USMANU DANFODIYO UNIVERSITY, SOKOTO
(POSTGRADUATE SCHOOL)

EFFECT OF *VITELLARIA PARADOXA* STEM BARK EXTRACTS ON SOME CYTOKINES IN DIABETIC RATS

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By

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DEDICATION

This research work is dedicated to: (i) my late mother, Hajiya Aishatu Usman who gave me her utmost moral, educational and financial support till her death. I pray to Almighty Allah (SAW) to reward her with Jannatul-Fiddausi, amen; and (ii) my father (Alhaji Hussaini Umar), my wife (Fauziya Isah) and my beloved two living sons (Muhammad Mahdi and Muhammad Humaid).

CERTIFICAION

This Dissertation by **Kabiru Hussaini** (Adm. No:15211227021), has met the requirement for the award of the degree of Master of Science in Medical Laboratory Sciences (Immunology) of the Usmanu Danfodiyo University, Sokoto and is approved for its contribution to knowledge.

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LIST OF ACRONYMS

AB	Antibody
ADA	American Diabetes Association
ADSCs	Adipose-derived mesenchymal stem cells
Akt	Protein kinase B
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APC	Antigen Presenting Cells
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
BM-MSCs	Bone Marrow Mesenchymal Stem Cells
BSF-2	B-cell Stimulatory Factor 2
CD	Cluster of Differentiation
CDC	Center for Disease Control
CDK4	Cyclin-Dependent Kinase 4
CKD	Chronic Kidney Disease
CRP	C-Reactive Protein
CTL	Cytotoxic T lymphocyte
CVD	Cardiovascular Diseases
DD	Dead Domain
DM	Diabetes Mellitus
DNA	Deoxyribose Nucleic Acid

DPP-4	Dipeptidyl Peptise-4
DR	Diabetes Retinopathy
EDTA	Ethylene diamine Tetraacetic Acid
ELISA	Enzymes Link Immunosorbent Assay
FADD	Fas Associated Dead Domain Protein
FBG	Fasting Blood Glucose
FFBG	Final Fasting Blood Glucose
GAD	Glutamic Acid Decarboxylase
GLP1	Glucagon-Like Peptide-1
GLUT 2	Glucose Transporter-4
H_1	Alternative Hypothesis
H_2SO_4	Sulphuric Acid
HbA1c	Glycated Haemoglobin
HCl	Hydrochloric Acid
HGF	Hepatocyte Growth Factor
HGF	Hybridoma Growth Factor
HLA	Human Leukocyte Antigens
H_o	Null Hypothesis
HRP	Avidin-Horseradish Peroxidase
HSF	Hepatocyte-Stimulating Factor
IA -2	Islet Antigens
IAA	Insulin Autoantibody
ICAM	Intercellular Adhesion Molecule-1

IDDM	Insulin Dependent Diabetes Mellitus
IDF	International Diabetes Federation
IFG	Impaired Fasting Glucose
IFN-γ	Interferon Gamma
IgG	Immunoglobulin
IL	Interleukin
INGAP	Islet Neogenesis Associated Protein
IR	Insulin Receptors
IRF-1	Interferon Regulatory Factor-1
IRS	Insulin Receptor Substrate
IRS-1	Insulin Receptor Substrate-1
LD_{50}	Lethal Dose
MCP-1	Monocyte Chemotactic Protein-1
MHC	Major Histocompatibility Complex
MIC	Minimal Inhibition Concentration
MRDM	Malnutrition Related Diabetes Mellitus
mRNA	Messenger Ribose Nucleic Acid
MSCs	Mesenchymal Stem Cells
NCD	Non-Communicable Disease
NF-$_K$B	Nuclear Factors
Ngn3	Neurogenin 3
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NLR	Neutrophils To Lymphocyte Ratio

NLRP3	NLR Family, Pyrin Domain Containing 3
NO	Nitric Oxide
NOD	Non-Obese Diabetic
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
OD	Optical Density
OECD	Economic Cooperation And Development
Pdx1	Pancreatic Duodenal Homeobox-1
PMSCs	Pancreatic Mesenchymal Stem Cells
ROS	Reactive Oxygen Species
SAA	Serumamyloid A
SBE	STAT-Binding Elements
SNPs	Single Nucleotide Polymorphisms
STAT-1	Signal Transducer And Activator of Transcription-1
STAT3	Signaling Pathway That Activates The Transcription Factor
T1D	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes Mellitus
TGF	Transforming Growth Factor
TH	T-Helper
TLR	Toll-Like Receptor
TNFR	Tumor Necrosis Factor Receptor
TNF-α	Tumour Necrosis Factor- Alpha
TRADD	TNF Receptor Associated Dead Domain Protein
TXNIP	Thioredoxin-Interacting Protein

U.S.A	United State of America
UCBhMSCs	Umbilical Cord Blood Mesenchymal Stem Cells
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low-Density Lipoproteins
VPSBE	*Vitellaria Paradoxa* Stem Bark Extract
WHO	World Health Organization
ZnT8	Zinc Transporter 8

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ABSTRACT

The pathogenesis of diabetes mellitus involves a low grade inflammatory process due to the increase blood glucose. Effects of *Vitellaria paradoxa* stem bark extracts (VPSBE) in diabetic rats through serum cytokine quantification were investigated. Thirty *Wistar* rats were rendered diabetic by peritoneal injection of Alloxan (80mg/kg/b.wt.) for 3 three consecutive days. The rats were randomly divided into five respective groups as: Non-DM group, DM group, DM + 100VPSBE group, DM + 200VPSBE group and DM + 200Metformin group. The animals were treated with VPSBE for 28 consecutive days. Animals were euthanized humanely and blood sample was obtained through cardiac puncture. Serum TNF- α, IL 6 and IL-10 were measured through ELISA technique and neutrophils to lymphocyte ratio (NLR) were calculated. Our result showed that, VPSBE induced a significant decrease in blood glucose more than the standard drug metformin, stabilized food intake and improved body weight loss in diabetic rats. The concentrations of TNF-α and IL-6 significantly increased while those of IL-10 and NLR diminished in diabetic rats compared to control animals. Interestingly, after treatment with VPSBE IL-10 levels and NLR significantly increased in diabetic rats, while TNF-α and IL-6 concentrations decreased. Phytochemical analysis of the VPSBE revealed the presence of ten (10) bioactive compounds and there was no mortality recorded in the acute toxicity study. In conclusion VPSBE contains potent phytochemical constituents that offer safe hypoglycaemic agent, reduced pro-inflammatory cytokines, improved regulatory cytokines and may, therefore play a significant role in the management of diabetes and diabetic complication.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

The balance of cytokines is important for normal immune responses. Irregular cytokine levels can shift the immune responses from being beneficial to being harmful (Bijjiga and Martino 2013; Talaat *et al.,* 2016)

International Diabetes Federation has recently declared diabetes mellitus (DM) as a universal disaster, seriously affects the quality of patients' life and the national healthcare systems. There are nearly 415 million adults living with diabetes globally, with a prevalence of 17.5% in 2015 which is expected to rise to 642 million or multiply three times by 2040 (ADA, 2015; IDF, 2015)

Diabetes mellitus is a metabolic disorder characterized with hyperglycaemia, increased thirst, polyuria and weight loss as a result of inadequate insulin secretion or, insulin receptor dysfunction or both (ADA, 2015). The three types of diabetes are: (i) Type 1 diabetes mellitus (insulin dependent DM or T1DM) which is an autoimmune disease characterized by beta cell destruction cause by aggressive autoreactive T-lymphocyte via production of IFN-γ and TNF-α (Li *et al,* 2014; Ferreira-Hermosillo *et al.,* 2015). Cellular infiltration and inflammatory responses in the Islets of Langerhans is associated with T1DM (Alnek *et al.,* 2015). The cellular components of this infiltrate include monocytes, macrophages, CD4+ and CD8+ T cells (Pestana, *et al.,* 2016), and the balance between Th1 and Th2 cytokines is crucial in the pathogenesis of this disease (Talaat *et al.,* 2016). (ii) Type 2 diabetes (non-insulin dependent DM), which is more common, is a problem of progressively impaired glucose regulation due to one or combination of dysfunctional pancreatic beta cells and insulin resistance (iii)

Diabetes in pregnancy that is known as gestational DM which resolves after delivery (ADA, 2016).

In all types of diabetes, breakdown of all major nutrients develops disorder (Mardiah *et al.,* 2015). The efficiency of the use and uptake of these nutrients by most of the body cells reduce by resistance or absence of insulin. Disorders in either the secretion or activity of insulin in diabetes mellitus could be caused by non-enzymatic glycation mechanism, which is the processes of glucose chemically binding to the free amino group on the protein without helping of enzymes, as well as increasing of inflammation. Inflammation is a physiological response of the body against damage or disturbance outside factors. Hyperglycaemia condition caused the response of inflammatory compounds that mediated by cytokines. The presence of cytokines will damage the insulin sensitivity and glucose balance (Lewis *et al.,* 2014; Mardiah *et al.,* 2015). Inflammation occurs after increasing blood glucose that is marked by an increase of various markers of inflammation, such as high sensitivity C reactive protein (hs-CRP), interleukin-6 (IL 6), tumor necrosis factor-α (TNF-α) and interleukin-18 (IL -18) (Mardiah *et al.,* 2015). Inflammation can be triggered through increase of ROS (reactive oxygen species) during diabetes mellitus. Reactive oxygen species (ROS) can activate NF-κB, which is a transcription factor that regulates the expression of proinflammatory genes such as TNF-α, IL-6 and C reactive protein. In addition, the condition of diabetes can increase the availability of free fatty acids due to lipolysis process (Bolajoko *et el.,* 2017; Elizabeth *et al.,* 2017; Qiao *et al.,* 2017). The increase of free fatty acids will activate the immune system for releasing cytokines IL-6, TNF-α, IL-1β. It also explains the link between obesity and the increase of inflammation. Moreover different methods of diabetes induction on Wistar rats are reported in literature to cause elevated level of proinflammatory mediators (Mardiah *et al.,* 2015;

Bolajoko *et el.,* 2017; Elizabeth *et al.,* 2017; Qiao *et al.,* 2017), however anti-inflammatory cytokines depressed on diabetic animal model (Furudoi *et al.,* 2013; Fachinan *et al.,* 2017)

Tumor necrosis factor-α plays part in apoptosis of macrovascular and microvascular in DM type 1 and 2 and was involved in the pathogenesis of diabetic nephropathy, neuropathy, retinopathy and hepatic complication (Ingaramo, *et al.,* 2011; Dell'Omo *et al* 2013; Satoh *et al.,* 2013; Nadeem *et al.,* 2013; Elizabeth *et al.,* 2017). Tumor necrosis factor-α is well acknowledged to cause damage to renal cells by enhancing renal hypertrophy, hemodynamic imbalance, albumin permeability (Rivero *et al.,* 2009). The harmful effects of these responses lead to the development of renal disease in patients with T2DM, hence resulting in the progression of renal failure (Gautam *et al.,* 2017); TNF-*α* increases retinal endothelial permeability by down regulating the expression of tight junction proteins and the increased permeability can lead to rupturing of the blood retinal barrier (Aveleira *et al.,* 2010). Interleukin (IL)-6 causes not only insulin resistance which can worsen the condition of diabetes but also dysfunction of β cells (Ikmal *et al.,* 2013). The elevated level of IL-6 is highly related to increased blood glucose, decreased glucose tolerance, and decreased insulin sensitivity (Gomes, 2017). Furthermore, IL-6 can lead to a permanent damage to pancreatic β cells through promoting B lymphocytes differentiation and activating killer T cells (Chen *et al*. 2017). Interleukin (IL)-10 is considered primarily to have an anti-inflammatory effect and does so by suppressing the production of inflammatory cytokines (Khan *et al.,* 2017).

Diabetes is a frequent, unbearable metabolic disorder that required lifelong management and greatly increases the risk of severe complications, which are usually managed with drugs like insulin injection, glibenamide and metformin. However, the cost of management therapy and accompanied side effects of these drugs remain a major global concern. Therefore there is

need for an alternative, cost–effective way of managing the disease. The use of medicinal plants could be an alternative means to improve health care globally particularly in poor resource countries (Malviya *et al.,* 2010). Medicinal plants are locally available, easily accessible regardless of social status (Maghrani *et al.,* 2005). Phytochemicals (plant chemicals with protective or disease preventive activity) which originate from plants offer a notable prospect for the exploration of new varieties of therapeutics. As a result, efforts are being geared globally towards the exploitation of these medicinal plants which possess significant amount of Phytochemicals exhibiting diverse beneficial effects in tackling diabetes and associated complications (Atmakuri & Dathi, 2010).

The *Vitellaria paradoxa* tree is available in the most part of Nigeria with a variety of ethnomedicinal importance. *Vitellaria paradoxa* stem bark are dried, grounded to paste and soaked in boiled water, the hot filtrate is used locally in the Hausa land of the North Western region of Nigeria in the treatment of abnormal lump (tumour) around mouth cavity, neck and the breast.

In this study the ability of the VPSBE toward decreasing levels of inflammatory compounds existing in diabetic rats possibly through increase level of anti-inflammatory cytokine was determined. Benefits of the research were to provide information about VPSBE as immunomodulatory agent that will contribute to the VPSBE functions on diabetes and it complications.

1.2 Statement of Research Problem

The increasing prevalence of diabetes and its complications in rapid successions has become a major concern Worldwide and there is a need to prevent or manage the disease (Stumvoll *et al.,* 2005). Various hypoglycemic medications have been formulated, but have been found to display serious side-effects, prompting the need for the development of indigenous and

inexpensive herbal sources for management of diabetes (Atmakuri & Dathi, 2010). These limitations have stimulated the need to explore the potential anti-diabetic and anti-inflammatory activities of *Vitellaria paradoxa* stem bark (VPSBE). Increasing evidence suggests that individuals who progress to diabetes mellitus display features of inflammation years before the disease onset (Gabbay *et al.*, 2012). In addition, studies in recent years have shown that inflammatory cytokines, are determinant factors in the development of microvascular diabetic complications, including neuropathy, retinopathy, and nephropathy. (Qiao *et al.*, 2017), which constitutes the most frequent cause of organs failure there by produces significant social and economic burdens. Hence, this research was designed to examine the immunomodulatory effects of VPSBE for the management of diabetes.

1.3 Justification of Study

Considering the involvement of inflammatory cytokines and its clinical significance in diabetes mellitus and diabetic microvascular complications, there is a need to develop desirable therapeutic strategies that attempt to restore the cytokines balance disturbed by pathological overproduction of cytokines in diabetes in order to prevent, delaying or even revert microvascular complication. Interestingly, however natural product posses significantly bioactive component that have been confirmed to inhibit cytokines over expression, elicit antibodies production or contents agents blocking cytokines receptors to improve insulin resistance through suppression of inflammatory signaling pathways with less or no side effect (Anm *et al.*, 2014). Recent studies have found that VPSBE is rich in bioactive component with good anti-bacteria effect (Fodouop *et al.*, 2017), anti-inflammatory (Foyet *et al.*, 2015). However the anti-diabetic effect of VPSBE has not been establish, to date no study has been reported on the possible effect of VPSBE in modulating inflammatory mediators in diabetic

rats. The finding of this study could provide a better approach for the management of diabetes and prevention, delaying or reverting microvascular complications.

1.4 Aim and Objectives

1.4.1 Aim

The aim of this study was to determine the effect of *Vitellaria paradoxa* stem bark extract (VPSBE) on some pro-inflammatory cytokines (IL-6 and TNF-α) and anti-inflammatory cytokines (IL-10), in diabetic rats

1.4.2 Objectives:

The objectives of the study were to:

1. Determine the phytochemical composition and LD_{50} VPSBE
2. Determine the effect of hyperglycaemia on serum level of IL-6, IL-10 and TNF-α in diabetic rats
3. Determine the effect of VBSBE on serum level IL-6, IL-10 and TNF-α in diabetics rats
4. Determine the effect glycaemic status, weight gain and food intake after the diabetic rats was fed with VPSBE

1.5 Research Question

1. What are the bioactive components and LD_{50} of VPSBE?
2. What are the serum level IL-6, IL-10 and TNF-α in diabetic rat?
3. What are the potential effects of VBSBE on serum level IL-6, IL-10 and TNF-α in diabetic rat?
4. What is the potential effect of VPSBE on glycaemic status, weight gain and food intake after the diabetic rats was fed with VPSBE?

1.6 Research Hypothesis

H_o: the VPSBE might not contain *invitro* phytochemical substances with *invivo* anti-inflammatory activities that can down regulate the expression of inflammatory mediators and up regulate some regulatory cytokines in alloxan induced diabetic rats.

H_1: the VPSBE might contain *invitro* phytochemical substances with *invivo* anti-inflammatory activities that can down regulate the expression of inflammatory mediators and up regulate some regulatory cytokines in alloxan induced diabetic rats

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Diabetes Mellitus

The word diabetes originates from a Greek word "siphon". Aretus the Cappadocian described the condition known as *diabainein* - passing too much water (polyuria). Thomas Willis added mellitus to the term in 1675, drawing reference from the term “Mel” which means "honey" in Latin. Taking cognizance of the fact that the blood and urine of diabetic individuals have excess glucose, DM could literally be taken to mean "siphoning off sweet water". The term "Sweet Urine Disease" was coined when ants were observed to be attracted to some individuals’ urine, because of glucose content in the urine (Maddur, 2012). Diabetes is associated with the destruction of pancreatic β-cells which consequently leads to insulin deficiency resulting in insulin resistance (ADA, 2016).

Diabetes Mellitus (DM) is a metabolic disorder of multiple aetiology characterised by persistent hyperglycaemia, polyuria, polyphagia and polydipsia with alter normal carbohydrate, fat and protein metabolism due to defects in insulin secretion, insulin action or both (ADA, 2016; IDF, 2016). DM is the most common non-communicable disease (NCD) and life threatening diseases Worldwide and it severe forms, it may lead to ketoacidosis or non-ketotic hyperosmolar state leading to stupor, coma, and if not effectively managed may be a sentence to death (Diabetes, 2015). The effects of diabetes mellitus include long term damage, dysfunction and failure of some organs. Thus long term effects of diabetes include progression into complications such as retinopathy with potential of causing blindness, nephropathy which may lead to kidney failure, and/or neuropathy with the risk of foot ulceration, limb amputation, and sexual dysfunction among others (ADA, 2016)

2.1.1 Prevalence of Diabetes Mellitus

The prevalence of diabetes for all age groups worldwide was estimated at 2.8% in the year 2000. This was projected to reach 4.4% of the World population by the year 2030 (Wild *et al.*, 2004). The prevalence is higher for adults (aged 20-79 years), with an estimate of 415 million in 2015 (Diabetes, 2015) and projection of 439 million adults (7.7%) by 2030 (Shaw *et al.*, 2010) and 642 million, representing 10% of global adult population by 2040 (Diabetes, 2015). The prevalence varies greatly between communities and ages. In Britain, about 4 million people representing 6.25% of the total population have diabetes (Diabetes, 2015) in U.S.A 29.1 million people (9.3%) of the population have diabetes (CDC, 2016).

The prevalence of diabetes in developing countries is increasing at an alarming rate. It is estimated that between 2010 and 2030, there will be a 69% increase in number of adults with diabetes in developing countries as compared to a 20% estimated increase in developed countries (Shaw *et al.,* 2010). The sudden rise in the diabetes population in African communities and other developing nations of the World is attributed to ageing of the population and life style changes associated with urbanization, increased physical inactivity, changes in dietary habits and increasing obesity (Sobngwi *et al.,* 2001). In the traditional rural Africa communities, the prevalence is still low (about 2%), except in some specific high risk groups, where the prevalence rate is as high as 13% (Sobngwi *et al.,* 2001). By and large the prevalence of diabetes has skyrocketed from barely 4 million diabetic populations in 1980, representing 3.1% to 25 million Africans in 2014, representing 7.1% (WHO, 2016). It is estimated that the prevalence of diabetes in Africa will triple in the next two decades. The African region is expected to have the largest proportional increase in adult diabetes numbers by 2030, although North America will continue to have the world's highest prevalence (Shaw *et al.,* 2010). The situation is more disturbing if one considers the fact that the urban

population in developing countries is projected to double in the next two decades (Wild *et al.*, 2004).

The incidence of diabetes in Nigeria increased over the years, with the prevalence put at between 3.9 and 4.7% in 2010 and projected to reach 4.3-5.5% in 2030 (Shaw *et al.*, 2010). With an adult (age 30-70 years) population of 27.7% (WHO, 2014), it is estimated that over 5.3 million Nigerian adults will have diabetes in 2030 (Shaw *et al.*, 2010). The must worrying aspect is the raising prevalence of diabetes mellitus in rural communities of Nigeria (Isara and Okundia, 2015).

2.1.2 Health Burden of Diabetes Mellitus

Non-communicable diseases (NCDs) such as diabetes are the leading causes of death globally, killing more people each year than all other causes put together. Ironically, nearly 80% of NCD deaths occur in low- and middle-income countries contrary to the population opinion (WHO, 2010). It is pertinent to note, however, that despite their rapid growth and inequitable distribution, much of the human and social impact caused each year by NCD-related deaths could be averted through well understood, cost effective and feasible interventions (WHO, 2010).

In 2008 for example, out of 57 million deaths that occurred globally, about two third were due to NCDs, comprising mainly cardiovascular diseases, cancers, diabetes and chronic lung diseases (WHO, 2010). The combined burden of these diseases is rising fastest among lower-income countries including Nigeria between the year 2000 and 2014. Of the 24% proportional mortality due to Non Communicable Diseases, 8.33% (i.e. 2% of all death) were attributable to diabetes mellitus (WHO, 2014).

2.1.3 Classification of Diabetes Mellitus

Diabetes is classified based on number of criteria set by different bodies, but the most widely acceptable classification is that of World Health Organization (Ahmad, 2002; Jeon and Murray, 2008). This classification, which is based on clinical descriptive criteria, includes a number of clinical cases and two increase-risk categories (designated as statistical risk classes) (Kerner and Bruckel, 2014). Based on this classification, clinical types of diabetes mellitus are of four (4) main types: insulin dependent diabetes mellitus (IDDM), non- insulin dependent diabetes mellitus (NIDDM). This classification is informed by the aetiology, clinical profile, natural history of diabetes mellitus and its response to insulin therapy (Jeon and Murray, 2008)

2.2 Type 1 Diabetes Mellitus

Type 1 diabetes (T1D) is an autoimmune disease marked by infiltration of the pancreatic Islets by Islet-autoreactive $CD4^+$ and $CD8^+$ T cells, which mediate the selective destruction of the insulin-producing beta cells. Clinical onset of T1D in man represents the peak of a prodromal period of chronic beta cell autoimmunity, inflammation, destruction and antigen spreading. Clinical onset of disease takes place when the majority of beta cells have been destroyed and remaining beta cells are no longer able to produce sufficient insulin for maintaining glucose homeostasis. One or more circulating autoantibodies can be detected in serum months to years prior to the clinical onset of T1D. However, it is the Islet autoreactive T cells, rather than Islet cell autoantibodies, which appear to mediate beta cell destruction. Autoimmune B lymphocytes may, however, play a critical role as antigen presenting cells fueling a chronic autoimmune response, due to their ability to take up and concentrate minute amounts of autoantigen from disappearing beta cells through antigen specific membrane bound immunoglobulin. Furthermore, a growing body of evidence implicates a role for beta cells themselves in aiding their own destruction (Baekkeskov *et al.*, 2017). In particular, cytokine or

inflammation-induced endoplasmic reticulum (ER) stress may play a pivotal role in both beta cell apoptosis and initiation of autoimmunity (Baekkeskov *et al.*, 2017).

The auto-immune response of the Islet β-cells usually occurs in persons with certain susceptibility alleles who lack other protective alleles of the Major Histocompatibility Complex (MHC) genes or the Human Leukocyte Antigens (HLA). This leads to the emergence of the islet β-cell auto-reactive T cells, with the resultant destruction of the β-cells (Lampeter *et al.,* 1993; 1992; Horwitz *et al.,* 2002). The T-cells drive the destruction of the autoimmunity of β-cells, supported in the mouse models by a reduction of the CD40 or CD60 populations which prevent the type 1 diabetes, while replacing the population that reverses the disease (Yoon and Jun, 2001). The T-cells produced in the local lymph node infiltrate the pancreatic Islets and destroy the β-cell cytokines (CD8T and natural killer cells) or the pro-inflammatory cytokines (CD4T) (van Belle *et al*., 2011). The lymphocytes produced by antibodies are known to enhance the damage of β-cells, but the mode of mechanism of action is under debate (Pescovitz *et al.,* 2009). Endogenous proteins (proinsulin, glutamate acid decarboxylase, protein-tyrosine phosphatase-like protein and the zinc transporter) are attacked by the auto-antibodies produced in the plasma cells that contribute to the development of type 1 diabetes (Wenzlau *et al.,* 2007). However, apart from the B-cells and T-cells, some other immune cells are involved in the development of the type 1 diabetes, such as, the natural killer cells, dendrites and macrophages. The elimination of natural killer cells in mice models prevents diabetes symptoms although the mode of action is poorly understood (Cardell, 2006).

