *Cucumis sativus* in alloxan induced diabetic rats. *Journal of Medicinal Plants Research*, 8(39), pp.1173-1178.

Sales, P.M., Souza, P.M., Simeoni, L.A., Magalhães, P.O. and Silveira, D., 2012. α-Amylase inhibitors: a review of raw material and isolated compounds from plant source. *Journal of Pharmacy & Pharmaceutical Sciences*, 15(1), pp.141-183.

Samuel, V.T. and Shulman, G.I., 2012. Mechanisms for insulin resistance: common threads and missing links. *Cell*, 148(5), pp.852-871.

Samuel, V.T. and Shulman, G.I., 2016. The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. *Journal of Clinical Investigation*, 126(1), p.12.

Sarkar, S., Pranava, M. and MARITA, A.R., 1996. Demonstration of the hypoglycemic action of *Momordica charantia* in a validated animal model of diabetes. *Pharmacological Research*, 33(1), pp.1-4.

Sedigheh, A., Jamal, M.S., Mahbubeh, S., Somayeh, K., Mahmoud, R.K., Azadeh, A. and Fatemeh, S., 2011. Hypoglycaemic and hypolipidemic effects of pumpkin (*Cucurbita pepo L.*) on alloxan-induced diabetic rats. *African Journal of Pharmacy and Pharmacology*, 5(23), pp.2620-2626.

Semwal, D.K., Bamola, A. and Rawat, U., 2007. Chemical constituents from some antidiabetic plants. *Univ J Phytochem Ayur Heig*, 2(3), pp.40-48.

Shih, C.C., Shlau, M.T., Lin, C.H. and Wu, J.B., 2014. Momordica charantia ameliorates insulin resistance and dyslipidemia with altered hepatic glucose production and fatty acid synthesis and AMPK phosphorylation in high-fat-fed mice. *Phytotherapy research*, 28(3), pp.363-371)

Shrestha, S., Gyawali, P., Shrestha, R., Poudel, B. and Sigdel, M., 2008. Serum urea and creatinine in diabetic and non-diabetic subjects. *Journal of Nepal Association for Medical Laboratory Sciences* P, 11, p.12.

Shu, H.J., Isenberg, K., Cormier, R.J., Benz, A. and Zorumski, C.F., 2006. Expression of fructose sensitive glucose transporter in the brains of fructose-fed rats. *Neuroscience*, 140(3), pp.889-895.

Shulman, G.I., 2014. Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease. *New England Journal of Medicine*, 371(12), pp.1131-1141.

Simopoulos, A., 2013. Dietary omega-3 fatty acid deficiency and high fructose intake in the development of metabolic syndrome, brain metabolic abnormalities, and non-alcoholic fatty liver disease. *Nutrients*, 5(8), pp.2901-2923.

Singh, A., Bhat, T.K. and Sharma, O.P., 2011. Clinical biochemistry of hepatotoxicity. *Journal of clinical toxicology*, 4(0001), pp.1-9.

Singh, S.S., Pandey, S.C., Srivastava, S., Gupta, V.S., Patro, B. and Ghosh, A.C., 2003. Chemistry and medicinal properties of *Tinospora cordifolia* (Guduchi). *Indian journal of pharmacology*, 35(2), pp.83-91.

Skovsø, S., 2014. Modeling type 2 diabetes in rats using high fat diet and streptozotocin. *Journal of diabetes investigation*, 5(4), pp.349-358.

Sone, Y., Kido, T., AINUkI, T., Sonoda, M., Ichi, I., Kodama, S., Sone, H., Kondo, K., Morita, Y., Egawa, S. and Kawahara, K., 2013. Genetic variants of the fatty acid desaturase gene cluster are associated with plasma LDL cholesterol levels in Japanese males. *Journal of nutritional science and vitaminology*, 59(4), pp.325-335.

Sood, A., Kaur, P. and Gupta, R., 2012. Phytochemical screening and antimicrobial assay of various seeds extract of *Cucurbitaceae* family.

Srinivasan, K., Viswanad, B., Asrat, L., Kaul, C.L. and Ramarao, P., 2005. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening. *Pharmacological research*, 52(4), pp.313-320.