Environmental impact in the development of the disease has given rise to the “hygiene hypothesis”: the poor management of western lifestyle diseases led to hyper-immune defenses, which develop into auto-reactivity. This hypothesis indicates that a range of infections poorly

managed develop into diseases due to hyperactivity of the immune response (van Belle *et al.*, 2011). Viral infections contribute to the development of type 1 diabetes, through the auto reactivity of T-cells (Antovorskov *et al.*, 2012). Diet plays a crucial role in the development of type 1 diabetes. A gluten-rich diet and milk albumin are known to contribute to the development of immune imbalance side effects. The relationship between gluten and type 1 diabetes is supported by the frequent development of celiac diseases in type 1 diabetes patients (Antovorskov *et al.*, 2012).

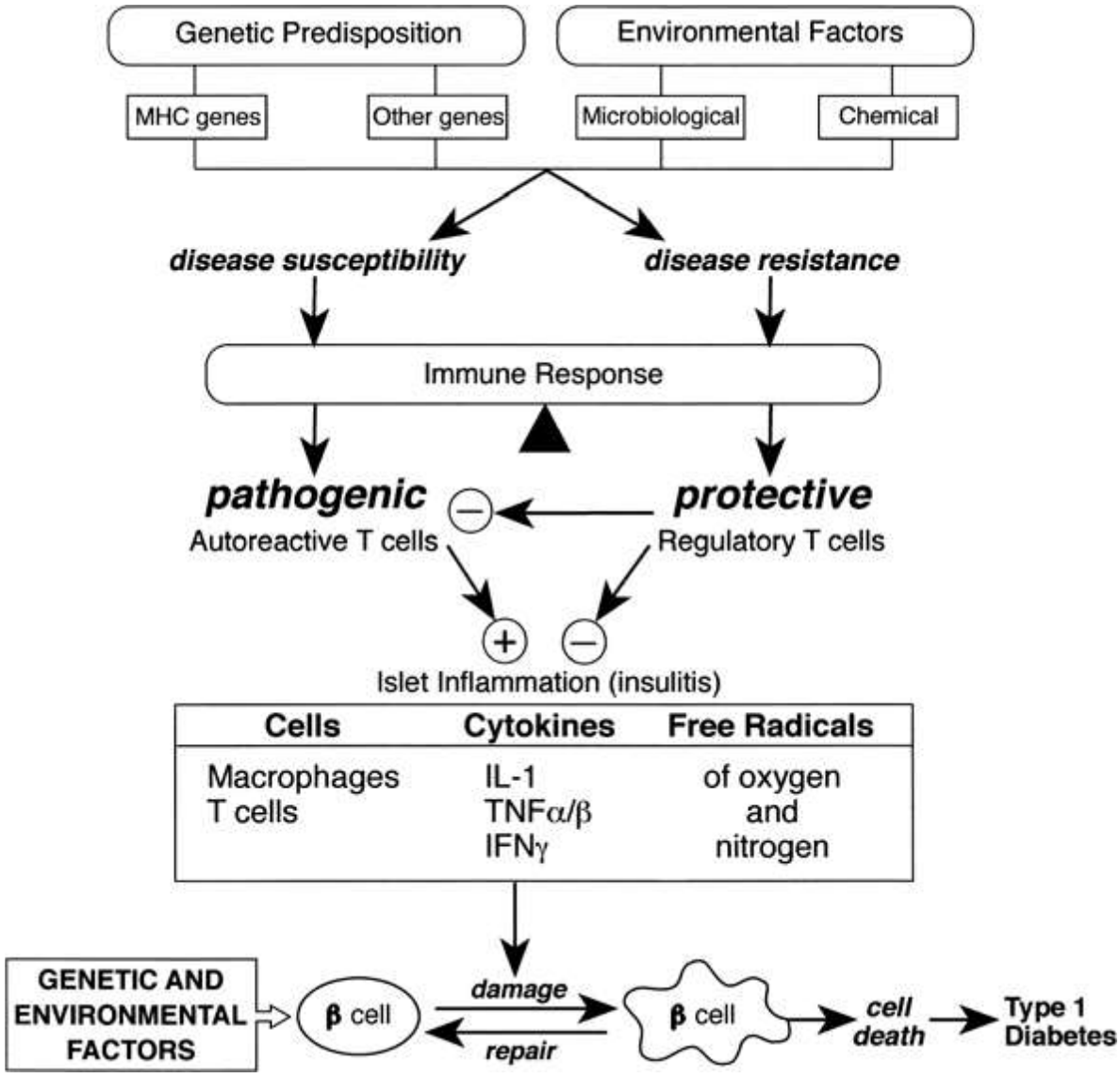

Figure 2.1: Mechanism of Type 1 Diabetes (Adopted from Bolajoko *et al.*, 2017)

2.2.1 Pathogenesis of Type 1 Diabetes (T1D)

Several mechanisms may contribute to β cell destruction, including inflammation mediated by CD4+ T H1 cells reactive with islet antigens (including insulin), CTL-mediated lysis of Islet cells, local production of cytokines (TNF and IL-1) that damage islet cells, and autoantibodies against islet cells (Goodnow, 2007). Cases in which the pancreatic lesions (insulitis) have been examined at the early active stages of the disease, the islets show cellular necrosis and lymphocytic infiltration consisting of both CD4+ and CD8+ T cells (Maddur *et al.,* 2012). Autoantibodies against islet cells and insulin are also detected in the blood of these patients. In susceptible children who have not developed diabetes, the presence of antibodies against Islet cells is predictive of the development of type 1 diabetes. An informative animal model of the disease is the non-obese diabetic (NOD) mouse, which develops spontaneous diabetes, in this model; there is evidence for defective survival and function of regulatory T cells as well as resistance of effector T cells to suppression (Nagata *et al.,* 2010).

A great deal of attention has been devoted to the role of HLA genes in the pathogenesis of T1D. Between 90% and 95% of Caucasians with type 1 diabetes has HLA-DR3, or DR4, in contrast to about 40% of normal subjects, and 40% to 50% of patients are DR3/DR4 heterozygotes, in contrast to 5% of normal subjects. Several non-HLA genes also contribute to the disease. The first of these to be identified is insulin, with tandem repeats in the promoter region being associated with disease susceptibility. The mechanism of this association is unknown; it may be related to the level of expression of insulin in the thymus, which determines whether insulin-specific T cells will be deleted (negatively selected) during maturation. Several other polymorphisms have been identified in patients and in NOD mice, including in the *IL2* and *CD25* genes. The functional consequences of these polymorphisms are not known. Some studies have suggested that viral infections (e.g., with coxsackievirus

B4) may precede the onset of type 1 diabetes, perhaps by initiating cell injury, inducing inflammation and the expression of costimulators, and triggering an autoimmune response (Goodnow, 2007). However, epidemiologic data suggest that repeated infections protect against type 1 diabetes, and this is similar to the NOD model. In fact, it has been postulated that one reason for the increased incidence of type 1 diabetes in developed countries is the control of infectious diseases.

2.2.1.1 Islet Autoantigen in the Pathogenesis of T1D

The identification of Islet autoantibodies has important implications in the diagnosis and prediction of T1D. Autoantibodies directed against islet autoantigens such as insulin, glutamic acid decarboxylase 65 (GAD 65), islet antigen-2 (IA-2) and Zinc transporter 8 (ZnT8) have been demonstrated to be markers of the islet autoimmunity that precede clinical onset of T1D (Long *et al.,* 2012)

(A) Insulin

Insulin is a critical autoantigen specifically expressed on the β-islet cells, which is perceived as the target antigen to cause autoimmune diabetes for a long time (Li *et al.,* 2014). It has been reported that insulin peptide A:1-12 and B:9-23 might be essential targets of the immune destruction for human and non-obese diabetic (NOD) mouse respectively (Odumosu *et al.,* 2011). Studies of multiple countries have reported that insulin autoantibody (IAA) takes an important role in diabetes prediction (Li *et al.,* 2014). In man, IAA was frequently present as early as 9 months of age (Li *et al.,* 2014). Non-obese diabetic mice had high levels of IAA at 8 weeks of age, which strongly correlated with early development of diabetes, and, in a similar manner, children persistently expressing IAA early in life progressed to diabetes much earlier (Li *et al,* 2014). In addition, recent experiments have shown that mucosal administration of

insulin or gene disruptions of insulin prevent the onset of diabetes in the NOD model of diabetes (Harrison *et al.,* 2013).

(B) GAD

The enzyme GAD is of great importance for the neurotransmission in the central nervous system and for treatment of pain and neuro-logical disease, which is also released in pancreas (Li *et al,* 2014). GAD exists in two isoforms, GAD-65 and GAD-67, which are the products of two different genes and differ substantially only at their N-terminal regions (Li *et al,* 2014). Only GAD65 is expressed in the β-cells of human islets, the autoantibody response is primarily to this isoform, and GAD67 antibodies add little to the detection of T1D (Li *et al,* 2014). Autoantibodies to GAD65 are observed months to years before the clinical onset of diabetes and are present in the sera of 70–80% of patients with T1D (Wang *et al.,* 2012). A few earlier reports indicate that treatment using GAD 65 formulated with aluminium hydroxide (GAD alum) have significant beneficial effects on T1D, however, in the latest trials, treatment with GAD-alum did not significantly improve clinical outcome (Boettler *et al.,* 2013)

(C) IA-2

IA-2 and its paralog, IA-2 b, are major autoantigen found after GAD in T1D, which are transmembrane protein-tyrosine phosphatase-like proteins belonging to an evolutionarily conserved family (Li *et al.,* 2014). IA-2 b is similar in many respects to IA-2, especially in its intracellular domain, which is 74% identical to IA-2 (Li *et al,* 2014). IA-2-deficient ($IA\text{-}2^{-/-}$) mice showed impaired insulin secretion after intraperitoneal injection of glucose as well as elevated glucose level in a glucose tolerance test (Li *et al.,* 2014). It is estimated that about 65% (range 55 - 75%) of newly diagnosed type 1 diabetic patients have autoantibodies to IA-2 and between 35% and 50% of type 1 diabetic patient have autoantibodies to IA-2 b (Li *et al.,*

2014). In particular, novel autoantibodies, such as those against the initial 277 amino acid residues of extracellular domain of the neuroendocrine antigen IA-2, had a predictive rate of 100% in a 10-year follow-up (Khadra *et al.,* 2011).

(d) ZnT8

ZnT8 is an islet β-cell secretory granule membrane protein recently identified as an autoantibody antigen in T1D (Skarstrand *et al.,* 2013), which is highly β-cell specific unlike GAD and IA-2. ZnT8 contains six transmembrane domains and a histidine-rich loop between transmembrane domains IV and V, like the other ZnT proteins (Li *et al.,* 2014). A high ranking candidate, the ZnT8 was targeted by autoantibodies in 60–80% of new-onset T1D compared with <2% of controls (Li *et al,* 2014). ZnT8107–115, ZnT8115–123 and ZnT8145–153 derived from ZnT8 might be capable of inducing specific CTLs and played a vital role in T1D (Li *et al.,* 2013) Vaziri *et al*. have further reported that the assay of ZnT8-TripleA would be more suitable to analyze patients with newly diagnosed diabetes as this assay demonstrated high sensitivity and very high specificity (Vaziri-Sani *et al.,* 2011). Studies in humans have shown that reagents that target ZnT8-specific T cells could have therapeutic potential in preventing or arresting the progression of this disease (Long *et al.,* 2012). Unlike GAD and IA2, ZnT8 is highly β-cell specific, and thus, ZnT8 antibodies measurements may be useful in monitoring islet destruction after onset and in evaluating therapeutic interventions that limit β-cell-specific auto reactivity or restore β-cell mass (Li *et al,* 2014)

2.2.1.2 $CD4^+$ T-Lymphocytes in the Pathogenesis of T1D

CD4+ T lymphocytes are mainly involved in cellular immune response, and play important roles in the activation and proliferation of CD8+ T lymphocytes and B cells. Human CD4+

cells from the pancreatic lymph nodes of patients with T1D respond to the first 15 amino acids of the insulin A-chain (Li *et al*, 2014). Insulin-autoreactive CD4+ T cells have also been described in T1D patients, and there is evidence suggesting that high-avidity insulin-reactive thymocytes may evade central tolerance in such patients (Santamaria, 2010). CD4+ T cells can be divided into type 1 T helper (Th1), Th2, Th17, regulatory T cells (Tregs) and so on according to their secretion of cytokines. Recently, there have been considerable insights into the effects of Tregs in the pathogenesis of T1D, which have evoked great interest. It is essential to understand more clearly the role of each CD4+ T cell subset in the protection or exacerbation of various pathologies in T1D. Thus:

(A)Th1 CELLS

Th1 cells are responsible for cell-mediated immunity and phagocyte-dependent protective responses, which can also destroy the islet β- cells and accelerate the course of T1D via production of interferon (IFN)-γ and interleukin (IL)-2 However, the role of these cytokines in the pathogenesis of T1D is complex. For example, IFN-γ plays a dual role in destruction of β-cells via the signal transducer and activator of transcription-1 (STAT-1) pathway and in protection via the interferon regulatory factor-1 (IRF-1) pathway (Singh *et al.,* 2011). And IL-2 may have therapeutic efficacy in T1D by promoting the survival and function of Tregs.

(B) Th2 CELLS

Th2 cells mainly produce IL-4 and IL-10, which are responsible for strong antibody production, eosinophil activation and inhibition of several macrophage functions (Lin *et al.,* 2011). Immunotherapeutic approaches like anti-CD28 stimulation, which promote and enhance the function of intra islet Th2 cells and secretion of IL-4 by these cells can effectively

prevent the onset of T1D (Li *et al.*, 2014). Transgenic NOD mice expressing IL-4 in the pancreatic islets are protected from the development of diabetes (Li *et al.*, 2014). The onset of hyperglycaemia in NOD mice has reduced after regulated delivery of IL-4 to pancreatic β-cells in vivo using an adenoassociated vector expressing IL-4 under the control of the mouse insulin promoter (Li *et al.*, 2014). Similarly, IL-10 is an immunoregulatory cytokine that has multifunctional effects. Several lines of evidence suggested that IL-10 was important in establishing immune tolerance in NOD mice. There is now widespread recognition that Th1 cells regulate cellular immunity, whereas Th2 cells mediate humoral immunity and allergic responses (Wang *et al.*, 2015). Th1 cytokines are generally believed to exacerbate, while Th2 cytokines protect from, T1D.

(C)Th17 CELLS

Th17 are a subset of T helper cells producing IL-17, which are distinct from Th1 and Th2 cells. Th17 play a key role in a variety of infectious diseases, cancer occurrence and many autoimmune diseases, such as T1D, rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus (Li *et al.*, 2014). One report demonstrated that in T1D, Th17 might induce local inflammation, which in turn might hasten the development of diabetic complications (Ryba-Stanislawowska *et al.*, 2013). Increasing evidence has shown that therapeutic agents targeting the IL-17 molecule or directly inhibiting IL-17-producing cells regulate autoimmune diabetes, suggesting that IL-17 is involved in the pathogenesis of T1D (Lee *et al.*, 2013). Increased production of IL-17 by peripheral blood T cells has furthermore been detected in children with T1D (Li *et al.*, 2014). In animal studies, a function for Th17 in T1D is supported by the observation that IL-17 is expressed in pancreas of NOD mice and that inhibition of IL-17 in this model leads to delayed onset of T1D during the effectors phase of the disease (Li *et al.*,

2014). Meanwhile, transfer of highly purified Th17 cells could cause diabetes in NOD/SCID recipients with similar rates of onset as in transfer of Th1 cells (Li *et al.*, 2014).

(D) T - REGS

Tregs, suppressors of antigen-activated immune responses to self and non-self antigens, were first described in 1975 (Li *et al.,* 2014). Tregs play an indispensable role in maintaining immunological unresponsiveness and in suppressing excessive immune responses through cell contact- dependent mechanisms, by secretion of cytokines such as transforming growth factor (TGF)-β, IL-10 and IL-35 (Petzold *et al.*, 2013). Transforming growth factor-β regulates multiple functions of T cell development, which plays a major role in T effector cells resistance to regulation and Tregs dysfunction (Kawamoto *et al.*, 2012). Furthermore, the autocrine/paracrine TGF-β signalling in diabetogenic CD4+ T cells is essential for the control of T1D development (Ishigame *et al.,* 2013). In addition, IL-10 was believed to be a potent anti-inflammatory cytokine and ablation of IL-10 exacerbates autoimmune diseases. In vitro and in vivo, IL-35 has two well-known biological effects: suppression of the proliferation of T cells and the conversion of naive T cells into a strongly suppressive induced Tregs, which has the capacity to protect β cells from autoimmune attack under certain circumstances (Collison *et al.,* 2012). Adoptive transfer of Tregs has been shown to offer protection from T1D, whereas their experimental depletion or genetic deficiency in their numbers or activity promotes a more aggressive disease (Kawamoto *et al.,* 2012).

2.2.1.3 $CD8^+$ T-Lymphocytes in the Pathogenesis of T1D

$CD8^+$ T cells, which recognize pathogen-derived peptides presented by Major Histocompatibility Complex (MHC) class I molecules, were activated to proliferate and

differentiate into cytotoxic T cell (CTL) and respond to infection by a number of intracellular bacteria (Sutherland *et al.*, 2013). In addition, effective CTL immunity is associated with long-term protection against chronic or subsequent exposure to the virus or tumour, through the stable induction of antigen-specific $CD8^+$ T cell memory (Ghazarian *et al.*, 2013). Previous studies have generally considered that both $CD4^+$ and $CD8^+$ T cells are involved in the pathogenesis of T1D and are thus capable of inducing β-cell death. However, pancreatic β cells express MHC class I, but lack MHC class II proteins, suggesting that direct cytotoxicity can only be mediated by $CD8^+$ CTL that recognize peptide antigen: MHC class I complexes displayed on β-cells (Bulek *et al.*, 2012). For example, under histopathological examination $CD8^+$ T cells were indeed found in the 'insulitis' of patients who died at onset of T1D, or in islets of monozygotic twins with recurrent T1D, after segmental pancreas transplantation from their non-diabetic co-twin (Fierabracci, 2011). Moreover, NOD mice deficient in MHC class I or MHC class I associated-β 2-microglobulin are protected from both insulitis and T1D, demonstrating that MHC class I presentation to CTL is necessary for disease initiation and progression to T1D (Li *et al.*, 2014). Other studies have shown that IL-21 was required for efficient initial activation of autoreactive $CD8^+$ T cells, which could rapidly kill β cells and therefore contribute to the development of T1D (Chen, *et al.*, 2013). Key factors that can lead to β-cell death are cytotoxic $CD8^+$ lymphocytes secreting perforin, direct action of cytokines such as IFN-γ, TNF-α and IL-1b, Fas–Fas-L interactions and nitric oxide synthesis (Li *et al.*, 2014). Also noteworthy is the fact that, a population of $CD8^+$ T cells recognizing an insulin-derived epitope (B: 15–23) appears in the islets of NOD mice as early as 3 weeks of age (Santamaria, 2010). The size of this population declines quickly with age and is replaced by other specificities, which targets a peptide from islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP206–214) and are highly diabetogenic (Santamaria, 2010).

Furthermore, in the majority of T1D patients tested, there was a specific defect in CD8+ T cell recognition of HLA-E/Hsp60sp, which was associated with failure of self/non-self discrimination (Jiang *et al.,* 2010). A failure of T-suppressor $CD8^{+}CD28^{-}$ T cell population was recognized in T1D (Li *et al.,* 2014).

2.2.1.4 B-Lymphocytes in the Pathogenesis of T1D

B lymphocytes and their products are not directly pathogenic to β cells, emerging evidence has revealed that they could promote autoimmunity by several mechanisms including: production of autoantibodies with consequent generation of immune complexes, antigen presentation to generate primary autoreactive T cell responses, contribution to the maintenance of $CD4^{+}$T cell memory or production of pro-inflammatory cytokines (Li *et al.*, 2014). Many studies have shown that autoantibodies are present in pre-diabetic and newly diagnosed patients with diabetes (Hampe, 2012). These include antibodies to proteins such as insulin, GAD, islet-cell antibodies, IAA, IA-2 and IA-2 β, which are also good markers for disease progression (Li *et al.,* 2014). Moreover, a recent study demonstrated the necessity for B cells in the islets to promote survival of activated $CD8^{+}$ T cells at the CTL transition stage, thereby accelerating disease progression (Li *et al.,* 2014). On the other hand, B cells are crucial antigen-presenting cells in the initiation of T cell autoimmunity to islet β -cell autoantigens in T1D, although they do not present antigens as efficiently as dendritic cells. Migration of B cells into pancreatic lymph nodes in NOD mice is mediated predominantly by a $\alpha_4\beta_7$ integrin/mucosal addressin cell adhesion molecule 1 pathway and partially by L-selectin/peripheral node addressin pathway and leucocyte adhesive protein-1 (Xu *et al.,* 2010). Furthermore, B cells could play other roles such as promoting normal lymphoid architecture and follicular dendritic cell formation (Li *et al.,* 2014). Chronic depletion of B cells abrogates the destructive mononuclear cell infiltration of the pancreatic islets. B-cells depletion also exerted a similar protective effect

and completely abrogated the development of insulitis in NOD mice (Li *et al.*, 2014). Hu et al. recently demonstrated that combined treatment with intravenous anti-human CD20 and oral anti-CD3 reversed diabetes in >60% of mice newly diagnosed with diabetes, providing important pre-clinical evidence for the optimization of B cell-directed therapy for T1D (Hu *et al.*, 2013). In human, it has been reported that T1D developed in the absence of B cells, as seen in a patient who had X-linked agammaglobulinemia. This individual had very low serum levels of all classes of immunoglobulin and markedly decreased numbers of B cells in peripheral blood, but still developed T1D (Li *et al.*, 2014). Taken together, B cells play an important role in disease development, especially in the animal models of T1D. Although it seems that B cells are not indispensable in human T1D, we would predict that B cells might assist the development of the T1D in other ways.

2.2.2 Prevention Trials and Therapy for Type 1 Diabetes Mellitus

Patients with type 1 diabetes can have a near-normal life relying on the administration of exogenous insulin by daily injections, continual pump therapy, or islet transplantations. Through these methods, diabetic patients can optimize the glycemic control and decrease the incidence of diabetes complications. In order to optimize the treatment for this disease, large investigations have been designed and conducted to evaluate prevention trials (Wu e*t al.*, 2013) and therapy for type 1 diabetes. Based on current concept of the disease, it seems to be possible to delay or prevent type 1 diabetes

2.2.2.1 Primary Prevention Trials

Primary prevention is treatment in infants with increased genetic risk. These studies include several dietary manipulations, such as free of cow's milk, delayed exposure of gluten-containing foods, and vitamin D supplementation. To date, all primary prevention trials have been dietary interventions designed to interrupt putative environmental factors of type 1

diabetes. Because none of the specific dietary factors has been shown to be an unequivocal risk factor for type 1 diabetes, and their effects have still been contradictory, it is difficult to evaluate the efficiency of the primary interventions (Knip *et al.,* 2010; Wu e*t al.,* 2013).

2.2.2.2 Secondary Prevention Trials

Secondary prevention is targeted at individuals with persistent islet autoantibodies. These prevention trials involve the use of nicotinamide or antigen-specific therapies. In animal studies nicotinamide has been shown to increase insulin synthesis and inhibit the development of diabetes if administered prior to the onset of disease (Wu e*t al.,* 2013). The results obtained from participants either to nicotinamide or placebo groups showed that the rates of type 1 diabetes development were essentially the same (Skyler, 2013).

Antigen-specific therapy is based on the concept that the appropriate administration of a diabetes autoantigens controls the autoimmune response. This kind of therapy, according to idea, changes immune system to a protective rather than destructive response (Wu e*t al.,* 2013). Antigens used in this trial were parenteral insulin, oral and nasal insulin or intradermal administration of proinsulin peptides, and a vaccine with GAD.

In the case of parenteral insulin, the intervention included low-dose subcutaneous ultravalente insulin twice every day with a total dose of 0.25 units/kg bodyweight per day (Szablewski, 2014). The results failed to demonstrate the delay or prevention in type 1 diabetes. As only one dose of insulin was tested, it was impossible to evaluate the effect of insulin in the protection of the β-cells. The study with subjects positive for anti-insulin autoantibodies that received oral insulin demonstrated that it was no delay in the clinical onset of type 1 diabetes as compared to participants treated with placebo (Szablewski, 2014). The trial showed that the nasal insulin had no effect on the protection of the disease (Nonto-Salonen *et al.,* 2008). A pilot safety study with a single proinsulin peptide administration has been performed in

patients with established type 1 diabetes (Thrower *et al.,* 2009) and recently, several trials with various proinsulin peptides are underway (Wu e*t al.,* 2013). Whether a vaccine using glutamic acid decarboxylase can promote the prevention of type 1 diabetes is being assessed by the Diabetes Prevention-Immune Tolerance Study in Southern Sweden (Skyler, 2013). It is to note that a French pilot trial indicated that immunosuppression with low-dose cyclosporine may delay the development of type 1 diabetes (Eisenbarth, 2010).

2.2.2.3 Pancreatic B-cell Regeneration

The key of treating type 1 diabetes is to replenish the lost β-cells. During the last two decades, much effort has been made into exploring β-cell regeneration. It is commonly accepted that the total number of pancreatic islets remains constant in the life time while the size of islet increases with age. Moreover, pancreatic β-cells which have longer life-span do not undergo proliferation frequently (Desgraz and Herrera, 2009). On the other hand, many preclinical studies revealed that given proper stimulation some β-cells may regain the potential to proliferate (Wu *et al.,* 2013); however, only a small group of specialized β-cells will regain the potential to proliferate under certain conditions (Smukler *et al.,* 2011)

Two categories of factors or hormones have been used to promote β-cell regeneration. The first category includes factors involved in the development and differentiation of pancreatic β-cells such as cyclin D1/D2, cyclin-dependent kinase 4 (CDK4) (Szablewski, 2014), glucagon-like peptide-1 (GLP1), neurogenin 3 (Ngn3) (Van de casteele *et al.,* 2013) or pancreatic duodenal homeobox-1 (Pdx1) (Szablewski, 2014). From mentioned factors, CDK4, GLP-1 and Ngn3were demonstrated to be effective to promote β-cell regeneration (Van de casteele *et al.,* 2013; Lee *et al.,* 2013). As a second category there are mitogenic factors: vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF). These factors seemed

to work through combination with the native mechanism of β-cell regeneration in a synergistic or additive manner (Wu *et al.,* 2013). A hormone called betatrophin, produced by liver and fat, was identified (Yi *et al.,* 2013). According to author's observations, this hormone worked specifically on pancreatic β-cells to promote replication. The injection of betatrophin led to a 17-fold increase in β-cell proliferation in comparison to the control (Yi *et al.,* 2013).

2.2.2.4 Stem Cell Transplantation Therapy

Stem cells with proliferation capacity and transdifferentiation potential are being explored as a promising new treatment for tissue regeneration, although their immunomodulatory properties are not yet completely understood. These cells were used to repair bone defect (Grayson *et al.,* 2010) and heart infarct (Szablewski, 2014) and can be used to promote β-cell regeneration (Ezquer *et al.,* 2008). Low immunogenic potential of mesenchymal stem cells (MSCs) together with their effects on immune response makes them a promising therapeutic tool for severe refractory immune diseases, such as type 1 diabetes (Vija *et al.,* 2009). Stem cells therapy, based on the generation of insulin-producing cells derived from mesenchymal stem cells, represents an attractive possibility. As shown by in vitro experiments,

MSCs can modulate the immunological activity of different cells. The most important being their inhibitory effect on T cell proliferation and dendritic cell differentiation key factors for activating autoimmune disorders. These cells inhibit proliferation of CD4, CD8, memory and naïve T cells (Szablewski, 2014), and can also stimulate the production $CD8^+$ Treg which inhibits lymphocyte proliferation in allogenic transplants (Szablewski, 2014). It is important that MSCs can be differentiated into insulin-producing cells by using a specific culture medium. Insulin-producing cells, obtained From MSCs, express gene related to pancreatic development and function, such as insulin I and II, GLUT 2, glucose kinase, Islet amyloid polypeptide, and pancreatic duodenal homeobox. These cells synthesize C peptide and insulin

(Szablewski, 2014). As a source of insulin producing cells there are bone marrow MSCs (BM-MSCs), adipose-derived MSCs (ADSCs), umbilical cord blood MSCs (UCBhMSCs), pancreatic MSCs (Vija *et al.,* 2009), and amniotic stem cells (Liu *et al.,* 2013). Several studies have reported on the treatment of newly diagnosed type 1 diabetes using stem cell transplantation. For example, newly diagnosed type 1 diabetic patients treated with autologous hematopoietic stem cell transplantations achieved good glycemic control (Zhang *et al.,* 2012). The majority of children with Fanconi anemia had normal glucose tolerance and normal β-cell function after hematopoietic cell transplantation (Polgreen *et al.,* 2009). Patients with insulinopenic diabetes showed a 30–50% decrease in insulin requirements after human adipose-derived MSCs transplantation (Trividi *et al.,* 2008). Stem cell therapy which is still under preclinical studies has faced more practical challenges. Stem cell therapy has demonstrated its potential in many reports, as a potent immunosuppressant and trophicmediator. These results suggest the use of MSCs in clinical practice as a new therapeutic option to treat type 1 diabetes (Trividi *et al.*, 2008; Liu *et al.,* 2013).

2.2.2.5 Pancreatic or Islet Transplantation

β -cell replacement therapies represent a therapeutic alternative for insulin injections with careful blood glucose monitoring (Szablewski, 2014). Human pancreatic transplantation has demonstrated partially successful results in reversing long-term renal and neural complications in selected type 1diabetic patients. This therapy also offers glycemic control and sufficient prevention of hypoglycemia without the need for exogenous insulin administration (Szablewski, 2014).

2.2.3 New Therapies for Type 1 Diabetes

The most interesting new therapeutic strategies for type 1 diabetes are focused on inducing tolerance with diabetogenic peptides from islet antigens (such as insulin) or generating or

giving regulatory T cells to patients. These clinical trials are in their infancy (Shwab and Nimmerjahn, 2013).

2.3 Type 2 Diabetes Mellitus

Type 2 diabetes, also known as non-insulin dependent diabetes mellitus is common in adults. It accounts for about 90 % of all the diabetic complications. It is usually characterized by progressive decline in β-cell function or impaired pancreatic insulin secretion and chronic insulin resistance or impaired insulin action, which is associated with impaired fibrosis, impaired insulin signaling, and inflammation (Umar *et al.,* 2017). Studies have revealed that abnormalities in the key molecules of the insulin-signaling pathways may result in insulin resistance and also enhances the over-expression of phosphatases and activation of protein kinase cascade (Avramoglu *et al.,* 2006). These actions initiate abnormalities in the expression and action of various peptides, cytokines, growth factors, and also result in the over-production of very low-density lipoproteins (VLDL) (Fonseca *et al.,* 2004). More so, insulin resistance may be responsible for the over-production of pro-inflammatory cytokines such as C-reactive protein (CRP), tumor necrosis factor (TNF), interleukin-6 (IL-6) and a relative deficiency of anti-inflammatory cytokines such as adipokines, which arise from adipose tissues due to obesity (Eckel *et al.,* 2005). Adipokines initiate the production of reactive oxygen species (ROS), leading to a process known as oxidative stress.

The pathology is mainly Insulin resistance in peripheral tissues with associated Insulin secretory defect of beta cells. There is Strong genetic predisposition in this case. The aetiology is multifactorial – interaction between genetic & environmental factors. Some risk factors for Type 2 DM includes, Genetic factors (Genetic markers, family history, “thrifty” gene), Advancing age, Ethnicity (blacks, Native Americans), Obesity, Physical inactivity, Diet,

Urbanization/modernization, Metabolic determinants (Impaired glucose tolerance, Insulin resistance) (Saidu, 2017).

2.3.1 Factors that potentiate tissue inflammation in T2D

Hyperglycemia, dyslipidemia, and oxidative stress (Akash *et al.,* 2012a) are considered to be directly involved in tissue specific inflammation. Here in following paragraphs, we have tried to describe that how these phenomena can potentiate tissue specific inflammation.

2.3.1.1 Hyperglycaemia

Hyperglycemia refers to the constantly elevated levels of blood glucose that imparts its damaging effects on normal functioning of β-cells finally decreasing insulin secretion. Augmented level of glucose in plasma is a primary motive for pathogenesis of T2DM. High levels of glucose are very toxic to β-cells (Akash *et al.,* 2013); once, it enters into the β-cells of pancreatic islets along FFAs (Dinarello, 2010), it induces the stimulation of various proinflammatory mediators such as IL-1b, TNF-a, IL-6, and various other IL-1 dependent cytokines and chemokines (Akash *et al.,* 2012a). As these pro -inflammatory mediators are provoked, they might cause tissue specific inflammation.

2.3.1.2 Dyslipidaemia

The term dyslipidemia is used when circulating levels of various lipids are changed accordingly in response to insulin resistance and over nutrition. The effect of change in concentration of lipids on β cells depends on specific lipid profile. Some saturated fatty acids (palmitate) may act as pro-apoptotic for β-cells whereas; monosaturated fatty acids (oleates) protect β-cells from harmful effects of saturated fatty acids and glucose (Akash *et al.,* 2013). As the insulin resistance in peripheral tissues increases, the circulating levels of FFAs are also increased (Akash *et al.,* 2013). Once circulating levels of FFAs are augmented, they subsequently migrate to β-cells of pancreatic islets where they cause the disruption of β cells

by inducing the secretion of IL-1b (Akash *et al*, 2013). Like fatty acids, lipoproteins may also exert their effects on the survival and normal functioning of β-cells. Very low density lipoproteins (VLDL) and low density lipoproteins (LDL) are very harmful and behave as pro-apoptotics for β-cells like saturated fatty acids whereas, the role of high density lipoprotein (HDL) is to protect the β-cells from harmful effects of other lipoproteins, saturated fatty acids and glucose (Akash *et al*, 2013).

2.3.1.3 Oxidative Stress

The role of oxidative stress in pathogenesis of T2DM is well recognized. It may cause tissue damage that accompanies chronic hyperglycemia. Several factors induce oxidative stress that may demolish the structural and functional integrity of β-cells of pancreatic islets. Oxidative stress may also potentiate the generation of ROS along with other proinflammatory cytokines and chemokines around the β-cells (Akash *et al.,* 2012a) that disrupts the blood flow into the β-cells and abolishes its function (Ehses *et al.*, 2007). As anti-oxidative enzymes (Cu/Zn superoxide dismutase, Mn superoxide dismutase, catalase, and glutathione Peroxidase) are not sufficiently present in β-cells, these cells are highly vulnerable to oxidative stress (Akash *et al.*, 2012b). In addition to β-cells destruction, oxidative stress may also potentiate inflammation and insulin resistance (Akash *et al.*, 2012a) in peripheral tissues along with generation of ROS in endothelial cells by abolishing their normal functions (Akash *et al*, 2013).

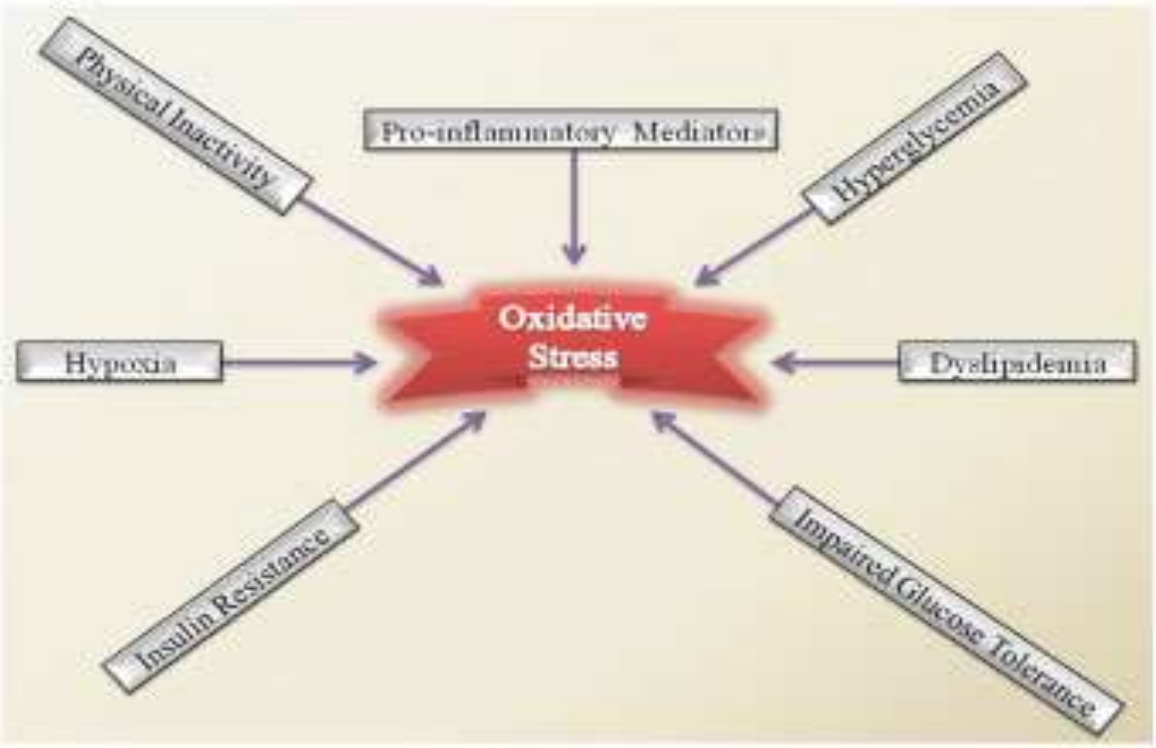

Figure 2.2: Production of oxidative stress in individuals with T2DM (adopted from Akash *et al.*, 2013)

Multiple risk factors are decisively involved to provoke oxidative stress. The optimal factors include pro-inflammatory mediators, insulin resistance, and glucolipotoxicity (hyperglycemia and dyslipidemia) whereas; physical inactivity, impaired glucose tolerance, and hypoxia are account for partial optimal factors. Once oxidative stress is generated, it may cause further insulin resistance and activation of pro-inflammatory mediators that lead to onset of T2DM.

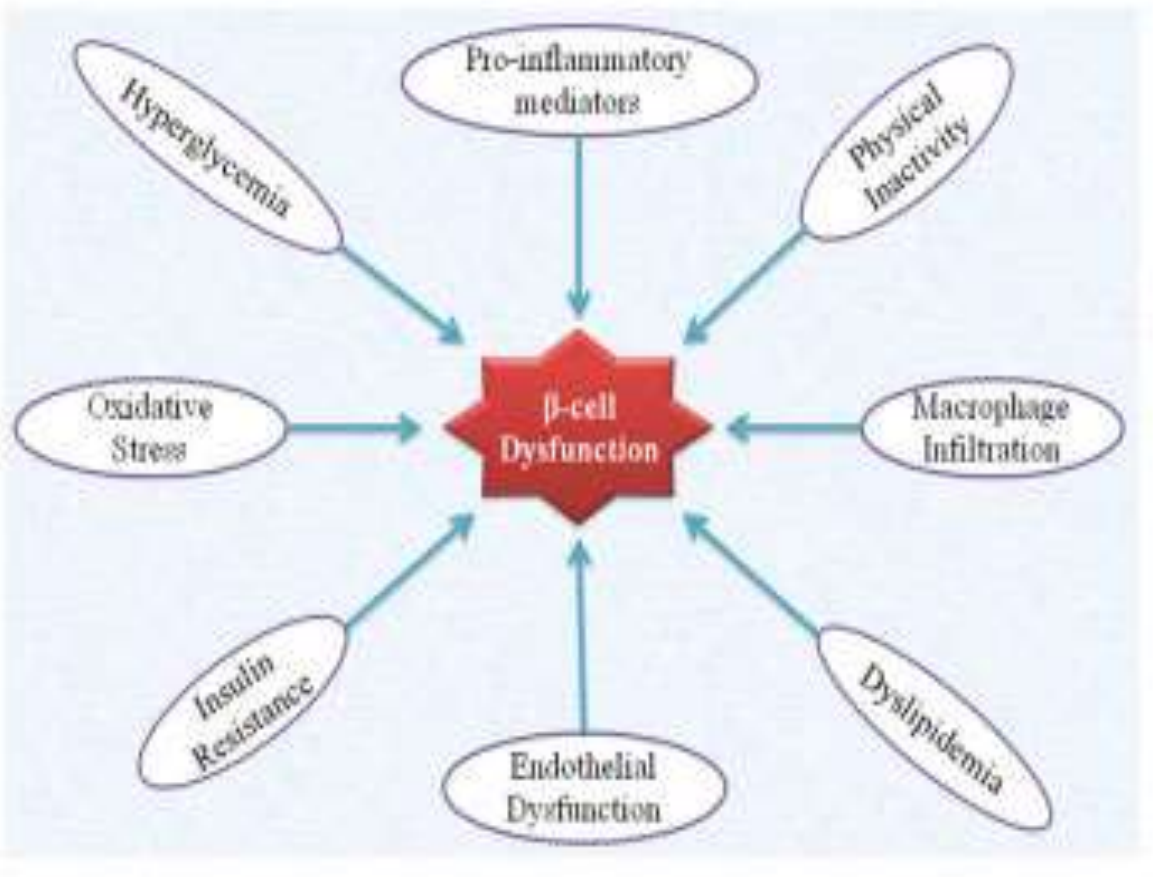

Figure 2.3: Mechanism of β-cell dysfunction. Adopted from Akash *et al.*, 2013)

Multiple risk factors are involved in induction of β-cell dysfunction. These include optimal glucolipotoxicity (hyperglycemia and dyslipidemia), which can impact the development of insulin resistance, oxidative stress and/or endothelial cells dysfunction, as well as the activation of pro-inflammatory mediators and macrophage infiltration. Collectively, these factors may lead to β-cell dysfunction due to which impairment of insulin secretion occurs that may provoke the onset of T2DM.

2.3.2 Mechanisms of Inflammatory Mediators in T2D

Many studies have been conducted in order to develop the relationship between various inflammatory mediators and T2D, and have found abnormally high levels of various cytokines, plasminogen activator inhibitor, chemokines, acute phase proteins (such as CRP) in type 2 diabetic patients (Spranger *et al.,* 2003; Herder *et al.,* 2009) concluding that high circulating levels of IL-1b, IL-6, and CRP can be the main predictive indicators for progression of T2D (Spranger *et al.,* 2003). These high levels of numerous cytokines and CRPs may induce the activation of innate immune system in type 2 diabetic patients due to over nutrition. All compounds that are necessary for normal body functions and development are the main inducer of inflammatory mediators and CRPs. In patients with T2D, augmented circulating levels of various proinflammatory cytokines and chemokines along with overt tissue inflammation have been detected (Ehses *et al.,* 2007). Consequently, one may not predict the degree and extent of inflammation in specific tissue by only observing the circulating levels of these pro-inflammatory mediators. It has been clear from the above-mentioned facts that inflammation plays a crucial role in the dissemination of T2D. Thereby, T2DM may be stated as a chronic form of auto inflammatory disease producing IL-1b from β-cells of pancreatic islets; which eradicates β-cells themselves (Dinarello, 2010) leading to β-cell dysfunction. Following are the potential inflammatory responses that play their pivotal role in inflammatory mechanism for pathogenesis of T2D.

2.3.2.1 Reduced oxygenation

It also known as hypoxia and occurs when oxygen supply is limited. To overcome this, a compensatory phenomenon known as angiogenesis is stimulated through the secretion of numerous angiogenic factors in order to compensate the required amount of oxygen in rapidly growing tissues (e.g., cancerous tissues) (Carmeliet, 2005). Similar type of phenomenon has

also been observed in animal models of obesity (Hosogai *et al.,* 2007; Yin *et al.,* 2009) and in human adipose tissues that may cause tissue dysfunction (Pasarica *et al.*, 2010). Hypoxia may also stimulate the induction of various pro-inflammatory genes in macrophages. Macrophages accumulate at the site of hypoxia and endow a link between rapidly growing adipose tissues and commencement of inflammation (Burke *et al.,* 2003). Moreover, hypoxia has been also known for initiating ER-stress in pancreatic islets most probably by influencing the ER redox potential and decreasing the concentration of ATP which results in β-cell death (Tu and Weissman, 2004).

2.3.2.2 Transcriptional Pathways

There are many metabolic pathways that cause insulin resistance in peripheral tissues. They provoke inflammation and stress-induced kinases such as IkB kinase-b (IKKb) and JUN N-terminal kinase (JNK). These kinases are known to efficiently participate in pathogenesis of diabetes (Shoelson *et al.,* 2006). IKKb may potentiate the activation of nuclear factor-kB (NF-kB), which in turn induces pro-inflammatory cytokines (TNF-α and IL-1b) in liver and adipose tissues. These cytokines result in insulin resistance in peripheral tissues (Arkan *et al.*, 2005). However, JNK potentiates activating transcription factor-2 (ATF2) and ELK1. Although, the role of JNK stimulated transcription factors is not known (Solinas and Karin, 2010) but some experimental studies have provided ample evidences that JNK plays its crucial role in inflammatory responses for pathogenesis of T2DM. TNF-α and IL-1b, which are produced by the activation of NF-kB are also known to stimulate both NF-kB and JNK in response to feed-forward mechanism through the involvement of their particular receptors (Donath and Shoelson, 2011). Other then NF-kB and JNK pathways, FFAs and advanced glycation end-products may promote insulin resistance and overt T2DM by the activation of toll like receptors (TLRs) and receptors for advanced glycation end-products (RAGE) (Shi *et*

al., 2006). These extracellular stimuli bind these cell surface receptors by activating intracellular pathways that unite on both JNK and NF-kB. Activation of these pathways takes place in liver and adipose tissues and upregulates the production of TNF-a, IL-1b, and IL-6 (Sabio *et al.,* 2008). Hyperglycemia and over production of IL-1b in β -cells of pancreatic islets also activate NF-kB. Blockade of NF-kB activation by naturally occurring anti-inflammatory cytokine interleukin-1 receptor antagonist (IL-1Ra) protects β-cells from various deleterious effects (Akash *et al.*, 2012a). Since, these NF-kB and JNK pathways are activated in many tissues and play crucial role in tissue inflammation, blocking the activity of these pathways may stop the prevalence of inflammation.

Figure 2.4: Inflammatory mechanisms that lead to the pathogenesis of T2DM (adopted from Akash *et al.,* 2013)

Hyperglycaemia and dyslipidemia decisively provoke oxidative stress and endoplasmic reticulum stress (ER stress). Oxidative and ER stress may also potentiate the effects of each other. Once oxidative stress is produced, it induces the generation of various proinflammatory mediators. These pro-inflammatory mediators may further cause inflammation in pancreatic islets and peripheral tissues. Due to inflammation, insulin resistance is developed in peripheral tissues. Inflammation in pancreatic islets impairs normal functions of β-cells that ultimately lead to cell death. Hence, T2DM occurs.

2.3.2.3 Cytokines

The most promising part among multifactorial pathophysiology for dissemination of T2DM is played by numerous pro-inflammatory cytokines such as IL-1b, TNF-α, and IL-6. These cytokines released from adipose tissues induce inflammation not only in the corresponding tissue but also in the β-cells of pancreatic islets and ultimately leads to insulin resistance (Ehses *et al*., 2009a).

TNF-α also plays an essential role by creating a linkage among insulin resistance, obesity, and inflammation (Tilg and Moschen, 2008) to be precise, TNF-α has been recognized as a key factor linking inflammation and insulin resistance. It modulates the activities of IKKb/NF-kB and JNK pathways regulating insulin resistance (Tilg and Moschen, 2008). Overproduction of TNF-α in adipose tissues causes insulin resistance in peripheral tissues by the induction of inflammation and β-cell death in pancreatic islets (Rosenvinge *et al.,* 2007).

The role of IL-6 in T2DM is considered to be complex and controversial, however, various experimental studies have confirmed that IL-6 induces insulin resistance in peripheral tissues (Akash *et al.*, 2012a), apoptosis in pancreatic islets together with other inflammatory cytokines (Akash et al., 2012a) and stimulates the inhibition of cytokine's signaling proteins (Pradhan *et al.,* 2001). Due to these deleterious effects, IL-6 is considered as an independent risk factor and acts as predictor and pathogenic marker for insulin resistance and progression of T2DM (Tilg and Moschen, 2008).