Sudheesh, S. and Vijayalakshmi, N.R., 1999. Lipid-lowering action of pectin from *Cucumis sativus*. *Food chemistry*, 67(3), pp.281-286.

Suman, R.K., Mohanty, I.R., Maheshwari, U., Borde, M.K. and Deshmukh, Y.A., 2016a. Metformin ameliorates diabetes with metabolic syndrome induced changes in experimental rats. *Int J of Biomed & Adv Res*, 7(2), pp.55-65.

Suman, R.K., Ray Mohanty, I., Borde, M.K., Maheshwari, U. and Deshmukh, Y.A., 2016b. Development of an experimental model of diabetes co-existing with metabolic syndrome in rats. *Advances in pharmacological sciences*.

Sun, N.N., Wu, T.Y. and Chau, C.F., 2016. Natural dietary and herbal products in anti-obesity treatment. *Molecules*, 21(10), p.1351.

Ta, S., 2014. Diagnosis and classification of diabetes mellitus. *Diabetes care*, 37, p.S81.

Tan, M.J., Ye, J.M., Turner, N., Hohnen-Behrens, C., Ke, C.Q., Tang, C.P., Chen, T., Weiss, H.C., Gesing, E.R., Rowland, A. and James, D.E., 2008. Antidiabetic activities of triterpenoids isolated from bitter melon associated with activation of the AMPK pathway. *Chemistry & biology*, 15(3), pp.263-273.

Tangvarasittichai, S., 2015. Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. *World journal of diabetes*, 6(3), p.456.

Teklay, A., Abera, B. and Giday, M., 2013. An ethnobotanical study of medicinal plants used in KilteAwulaelo District, Tigray Region of Ethiopia. *Journal of ethnobiology and ethnomedicine*, 9(1), p.1.

Teklehaymanot, T., 2009.Ethnobotanical study of knowledge and medicinal plants use by the people in Dek Island in Ethiopia. *Journal of Ethnopharmacology*, 124(1), pp.69-78.

Teklehaymanot, T., and Giday, M., 2007. Ethnobotanical study of medicinal plants used by people in Zegie Peninsula, Northwestern Ethiopia

Telford, I.R., Sebastian, P., Bruhl, J.J. and Renner, S.S., 2011. *Cucumis* (*Cucurbitaceae*) in Australia and Eastern Malesia, including newly recognized species and the sister species to C. melo. *Systematic Botany*, 36(2), pp.376-389.

Teoh, S.L., Latiff, A.A. and Das, S., 2010. Histological changes in the kidneys of experimental diabetic rats fed with *Momordica charantia* (bitter gourd) extract. *Rom J Morphol Embryol*, 51(1), pp.91-5.

Thongprayoon, C., Cheungpasitporn, W. and Kashani, K., 2016. Serum creatinine level, a surrogate of muscle mass, predicts mortality in critically ill patients. *Journal of thoracic disease*, 8(5), p.E305.

Tolman, K.G., Fonseca, V., Dalpiaz, A. and Tan, M.H., 2007. Spectrum of liver disease in type 2 diabetes and management of patients with diabetes and liver disease. *Diabetes care*, 30(3), pp.734-743.

Triplitt, C.L., 2012. Understanding the kidneys' role in blood glucose regulation. *The American journal of managed care*, 18(1 Suppl), pp.S11-6.

Tsuchitani, M., Sato, J. and Kokoshima, H., 2016. A comparison of the anatomical structure of the pancreas in experimental animals. Journal of toxicologic pathology, 29(3), pp.147-154.

Umer, S., Tekewe, A. and Kebede, N., 2013.Antidiarrhoeal and antimicrobial activity of *Calpurnia aurea* leaf extract. *BMC complementary and alternative medicine*, 13(1), p.1.

Usman J.G., Sodipo, O.A., Kwaghe, A.V. and Sandabe, U.K., 2015. Uses of *Cucumis metuliferus*: A Review. *Cancer Biology*, 5(1):pp.24-34.