2.3.2.4 Chemokines

Adipocytes secrete various chemokines that recruit monocytes and macrophages. It has been demonstrated that the adipose tissues in obese individuals secrete more chemokines as compared to nonobese individuals (Tilg and Moschen, 2008). There are various types of

chemokines such as CCL2 (MCP1), CCL3 (MIP-1a), CCL6, CCL7, CCL8, and CCL9. These chemokines are released from adipose tissues, pancreatic islets (Akash *et al.,* 2012a), and endothelial cells (Ehses *et al.,* 2007). Increased production of these chemokines has also been confirmed in various diabetic animals (Ehses *et al.,* 2009b). Some experimental studies have observed that these chemokines play their role in the pathogenesis of T2DM along with proinflammatory cytokines however, the precise mechanism of these chemokines still remains to be elucidated (Ehses *et al.,* 2009a).

2.3.2.5 Adipocytokines

Leptin and adiponectin are well known as adipocytokines and are usually produced from adipocytes. These adipocytokines exert their immunomodulatory effects in T2DM. Due to the structural similarity of leptin with other pro-inflammatory cytokines, it is also considered as pro-inflammatory cytokine (Otero *et al.,* 2005). Leptin induces apoptosis in pancreatic islets via induction of IL-1b secretion and suppression of IL-1Ra (Ehses *et al.,* 2009b), which impairs insulin secretion from β-cells (Zhao *et al.,* 2006). Leptin does not play its role in obesity-induced inflammation (Donath and Shoelson, 2011). The role of adiponectin in T2DM is totally opposite to that of leptin. It exerts its anti-inflammatory effects on endothelial cells through inhibition of TNF-α induced adhesion molecule expression and NF-kB, and activation of IL-1Ra (Wolf *et al.,* 2004) whereas in obese animals, it diminishes the levels of glucose by improving insulin sensitivity.

2.3.3 Cellular Immunopathogenesis of Type 2 Diabetes

Macrophages are the major inflammatory cell type in the glucose-utilizing tissues such as adipose tissue and liver. For example, macrophage in the adipose tissue increased from 5% in lean subjects to a level of up to 50% of all adipose tissue cells in obese individuals (Boutens Stienstra, 2016). Therefore, early studies on inflammatory regulation of diabetes have been

focused on innate immune function. Recent studies, however, suggest adaptive immune system, especially T lymphocyte, also plays a pivotal role in the pathogenesis of T2DM. There has been a rapid growth in our understanding of the role of T cell in the pathogenesis of T2DM in recent years.

2.3.3.1 Th1 and Th2 Cells

Th and Th are the among the subset of $CD4^+$ effector T cells, once activated, Th1 and Th2 cells show many of the significant signs of inflammation, such as releasing large amount of cytokines. Th1 cells could produce interferon- (IFN-) gamma, interleukin-2 (IL 2), and tumor necrosis factor- (TNF-) beta, triggering cell mediated immunity and phagocyte-dependent inflammation. Th2 cells, in contrast, produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 to regulate antibody responses (Xia *et al.,* 2017).

Increasing evidence suggests a pathological role for CD4+ T cells in obesity and insulin resistance. Obesity is a major important risk factor for Type 2 DM. A recent study by Shirakawa *et al,* (2016) established that activated CD4+ T cells ($CD4^+CD44^{hi}CD62L^{lo}$) increased in the visceral adipose tissue of obese mice; these cells expressed PD-1 and CD153 and displayed characteristics of cellular senescence (Shirakawa *et al,*. 2016). It has also been revealed that obesity induces MHC class II expression on adipocytes and thus activates $CD4^+$ T cells to initiate adipose tissue inflammation (Deng *et al.,* 2013). These studies suggest $CD4^+$ T cells may play an important role in obesity and obesity-induced insulin resistance through inducing proinflammatory cytokines in metabolic organs, such as the adipose tissue, liver, muscle, and pancreas.

Several clinical studies have confirmed that Th1 was upregulated in the adipose tissue and peripheral blood from the individuals with prediabetes or T2DM (McLaughlin *et al.*, 2014). Zeng and coworkers reported an imbalance of $CD4^+$ T helper cell subsets including Treg, Th1,

and Th17 in the patients with T2DM (Zeng *et al.,* 2012). In contrast, naive $CD4^+$ T cells were reported to decline in the patients with T2DM, which may be associated with adaptive immune activation and chronic inflammation during the pathogenesis of T2DM (Nekoua *et al.,* 2016). Similar findings were observed in lymphocyte-deficient mice, which displayed a less severe phenotype of insulin resistance on short-term high-fat diet (Xia *et al.,* 2017). Th1 and $CD8^+$ lymphocytes in the adipose tissue were significantly increased in response to a high fat diet, while anti-inflammatory Th2 and Treg cells were decreased (Xia *et al.,* 2017). $CD4^+$ helper T cells also play a critical role in a series of complications associated with T2DM. Activated T lymphocytes and the inflammatory cytokines increased in the kidneys in patients with T2DM (Abouzeid and Sherif, 2015). Experimental evidence indicates that the activation of Th2 cell-mediated immunity is delayed and impaired in diabetes (Xia *et al.,* 2017). It has been shown that Th1-associated cytokines induce hyperinflammatory response and subsequently lead to progressive innate immune response (Francisco *et al.,* 2016). It is reported that the circulating levels of Th1-associated cytokines increased in diabetic patients (Francisco *et al.,* 2016). The levels of T cell-related cytokines such as IL-10 and IL-17 were significantly higher in patients with T2DM, suggesting an involvement of T cells in diabetes (Xia *et al.,* 2017). In consistency, oral anti-CD3 plus glucosylceramide (an NKT cell target antigen) treatment was shown to induce the production of IL-10 and TGF-β, which was associated with improved fasting glucose, visceral adipose tissue inflammation, liver enzymes, and hepatic steatosis in ob/ob mice (Xia *et al.,* 2017).

These findings suggest both Th1 and Th2 are closely associated with insulin resistance and chronic inflammation in T2DM.

2.3.3.2 Th17 Cells

Th17, an important proinflammatory $CD4^+$ T cell subtype secreting IL-17, has also been associated with T2DM (Zhang *et al.*, 2014). A recent study examined the differentiation of different $CD4^+$ T cell subsets in T2DM by analyzing the cytokine production by PBMCs (Garidou *et al.*, 2015) The results demonstrated that Th17 cells increased in T2DM patients and might be associated with dysregulated lipid metabolism (Garidou *et al.*, 2015). Studies have shown that IL-17 could stimulate the production of TNF-α, the first cytokine being associated with obesity and insulin resistance.

It is reported that intestinal Th17 cells may induce AMPs, specifically Reg3b and Reg3g, contributing to increased intestinal permeability in high-fat diet-fed mice (Garidou *et al.*, 2015). Th17 has also been shown to increase in diabetic complications such as diabetic nephropathy (Abouzeid and Sherif, 2015). The function of Th17 cells could not be ignored in the progress of complications associated with T2DM. The result showed that Th17 counts and Th17/Treg ratio increased in diabetic nephropathy patients compared with healthy individuals and diabetic patients without nephropathy (Abouzeid and Sherif, 2015)

2.3.3.3 Regulatory T cells

Treg cells, a small subset of T lymphocytes constituting only 5–20% of the CD4+ compartment, are thought to be important to prevent excessive inflammatory responses and limit tissue impairment (Xia *et al.*, 2017). Typically, they regulate the response of other T cell subtypes but can also influence the activities of innate immune cells (Xia *et al.*, 2017). In T2DM, Treg cells could suppress Th1, Th2, and Th17 response to improve insulin resistance. Treg could inhibit the inflammatory response by various pathways, such as surpassing cytokine secretion, modulating the microenvironment, and changing the expression of surface receptors (Guzm´an-Flores and Portales-P´erez, 2013). The appropriate balance between

proinflammatory (Th17 orTh1) and anti-inflammatory (Treg) subsets of T cells is vital to maintain host immunity and control inflammatory damage (Xia *et al.*, 2017). It was found that the amount of Treg cells decreased in patients with T2DM. Zeng *et al*. also reported that Treg/Th17 ratio and Treg/Th1 ratio decreased in patients with T2DM (Zeng *et al.*, 2012). Further analysis suggests peripheral induced Treg but not natural Treg produced in the thymus reduced in T2DM, possibly due to due to decreased bcl2/bax and low HDL level (Zeng *et al.*, 2012).

2.3.3.4 $CD8^+$ Cytotoxic T cells

The inflammation of adipose tissue is considered as a key event leading to the metabolic syndrome, diabetes, and atherosclerotic cardiovascular disease. It was reported that, within two weeks of high-fat diet feeding, the $CD8^+CD4^-$ T cells were significantly increased in C57BL/6 mice (Xia *et al.*, 2017). The amount of $CD8^+CD4^-$ T cells kept increasing until 15 weeks. In contrast, Treg cells and $CD4^+$ T cells were reduced upon high-fat diet (Xia *et al.*, 2017). Unlike in wild type mice, there were no significant increases in the M1 or M2 macrophage fraction in CD8-deficient mice under high-fat diet although both body weight and epididymal fat mass significantly increased on high-fat diet (Xia *et al.*, 2017). More importantly, it was shown that $CD8^+$ T cells were essential for induction of macrophage activation and migration to adipose tissue by secreting MCP-1, MCP-3, and RANTES (regulated on activation, normal T cell expressed and secreted) (Xia *et al.*, 2017). These studies indicate that $CD8^+$ T cells were crucially involved in evoking inflammatory cascades in obese adipose tissue. Another research indicates that $CD8^+$ T cells increased in both the small bowel and colon in some obese individuals, compared with lean humans (Luck *et al.*, 2015). This study also showed the level of IFN-γ produced by $CD8^+$ T cells increased in obese individuals (Luck *et al.*, 2015). The increased intraepithelial $CD8^+$ T cells may have the

potential to modulate the insulin sensitivity of enterocyte (Luck *et al.*, 2015). CD137-CD137L interaction contributes to $CD8^+$ cytotoxic T cell proliferation and secretion of IFN-γ, TNF-α, IL-2, and IL-4 (Tu *et al.,* 2014). The expression of CD137 was upregulated in obese humans and mice (Tu *et al.,* 2014). CD137-CD137L could promote monocyte and T cell recruitment to the adipose tissue (Tu *et al.,* 2014). The inflammation in adipose tissue decreased in $CD137^{-/-}$ mice, but glucose tolerance was strengthened (Xia *et al.*, 2017). Therefore, CD137 contributes to abnormal glucose and lipid metabolism, which may involve the expansion and activation of $CD8^+$ T cells.

2.3.3.5 Gamma and Delta ($\gamma\delta$) T Cells

T cells are subdivided into two major populations according to their surface expression of $\alpha\beta$ and $\gamma\delta$ T cell receptors (TCR). Activated $\gamma\delta$ T cells could produce a vast variety of cytokines such as IFN-γ and TNF-α to regulate the function of other immune cells. In recent years, there is increasing evidence indicating that $\gamma\delta$ T cells may interact with macrophages, CTLs, Th1/Th2 cells, Treg, and Th17 cells depending on specific microenvironment, bridging innate and adaptive immunity. $\gamma\delta$ T cells could produce IL-10 and IL-17 (Xia *et al.*, 2017). In addition, they also secret TNF-α to regulate the activation of $CD8^+$ T cells; obesity is an important risk factor for chronic inflammatory diseases, such as T2DM and cardiovascular disease (Xia *et al.*, 2017) $\gamma\delta$ T cells have been suggested to play a critical role in chronic inflammatory disease. It was found that obese patients exhibited a decreased level of Vγ9Vδ2 T cells and the level of secreting IFN-γ was also declined. These Vγ9Vδ2 T cells could prefer to differentiate mature T effecter memory cluster of differentiation 45RA ($CD45RA^+$) cells, indicating $\gamma\delta$ T cells are also involved in inflammation in obesity and diabetes (Dimova *et al.*, 2015). A recent study reported that IL-2 treatment rescued Vγ9Vδ2 T cell cytokine production

(Costanzo *et al.,* 2015), which provides a novel insight into the pathogenic role $\gamma\delta$ T cells in the development of T2DM.

2.3.3.6 Circulating Natural Killer (iNKT) Cells

Previous studies have demonstrated that circulating natural killer cells (iNKT cells) were decreased and their function was suppressed in obese individuals, compared with lean individuals (Xia *et al.,* 2017). It has been identified that iNKT cells are enriched in human and murine adipose tissue (Lynch *et al.,* 2012). iNKT cells could recognize lipid antigens presented by CD1d but not peptide through MHC molecules in the innate immune system (Xia *et al.,* 2017). Further investigation has been shown that adipose iNKT cells are a tissue resident with little influx from the circulation and play an important anti-inflammatory regulatory role in congenic parabiotic mice (Lynch *et al.,* 2015). However, adipose iNKT cells are obviously decreased in obesity (Schipper *et al.,* 2012). iNKT cells can regulate the cross talk between innate and adaptive immunity as potent transactivators. Most studies reported that iNKT cell-deficient mice have worse metabolic disorder and weight gain (Schipper *et al.,* 2012). α-Galactosylceramide (αGalCer), a potent lipid ligand, could activate iNKT cell. It is shown that injection of αGalCer in obesity increases the iNKT cell amount and induces weight loss rapidly, anti-inflammatory macrophage differentiation, and reversal of glucose and insulin sensitivity without hypoglycemia (Hams *et al.,* 2013). The majority of findings suggest that iNKT cells in adipose tissue play a critical role in regulating local inflammation and protecting against metabolic disorder in obesity (Kohlgruber and Lynch, 2015).

2.4 Malnutrition Related Diabetes Mellitus (MRDM)

MRDM also called tropical diabetes (Sobngwi *et al.,* 2001). Is a form of diabetes that is more common in the tropics, where young people with diabetes may present with a constellation of clinical features including onset at less than 30 years of age, body mass index of <20,

moderate to severe hyperglycaemia, no-proneness to ketosis in the absence of precipitating factors such as infection, the requirement of a large dose of insulin for metabolic control, most often than not a history of malnutrition at early infancy and early childhood or poor economic status, inconsistence abdominal pains, pancreatic calcification in the absence of heavy alcohol intake, hyperparathyroidism or gallstones (Sobngwi *et al.,* 2001). Though called MRDM, the actual pathogenesis is not clear (Sobngwi *et al.,* 2001).

2.5 Gestational Diabetes

Gestational diabetes mellitus in which hyperglycaemia develops only during pregnancy and can resolve immediately after delivery, although, may come up with type 2 DM letter in life (CDC, 2016).

Other types of diabetes are due to some associated causes such as genetic defects in beta cell normal physiology, genetic defects in insulin function, diseases affecting the islets or exocrine pancreas, beta cell cytotoxic chemicals or steroid therapy (ADA, 2016; IDF, 2016).

2.6 Diabetic Symptoms and Complications

Diabetic symptoms include excessive thirst (polydipsia), excessive weight gain or loss, blurred vision, excessive hunger (polyphagia), fatigue, frequent infections, frequent urination (polyuria), muscle loss, dehydration, slow healing of sores and wounds, trembling, depression, dizziness, erectile dysfunction, and numbness (Ciechanowski *et al.,* 2003; Genuth *et al.,* 2003). There are chronic diabetic complications arising from hyperglycaemia which could possibly lead to glucotoxicity causing damaging effects to various cells in the body (Wu and Yan, 2015). These damaging effects are associated with pancreatitis, diabetic retinopathy, nephropathy, hepatopathy and neuropathy (Matsuda & Shimomura, 2013). Other complications implicated in diabetes include stroke, coronary and peripheral arterial diseases (Brownlee, 2001).

2.6.1 Diabetic Pancreatitis

Pancreatitis occurs when the pancreas is inflamed. Acute pancreatitis is a known complication of diabetes, although there are no studies linking diabetes as a causative of acute pancreatitis (Blomgren *et al.,* 2002). The pancreas synthesizes digestive enzymes and hormones that help regulate glucose and metabolism of fats and protein (Mahadevan, 2016). Glucagon and insulin are produced by the pancreas which aids in glucose regulation. In type 1 diabetes, β-cells are destroyed leading to insufficient insulin production (Lumelsky *et al.,* 2001). Transient hyperglycaemia might be seen in individuals with pancreatitis (Khan *et al.,* 2017). Studies revealed that individuals with type 2 diabetes may be at higher risk of acute pancreatitis and biliary disease than individuals without diabetes (Girman *et al.,* 2010). Severe acute pancreatitis results in low blood pressure, dehydration and can cause failure of vital organs (Mahadevan, 2016).

2.6.2 Diabetic Neuropathy

Diabetes is a major cause of peripheral neuropathy, with about 30% of individuals with diabetes developing neuropathy (Lindsay *et al.,* 2010). Diabetic neuropathy occurs when there is damage to the nerves of the peripheral nervous system resulting in severe diabetic complications (Callaghan *et al.,* 2012). In diabetic conditions, the walls of the tiny blood vessels responsible for the supply of nutrients and oxygen to the nerves in organs, hands and feet are damaged (Sen *et al.,* 2016). Diabetic neuropathy is associated with hyperglycaemia, low insulin production, distal symmetrical polyneuropathy and mechanical injury to the nerves (Vinik *et al.,* 2013). Current reports have indicated diabetic neuropathy as the most common complications of diabetes pose a high health risk globally (Jack & Wright, 2012, Hinder *et al.,* 2013).

2.6.3 Diabetic Retinopathy and Nephropathy

Diabetes contributes to an increase in oxidative stress, which subsequently plays a pivotal role in the pathogenesis of diverse diabetic complications (Feldmen, 2003; Kowluru, 2003; Brownlee, 2005). Diabetic effects on structural proteins of the retina leading to diabetic retinopathy, cataracts and glaucoma have been documented (Ahmad, 2002; Feldman, 2003). Enduring cycle of metabolic stress, damage of tissue and cell death may amplify the oxidative stress, nitrative stress and lipid peroxidation status resulting in increased damage to DNA and free radicals production (Kowluru, 2003).

Diabetic nephropathy is a life-threatening complication of type 1 diabetes and continues to increase worldwide (Chakraborty *et al.,* 2012). It causes progressive damage to the capillaries of the kidney's glomeruli leading to endothelial dysfunction, changes in inflammatory biomarkers and oxidative stress (Toma *et al.,* 2012; Forbes & Cooper 2013). Diabetes nephropathy has been investigated in diabetic individuals revealing elevated level of microalbuminuria which precedes the development of diabetic nephropathy (Marketou *et al.,* 2016). It is characterised by the presence of micro-albuminuria with a low abnormal level of albumin in the urine which could lead to further kidney damage, cardiovascular morbidity and eventually mortality (Huang *et al.,* 2011; Navarro-González and Mora-Fernandez, 2016).

2.6.4 Diabetic Hepatopathy

The liver plays a vital role in the regulation of carbohydrate metabolism. It supplies glucose to other organs that require glucose as an energy source and is one of the organs affected by diabetes. In the diabetic state, there is an increased generation of ROS which triggers a chain of reaction leading to the peroxidation of lipids, lipoprotein modifications and several cellular mutations of biomolecules (Miller *et al,* 2011; Mbikay, 2012). This peroxidation leads to

oxidative stress which is an important risk factor in the pathogenesis of cardiovascular and chronic diseases such as diabetic and associated complications (Forbes & Cooper, 2013).

Moreover, there is increasing evidence from clinical studies showing oxidative stress as a major player in diabetic pathophysiology leading to dyslipidemia, impaired glucose tolerance, β-cell dysfunction, and ultimately resulting in liver malfunction (Ghosh *et al.,* 2015; Tangvarasittichai, 2015). Diabetic hepatopathy is linked to liver failure and liver cirrhosis. Glycogenic hepatopathy has been reported in other studies conducted which show pathological overloading of the hepatocytes with glycogen and transaminase (Torbenson *et al.*, 2006). Torbenson (2006) also diagnosed hepatomegaly in individuals with poorly controlled type 1 diabetes.

2.6.5 Cardiovascular Diseases

Cardiovascular diseases (CVD) are the number one causative of mortality globally; with diabetic individuals at higher risk (Beulens et al., 2010). Oxidative stress is involved in the onset and progression of diabetes (Matough *et al.,* 2012), leading to Cardiovascular Complication, Ischemic Heart Disease and Myocardial Dysfunction (Mbikay, 2012; Patel *et al.*, 2012b; Ezuruike and Prieto, 2014). The low antioxidant defence as a result of diabetic complications may contribute to cardiovascular disease, nerve damage, and nephropathy (He & King, 2014). Therapeutic interventions are currently been employed in the prevention and treatment of cardiovascular diseases (Rader & Hovingh, 2014)**.**

2.7 Diagnostic Criteria for Diabetes

The disease is usually diagnosed based on plasma glucose criteria, either the fasting blood sugar level of ≥ 126 mg/dL (≥ 7.0 mmol/L) or the two hour plasma glucose value ≥ 200 mg/dL (≥ 11.1 mmol/L) after a 75g glucose tolerance test. Recently, an International Expert Committee has added the glycated hemoglobin level of ≥ 6.5% with any of the following

symptoms, polyuria, polydipsia, weight loss (ADA, 2016; WHO, 2016). The vivid criteria for the diagnosis of diabetes mellitus are as follows:

1. When a patient present with signs and symptoms of diabetes, namely, polyuria, polydipsia, polyphagia and unexplained weight cost, plus random plasma glucose concentration ≥200mg/dl or (11.1mmol/L), and confirmed on a subsequent day, to give a similar outcome, the diagnosis of diabetes mellitus is made.

2. When a patient presents with sign and symptoms of diabetes listed above, with fasting plasma glucose concentration of ≥ 126mg/dl (7.2mmol/L), also confirmed on a subsequent day, to give similar outcome.

3. When a patient presents with sign and symptoms of diabetes mellitus, with 2 ours plasma glucose concentration of 200mg/dl (11.1mmol/L) during an oral glucose tolerance test as described by W.H.O., using a glucose load containing the equivalent of 75g anhydrous glucose, dissolved in water. This however, is not routinely used in the diagnosis of diabetes mellitus.

4. An intermediate group of subjects with glucose concentration that do not meet the above criteria for diabetes, and nevertheless, too high to be considered normal exists (ADA 2016; IDF, 2016). This group of subjects had fasting plasma glucose level > 110mg/dl (6.1mmol/L), but <126mg/dl (7.0mmol/L). To avoid the stigmatization attached to the name diabetic, they are classified as subjects with impaired fasting glucose (IFG). With time, these class of subjects may progress to full blown diabetes, or revert to normal plasma glucose concentration (WHO, 2010; ADA, 2014; IDA, 2016).

2.8 Diabetes and Inflammation

Chronic Inflammation is associated with diabetes and has been reported to cause severe organ damage in diabetic rats (Mohamed *et al.,* 2016b). Individuals with diabetes have a high level

of inflammatory cytokines, activation of leukocytes and increased tissue fibrosis (Donath & Shoelson, 2011). Inflammatory cytokines; C-reactive protein (CRP), interleukin-1 α (IL-1α), interleukin-6 (IL-6), interleukin-12 (IL-12), interleukin-18 (IL-18), tumour necrotic factor-α (TNF-α) and chemokine; monocyte chemotactic protein-1 (MCP-1) concentrations are high in diabetic conditions. Inflammation in tissues occurs in response to harmful stimuli or damage to cells. IL-6 and TNF- α cytokines play a crucial role in hyperglycaemia-induced kidney injury and are associated with the development of diabetes. Increasing levels in patients with diabetic nephropathy have been reported, suggesting that these cytokines play significant roles in the pathogenesis of diabetic nephropathy (Navarro- González & Mora-Fernández, 2016). Seca *et al.* (2014) noted the presence of high concentration of inflammatory proteins which negatively impacted on the function of insulin.