Van Stee, M.F., de Graaf, A.A. and Groen, A.K., 2018. Actions of metformin and statins on lipid and glucose metabolism and possible benefit of combination therapy. *Cardiovascular diabetology*, 17(1), p.94.

Vergès, B., 2010. Abnormalities in lipoprotein kinetics in Type 2 diabetes. *Clinical Lipidology*, 5(2), pp.277-289.

Verma, J., Rathore, D.S., Agarwal, S. and Tripathi, V., 2015. Effects of *Citrullus Colocynthis* and *Cucumis Callosus* Extract on Blood Glucose Levels in Alloxan-Induced Diabetic Rats. *SGVU Int. J. Env. Sci. Technol*, 1(1), pp.50-56.

Vinayagam, R. and Xu, B., 2015. Antidiabetic properties of dietary flavonoids: a cellular mechanism review. *Nutrition & metabolism*, 12(1), p.60.

Vishwakarma, V.K., Gupta, J.K. and Upadhyay, P.K., 2017. Pharmacological importance of *Cucumis melo L*.: An overview. *Asian Journal of Pharmaceutical and Clinical Research*, 10(3), pp.8-12.

Vodičar, J., Pajek, J., Hadžić, V. and Bučar Pajek, M., 2018. Relation of Lean Body Mass and Muscle Performance to Serum Creatinine Concentration in Hemodialysis Patients. *BioMed research international*, 2018.

Vouldoukis, I., Lacan, D., Kamate, C., Coste, P., Calenda, A., Mazier, D., Conti, M. and Dugas, B., 2004. Antioxidant and anti-inflammatory properties of a *Cucumis melo LC*. extract rich in superoxide dismutase activity. *Journal of Ethnopharmacology*, 94(1), pp.67-75.

Wang, C.Y. and Liao, J.K., 2012. A mouse model of diet-induced obesity and insulin resistance. *In mTOR* (pp. 421-433). Humana Press.

Wang, L., Yamasaki, M., Katsube, T., Sun, X., Yamasaki, Y. and Shiwaku, K., 2011. Antiobesity effect of polyphenolic compounds from molokheiya (*Corchorus olitorius L.*) leaves in LDL receptor-deficient mice. *European journal of nutrition*, 50(2), pp.127-133.

Weyer, C., Bogardus, C., Mott, D.M. and Pratley, R.E., 1999. The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *The Journal of clinical investigation*, 104(6), pp.787-794.

WHO (World Health Organization), 2010. *Report of Traditional Medicine.* Geneva,Switzerland: World Health Organization.

WHO (World Health Organization), 2016. *Global report on diabetes*. Geneva, Switzerland: World Health Organization.

Williams, K.H., Shackel, N.A., Gorrell, M.D., McLennan, S.V. and Twigg, S.M., 2012. Diabetes and nonalcoholic fatty liver disease: a pathogenic duo. *Endocrine reviews*, 34(1), pp.84-129.

Wilson, R.D. and Islam, M.S., 2012. Fructose-fed streptozotocin-injected rat: an alternative model for type 2 diabetes. *Pharmacological Reports*, 64(1), pp.129-139.

Wong, S.K., Chin, K.Y., Suhaimi, F.H., Fairus, A. and Ima-Nirwana, S., 2016. Animal models of metabolic syndrome: a review. *Nutrition & metabolism*, 13(1), p.65.

Xia, T. and Wang, Q., 2006. D-chiro-Inositol found in *Cucurbita ficifolia* (*Cucurbitaceae*) fruit extracts plays the hypoglycaemic role in streptozocin-diabetic rats. *Journal of pharmacy and pharmacology*, 58(11), pp.1527-1532.

Xia, T. and Wang, Q., 2007. Hypoglycaemic role of *Cucurbita ficifolia* (*Cucurbitaceae*) fruit extract in streptozotocin-induced diabetic rats. *Journal of the Science of Food and Agriculture*, 87(9), pp.1753-1757.

Xiang, X., Wang, Z., Zhu, Y., Bian, L. and Yang, Y., 2010. Dosage of streptozocin in inducing rat model of type 2 diabetes mellitus. *Wei sheng yan jiu= Journal of hygiene research*, 39(2), pp.138-142.