2.8.1 Mechanisms Regulating Inflammation in Diabetes

As we stated earlier, multiple mechanisms are thought to contribute to β-cell dysfunction, insulin resistance, and vascular complications of diabetes (Donath *et al.,* 2013). In diabetes, hyperglycaemia and elevated free fatty acids may promote inflammation by stimulating glucose utilization along with alterations in oxidative phosphorylation (Pollack *et al.*, 2016). Such metabolic dysregulation has been shown to induce a proinflammatory trait in macrophages residing or invading the adipose tissue and other tissues including the islets and vasculature (Vandanmagsar *et al.*, 2011). Glucotoxicity and lipotoxicity might also exert oxidative and endoplasmic reticulum stress, which in turn elicits an inflammatory response by activating thioredoxin-interacting protein (TXNIP) and the NLR family, pyrin domain containing 3 (NLRP3) inflammasome, which increase the release of active interleukin (IL)-1b (Vandanmagsar *et al.*, 2011). IL-1b further amplifies inflammation by inducing the expression

of various cytokines and chemokines, resulting in the recruitment of immune cells including macrophages ("auto-stimulation") (B¨oni-Schnetzler *et al.,* 2008). Similar mechanisms have been reported in diabetic β-cells, adipose tissue, and blood vessels (Pollack *et al.,* 2016). In type 2 diabetes, oligomers of islet amyloid polypeptide deposit in the pancreas and may trigger inflammation by stimulating the NLRP3 inflammasome and the generation of mature IL-1b (Masters *et al.,* 2010). Stress and inflammation may eventually lead to apoptosis and contribute to β-cell dysfunction, insulin resistance, and atherosclerosis. In addition, obesity is associated with alterations in the gut microbiome along with increased gut leakiness of bacterial wall lipopolysaccharides (endotoxins) that may further promote tissue inflammation (Pollack *et al.,* 2016). Endotoxins, free fatty acids (probably in conjunction with fetuin), and cholesterol induce inflammation by activating Toll-like receptor (TLR) pathways and, subsequently, nuclear factor-kB (NF-kB)-mediated release of a broad range of cytokines and chemokines including tumor necrosis factor (TNF), IL-1b, IL-8, and MCP-1 that promote the accumulation of various immune cells in different tissues (Vandanmagsar *et al.,* 2011). It has recently been reported that in obesity, alterations of the gut microbiome might stimulate not only the innate immune system but also the adaptive immune system, which might contribute to insulin resistance (Sell *et al.,* 2012). Adipose tissue inflammation can also be triggered by local hypoxia caused by rapid expansion of adipose tissue with insufficient vascularadaptation (Pollack *et al.,* 2016). The renin-angiotensin system may also play a role in inflammation, insulin resistance, and vascular damage (Van der Zijl *et al.,* 2012). Recent data suggest that this system may have a role in islet inflammation and β-cell dysfunction, independent of its effects on glucose metabolism. Angiotensin II has been shown to induce expression of chemokine MCP-1 and IL-6, leading to impaired mitochondrial function and insulin secretion, as well as increased β-cell apoptosis (Sauter *et al.,* 2015). These findings shed new light on the

mechanisms of inflammation in obesity and diabetes and open new venues for prevention of inflammation by modifying the proinflammatory microbiota or by using inhibitors of the rennin angiotensin system. Alternatively, it is possible to use treatments that target key molecules that regulate the inflammatory response.

2.8.2 Targeting Inflammation in Management of Diabetes Mellitus

Inflammation has been recognized as a common mechanism in the pathophysiology of diabetes and obesity conditions. Inflammation increases insulin resistance and islet cell inflammation, which leads to defects in beta cell secretion both of which lead to diabetes. Inflammation may also be the underlying mechanism in the increased risk of cardiovascular disease in subjects with diabetes and/or obesity. Hence, targeting inflammation may be a new therapy in the already expanding options for the management of diabetes mellitus and its complications.

2.8.2.1 Etanercept

Etanercept (934 amino acids, 150 kilo Dalton) is a dimeric fusion protein with an extracellular ligand binding domain of the Human Tumor Necrosis Factor Receptor (TNFR) linked to the Fc component of human IgG1. It is produced by a recombinant DNA technique in Chinese Hamster Ovary cells. Etanercept has been shown to decrease insulin resistance by blockade of TNF-α receptor in obese rats (Rajpathak *et al.,* 2009). A human trial of Etanercept failed to improve insulin sensitivity in subjects with the metabolic syndrome despite lowering CRP (Bernstein *et el.,* 2006). This may have been due to the fact that the concentration of TNF-α intracellularly is almost twice that in the extracellular space, and it is the intracellular TNF-α that is responsible for insulin resistance via paracrine effects which were not blocked by Etanercept.

2.8.2.2 Anakinra

Anakinra (153 amino acids, 17.3 kilo Dalton) is a non glycosylated form of the Human IL-1 Receptor antagonist (IL-1Ra) from which it differs only by the addition of a single methionine residue at the amino terminus. It is produced by a recombinant DNA technique in E. coli. IL-1 contributes to impaired insulin secretion, decreased cell proliferation, and apoptosis of pancreatic β cells. The IL-1Ra is endogenously produced, and its concentrations are reduced in the pancreatic islets of patients with T2DM. Anakinra was studied in T2DM and showed promise in increasing beta cell secretory function, and reducing glycemia and markers of systemic inflammation (Akash *et al.*, 2012b). Definitive conclusions on the possible clinical utility of IL-1Ra in the prevention of diabetes are awaited from the large ongoing Canakinumab Antiinflammatory. Thrombosis Outcomes Study phase III clinical trial (Ridker *et al.*, 2011). The study is being conducted in more than 40 countries around the world and is specifically testing whether blocking the pro-inflammatory cytokine IL-1β with Canakinumab, as compared to placebo, can reduce rates of recurrent myocardial infarction, stroke, and cardiovascular death among patients with a history of myocardial infarction who remain at high risk due to a persistent elevation of the inflammatory biomarker hsCRP (≥ 2 mg/L) despite best medical care.

2.8.2.3 Salsalates

Salsalates belong to the class of Nonsteroidal Anti-inflammatory Drugs (NSAIDs) which exert their antiinflammatory effect through inhibition of prostaglandin G/H synthase, or cyclooxygenase.These enzymes catalyse the transformation of arachidonic acid to prostaglandins and thromboxanes. NSAIDs also inhibit the expression of cell adhesion molecules, which play a role in targeting circulating cells to inflammatory sites and directly inhibit activation and function of neutrophils. Trials with high dose Salsalates in rodents

(Yuan *et al.*, 2010) and in subjects with diabetes (Goldfine *et al.*, 2008) have shown that Salsalates by inhibiting the inhibitor of nuclear factor kappa-B kinase subunit beta decreases glucose intolerance and increases insulin sensitivity. In an open label study, Salsalates, a pro drug form of salicylate, reduced fasting and post challenge glucose levels and increased glucose utilization in euglycemic, hyperinsulinemic clamp studies (Goldfine *et al.*, 2008). Circulating FFAs were reduced and adiponectin levels were increased. In another study, Salsalates, when compared with placebo, reduced fasting glucose by 13% ($P < 0.002$), glycemic response after an oral glucose challenge by 20% ($P = 0.004$), and glycated albumin by 17% ($P < 0.0003$). Although insulin levels were unchanged, fasting and oral glucose tolerance test and C-peptide levels decreased in the Salsalates-treated subjects compared with placebo ($P < 0.03$), consistent with improved insulin sensitivity and a known effect of salicylate to inhibit insulin clearance.

Adiponectin increased by 57% after Salsalates treatment compared with placebo ($P < 0.003$). Additionally, within the group of Salsalates-treated subjects, circulating levels of CRP were reduced by 34% ($P < 0.05$) (Fleischman *et al.*, 2008). These findings prove that Salsalates reduces glycemia and may improve inflammatory cardiovascular risk indices in overweight individuals. These data support the hypothesis that sub-acute to chronic inflammation contributes to the pathogenesis of obesity-related dysglycemia and that targeting inflammation may provide a therapeutic option for diabetes prevention. However, the effects of Salsalates on inflammation are controversial as shown by another study in which Salsalates did not change flow mediated dilatation in peripheral conduit arteries in patients with T2DM despite lowering HbA1c. This finding suggests that Salsalates does not have an effect on vascular inflammation (Goldfine *et al.*, 2013).

2.8.2.4 Chloroquine

Chloroquine is a weak base and carries a positive charge at acidic pH. It is this property of the drug that makes it selectively accumulate in lysosomes and generate a concentration gradient of a high order. This lysosomatotrophic action is responsible for the hepatic retention of insulin. Another action of the drug is decreased degradation of insulin in the muscle tissue. A retrospective study suggested that the use of Chloroquine to treat rheumatoid arthritis is associated with a lower incidence of T2DM (Wasko *et al.,* 2007). However, this study included a specific group of patients who required the drug for another indication. Prospective studies of Chloroquine are ongoing and the results are awaited.

2.8.2.5 Diacerein

Diacerein is a semi-synthetic anthraquinone derivative which directly inhibits IL-1 synthesis and release *in vitro* and down regulates IL-1 induced activities. It has been shown to possess a disease modifying effect in osteoarthritis. In a randomized double-blind, placebo-controlled trial, 2-mo treatment of drug-naive T2DM patients with diacerein increased insulin secretion without changes in insulin sensitivity (Ramos-Zavala *et al.,* 2011). This implies a direct effect of the drug on beta cell function.

2.8.2.6 Inhibition of 12-lipo Oxygenase

Twelve-Lipo oxygenase (12-LO) produces pro-inflammatory arachidonic acid products and is upregulated in islets of both T1DM and T2DM patients (Dobrian *et al.,* 2011) leading to insulin resistance and islet cell dysfunction. Hyperglycaemia and inflammatory cytokines increase the expression of 12-LO (Ma *et al.,* 2012; Dobrian *et al.,* 2011). The activation of 12-LO has also been implicated in causing adipose tissue inflammation and insulin resistance. In NOD mice (T1DM model), Zucker diabetic fatty rats (T2DM model), and diet-induced obese mice (T2DM model) gene deletion and pharmacological suppression of 12-LO prevented the

development of diabetes (Tersey *et al.,* 2012). These findings point towards inhibition of 12-LO being a promising target in both T1DM and T2DM for decreasing insulin resistance, β cell dysfunction and cardiovascular complications.

2.9 New Trends in Diabetes Mellitus Managements

2.9.1 The Bio-artificial Pancreas

A cross section of bio-engineered tissue with encapsulated islet cells delivering endocrine hormones in response to glucose is implanted (Barkai *et al.,* 2016). Encapsulation of the islet cells in a protective coating that has been developed to block the immune response to transplanted cells, which relieves the burden of immunosuppression and benefits the longevity of the transplant. This approach has had very positive clinical studies and is currently underway in human trials. So far, treatment using this method of cell encapsulation has been proven safe and effective and is the first to achieve insulin independence in human trials without immunosuppressant drugs (Barkai *et al.,* 2016; Pothuloori and Chaidarun, 2015).

2.9.2 Islet cell Regeneration

Researchers have discovered a protein they referred to as INGAP Islet Neogenesis Associated Protein (INGAP). INGAP seems to be the product of a gene responsible for regenerating the Islets that make insulin and other important hormones in the pancreas. As of 2008, the protein had undergone Phase 2 Human Clinical Trials, and developers were analyzing the results (Assouline-Thomas *et al.,* 2015). This trial will be unique in that patients who are beyond the 'newly diagnosed' period will be included in the study. Most current trials seeking to treat people with type 1 diabetes do not include those with established disease (Flores *et al.,* 2014).

2.9.3 Stem Cells

Stem cell research has been suggested as a potential avenue for a cure since it may permit regrowth of Islet cells which are genetically part of the treated individual, thus perhaps

eliminating the need for immunosuppressants. This new method autologous nonmyeloablative hematopoietic stem cell transplantation was developed by a research team composed by Brazilian and American scientists and it was the first study to use stem cell therapy in human diabetes mellitus. This was initially tested in mice and in 2007 there was the first publication of stem cell therapy to treat this form of diabetes (He *et al.,* 2015). In summary it is a kind of "immunologic reset" that blocks the autoimmune attack against residual pancreatic insulin-producing cells. It is too early to say whether the results will be positive or negative in the long run. In September 2008, scientists from the University of North Carolina at Chapel Hill School of Medicine have announced their success in transforming cells from human skin into cells that produce insulin. The skin cells were first transformed into stem cells and then had been differentiated into insulin secreting cells (Mellado-Gil *et al.,* 2012).

2.9.4 Gene Therapy

Technology for gene therapy is advancing rapidly with such pathways possible to support endocrine function, with potential to practically cure diabetes. Gene therapy might eventually be used to cure the cause of beta cell destruction, thereby curing the new diabetes patient before the beta cell destruction is complete and irreversible (Zulewski *et al.,* 2001). Gene therapy can be used to turn duodenum cells and duodenum adult stem cells into beta cells which produce insulin and amylin naturally. By delivering beta cell DNA to the intestine cells in the duodenum, a few intestinal cells will turn into beta cells and subsequently adult stem cells will develop into beta cells (Kawser-Hossein *et al.,* 2016). This makes the supply of beta cells in the duodenum self-replenishing, and the beta cells will produce insulin in proportional response to carbohydrates consumed. Transplants of exogenous beta cells have been performed experimentally in both mice and humans, but this measure is not yet practical in regular clinical practice partly due to the limited number of beta cell donors. Thus far, like any

such transplant, it has provoked an immune reaction and long-term immunosuppressive drugs have been needed to protect the transplanted tissue (Kawser-Hossein *et al.,* 2016).

2.9.5 Natural Products

Recent findings suggest that natural approaches to the DM may help supplement current standard medications for socio-economic alleviation and good glycaemic control (Schultz *et al.,* 2016). Thus, establishing an intellectual property protection of medical treatments comprising of natural products is existing in public domain and currently promoted as dietary supplements in the management of DM (Bulaj *et al.,* 2016). Additionally, some flavonoids and polyphenols are found to be effective in DM management (Jung *et al., 2006;* Patel *et al.,* 2016). However, the safety and efficacy of natural products supplementation remains to be established.

2.10 Diabetes and Apoptosis

Apoptosis, also known as programmed cell-death, is executed by caspases which are critical for maintaining tissue homeostasis. Apoptosis involves the prompt removal of unwanted cells for maintenance of specific organs (Nakanishi *et al.,* 2009), and is essentially a prominent feature in some disease state including diabetes. ROS generation results in immune response inducing apoptotic cell death via membrane receptors and intracellular stress (Matés & Sánchez-Jiménez, 2000). Diabetes is also associated with the production of pro-apoptotic factors (Barclay *et al.,* 2015). Some apoptotic markers are NFKβ, p53, caspase 3, caspase 7, caspase 9 and BCL-2. Caspase 3 is an executioner caspase, with a high level seen in a diabetic state where the neural cells are lost and increases excessive cell death and it is regulated by BCL-2 (Hui *et al.,* 2004). Fundamentally, BCL-2 is an anti-apoptotic protein that regulates cell death and promotes cell survival. It determines the commitment of cells to apoptosis (Ahmad *et al.,* 2009; Wang, 2015).

2.11 Drugs used in the Management of Diabetes

Oral medications, other than insulin injections used in the treatment of diabetes, include sulfonylureas, metformin, alpha-glucosidase, thiazolidinediones and meglinides.

Sulfonylureas works by enhancing the pancreas to increase insulin secretion thus reducing blood glucose levels, but is usually accompanied by side effects such as hypoglycaemia, hunger and weight gain.

Metformin reduces blood glucose by monitoring the amount of glucose released by the liver into the bloodstream. Associated side effects include nausea, abdominal discomfort, a metallic taste in the mouth and a loss of appetite.

Alpha-glucosidase inhibitors (starch blockers) reduce the digestion rate of carbohydrates by inhibiting the enzymes responsible for carbohydrate breakdown in the small intestine (acarbose and miglitol), thereby enhancing steady glucose uptake from food, with the resultant regulation of postprandial blood glucose. However, side effects from these inhibitors include abdominal pain, diarrhea and flatulence.

Thiazolidinediones reduce the body's ability to resist insulin, leading to low blood glucose levels. Possible side effects include heart attack, stroke, fluid retention and weight gain.

Meglitinides, on the other hand, excite the pancreas, increasing the release of insulin. Side effects include hypoglycaemia, back pain and joint pain.

On the whole, the costs, availability issues and serious side effects associated with these drugs have led to the clamour for alternative options for treatment using medicinal plants.

2.12 Cytokines of the Immune System

Cytokines, a large group of soluble extracellular proteins or glycoproteins, are key intercellular regulators and mobilizers. Cells of the immune system communicate with one

another by releasing and responding to chemical messengers called cytokines. These proteins are secreted by immune cells and act on other cells to coordinate appropriate immune responses. They are now seen to be crucial to innate and adaptive inflammatory responses, cell growth and differentiation, cell death, angiogenesis and developmental as well as repair processes (Wahab *et al.,* 2013). Cytokine plays a key role in modulation of immune responses. Many different cell types, in addition to immune cells, produce cytokines and express receptors for cytokines. Cell to cell communication is maintained via cytokine networks (Wahab *et al.,* 2013). Cytokines include a diverse assortment of interleukins, interferons, and growth factors and chemokines. Adverse effects of cytokines have been linked to many disease states and conditions with levels either being elevated or changed. Over secretion of cytokines can trigger a dangerous syndrome known as a cytokine storm (Dowlati *et al.,* 2010). Plasma levels of various cytokines may give information on the presence, or even predictive value of inflammatory processes involved in autoimmune diseases as well as immunomodulatory effects of foods or drugs (Swardfager *et al.,* 2010).

2.12.1 Tumor Necrosis Factor-Alpha (TNF-α): Sources, Gene and Function

Tumor Necrosis Factor-α, is a member of a group of cytokines that involved in systemic inflammation and it was discovered later independently as cachectin, a circulating mediator of wasting syndrome (cachexia) associated with chronic disease (Olszewski *et al*., 2007). The primary role of TNF-α is the regulation of immune cells because of its ability to induce fever, apoptotic cell death, inhibit tumorigenesis, viral replication, maintenance of secondary lymphoid organ structure, and host defense against various pathogens (Saud, 2014). So the TNF plays a critical role in bridging innate and adaptive immunity. Deregulation of TNF-α production has been implicated in a variety of human diseases including Alzheimer's disease,

cancer, major depression, and inflammatory disease (ID) (Swardfager *et al*., 2010). It is a 26 kilodalton transmembrane protein that is cleaved into a 17 kilodalton biologically active protein that exerts its effects via type I and type II TNF receptors. Within adipose tissue, TNF-α is expressed by adipocytes and stromovascular cells (Olszewski *et al*., 2007). Although initially suspected of playing a role in cachexia, TNF-α has now been implicated in the pathogenesis of obesity and insulin resistance. Adipose tissue expression of TNF is increased in obese rodents and humans and is positively correlated with adiposity and insulin resistance. Although circulating concentrations of TNF-α is low relative to local tissue concentrations, plasma TNF-α level have been positively correlated with obesity and insulin resistance (Fernandez-Real and Ricart, 2003). Chronic exposure to TNF- α induces insulin resistance both in vitro and in vivo (Ruan and Lodish, 2013). Several potential mechanisms for TNF-α metabolic effect have been described. First, TNF-α influences gene expression in metabolically important tissues such as adipose tissue and liver (Ruan *et al*., 2012), in liver, TNF-α suppresses expression of genes involved in glucose uptake and metabolism and fatty acid oxidation and increases expression of genes involved in synthesis of cholesterol and fatty acids (Ruan *et al*., 2012). Second, TNF-α impairs insulin signaling, and this effect is mediated by activation of serine kinases that increase serine phosphorylation of insulin receptor substrate-1 and -2, making them poor substrates for insulin receptor kinases and increasing their degradation.

2.12.1.1 Serum TNF-α Level and Diabetes Mellitus

TNF-α is highly involved with macrophage activation and increased serum TNF-α level have been observed in insulin resistance stages and diabetes mellitus development (Qiao *et al.,* 2017), The molecular mechanisms by which TNF-*α* induces insulin resistance are considered to be the following. TNF-*α* binds TNF receptor 1 and activates sphingomyelinase that

metabolizes sphingomyelin to ceramide (Qiao *et al.,* 2017). Ceramide increases serine phosphorylation of insulin receptor substrate-1 (IRS-1), which inhibits the insulin receptor tyrosine phosphorylation, resulting in attenuation of insulin signaling and a decrease in glucose transporter-4 (GLUT-4) translocation and glucose uptake.

TNF-α level may play an important role and many factors may contribute to the serum TNF-α level in diabetes. TNF-α, as the major physiological and pathophysiological regulators of vascular adhesion molecules, is a key proinflammatory cytokine with widespread metabolic effects, and directly regulate the production of several cardiovascular risk factors (Qiao *et al.,* 2017).

TNF-α via effects on soluble intercellular adhesion molecule-1, may promote vascular adhesion, otherwise plasma levels of TNF-α are associated with dyslipidaemia and increase blood pressure, adding to vascular disease risk, besides, the actions of TNF-α is probably modified by altered production of soluble receptors in type 1 diabetic patients (Qiao *et al.,* 2017). Increased TNF-α level through metabolic control exist in types 1 and 2 diabetic patients, which suggest that the control of diabetes improves the capacity of activation and maintenance of these pro-inflammatory cytokines (Qiao *et al.,* 2017). Other study demonstrated TNF-α level were elevated in T1DM which was correlated positively with HbA1c and inversely with HDL cholesterol levels (Qiao *et al.,* 2017).

Owning the various biological effects, TNF-α has been proved to have certain catabolic effects on fat cells, and neutralization of TNF-α in obese rats causes a significant increase in the peripheral uptake of glucose in response to insulin, which indicates an important role in the insulin resistance and diabetes that often accompany obesity (Gabbay *et al.,* 2012). TNF-α, through increasing the activities of the NF-κB transcriptional factor (Qiao *et al.,* 2017), protein kinase C, amino terminal kinase and inhibitor kinase, could cause serine/threonine

phosphorylation of the insulin receptor substrate, interfere with normal phosphorylation of tyrosine, and weaken signal transduction of insulin, resulting in insulin resistance (Yuan *et al.*, 2010), otherwise, TNF-α may be result in the destruction of pancreatic beta cells and lead to the development of T1DM (Yuan *et al.*, 2010).

Mardiah *et al.* (2015) reported a significant increase of serum TNF-α level on her paper published on anti-inflammatory purple Roselle extract in diabetic rats induced by streptozotocin. The TNF-α level in these diabetic rats was reduced or suppress after diabetic rats has been treated with Roselle. Bolajoko *et al.* (2017) demonstrate an evidence of an increased concentration of cytokine TNF-α in the kidney, liver and serum of diabetic rats when compared to the normal control and diabetic control. These increased in the concentration of TNF-α was significantly reduced after diabetic rats has been treated with *Moringa oleifera* extracts. Elizabeth *et al.*, (2017) also report similar finding of increased concentration of cytokines TNF-α in the serum of diabetic rats when compared to the normal control and diabetic control, Bolajoko *et al.*, (2017) similarly report increase in serum TNF-α levels in the diabetic rat compared to non-diabetic rat in his paper, Modulatory influence of *Parkia biglobosa* protein isolate on testosterone and biomarker of oxidative stress in brain and testes of streptozotocin-induce diabetic male rat. This increase in serum TNF-α level in the diabetic rat was reduced after diabetic rats has been treated with protein from *Parkia biglobosa.*

2.12.1.2 TNF-α in Chronic Diabetic Complication

Chronic hyperglycaemia activates macrophages (Satoh *et al.*, 2013) and stimulates in vivo TNF-α production (Satoh *et al.*, 2013). Enhanced TNF-α production in a diabetic state may promote the development of diabetic micro- and macroangiopathies through a variety of TNF-α bioactivities. For example, TNF-α increases the permeability of the endothelium through

release of nitric oxide (Satoh *et al.,* 2013) and increases thrombogenesis through plasminogen activator inhibitor-1 (PAI-1) overexpression (Satoh *et al.,* 2013). A role of TNF-α in angiopathies is supported by a report in which cerebral ischemia is reduced by neutralizing serum TNF-α with specific antibody in spontaneously hypertensive rats (Satoh *et al.,* 2013). Furthermore, TNF-α stimulates the expression of adhesion molecules on the endothelial cells (Satoh *et al.,* 2013). The serum levels of free adhesion molecules (vascular cell adhesion molecule-1, VCAM-1) significantly correlate with the intima-media complex thickness (IMT) of the carotid artery (Satoh *et al.,* 2013). These imply that TNF-α accelerates atherosclerosis by inducing the expression of adhesion molecules on the endothelial cells.