Xu, T., Brandmaier, S., Messias, A.C., Herder, C., Draisma, H.H., Demirkan, A., Yu, Z., Ried, J.S., Haller, T., Heier, M. and Campillos, M., 2015. Effects of metformin on metabolite profiles and LDL cholesterol in patients with type 2 diabetes. *Diabetes care*, 38(10), pp.1858-1867.

Yadav, R.H., 2013. Medicinal plants in folk medicine system of Ethiopia. *Journal of Poisonous and Medicinal Plants Research*, 1(1), pp.7-11.

Yuan, H., Ma, Q., Ye, L. and Piao, G., 2016. The traditional medicine and modern medicine from natural products. *Molecules*, 21(5), p.559.

Zabielski, P., Hady, H.R., Chacinska, M., Roszczyc, K., Gorski, J. and Blachnio-Zabielska, A.U., 2018. The effect of high fat diet and metformin treatment on liver lipids accumulation and their impact on insulin action. *Scientific reports*, 8(1), p.7249.

Zamani, A., Beni, M.A. and Abadi, M.A.Z.N., 2012. Relationship between body composition with Blood Lipids profile. *European Journal of Experimental Biology*, 2(5), pp.1509-13.

Zhang, X., Zhao, Y., Zhang, M., Pang, X., Xu, J., Kang, C., Li, M., Zhang, C., Zhang, Z., Zhang, Y. and Li, X., 2012. Structural changes of gut microbiota during berberine-mediated prevention of obesity and insulin resistance in high-fat diet-fed rats. *PloS one*, 7(8), p.e42529.

Zhang, Y., Zhang, Y., Bone, R.N., Cui, W., Peng, J.B., Siegal, G.P., Wang, H. and Wu, H., 2012. Regeneration of pancreatic non-β endocrine cells in adult mice following a single diabetes-inducing dose of streptozotocin. *PloS one*, 7(5), p.e36675.

Zheng, Y., Ley, S.H. and Hu, F.B., 2018. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nature Reviews Endocrinology*, 14(2), p.88.

Zhou, J., Massey, S., Story, D. and Li, L., 2018. Metformin: an old drug with new applications. *International journal of molecular sciences*, 19(10), p.2863.

Zhuo, J., Zeng, Q., Cai, D., Zeng, X., Chen, Y., Gan, H., Huang, X., Yao, N., Huang, D. and Zhang, C., 2018. Evaluation of type 2 diabetic mellitus animal models via interactions between insulin and mitogen-activated protein kinase signaling pathways induced by a high fat and sugar diet and streptozotocin. *Molecular medicine reports*, 17(4), pp.5132-5142.

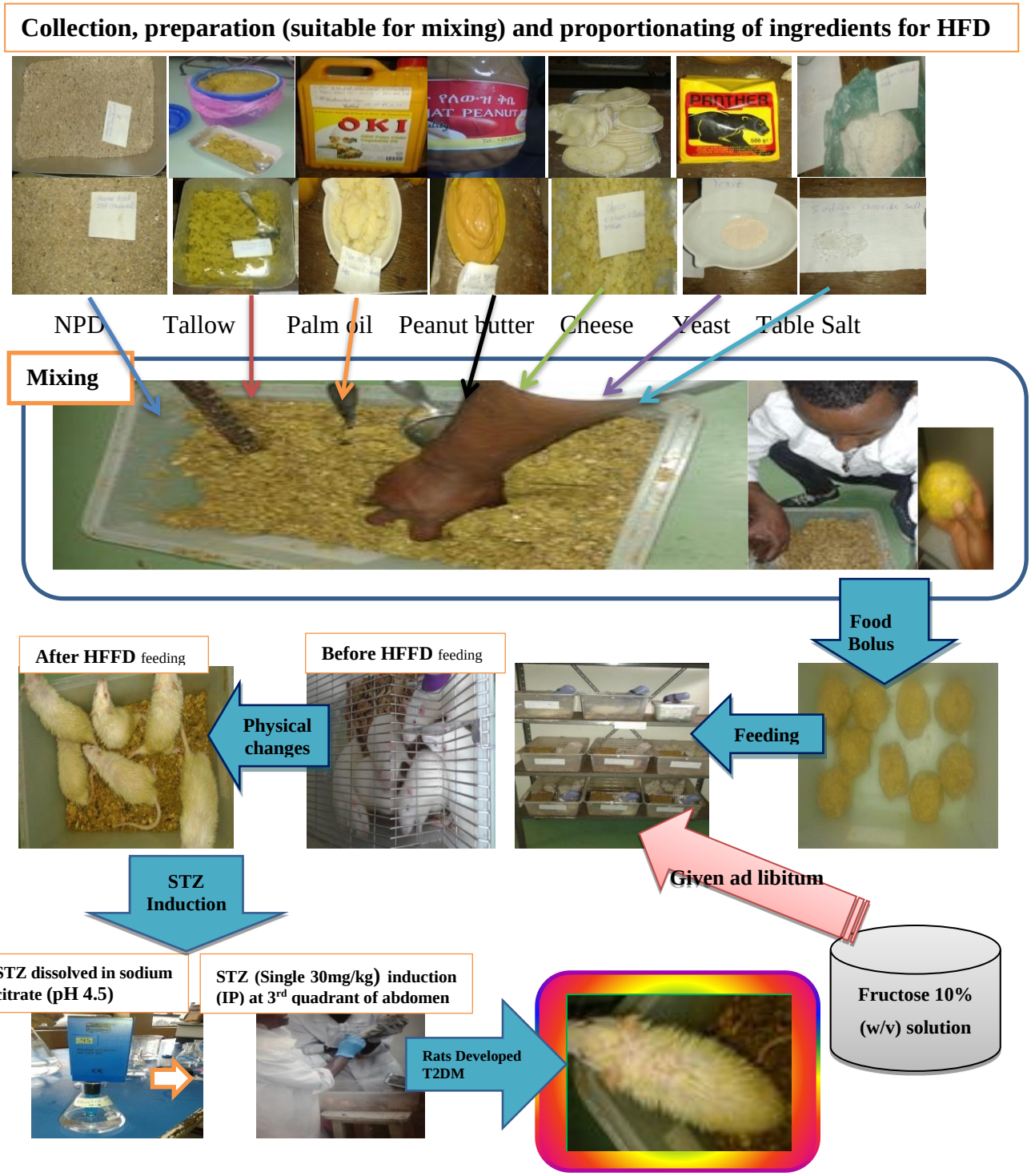
APPENDIXES

Annex-1: Some photographs depicting the basic experimental activities of this work carried out at Laboratory

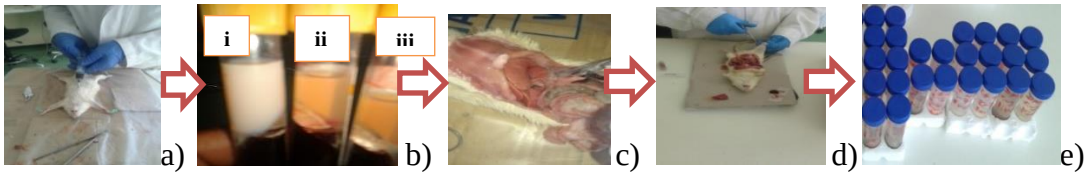
A) Phytochemical screening (qualitative)

Key: a) preparation of reagents for phytochemical screening; b) addition of particular screening reagents to the extract, and c & d) observation of presence or absence of specific phytochemical class

B) HFFD Preparation and feeding, and STZ-induction for Development of T2DM-Model



C) Sacrification and collection of blood and organ samples



Key: a) blood collection from cardiac puncture; b) Serum separation by centrifugation: where "i=cloudy serum" represent the serum of diabetic negative control, "ii & iii=clear serum"-represents the serum of metformin and *C. ficifolius* treated diabetic groups, respectively; c &d) abdominal operation & organs isolation; e) preservation of organs using 10% neutral formalin buffer.