2.12.1.3 TNF-α in Diabetic Nephropathy

In 1991, Hasegawa *et al.* reported that glomerular basement membranes from diabetic rats induced significantly greater amounts of TNF-α in cultured peritoneal macrophages than when these cells were incubated with basement membranes from nondiabetic rats (Hasegawa *et al.,* 1991). These new findings were the first to suggest that inflammatory cytokines may participate in the pathogenesis of diabetic nephropathy (Gautam *et al.,* 2017). Today, it is known that among inflammatory cytokines, TNF-α is relevant to the development of diabetic nephropathy, with diverse actions potentially involved in the development of complications. TNF-α is a well known proinflammatory cytokine associated with systemic inflammation (Rivero *et al.,* 2009). TNF-α acts *via* NF-κB signaling and mediates the transcription of various cytokines performing roles in cell survival, proliferation, inflammatory responses, cell adhesion and inflammation (Gautam *et al.,* 2017). A study has shown that there is upregulation of TNF-α expression in glomeruli of diabetic rats (Gautam *et al.,* 2017). TNF-α is well acknowledged to cause damage to renal cells by enhancing renal hypertrophy, hemodynamic

imbalance, albumin permeability (Rivero *et al.,* 2009). The harmful effects of these responses lead to the development of renal disease in patients with T2DM, hence resulting in the progression of renal failure.

2.12.1.4 TNF-α in Diabetic Retinopathy

TNF-α recruits inflammatory cells, which stimulate neovascularization in some circumstances and inhibit it in others. Therefore, the effect of TNF-α in various tissues and disease processes is difficult to predict and must be determined by experimentation (Dell'Omo *et al.,* 2013). TNF-α increases retinal endothelial permeability by down regulating the expression of tight junction proteins and the increased permeability can lead to rupturing of the brain retina barrier (BRB) (Aveleira *et al.,* 2010). TNF-α can also stimulate leukocyte adhesion and induce oxidation and production of reactive oxygen species due to the death of retinal ganglion cells and degeneration of the optic nerve (Dell'Omo *et al.,* 2013). Increased levels of TNF-α have been demonstrated in proliferative retinopathies and in animal models of retinal neovascularization. The increased levels of TNF-α in the presence of VEGF can stimulate the generation of new retinal vessels. TNF -α is also a chemoattractant for leukocytes (Majka *et al.,* 2002). TNF-α has been associated with the pathogenesis of several chronic inflammatory diseases, including type 2 diabetes (Kahn *et al.,* 2006). The intraocular production of TNF-α is higher than that at the systemic level, and both vitreous TNF-α levels and the TNF-α vitreous/serum ratios of diabetic patients were found to be higher than those of the non diabetic patients (Doganay *et al.,* 2002). Moreover, TNF-α is expressed in the endothelial cells and stromal cells of the fibrovascular membranes of diabetic patients with PDR (Goldberg, 2009). Recently, Costagliola *et al.*, have found that the TNF-α concentration in tears increases with the severity of pathology, the levels being lower in nondiabetic patients than in diabetic

subjects, and that the levels were highly correlated with DR severity (Costagliola *et al.*, 2013). The intravitreal injection of an inhibitor of TNF-α leads to a significant reduction in the loss of pericytes and capillary degeneration in diabetic mice (Behl *et al.*, 2009), and TNF-α -deficient mice show decreased vascular changes induced by diabetes (Huang *et al.*, 2011).

2.12.1.5 TNF-α in Diabetic Neuropathy

Diabetic Neuropathy develops as a result of hyperglycaemia-induced local metabolic, enzymatic and microvascular changes. Pro-inflammatory cytokines are produced locally by resident and infiltrating cells. These molecules exhibit pleiotropic effects on homeostasis of glia and neurons in the central, peripheral and autonomic nervous systems. Changes induced by chronic hyperglycaemia lead to dysregulation of these cytokines. It has been demonstrated that endogenous TNF-α production is accelerated in microvascular and neural tissues, which may undergo increased microvascular permeability, hypercoagulability and nerve damage, thus initiating and promoting the development of characteristic lesions of diabetic polyneuropathy (Satoh *et al.*, 2013).

2.12.1.6 TNF-α in Hepatic Complication

Diabetes is known to be a major disorder in which the TNF-*α* production have been implicated through several lines of evidence (Brownlee, 2001). TNF-*α* have been defined as an autocatalytic mechanism that can lead to programmed cell death (apoptosis) (Jones and Lo 2000). Regulation of cell death by apoptosis may be another determinant of liver structure and lesion formation (Koniaris and McKillop 2003). It has become increasingly clear that the process of cell death by apoptosis is a relatively everywhere phenomenon in a variety of cell types, including hepatic cells (Zhao, *et al* 2001). In a research recently it is demonstrated that

the diabetic state induces an increase of TNF-α and of its receptor TNF-R1 in the liver (Ingaramo, *et al.*, 2011).

Following TNF-α binding to the TNF-R1, an adaptor molecule (TRADD, TNF receptor associated DD protein), is recruited by the dead domain (DD) to form the first protein complex, which also includes TRAF2 (Wullaert *et al*, 2006). This complex then dissociates from TNF-R1 and forms a different complex in the cytosol, which binds FADD (Fas associated DD protein), and then recruits caspase-8. Cleavage of procaspase 8 allows the release of activated caspase-8 (Wullaert *et al.*, 2006). Caspase-8 can cleave Bid to form an active fragment, t-Bid (Zhao, *et al.*, 2003). Pro-apoptotic protein Bid promotes initiation of the mitochondrial death pathway with release of cytochrome c, and activation of effectors caspase-3 that ultimately induce apoptosis (Zhao, *et al* 2001).

2.12.2 Interleukin-6 (IL-6): Sources, Gene and Function

IL-6 is a pleiotropic cytokine performing a broad range of biological activities in inflammation, immune regulation, haematopoiesis, and oncogenesis (Mesquida *et al.*, 2017). IL-6 is essential for life, and under physiological conditions its production has tight negative regulatory mechanisms. However, abnormal overproduction of IL-6 has been found responsible for the pathogenesis of various autoimmune, chronic inflammatory diseases, and even cancers (Ho *et al.*, 2015). IL-6 exerts as a promoting cytokine that regulates insulin secretion (Chen *et al.*, 2017) Some studies elucidate that low concentration of IL-6 can induce insulin secretion; while it's high content inhibits insulin generation (Chen *et al.*, 2017) Furthermore, IL-6 gene may contribute to the genetic susceptibility to Type 1 DM (Chen *et al.*, 2017), IL-6 can lead to a permanent damage to pancreatic β cells through promoting B lymphocytes differentiation and activating killer T cells (Chen *et al.*, 2017). IL-6 induces the

synthesis of a variety of other inflammatory mediators in the liver, stimulates Neutrophil production in the bone marrow, and promotes the differentiation of IL-17–producing helper T cells (Latz *et al.,* 2013).

IL-6 is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts, and other cells in response to PAMPs and in response to IL-1 and TNF (Tanaka *et al.,* 2014) IL-6 is a homodimer that belongs to the type 1 cytokine family. Human IL-6 is made up of 212 amino acids, including a 28-amino-acid signal peptide, and its gene has been mapped to chromosome 7p21 (Ferreira-Hermosillo *et al.,* 2015). Although the core protein is approximately 20 kDa, glycosylation accounts for the size of 21–26 kDa of natural IL-6 (Tanaka *et al.,* 2014). The receptor for IL-6 consists of a cytokine-binding polypeptide chain and a signal-transducing subunit (called gp130) that is also the signaling component of receptors for other cytokines. The IL-6 receptor engages a signaling pathway that activates the transcription factor STAT3 (Tanaka *et al.,* 2014)

The distinct functions of IL-6 were studied and given distinct names based on their biological activity. For example, the name B-cell stimulatory factor 2 (BSF-2) was based on the ability to induce differentiation of activated B cells into antibody (Ab)-producing cells (Pestana *et al.,* 2016), the name hepatocyte-stimulating factor (HSF) on the effect of acute phase protein synthesis on hepatocytes, the name hybridoma growth factor (HGF) on the enhancement of growth of fusion cells between plasma cells and myeloma cells, or the name interferon (IFN)-b2 owing to its IFN antiviral activity. When the BSF-2 cDNA was successfully cloned in 1986, (Tanaka *et al.,* 2014) however, it was found that the molecules with different names studied by various groups were in fact identical, resulting in the single name IL-6 (Tanaka *et al.,* 2014)

2.12.2.1 Biological Effect of IL-6 on Inflammation and Immunity

After IL-6 is synthesized in a local lesion in the initial stage of inflammation, it moves to the liver through the bloodstream, followed by the rapid induction of an extensive range of acute phase proteins such as C-reactive protein (CRP), serumamyloidA A (SAA), fibrinogen, haptoglobin, and a1-antichymotrypsin (Tanaka *et al.,* 2014). On the other hand, IL-6 reduces the production of fibronectin, albumin, and transferrin. These biological effects on hepatocytes were at first studied as belonging to Hepatocyte-stimulating factor (HSF). When high-level concentrations of SAA persist for a long time, it leads to a serious complication of several chronic inflammatory diseases through the generation of amyloid A amyloidosis (Tanaka *et al.,* 2014). This results in amyloid fibril deposition, which causes progressive deterioration in various organs. IL-6 is also involved in the regulation of serum iron and zinc levels via control of their transporters. As for serum iron, IL-6 induces hepcidin production, which blocks the action of iron transporter ferroportin 1 on gut and, thus, reduces serum iron levels (Tanaka *et al.,* 2014) This means that the IL-6-hepcidin axis is responsible for hypoferremia and anemia associated with chronic inflammation. IL-6 also enhances zinc importer ZIP14 expression on hepatocytes and so induces hypozincemia seen in inflammation (Tanaka *et al.*, 2014). When IL-6 reaches the bone marrow, it promotes megakaryocyte maturation, thus leading to the release of platelets (Tanaka *et al.,* 2014). These changes in acute phase protein levels and red blood cell and platelet counts are used for the evaluation of inflammatory severity in routine clinical laboratory examinations.

2.12.2.2 IL-6 and Disease

An immediate and transient expression of IL-6 is generated in response to environmental stress factors such as infections and tissue injuries. This expression triggers an alarm signal and activates host defense mechanisms against stress. Removal of the source of stress from the

host is followed by cessation of IL-6-mediated activation of the signal-transduction cascade by negative regulatory systems such as ligand-induced internalization and degradation of gp130 and recruitment of SOCS (Naka *et al.* 2007), as well as degradation of IL-6 mRNA by regnase-1 leading to termination of IL-6 production. However, dysregulated and persistent IL-6 production of mostly unknown etiology, one of which may be the unbalance between Arid5a and regnase-1, in certain cell populations leads to the development of various diseases. This association of IL-6 with disease development was first shown in a case of cardiac myxoma. The culture of fluid obtained from the myxoma tissues of a patient who presented with fever, polyarthritis with positivity for antinuclear factor, elevated CRP level, and hypergammaglobulinemia, contained a large quantity of IL-6, which suggested that IL-6 may contribute to chronic inflammation and autoimmunity (Tanaka *et al.,* 2014). Subsequent studies have shown that dysregulation of IL-6 production occurs in the synovial cells of RA (Tanaka *et al.,* 2014), swollen lymph nodes of Castleman's disease (Yoshizaki *et al.* 1989), myeloma cells (Kimura and Kishimoto, 2010), and peripheral blood cells or involved tissues in various other autoimmune and chronic inflammatory diseases and even malignant cells in cancers (Nishimoto *et al.* 2008). Moreover, the pathological role of IL-6 in disease development has been shown in numerous animal models of diseases as well as the fact that IL-6 blockade by means of gene knockout or administration of anti-IL-6 or anti-IL-6R Ab can result in the preventive or therapeutic suppression of disease development. For example, IL-6 blockade resulted in a noticeable reduction in susceptibility to Castleman's disease–like symptomsin IL-6 transgenic mice (Katsume *et al.,* 2002). Similar effects were observed in models of RA (Fujimoto *et al.,* 2008), systemic lupus erythematosus (Mihara *et al.,* 2012), systemic sclerosis (Kitaba *et al.,* 2012), inflammatory myopathies (Okiyama *et al.,* 2009),

experimental autoimmune uveoretinitis (Haruta *et al.*, 2011), experimental autoimmune encephalomyelitis (Serada *et al.*, 2008), and many other diseases.

2.12.2.3 Serum IL-6 Levels in T1D

Several studies investigated the IL-6 levels in T1D. Alnek *et al*., (2015) observed that IL-6 decreased with age and tended to be lower in spring compared to summer, but no difference was observed between T1D and control groups. IL-6 levels were also similar in young T1D patients when compared to controls (Heier *et al.*, 2015). However, another study including young subjects observed significant higher IL-6 levels in T1D group (Talaat *et al.*, 2016). Bradshaw *et al*. (2009) also found marked increase IL-6 secreted by monocytes isolated from the blood cells of recent-onset T1D patients as compared to healthy subjects.

Pestana *et al*. (Pestana *et al.*, 2016) observed higher urinary IL-6 levels in T1D with micro- and macroalbuminuria when compared to T1D with normoalbuminuria or controls, but no difference was observed in plasma levels between the groups. Domingueti *et al*. (2016) showed higher IL-6 plasma levels in T1D with chronic kidney disease (CKD) when compared to patients without this complication. These results suggest that IL-6 may vary with the progression of nephropathy in T1D patients, and intrinsic renal cells are able to synthesize pro-inflammatory cytokines, but Hundhausen *et al*. (2016) did not consider the status of kidney disease, a limitation of this study.

Hundhausen *et al*. (2016) observed no difference in IL-6 mRNA between T1D and controls. Contrary, Ururahy *et al*. (2012) observed higher IL-6 mRNA levels in peripheral blood leukocytes from T1D when compared to control. In the former study, the authors also showed higher IL-6 expression in T1D patients with poor glycemic control (according to the values of glycated hemoglobin—HBA1c) when compared to control group. However, Hundhausen *et*

al., (2016) did not observed correlation between IL6-induced pSTAT3 signaling and HBA1c or blood glucose levels in T1D patients.

Kiec-Wilk *et al*., (2016) in a multiple linear regression analysis observed that the number of hypoglycemic episodes per 7 days was an independent predictor of high levels of IL-6. In another study, Gogitidze *et al*., (2010) evaluated T1D patients during either a 2-h hyperinsulinemic euglycemic or hypoglycemic clamp, where it was observed that IL-6 plasma levels were significantly increased during the 2 h of hyperinsulinemic hypoglycemia as compared with euglycemia subjects. These results suggest that acute hypoglycemia can result in activation of proinflammatory IL-6 in T1D patients, but this variable was not considered by Hundhausen *et al*., (2016), which could affect their results.

This study showed that dysregulation of IL-6 may be a marker of early disease. However, another work where biomarkers were measured at four time points over 20 years in 886 DCCT/EDIC participants with T1D (Hunt *et al.*, 2015) showed that IL-6 levels increased across the time, contrary observed by Hundhausen *et al*., (2016). Together, these data suggest that IL-6 signaling can change with the disease progression in T1D subjects and can be a bias in the studies.

The last aspect is that Hundhausen *et al*., (2016) study falls in investigating the IL-6 role in peripheral blood, but not in islet-specific T cells or pancreatic lymph nodes. However, we consider important results, which show that immune dysfunction play key role in the pathogenesis of T1D and open new perspectives in order to consider the IL-6 as a therapeutic target in the disease intervention.

2.12.2.4 Serum IL-6 Levels in Diabetes Nephropathy

Sekizuka *et al.* (1991) reported that serum levels of IL-6 were significantly higher in patients with type 2 diabetic nephropathy than the levels observed in diabetic patients without nephropathy, which suggests that this cytokine may play a role in the pathogenesis of diabetic nephropathy. After that report, Suzuki *et al.* (1991) analyzed kidney biopsies in patients with diabetic nephropathy by high-resolution *in situ* hybridization. These authors observed that cells infiltrating the mesangium, interstitium, and tubules were positive for mRNA encoding IL-6. Furthermore, they found a relationship between the severity of diabetic glomerulopathy (mesangial expansion) and expression of IL-6 mRNA in glomerular cells (mesangial cells and podocytes), which indicated that IL-6 may affect the dynamics of extracellular matrix surrounding those cells. More recent studies in type 2 diabetic patients demonstrate a significant association between IL-6 and glomerular basement membrane thickening, a crucial lesion of diabetic nephropathy and a strong predictor of renal progression (Dalla *et al.,* 2005). Recent studies show a significant overexpression of IL-6 in the diabetic rat kidneys, with an increase in the levels of mRNA encoding IL-6 in the renal cortex being directly associated with an elevation in its urinary excretion (Navarro *et al.*, 2006). Importantly, wet kidney weight, an accurate index of renal hypertrophy and one of the earliest renal changes during diabetes (Thomson *et al.,* 2008), is increased in diabetic rats and associated with renal mRNA expression of IL-6 and urinary excretion of this cytokine (Navarro *et al.*, 2006). Moreover, a direct correlation was observed between urinary levels and renal expression of IL-6 with urinary albumin excretion (Nosadini *et al.,* 2000). These results support previous findings on the development of renal injury mediated by IL-6, which has been related to alterations in

endothelial permeability, induction of mesangial cell proliferation, and increased fibronectin expression (Suzuki *et al.*, 1995).

2.12.3 Interleukin-10 (IL-10): Sources, Gene and Function

The anti-inflammatory Th2 cytokine interleukin-10 (IL-10) was discovered by Fiorentino and colleagues in 1989 for its ability to inhibit the synthesis of IL-2 and interferon-γ (IFN-γ) by Th1 cells (Sinuani *et al.,* 2013). To date, the IL-10 cytokine family includes nine members produced by cells, IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B and IL-29, and four viral homologs. IL-10 is produced by several T-cell subpopulations, such as Th2 and T-regulatory cells (Tregs), NK cells, and a variety of cell types, including macrophages, dendritic cells and B cells. In the kidneys, IL-10 is secreted primarily by the mesangial and endothelial cells. The viral homologs of IL-10 can be produced by Epstein-Barr virus, cytomegalovirus, ORF virus and Herpes type 2 viruses (Sabat, 2010). The gene encoding human IL-10 (5.1 kb pairs) is located on chromosome 1 and comprises five exons. The IL-10 promoter region contains several single nucleotide polymorphisms (SNPs) that influence IL-10 expression and function (Sabat *et al.,* 2010) -1082G/A SNP of the IL-10 gene, the -1082G/A, -819C/T, and -592C/A SNPs of the IL-10 promoter, while the -1087G/A, -824C/T, -597C/A SNPs, The -1082 SNP of the IL-10 gene are some of the examples of these SNPs (Romero, 2009).

Human IL-10 protein is a 35 kDa homodimer that is assembled from two non-covalently bound monomers. IL-10 acts through a specific receptor complex that consists of two subunits: IL-10R1 and IL-10R2. Binding of IL-10 to its receptor is a multistep process in which IL-10 initially binds to IL-10R1; the IL-10/IL-10R1 complex then binds to IL-10R2. Formation of the IL-10/IL-10R1 complex leads to modification of the cytokine's

conformation, enabling presentation of the binding site to IL-10R2 (Yoon *et al.,* 2016). While the IL-10R1 subunit is highly specific for initiating IL-10 effectors functions, the IL-10R2 subunit might bind other ligands, such as TNF-α and IFN-γ. Moreover, IL-10R2 is widely present in cells that do not express IL-10R1 and are thus unresponsive to IL-10 (Yoon *et al.,* 2010). Activation of the IL-10 receptor complex initiates a cascade of intracellular events. The first step involves activation of members of the Janus kinase family, Jak1 and Tyk2. Activation of Jak1 is related to IL-10R1, whereas Tyk2 binds to the IL-10R2 subunit. This step is followed by activation of members of the signal transducer and activator of transcription (STAT) family. STAT1, STAT3, and STAT5 molecules in their homo- or hetero-dimeric forms enter the nucleus and bind to STAT-binding elements (SBE) in the promoters of various IL-10-responsive genes. These events enhance the transcription of anti-apoptotic genes and genes associated with cell cycle progression, such as Bcl, Cyclin D1, Cyclin D2, Cyclin D3, Cyclin A, c-Myc, p19Ink and others (Finbloom and Winestock, 2015) IL-10 also induces activation of phosphatidylinositol 3-kinase and its downstream targets: p70 S6-kinase and Akt/protein kinase B. This pathway is required for the proliferative effect of IL-10 (Strle *et al.,* 2012). In addition, the IL-10 signaling cascade often interacts with other intracellular pathways. For example, IL-10 modulates the translation of TNF-α mRNA *via* the activation of p38MAPK, thereby increasing TNF-α production by mononuclear cells (Kontoyiannis *et al.,* 2011). In human monocytes, IL-10 up regulates the expression and activity of the general cell protective stress protein heme oxygenase-1 (Jung *et al.,* 2014). The complexity of IL-10 activities defines a broad spectrum of the properties of IL-10. The principal function of IL-10 is to control inflammation and instruct adaptive immune responses. IL-10 inhibits the activation and differentiation of antigen-presenting cells, such as dendritic cells and macrophages. IL-10 down-regulates the expression of major histocompatibility complex class

II and co-stimulatory B7-1/B7-2 molecules and decreases the secretion of pro-inflammatory cytokines, such as TNF-α, IL-12, IL-1β, and others. IL-10 also regulates the growth and/or differentiation of B cells, NK cells, cytotoxic T and T helper cells, mast cells, keratinocytes, and endothelial and mesangial cells (Hedrich and Bream, 2010; Sabat *et al.,* 2010). IL-10 protects the host from a variety of bacterial, parasitic, viral or fungal pathogens. Moreover, IL-10 has clear immunomodulatory properties (Saraiva and O'Garra, 2010)

2.13 Medicinal Plants

Despite the presence of many methods, approaches and therapies, the management of DM remains unsatisfactory (Singh *et al.,* 2011). The increasing prevalence of diabetes in both developed and developing countries has challenged scientists to further explore various therapeutic agents that can be used in the treatment and management of disease conditions possibly more efficiently (Gupta *et al.,* 2012). Antidiabetic drugs are expensive and not affordable for low socioeconomic individuals, thus the need to explore other inexpensive herbal sources for the treatment of diabetes (Singh *et al.,* 2011; Amod *et al.,* 2012).

Plants have played vital roles in the maintenance of human health and the improvement of the quality of life of people, serving as important components for medicines, beverages and seasonings (Elder, 2004). Interestingly, many indigenous plants contain Phytochemicals that exhibit both anti-diabetic and anti-inflammatory properties (Sánchez *et al.,* 2006; Dièye *et al.,* 2008).

2.13.1 *Vitellaria paradoxa*

The rapid discovery of various medicinal plants and natural products with anti-diabetic and hypoglycemic potentials has provided a remarkable intervention in the history of various diseases including diabetes (Elizabeth *et al.,* 2017). Ayurvedic medicine uses plants to

promote self-healing, to attain good health, and achieve longevity. *Vitellaria paradoxa* can provide therapeutic constituent to prevent and treat many disease conditions (Ndukwe *et al.*, 2007). *Vitellaria paradoxa* is plant of African origin, grows naturally in the wild in the dry savannah belt of West Africa from Senegal in the west to Sudan in the east, and onto the foot hills highland. It occur in 19 countries across African continent namely: Benin, Burkina Faso, Cameroon, Central Africa, Cameroon, Central Africa, Chad, Ethiopia, Ghana, Guinea Bissau, Cote D'ivore, Mali, Nigeria, Senegal, Sierra Leone, Sudan, Togo, Uganda, Congo and Guinea (El-Mahmood *et al.,* 2008).

The tree belongs to the Sapotaceae family and was formerly classified in the Genus *Butyrospermum*, meaning "butter seed". The species name Parkii honors Scottish explorer Mango Park who learned of tree while explaining Senegal. The tree is now classified using the binomial name *Vitellaria paradoxa* (Adamu *et al.,* 2013).

The common name is "Shiyiri" or Shisu in the Bamana language of Mali. This is the origin of the English word, and is correctly pronounced "Shea". The tree is called Ghariti in the Wolof language of Senegal which is the origin of French name (and its butter), "Karife" (Ayankunle *et al.,* 2012). The tree is also called "Kade" in Hausa language. *Vitellaria paradoxa* has been in existence for centuries, dating as far back as early 2000 BC and it is a very valuable plant because of its vast folk medicinal properties.