Annex-2: Worksheet for STT, OGTT and progressive glucose and body weight determination

A) Worksheet for Effect of <u>STT</u> of Crude *C. ficifolius* Root Extract on Blood Glucose

Group	Rat code	**Record of blood glucose in STT**					
		0 Hr(fasting)	1st Hr	2nd Hr	4th Hr	6th Hr	Remark
Normal control (group-1, G1)	G1C1	…./…/….	…./…/….	…./…/….	…./…/….	…./…/….	
	G1C2						
	G1C3						
	G1C4						
	G1C5						
	G1C6						
	G1C7						
Diabetic negative control +vehicle (group-2, G2)	G2C1						
	G2C2						
	G2C3						
	G2C4						
	G2C5						
	G2C6						
	G2C7						
. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Diabetic + *C. ficifolius* 400mg/Kg (group-6, G6)	G6C1	…./…/….	…./…/….	…./…/….	…./…/….	…./…/….	
	G6C2						
	G6C3						
	G6C4						
	G6C5						
	G6C6						
	G6C7						

B) Worksheet for Determination of Blood Glucose in Pre-&Post-treatment <u>OGTT</u>

Group	**Rat code**	**Blood glucose record inpre-treatment OGTT**							**Blood glucose record inpost-3-weeks-long treatment OGTT**					
		0Hr(fasting)	1st Hr	2nd Hr	4th Hr	6th Hr	Remark		0Hr	1st Hr	2nd Hr	4th Hr	6th Hr	Remark
Normal control (group-1, G1)	G1c1	**../../..**	**../../..**	**../../..**	**../../..**	**../../..**			**../../..**	**../../..**	**../../..**	**../../..**	**../../..**	
	G1c2													
	G1c3													
	G1c4													
	G1c5													
	G1c6													
	G1c7													
. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .			. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
Diabetic + *C. ficifolius* 400mg/ Kg (g6)	G6c1	**../.../..**	**../../..**	**../../..**	**../../..**	**../../..**			**../../..**	**../../..**	**../../..**	**../.../..**	**../.../..**	
	G6c2													
	G6c3													
	G6c4													
	G6c5													
	G6c6													
	G6c7													

C) Worksheet for Progressive Effect of Extract Treatment on <u>Fasting Blood Glucose</u> during Experiment

Groups	Rat code	Progressive (weekly) fasting blood glucose record						
		Before HFFD	After HFFD/Before STZ-induction	Week-0 (1st day of treatment)	Week-1	Week-2	Week-3	Remark
Normal control (Group-1, g1)	g1C1	**../.../…**	**../.../…**	**.../.../…**	**../.../…**	**../.../…**	**../.../…**	
	g1C2							
	g1C3							
	g1C4							
	g1C5							
	g1C6							
	g1C7							
. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	
D+ *C.ficifolius* 400mg/Kg (Group-6, g6)	g6C1	**../.../…**	**../.../…**	**../.../…**	**../.../…**	**../.../…**	**../.../…**	
	g6C2							
	g6C3							
	g6C4							
	g6C5							
	g6C6							
	g6C7							

D) Worksheet for Progressive Effect of Extract Treatment on <u>Fasting Body Weight</u> during Experimentation

Groups	Rat code	Progressive (weekly) fasting body weight record						
		Before HFFD	After HFFD/Before STZ-induction	Week-0 (1st day of treatment)	Week-1	Week-2	Week-3	Remark
Normal control (group 1, g1)	g1C1	**../.../…**	**../.../…**	**../.../…**	**../.../…**	**../.../…**	**../.../…**	
	g1C2							
	g1C3							
	g1C4							
	g1C5							
	g1C6							
	g1C7							
. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .	
D+ *C.ficifolius* 400mg/Kg)Group-6, g6)	g6C1	**../.../…**	**../.../…**	**../.../…**	**../.../…**	**../.../…**	**../.../…**	
	g6C2							
	g6C3							
	g6C4							
	g6C5							
	g6C6							
	g6C7							

Where; "**../../..**" Represent the three readings/repeated measures at a single measurement period

Annex-3: Figurative Abstract of This Research Work