Figure 2.5: *Vitellaria paradoxa* tree; identified by Botanist in the Herbarium of Faculty of Pharmaceutical Sciences of the Usmanu Danfodiyo University, Sokoto, Nigeria with a verification number PCG/UDUS/SAPO/0001

2.13.1.1 Phytochemicals in *Vitellaria paradoxa*

Studies on the phytochemical properties of crude extracts from the root stem bark and leaves of *Vitellaria paradoxa* by Ndukwe *et al.,* revealed the presence of carbohydrates, simple reducing sugars, soluble starch, saponins, alkaloids and tannins in all the plant parts studied. He reported the presence of saponins, tannins, alkaloids in all the plant parts studied (root, stem bark and leaves) calls for an in-depth study on the plant (Ndukwe *et al.,* 2007). Ukpanukpong *et al.* (2016) on his studies about Preliminary phytochemical screening on *Vitellaria paradoxa Nut,* revealed the presence of flavonoids, saponins, cardiac glycosides, terpenoids and steroids in the hexane extract. While tannins, anthraquinones, phlobatannins and cardenolides were absent in both two extracts. Polyphenolic compounds such as flavonoids, tannins and phenolic acids commonly found in plants which have been reported to have multiple biological effect including analgesic property (Ukpanukpong *et al., 2016*).This is supported by another literature work (Kanwaljit *et al.,* 2012).Terpenoid which is qualitatively present and served as heart-friendly phytochemical constituent which helps to reduce diastolic blood pressure and lowers the sugar level in the blood (Kanwaljit *et al.,* 2012). Flavonoids, glycosides and cardiac glycosides found in the extracts are suggestive of their antioxidant property. Flavonoids glycosides are reported to be antioxidants and used as anti-inflammatory in the treatment of capillary fragility (Akihisa *et al.,* 2010). Their presence in the extracts is a probable indication of the potent antioxidant and membrane-stabilizing properties of the fats sample.

2.13.1.2 Anti-bacterial activity of *Vitellaria paradoxa*

The anti-bacterial activity of extracts from root, stem and leaves of *Vitellaria paradoxa* were investigated by Ndukwe *et al.* (2007). The antimicrobial screening of the crude methanol

extract was carried out *in vitro* on the following clinical isolates; *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pnuemoniae and Salmonella typhi* showed that the crude methanol extracts had wider range of activity on these organisms than the petroleum ether extracts (Ndukwe *et al.,* 2007). The crude stem extracts inhibited the growth of *P. aeruginosa, K. pneumoniae, B. cereus and S. typhi* at concentration of 50mg/ml while the leaf had a minimal inhibition concentration (MIC) of 70 mg/ml on *S. aureus, E. coli and S. typhi* (Ndukwe *et al.,* 2007). The root had an MIC of 60 mg/ml on *S. aureus, E. coli, P. aeruginosa, K. pneumoniae* and *S. typhi* (Ndukwe *et al.,* 2007). Fodouop *et al.,* (2017) recently reported the *invivo* effect of *Vitellaria paradoxa* aqueous leaf administration on Salmonella typhimurium-infected rats. There finding support the ethnomedicinal use of *Vitellaria paradoxa,* and suggest that its leave can be used in the management antibacterial phytomedicine (Fodouop *et al.,* 2017).

2.13.1.3 Hepatotoxicity activity of *Vitellaria paradoxa*

The Hepatotoxicity effects of *Vitellaria paradoxa* stem bark extract has been reported by Mainasara *et al.* (2016), From present study on acute toxicity, no death or behavioural changes was recorded in all the groups within 24 hours and for up to 14 days. This indicates that the oral acute toxicity (LD50) of the *Vitellaria paradoxa* stem bark is greater than 5000 mg/kg (Mainasara *et al.,* 2016). Hepatotoxicity activity was studied and there was no significant difference in level of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein and albumin levels of experimental rats and the control rat with the exception of group 6 in which the albumin level was significantly higher than the control group but the values were within the reference range. They concluded that *Vitellaria*

paradoxa stem bark extract produced no harmful effect on liver function in Wistar rats (Mainasara *et al.*, 2016)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Site

The extraction and phytochemical screening of VPSBE was carried out in the pharmacognosy department at faculty of Pharmacy, Usmanu Danfodiyo University, Sokoto and sample was obtained and analyzed at Veterinary Research Laboratory Centre in the faculty of Veterinary, Usmanu Danfodiyo University, Sokoto; Sokoto is located at the extreme part of North-Western Nigeria between longitude 3^0 and 7^0 east and between latitude10^0 and 14^0 north of the equator. It shares borders with Niger Republic to the North, Kebbi State to the South-West and Zamfara State to the East (C-GIDD, 2008). The State covers a total land area of about 32,000 square kilometers and a population of 5,125,370 million based on 2017 projection (NPC, 2006). Sokoto State has semi-arid climate and vegetation is largely Sudan Savannah with an annual rainfall between 500-1300mm and temperature ranges between 15^0C and over 40^0C during warm days (C-GIDD, 2008).

3.2 Chemicals

All the reagents used for the study were of analytical grade. Alloxan monohydrate, methanol and metformin were purchased from Lab Tech Chemicals, India, Kits for the glucose, were obtained from Randox Laboratories, Switzerland. ELISA reagents (TNF-α, IL-6, IL-10 with catalog no.: E-EL-R0019, E-EL-R0015 and E-EL-R0013 respectively) was purchase from E-Lab Science Technology, USA.

3.3 Animals

Thirty (30) Adult healthy Wistar rats (weight 130–220 g) of both sexes were purchased from the Faculty of Pharmaceutical Sciences of the Ahmadu Bello University, Zaria, Nigeria. They were allowed to acclimate at the animal house of Faculty of Pharmaceutical Sciences of the

Usmanu Danfodiyo University, Sokoto, Nigeria for a week before commencement of the experiment. Rats were kept in their own cages at constant room temperature (21-28^{o}C) under a normal 12-h light/dark cycle with free access to food and water. These conditions were maintained constant throughout the experiments. The animals were housed according to regulations for the Welfare of experimented animals.

3.4 Plant

The *Vitellaria paradoxa* stem bark used in this study was collected from a public garden of Yargeda forest in Bakura local government of Zamfara State on 25th August, 2017 (during the rainy season) and identified by botanists in the herbarium of Faculty of Pharmaceutical Sciences of the Usmanu Danfodiyo University, Sokoto, Nigeria with a verification number PCG/UDUS/SAPO/0001

3.5 Methanol Preparation of VPSBE

Vitellaria paradoxa stem bark methanol extract was prepared by maceration method. 300g of *Vitellaria paradoxa* stem bark powder was macerated in 1500ml methanol for 72hours with continuous shaking kept at room temperature (21-28^{o}C). Supernatant was filtered using Whatman number 1 filter paper and then the filtrate was concentrated in an oven at 48°C to obtain 32 g brown powder extract. The dried crude extract was stored in a refrigerator at low temperature (4°C) in sterile plastic bottles, at the Faculty of Pharmaceutical Sciences, UDUS, until required for use.

3.6 Phytochemical Screening

Qualitative test for the phytochemical screening were carried out for the VPSBE as per the standard methods described by Prashant *et al.* (2011).

3.6.1 Test for Flavonoids

Three milliliter (3ml) of VPSBE was treated with 1ml of 10% sodium hydroxide solution, intense yellow color was formed which became colorless on addition of dilute hydrochloric acid; this indicated the presence of flavonoids

3.6.2 Test for Tannins

Five percent (5%) Ferric chloride solution was added drop by drop to a 2ml of VPSBE, white precipitate was formed, which indicated the presence of tannins.

3.6.3 Test for Saponins

A mixture of 5ml of VPSBE and 5ml of water in a test tube was shakes vigorously. The whole tube was filled up with foam; the persistent of foam for several minutes indicated the presence of saponins.

3.6.4 Test for Glycosides

Two and half milliliter (2.5ml) of 50% H_2SO_4 was added into a test tube containing 5ml of VPSBE. The mixture was heated in boiling water for 15 minutes, which was allowed to cool. It was then neutralized with 10% NaOH; 5ml of Fehling's solution was added and the mixture was boiled again. A brick-red precipitate was formed which indicated the presence of glycosides.

3.6.5 Test for Alkaloids

The VPSBE was dissolve individually in dilute hydrochloric acid and filtered. The filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). A yellow colored precipitate was observed which indicates the presence of alkaloids

3.6.6 Test for Cardiac glycosides

One milliliter (1ml) of VPSBE was added into a test tube contained 2ml of 3.5% ferric chloride solution and was allowed to stand for one minute. Then 1ml of concentration H_2SO_4

was poured carefully down the wall of the tube so as to form a lower layer. No reddish brown ring was formed at the interface, which indicates the absence of cardiac glycosides.

3.6.7 Test for Steroids (Salkowski)

Half milliliter (0.5ml) of VPSBE was dissolved in 2ml of chloroform. 2ml of sulphuric acid was carefully added, a lower layer was formed. A reddish brown color at the interface was observed which indicated the presence of steroidal ring.

3.6.8 Test for Saponins glysides

Two and half milliliter (2.5ml) of VPSBE was added into a test tube containing 2.5ml of Fehling's solution A and B. A bluish green precipitate was formed, which indicated the presence of saponins glycosides

3.6.9 Test for Balsams

The VPSBE was mixed with equal volume of 90% ethanol. 2 drops of alcoholic ferric chloride solution was added to the mixture. A dark green colour was formed which indicated the presence of balsams.

3.6.10 Test for Anthraquines

Half gram (0.5g) of VPSBE was sharked with 10ml benzene and 5ml of 10% ammonia solution was also added. The mixture was shaking again and the formation of a pink colour in lower phase was observed which indicated the presence of anthraquinones.

3.6.11 Test for Volatile oils

One milliliter (1ml) of VPSBE was mixed with dil. HCl, a white precipitate was formed which indicated the presence of volatile oils`

3.7 Lethal Dose (LD_{50})

Lethal Dose was performed in accordance with the procedure outlined by Lorke (1983). The method has 2 two phase, 1and 2 respectively. Sixteen (16) Wistar rats of both sexes were used for this study. For the phase I, twelve (12) rats were randomly divided into four (4) groups comprising three (3) animals each with the first group as control. The extract was administered to rats in groups 2-4 in single oral doses of 10mg/kg, 100mg/kg and 1000mg/kg body weight respectively, using oral cannula. The control group (group 1) received an equal volume of distilled water. For the phase II, four (4) rats were randomly divided into four (4) groups comprising three (3) animals each with the first group as control. The extract was administered to rats in groups 2-4 in single oral doses of 1600mg/kg, 2900mg/kg and 5000mg/kg body weight respectively, using oral cannula. The control group (group 1) received an equal volume of distilled water. Observation of toxic symptoms were made and recorded within the first hour, four hours, twelve hours and subsequently for 24 hours after the administration of the extract. Behavioral parameters and mortality were also monitored.

3.8 Induction of Diabetes

Experimental Wistar rats were made diabetic by intraperitoneal injection of multiple low dose of 80mg/kg of the body weight of alloxan monohydrate for three (3) consecutive days in cold citrate buffer (pH 4.5) (Wali *et al.*, 2013). After a week from last injection, the animals were observed for polydipsia, polyuria and polyphagia as well as general reduction of body weight by physical examination using diabetic cage. The animals were then allowed to fast overnight and the fasting blood glucose was estimated using a commercial glucose kit. Only rats that had

fasting blood glucose level of greater or equals to 11.0mmol/l (200mg/dl) were included in the study.

3.9 Experimental Designs

Thirty (30) Wistar rats were randomly allotted to five experimental groups (n = 6 per group) as follows: normal healthy control rats (non-DM group) which was fed orally with physiological saline; diabetic rats (DM group) which was fed orally with physiological saline; diabetic rats which was fed orally with 100mg/kg body weight/day of VPSBE (DM + 100VPSBE group); diabetic rats which was fed orally with 200mg/kg body weight/day of VPSBE (DM + 200VPSBE group); diabetic rats which was fed orally with 200mg/kg body weight/day of Metformin (DM + 200Metformin group). These treatments were continued to the end of the study (for 4 weeks).

TABLE 3.1: Experimental Designs

Groups	Treatment	No of animals
1 (Non-DM)	Distilled water	6
2 (DM)	Distilled water	6
3 (DM + VPSBE)	100mg/kg of extract	6
4 (DM +VPSBE)	200mg/kg of extract	6
5 (DM + Metformin)	200mg/kg of metformin	6

Before treatment start, rats adapted throughout the first seven days. During adaptation period, all rats were treated with the same standard diet. After seven days, treatment rats were given treatment based on five (5) selected groups for 28 days. Drinking was given ad libitum. Feed

for a rat was given more or less 30g per day. Residual feed was measured every day for checking how much treatment feed consumed every day. Rats were weighted once a week.

These treatments were continued to the end of the study (for 4 weeks). Blood glucose level and body weights were recorded at weekly intervals. At the end of the 4-weeks period, animals were fasted overnight and euthanized humanely. Blood sample was obtained through cardiac puncture and divided into fluoride oxalate containers, ethylenediamine tetraacetic acid (EDTA) containers and plane containers.

3.10 Laboratory Analysis

3.10.1 Determination of Blood glucose level

Glucose concentrations were measured with the Ames One Touch glucometer (One-Touch Basic; Lifescan, Johnson and Johnson, New Brunswick, NJ) in rat tail vein blood.

3.10.1.1 Principle

Blood Glucose Test Strips use biosensor technology. The sample is applied to the target area, covering both the working electrode and the reference electrode. This area is coated with enzymes that react in the presence of glucose to make a small electric current. The size of the current generated is proportional to the amount of glucose present in the blood drop.

3.10.1.2 Procedure

The tail was washed with warm soapy water and dried thoroughly, the test strip was inserted into the meter in the direction of the arrow and the meter turned on, preparing to test appeared. A drop of blood was obtained by pressing firmly against the side of the tail; the blood flow was assisted by gently squeezed of the tail. The end of the strip was touched to the blood drop. Analyzing appeared and the test result was displayed on the screen.

3.10.1.3 Result

The blood glucose level was displayed in mg/dl

3.10.2 Determination of Serum IL-6

Serum IL-6 was determined according to the method of Chard (1990) using IL-6 ELISA kit procured from E-lab. Science Technology (USA)

3.10.2.1 Principle

This uses Sandwich-ELISA as the method. The micro ELISA plate has been pre-coated with an antibody specific to IL-6. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for IL-6 and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away, and the substrate solution is added to each well. Only those wells that contain IL-6, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of IL-6.

3.10.2.2 Procedure

One hundred microliter (100μL) of Standard, Blank, and Sample were added per well. The blank were added with Reference Standard & Sample diluents. Solutions were added to the bottom of micro ELISA plate well, avoided inside wall touching and foaming as possible. These were mixed gently and covered the plate with sealer provided; these were incubated for

90 minutes at 37^0C. The liquid were removed from each well, after incubation and immediately without washing, One hundred microliter (100µL) of Biotinylated Detection Ab working solution were added to each well. The Plate was covered with sealer and mixed thoroughly by gentle tapped; these were Incubate for 1 hour at 37°C. Each wells was aspirated and washed repeatedly three (3) times with wash buffer. The plates were inverted and hit against thick clean absorbent paper. One hundred microliter (100µL) of HRP Conjugate working solution were added to each wells and the plate were covered with sealer; these were incubated for 30 minutes at 37°C. After incubation the plates were washed five times. 90µL of Substrate Solution was added to each well which was covered with a new plate sealer and Incubated for about 15 minutes at 37°C. The reaction was terminated with addition of 50µL of Stop Solution to each well. The optical density (OD value) was determined from each well at once, using a micro-plate reader set to 450 nm.

3.10.2.3 Result

The quantity of IL-6 in the test sample was interpolated from the standard curve constructed from the standards, and corrected for serum dilution.

3.10.3 Determination of Serum IL-10

3.10.3.1 Principle

This uses Sandwich-ELISA as the method. The micro ELISA plate has been pre-coated with an antibody specific to IL-10. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for IL-10 and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away, and the substrate

solution is added to each well. Only those wells that contain IL-10, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of IL-10.

3.10.3.2 Procedure

One hundred microliter (100µL) of Standard, Blank, and Sample were added per well. The blank were added with Reference Standard & Sample diluents. Solutions were added to the bottom of micro ELISA plate well, avoided inside wall touching and foaming as possible. These were mixed gently and covered the plate with sealer provided; these were incubated for 90 minutes at 37^0C. The liquid were removed from each well, after incubation and immediately without washing, One hundred microliter (100µL) of Biotinylated Detection Ab working solution were added to each well. The Plate was covered with sealer and mixed thoroughly by gentle tapped; these were Incubate for 1 hour at 37°C. Each wells was aspirated and washed repeatedly three (3) times with wash buffer. The plates were inverted and hit against thick clean absorbent paper. One hundred microliter (100µL) of HRP Conjugate working solution were added to each wells and the plate were covered with sealer; these were incubated for 30 minutes at 37°C. After incubation the plates were washed five times. 90µL of Substrate Solution was added to each well which was covered with a new plate sealer and Incubated for about 15 minutes at 37°C. The reaction was terminated with addition of 50µL of Stop Solution to each well. The optical density (OD value) was determined from each well at once, using a micro-plate reader set to 450 nm.

3.10.3.3 Result

The quantity of IL-10 in the test sample was interpolated from the standard curve constructed from the standards, and corrected for serum dilution.

3.10.4 Determination of Serum Tumour Necrosis factor-α

3.10.4.1 Principle

This uses Sandwich-ELISA as the method. The micro ELISA plate has been pre-coated with an antibody specific to TNF-α. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for TNF-α and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away, and the substrate solution is added to each well. Only those wells that contain TNF-α, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of TNF-α.

3.10.4.2 Procedure

One hundred microlitre (100µL) of Standard, Blank, and Sample were added per well. The blank were added with Reference Standard & Sample diluents. Solutions were added to the bottom of micro ELISA plate well, avoided inside wall touching and foaming as possible. These were mixed gently and covered the plate with sealer provided; these were incubated for 90 minutes at 37^0C. The liquid were removed from each well, after incubation and immediately without washing, One hundred microlitre (100µL) of Biotinylated Detection Ab

working solution were added to each well. The Plate was covered with sealer and mixed thoroughly by gentle tapped; these were Incubate for 1 hour at 37°C. Each wells was aspirated and washed repeatedly three (3) times with wash buffer. The plates were inverted and hit against thick clean absorbent paper. One hundred microliter (100µL) of HRP Conjugate working solution were added to each wells and the plate were covered with sealer; these were incubated for 30 minutes at 37°C. After incubation the plates were washed five times. 90µL of Substrate Solution was added to each well which was covered with a new plate sealer and Incubated for about 15 minutes at 37°C. The reaction was terminated with addition of 50µL of Stop Solution to each well. The optical density (OD value) was determined from each well at once, using a micro-plate reader set to 450 nm.

3.10.4.3 Result

The quantity of tumuor necrosis factor-α µL in the test sample was interpolated from the standard curve constructed from the standards, and corrected for serum dilution.

3.10.5 Neutrophils to Lymphocyte Ratio (NLR) Calculation

Anticoagulated whole blood from laboratory animal were processed and measured using an automated complete blood cell counter (model XT 2000i; sysmex, Kobe, Japan), which simultaneously provided values for total white blood cell count, absolute neutrophils count and absolute lymphocyte count. NLR was calculated as a simple ratio absolute neutrophils count to absolute lymphocyte count.

3.11 Statistical Analysis

All experiments were carried out in duplicate. One way analysis of variance (ANOVA) was performed and Tukey post hoc test was used for multiple comparisons of mean. Statistical

analyses were performed using the Graphite InStat 3.0 programme. The results are expressed as mean ± standard deviation (SD). Differences of $p < 0.05$ were considered significant.

3.12 Quality Control

To ensure the reliability of the results, the quality control measures taken include the following:

1. Standard Operating Procedure (SOP) for sample collection and processing were followed.
2. Immediate separation of serum and keeping it at -20 0C until the time of analysis were ensured.
3. All the reagents used for the work were kept at the appropriate temperature base on the manufacturer guideline.
4. All the analyses were carried out strictly based on the manufacture instruction.
5. During the analysis, the sample and reagents used were allowed to thaw at room temperature before the analysis and multiple thawing was avoided.
6. During the analysis, correct pipetting procedure were ensured and in order to avoid prolong pipetting time multi-channel pipettes were used.
7. During the analysis and in the process of incubation, exposure of reagents to strong light was avoided.
8. During the analysis correct incubation time and wavelength were ensured for accurate results.
9. Quality control sera were run along with the test to ensure reliability of results.

CHAPTER FOUR

4.0 RESULTS OF THE ANALYSIS

The physical properties of crude extracts of VPSBE were 10.8% of soft gum. The absolute methanol extraction produced red brown crude extract.

Table 4.1 shows the phytochemical screening of VPSBE. The phytochemical screening test revealed the presence of the entire eleven (11) phytochemical compounds tested except that of cardiac glycoside (Table 4.1), and the intensity of the colour changed was more pronounced in Saponins, Glycosides, Alkaloids, Steroid, and Volatile Oil. Flavonoids and Tannins revealed moderately colour intensity, while Saponins-glycoside, Balsams and Anthraquines has the least intensity colour.

Table 4.2 and 4.3 shows phase 1 and 2 of acute toxicity study (LD_{50}). The behavioural changes observed for gathering, locomotion, reaction to noise, state of the tail, consistency of the excrement and the mortality (within 24h) after the administration of the crude VPSBE at various doses are summarized in Table 4.2 and 4.3. No behavioural changes were observed in mice receiving the extract at doses $\leq$ 1000mg/kg as compared to the controls. Gathering, locomotion, and state of tail were not affected. Furthermore, there was no decrease in the reaction to noise and reaction to pinch at doses $\leq$ 5000mg/kg. The animals in all the groups had normal tail (flexible). There was no mortality at doses $\leq$ 5000mg/kg, therefore the LD_{50} value was greater than 5000mg/kg.

Table 4.4 shows the effect of VPSBE on total food intake among the diabetic treated group. The mean value for the total food intake in DM group was high, that is significantly increase compared to non-DM group ($p = 0.001$). Treating diabetes rats with 100 or 200mg of VPSBE

and 200mg of Metformin/kg body weight in their respective groups, significantly (P= 0.001, P= 0.001, P= 0.001 respectively) decreased the total food intake compared to DM group. Treating diabetic rats with the higher dose of VPSBE in DM + 200VPSBE and standard drug Metformin in DM +200Metformin groups was significantly (P= 0.001, P= 0.001 respectively) more effective than that of low dose of VPSBE in DM + 100VPSBE group. Furthermore treating diabetic rats with the higher dose of VPSBE in DM + 200VPSBE group was significantly (P= 0.04) more effective than that of standard drug Metformin in DM +200Metformin group.

Table 4.4 shows also the effect of VPSBE on body weight gain among the diabetics treated groups. The mean value for the body weight gain in DM group was low, that is, significantly decreased compared with that of the non-DM group (P= 0.000). Treating these diabetic rats with 100 or 200mg of VPSBE and 200mg of Metformin per kilogram body weight in their respectively groups, significantly (P= 0.001, P= 0.001, P= 0.001 respectively) increased the body weight gain values compared to DM control group. However, treating diabetic rats with the higher dose of VPSBE in DM + 200VPSBE group and standard drug Metformin in DM +200 Metformin group was not differ significantly (P= 0.09, P= 0.12 respectively) more effective than that of lower dose of VPSBE in DM +100 VPSBE. Furthermore treating diabetic rats with the higher dose of VPSBE in DM + 200VPSBE group was not differ significantly (P= 0.08) more effective than that of standard drug Metformin in DM +200Metformin group.

Table 4.5 shows the effect of VPSBE on fasting glucose level (FBG) among diabetic treated group. The FBG level in non-DM group was constant throughout the experimental period and the baseline FBG level before the treatment was significantly higher among the groups

compared with non-DM group. After treatment the mean value for the final fasting blood glucose (FFBG) in DM control group was high, that is significantly increase ($P= < 0.000$) compared to non-DM control group. Treating these diabetic rats with 100 or 200mg of VPSBE and 200mg of Metformin/kg body weight in their respectively groups, significantly ($P= 0.001$, $P= 0.000$, $P= 0.001$ respectively) decreased FBG levels compared with DM control group. Treating diabetic rats with the higher dose of VPSBE in DM + 200VPSBE group and standard drug Metformin in DM + 200Metformin group was significantly ($P= 0.000$, P= 0.001 respectively) more effective than that of lower dose of VPSBE in DM +100VPSBE. However treating diabetic rats with the higher dose of VPSBE in DM + 200VPSBE group was significantly ($P= 0.001$) more effective than that of standard drug Metformin in 200 +Metformin group

TABLE 4.1: Phytochemical Screening VPSBE

Chemical Compounds Classes	Test	VPSBE
Saponins	Foam test (foam produced greater ≥ 10mins)	+++
Glycosides	Modified Borntrager's Test	+++
Alkaloids	General test: Dragendorff reagent	+++
Steroids	Liebermann-Burchardt Test	+++
Volatile oil	Based on Formation of white precipitates	+++
Flavonoids	Alkaline Reagent Test	++
Tannins	Gelatin test	++
Saponins glycosides	Froth Test	+
Balsams	Based on colour intensity	+
Anthraquines	Borntrager's Test	+
Cardiac glycoside	Legal's Test	ND

ND = not detected, + = Low, ++ = moderate, +++ = high, VPSBE: *Vitellaria paradoxa* stem bark extracts

TABLE 4.2: Phase 1 of Acute Toxicity (LD_{50}) Study of VPSBE in Wistar Rats

Groups	**No. of animals**	**Dosage in mg/kg body weight.**	**Volume of extracts (ml)**	**Observational period (hours)**	**Behavioral changes**	**Mortality**
1	3	Distilled water	1	24	None	None
2	3	10	1	24	None	None
3	3	100	1	24	None	None
4	3	1000	1	24	None	None

kg = kilogram, ml = milliliter

TABLE 4.3: Phase II of Acute Toxicity (LD_{50}) Study of VPSBE in Wistar Rats

Groups	**No. of animals**	**Dosage in mg/kg body weight.**	**Volume of extracts (ml)**	**Observational period (hours)**	**Behavioral changes**	**Mortality**
1	1	Distilled water	1	24	None	None
2	1	1600	1	24	None	None
3	1	2900	1	24	None	None
4	1	5000	1	24	None	None

kg = kilogram, ml = milliliter

TABLE 4.4: Effect of VPSBE on total food intake and body weight gain among the groups

Group	Total food intake (g)	Body weight gain (g)
Non DM	655.23 ± 51.68	56.83 ± 27.86
DM	1134.00 ± 101.15[a]	-9 ± 22.30[a]
DM + 100VPSBE	972.98 ± 37.30[ab]	41.00 ± 3.87[b]
DM + 200VPSBE	711.11 ± 9.87[abc]	58 ± 7.48[b]
DM + 200Metformin	807 ± 19.30[abcd]	51.67 ± 10.65[b]

Data in the table above are expressed as Mean ± SD. and (n=6 per group). The significance level is given at P<0.05. (One-way ANOVA followed by Tukey post hoc-test). DM= diabetes mellitus, VPSBE= *Vitellaria Paradoxa* stem bark extracts.

a superscript represent p value < 0.05 compared to non-DM group; b superscript represent p value < 0.05 compared to DM group; c superscript represent p value < 0.05 compared to DM + 100VPSBE group; d superscript represent p value < 0.05 compared to DM + 200VPSBE group;

TABLE 4.5: Effect of VPSBE on fasting blood glucose among the groups

Groups	**BFBG (mmol/L)**	**FFBG (mmol/L)**
Non DM	4.92 ± 0.76	5.13 ± 0.74
DM	22.70 ± 0.41[a]	24.53 ± 4.17[a]
DM + 100VPSBE	23.17 ± 3.06[a]	14.57 ± 1.5[ab]
DM + 200VPSBE	23.23 ± 1.42[a]	4.87 ± 0.85[bc]
DM + 200Metformin	23.28 ± 1.37[a]	8.85 ± 1.224[bcd]

Data in the table above are expressed as Mean ± SD. and (n=6 per group). The significance level is given at P<0.05. (One-way ANOVA followed by Tukey post hoc-test). DM= diabetes mellitus, VPSBE= *Vitellaria Paradoxa* stem bark extracts.

a superscript represent p value < 0.05 compared to non-DM group; b superscript represent p value < 0.05 compared to DM group; c superscript represent p value < 0.05 compared to DM + 100VPSBE group; d superscript represent p value < 0.05 compared to DM + 200VPSBE group;

BFBG = baseline fasting blood glucose; FFBG = final fasting blood glucose

Table 4.6 shows the effect of hyperglycaemia and VPSBE diabetic treated rats on Neutrophils to lymphocyte ration (NLR) in peripheral whole blood. The mean value of NLR in DM group was markedly very low, that is significantly down regulated compared to non-DM group ($P=$ 0.000). Diabetes rats treated with 100 or 200mg of VPSBE/kg body weight in their respectively groups, significantly ($P=$ 0.001, P= 0.001 respectively) up regulated the peripheral NLR compared to DM group, however treatment with standard drug Metformin in DM +200Metformin group was not differ significantly compared to DM control group ($P=$ 0.09). Diabetic rats treated with the higher doses of VPSBE in DM + 200VPSBE was not differ significantly more effective than the low doses of VPSBE in DM + 200VPSBE group ($P=$ 0.07 for respective groups).

Table 4.7 shows the effect of hyperglycaemia and VPSBE diabetic treated rats on serum IL-6 level. The mean value of IL-6 in the DM group was significantly up regulated compared to non-DM group ($P=$ 0.001). Diabetic rats treated with 100 or 200mg of VPSBE and 200mg of Metformin/kg body weight in their respectively groups, significantly ($P=$ 0.001, $P=$ 0.001, $P=$ 0.001 respectively) down regulated IL-6 level compared to DM group. Diabetic rats treated with the higher dose of VPSBE in DM + 200VPSBE group and standard drug Metformin in 200 +Metformin group was not differ significantly (P = 0.11, P= 0.13 for respective groups) more effective than that of lower dose of VPSBE in DM +100VPSBE group. Furthermore treating diabetic rats with the higher dose of VPSBE in DM + 200VPSBE group was not differ significantly more effective than that of standard drug Metformin in DM +200Metformin group (P = 0.067).

Table 4.8 shows the effect of hyperglycaemia and VPSBE diabetic treated rats on serum TNF-α level. The mean value for the serum TNF-α in DM group was significantly up regulated

compared to non-DM group (P= < 0.000). Diabetic rats treated with 100 0r 200mg of VPSBE/kg body weight in their respectively group, significantly (P= 0.001, P= 0.001 respectively) decreased the serum TNF-α level compared to DM group, however diabetic rats treated with standard drug Metformin in DM +200Metformin group was not differ significantly compared to DM control group (P= > 0.15). Diabetic rats treated with the higher dose of VPSBE in DM + 200VPSBE group was significantly more effective than that of DM + 100VPSBE group (P= 0.001).

Table 4.9 shows the effect of hyperglycaemia and VPSBE diabetic treated on serum IL-10 level. The mean value for the serum IL-10 in DM group significantly down regulated compared to non-DM group (P= 0.001). Diabetic rats treated with 100 or 200mg of VPSBE and 200mg of Metformin/kg body weight in their respectively group; significantly (P= 0.001 P= 0.001, P= 0.001 respectively) up regulated the serum IL-10 level compared to DM group. Diabetic rats treated with the higher dose of VPSBE in DM + 200VPSBE group was significantly more effective than that of DM + 100VPSBE group (P= 0.001); however, diabetic rats treated with the standard drug metformin was not differ significantly compared to DM + 100VPSBE group (P= > 0.115). Furthermore diabetic rats treated with the higher dose of VPSBE in DM + 200VPSBE group was significantly more effective than that of standard drug Metformin in DM + 100Metformin group (P= 0.65).

TABLE 4.6: Effects of Hyperglyceamia and VPSBE Treatments on NLR among the Groups

Group	**NLR**
Non DM	1.85 ± 0.09
DM	0.04 ± 0.01[a]
DM + 100VPSBE	0.33 ± 0.02[ab]
DM + 200VPSBE	0.37 ± 0.01[ab]
DM +200Metformin	0.04 ± 0.02[a]

Data in the table above are expressed as Mean ± SD. and (n=6 per group). The significance level is given at P<0.05. (One-way ANOVA followed by Tukey post hoc-test). DM= diabetes mellitus, VPSBE= *Vitellaria Paradoxa* stem bark extracts.

a superscript represent p value < 0.05 compared to non-DM group; b superscript represent p value < 0.05 compared to DM group.

TABLE 4.7: Effects of Hyperglycaemia and VPSBE Treatments on Serum IL-6 Level among the Groups

Group	**Serum IL-6 (pg/ml)**
Non DM	23.20 ± 3.46
DM	154.13 ± 22.69[a]
DM + 100VPSBE	43.26 ± 13.82[b]
DM + 200VPSBE	29.45 ± 2.60[b]
DM +200Metformin	37.47 ± 5.67[b]

Data in the table above are expressed as Mean ± SD. and (n=6 per group). The significance level is given at P<0.05. (One-way ANOVA followed by Tukey post hoc-test). DM= diabetes mellitus, VPSBE= *Vitellaria Paradoxa* stem bark extracts.

a superscript represent p value < 0.05 compared to non-DM group; b superscript represent p value < 0.05 compared to DM group.

TABLE 4.8: Effects of Hyperglycaemia and VPSBE treatments on serum TNF-α Level among the Groups

Group	**Serum TNF-α (pg/ml)**
Non DM	490.10 ± 7.17
DM	1204.05 ± 150.91[a]
DM + 100VPSBE	943.95 ± 4.02[ab]
DM + 200VPSBE	680.64 ± 5.09[abc]
DM +200Metformin	1117.60 ± 8.14[a]

Data in the table above are expressed as Mean ± SD. and (n=6 per group). The significance level is given at P<0.05. (One-way ANOVA followed by Tukey post hoc-test). DM= diabetes mellitus, VPSBE= *Vitellaria Paradoxa* stem bark extracts.

a superscript represent p value < 0.05 compared to non-DM group; b superscript represent p value < 0.05 compared to DM group; c superscript represent p value < 0.05 compared to DM + 100VPSBE group.

TABLE 4.9: Effects of Hyperglycaemia and VPSBE treatments on Serum IL-10 level among the groups

Group	**Serum IL-10 (pg/ml)**
Non DM	146.61 ± 12.02
DM	72.16 ± 4.02[a]
DM + 100VPSBE	113.42 ± 2.97[ab]
DM + 200VPSBE	134.87 ± 2.70[abc]
DM +200Metformin	122.98 ± 1.1[abd]

Data in the table above are expressed as Mean ± SD. and (n=6 per group). The significance level is given at P<0.05. (One-way ANOVA followed by Tukey post hoc-test). DM= diabetes mellitus, VPSBE= *Vitellaria Paradoxa* stem bark extracts.

a superscript represent p value < 0.05 compared to non-DM group; b superscript represent p value < 0.05 compared to DM group; c superscript represent p value < 0.05 compared to DM + 100VPSBE group; d superscript represent p value < 0.05 compared to DM + 200VPSBE group.

CHAPTER FIVE

5.0 DISCUSSION

Preliminary phytochemical screening may be useful in the detection of bioactive compounds and subsequently may lead to the drug discovery and development. In this study, the phytochemical screening revealed the presence of several classes of compounds in VPSBE among which: alkaloids, saponins, glycosides, steroid, volatile oil, flavonoids, tannins, saponins glycosides, Balsams and anthraquines. This finding differ with one reported by Ndukwe, *et al.* (2007); Mainasara, *et al.* (2016); Ukpanukpong, *et al.* (2016); Fodouop, *et al.* (2017) in screening more phytochemical compounds, These specific differences are associated with either part of tree used, geographical location where the *Vitellaria paradoxa* tree are obtained, the edaphoclimatics conditions and the botanical source are important factors, which can affect the chemical composition of natural product (Silva *et al.,* 2015) Some of these secondary metabolites (Flavonoids, alkaloids saponins) have been shown for several modulatory properties including anti-diabetes, reducing the level of pro-inflammatory cytokine (IL-6 and TNF-α) and up regulate regulatory cytokines (Comalada *et al.*, 2006; Jeong *et al.,* 2009; Kao *et al.,* 2011; Kim *et al.,* 2014; Hsu *et al.,* 2013; Zhang *et al.,* 2015)

Acute toxicity study (LD50) was carried out in accordance with lokers method. From this study no death or behavioral changes was recorded in the entire group and among phases. This indicates that VPSBE is relatively safe; this finding is in agreement with the belief of the users, and no harmful effects have been observed among them. The organization for economic cooperation and development (OECD), (Paris and France) recommended chemical labeling and classification of acute systemic toxicity based on oral LD_{50} value as: very toxic, <5mg/kg; toxic, >5<50mg/kg; harmful, >50<500mg/kg and not toxic or harmful, >500mg/kg. Furthermore, in more recent development, Mainasara *et al.* (2016) reported on hepatotoxicity

assessment in Wistar rats exposed to VPSBE, they concluded that VPSBE was relatively safe and less likely to produced toxic effects.

In this study, hyperglycaemia was induced with a multiple low doses of freshly prepared Alloxan (80 mg/kg body weight) to overnight fasted rats. It has been reported that diabetes induced with multiple low doses of Alloxan represents a good model of autoimmune type 1 diabetes. Indeed, Alloxan when administered at a high single dose induces diabetes by the direct toxic effects on pancreatic β-islet cells (Furman, 2015; Yeray, *et al.,* 2016; Fachinan *et al.,* 2017) However, when Alloxan is administered at low doses during three consecutive days, it induces mild type 1 diabetes, through a T lymphocyte-dependent process, an autoimmune destruction of pancreatic β cells mediated by both $CD4^+$ and $CD8^+$ T cells (Fachinan *et al.,* 2017). The autoimmune process commences with the infiltration of T cells in pancreas tissue roughly on the 2^{nd} day after the last injection of Alloxan (Fachinan *et al.,* 2017)

Prior to VPSBE or Metformin treatment, the baseline-FBG levels were significantly higher in all alloxan administered groups compared to non-DM group. These results indicate the establishment of diabetic animal model; this may be due to the cytotoxic action of alloxan on pancreatic beta cells (Wali *et al.,* 2013) or may be due to autoimmune destruction of pancreatic β cells (Fachinan *et al.,* 2017). Beta cells are responsible in producing insulin in response to increased glucose level, and insulin is responsible for the reduction in blood glucose level.

The final Fasting Blood Glucose (FFBG) level in DM group was found to be sustained over the study period and significantly higher compared with FFBG level in non-DM group, this finding is similar with other studies by Babatunde *et al.*(2015) Umar and Mainasara, (2016). The mechanism of action of Alloxan in experimental animals was said to be the destruction of

beta cells of the pancreatic islet (Laenzen, 2008). Consequently, Alloxan toxicity causes modifications to cell function, ATP dephosphorylation reaction and helps xanthine oxidase to generate ROS (Laenzen, 2008); ROS- activate the transcription factor and the nuclear factor kappa B (NFκB). Ultimately, the activation of the transcription factor NFκB appears to play a pivotal role in the regulation of inducible enzymes, inflammatory cytokines, and other substances that are initiators or enhancers of the inflammatory process, causing damaging effects on various organs such as the liver, kidney and pancreas.

In this study, we observed that VPSBE induced a significant decrease of hyperglycaemia in both doses of VPSBE treated rats, suggesting that VPSBE has antihyperglycaemic in this diabetic animal model. This curative effect may be due to the active constituents present in VPSBE. It has been reported that, the antidiabetic activity of most natural products are due to the contents of Phenolics and Flavonoids (Victor *et al.,* 2017). The antidiabetic activity of the higher dose of VPSVE (200mg/kg body weight) was more effective than that of the lower dose (100mg/kg body weight). This result is consistent with that of Victor *et al.* (2017), who noticed that the antidiabetic effect was found to be increase with increasing concentrations. In addition our result showed that the higher dose of VPSVE (200mg/kg body weight) was more effective than that of the known antihyperglycaemic agent Metformin.

The decrease in body weight gain observed in DM group concur with other studies (Sivakumar *et al.,* 2010; Emordi *et al.,* 2016; Umar *et al.,* 2017) and the reason may be as a result of impaired utilization of nutrients. Persistent hyperglycaemia (another reason for decrease in body weight) may lead to the production of free radicals which attack insulin-producing cells (beta cells) leading to glucose deprivation, promoting protein breakdown (weight loss) in order to generate energy (DeFronzo, 2004). On the other hand, the increase in

body weight gain observed in non DM group of rats, can be attributed to the anabolic effect of insulin (Goodarzi *et al.*, 2010; Aranoff *et al.*, 2004). In contrast, DM rats supplemented with VPSBE and metformin significantly had higher body weight gain compared to DM group. This demonstrated a comparable antihyperglycaemic effect of VPSBE in preventing weight loss or improving the body weight gain.

The significant increases in total food intake observed in DM group compared to non-DM group concur with other study (Musabayane *et al.*, 2006). This increase in food intake may show the compensatory mechanism for the reduced energy from glucose despite the increased body catabolism, and also counter weight loss in diabetes (Dimitriadis *et al.*, 2011). However, treatment with VPSBE stabilized food intake in diabetic rats, this may be as a result of VPSBE anti-hyperglycaemic effect which in turn provide more glucose to the cells.

In this study, hyperglycaemia significantly, down regulates NLR compared to non-DM group. This finding it differs with the study reported by Xu *et. al.* (2017), where the NLR is significantly up regulated in patients with diabetic peripheral neuropathy as compared to patient with Type 2 diabetes mellitus. This difference may be due to the nature of disease condition and differences in the type of model used. However, the exact mechanisms of lowered NLR being related with diabetes are as yet unknown. Most likely, it can be explained in view of various functions of Neutrophils and Lymphocytes. This lower NLR was accompanied with leukocytosis, marked increase in number of lymphocytes population and marked decrease in number of neutrophils population. Marked decrease in number of neutrophils population may show an inactive non-specific inflammatory process. More recent studies showed that circulating neutrophil numbers decreased in patients with type 1 diabetes which might be associated with β-cell specific autoimmunity (Huang *et al.*, 2016). Reduced

numbers of blood neutrophils in type 1diabetes could be a result of abnormal neutrophil yield and maturation, peripheral consumption or damage, and tissue detainment. It was suggested that neutrophils restriction in the pancreas during diabetes, should account for the decreased peripheral blood neutrophils (Huang *et al.,* 2016). However, Li *et. al*. (2017) reported that, hyperglycaemia, leads to persistent activation of neutrophils, as evidenced by the increased activity of neutrophil alkaline phosphatase. The neutrophils of diabetes patients show increased necrosis and enhanced production of reactive oxygen species (Li *et al.,* 2017), and significantly lower neutrophil chemotactic responses (Li *et al.,* 2017). While in the other hand, the higher number of peripheral lymphocyte seen may indicates abnormal immune regulation as well as active immunity pathway, also this higher number of peripheral lymphocyte seen may represent the picture characteristic pathological appearance of βcells of islets of Langerhans similar to human Type 1 Diabetes that show relatively dense cellular infiltrate dominated by lymphocyte (Willcox *et al.,* 2009), CD8 T lymphocytes are the major cell type overall, and considerably outnumber CD4 T lympocytes (Willcox *et al.,* 2009). Previous studies have also shown that activation of leukocytes and their adhesion to the endothelium could cause endothelial injury (Li *et al.,* 2017). These leukocytes secrete cytokines and growth factors that can promote the migration and proliferation of smooth muscle cells, which can induce further vascular damage and cause most complications of atherosclerosis (Li *et al.,* 2017). However treatment with VPSBE significantly, increase the lowered NLR, and this effect may show VPSBE to possess the regulatory properties by stabilizing the number of neutrophils and lymphocytes population.

In this study hyperglycaemia significantly up regulate the serum IL-6 level in DM group compared to non-DM. This finding concurs with the a report by Elizabeth *et al.* (2017) and Bolajoko *et al.* (2017), this similarities may be due to similar in animal model and type of

disease induced. in contrast with preliminary study on human subject also indicates that serum IL-6 level in patients with type 2 diabetes mellitus was up regulated (Qiao *et al.,* 2016) However, some studies demonstrated that IL-6 levels of Type 1 DM patients did not differ significantly, as compared to controls (Pestana *et al.,* 2016). It has been established that the elevated levels IL-6 in diabetes and its associated complications, are as a result of hyperglycaemia and IL-6 have been considered to be the link between inflammation and insulin resistance (Gomes, 2017). IL-6 induced a number of glucocorticoid receptors, increased circulation of glucagon concentration and adipose paracrine effect to decrease insulin action. It has been reported that IL-6 causes insulin resistance through affecting both the proximal and distal events in hepatic insulin receptors (IR) signal transduction (Joseph *et al.,* 2013) Insulin signal transduction was not inhibited at the level of IR autophosphorylation. However, insulin-dependent tyrosine phosphorylation of IRS-1, association of the p85 subunit of PI 3-kinase with IRS-1, and activation of Akt were all comparably inhibited, both temporally and in magnitude, by IL-6 exposure in HepG2 cells at physiological concentrations of insulin. IL-6 was also capable of causing inhibition of insulin action in primary hepatocytes. Pretreatment of primary hepatocytes with IL-6 markedly inhibited both insulin-induced Akt activation and glycogen synthesis. This suggests that hepatocytes, in general, are physiologic targets for the inhibitory effect of IL-6 on insulin signaling (Joseph *et al.,* 2013).

VPSBE and Metformin down regulated the elevated levels of the IL-6 in the diabetic rats at the doses tested possibly through down regulating the pro-inflammatory gene. The observed effect suggests that both VPSBE and Metformin possess some immunomodulatory properties. The effects displayed by VPSBE may be due to the higher content of phytochemical constituent observed in this study, Zhao and Zang (2017) and Kim *et al.* (2014) reported a luteolin (flavonoids compounds) significantly reduced IL-6 by suppressing NF-κB activity.

Research by Rytter, (2012) showed that giving α-carotene, β-carotene, lutein, luteolin and lycopene reduce levels of IL-6 on patients of diabetes.

Our study provides evidence of an upward regulation of TNF-α level in the serum of diabetic rats compared to non-DM group. In a study of Omodanisi *et al.* (2017) demonstrate similar evidence of an upward regulation of TNF-α level in the kidney, liver and serum of diabetic rats when compared to the non-DM group. Elizabeth *et al.* (2017) also report similar finding of upward regulation of TNF-α level in the serum of diabetic rats compared to non-DM group. A paper on Modulatory influence of *Parkia biglobosa* protein isolate on testosterone and biomarker of oxidative stress in brain and testes of streptozotocin-induce diabetic male rat; similarly reported an upward regulation of serum TNF-α levels (Bolajoko *et al.*, 2017). In another yet study carried out on human subject, the result demonstrated an upward regulation in level of TNF-α in T1DM (Saud, 2014)

The reasons for the increase in TNF-α level in this our research, may be due to oxidative stress, Omodanisi *et al.* (2017) and Elizabeth *et al.* (2017); reported an increase in oxidative stress in diabetic rats. Oxidative stress increases expression of the pro-inflammatory gene by oxidant-mediated activation of transcription factors (Mardiah *et al.*, 2015). ROS is important in inflammatory response through the upregulation of redox-sensitive transcription factors, alteration of histones acetylation or deacetylation and thus pro-inflammatory gene expression.

The resultant activation of TNF-α is well acknowledged to cause damage to renal cells by enhancing renal hypertrophy, hemodynamic imbalance, albumin permeability (Rivero *et al.*, 2009). The harmful effects of these responses lead to the development of renal disease in patients with T2DM, hence resulting in the progression of renal failure. TNF-α increases retinal endothelial permeability by down regulating the expression of tight junction proteins

and the increased permeability can lead to rupturing of the brain retinal barrier (BRB) (Aveleira *et al.,* 2010). TNF-α can damage insulin receptor (IR) and insulin receptor substrate (IRS) and then it can inhibit insulin signal thereby causing insulin resistance (Mardiah *et al.,* 2015). TNF-α stimulated the expression of SOCS (suppressor of cytokine signal) which bonded either IRS1 or IRS2 and mediated damage and as a result, insulin could not take glucose into the muscle cells and adipose tissue, therefore glucose levels in blood plasma would increase (Mardiah *et al.,* 2015); as its compensatory mechanism, the β cells of the pancreas would produce excessively, insulin and caused hyperinsulinemia. All this condition can lead to vascular inflammation and finally insulin resistance (Mardiah *et al.,* 2015).

The serum TNF-α level were down regulated after VPSBE treatment in both doses as compared to non treated diabetic group, TNF-α down regulation in the diabetic rats treated with VPSBE was expected because of the high content of phytochemical that existed in VPSBE. According to Giriwono *et al.* (2011), Polyphenolic compounds decreased TNF-α on the plasma, Another reason for the decrease in TNF-α could be due to the upregulation of regulatory cytokine (IL-10) observed in the present study, in response to pro-inflammatory cytokine (TNF-α).

This study showed that giving VPSBE tended to have ability for down regulating inflammatory compounds. A decrease in TNF-α level could be expected to increase insulin receptor sensitivity and repairing blood sugar taking into cells. According to Donath *et al.* (2009) a down regulation of TNF-α in pancreatic β cells closely associated with the increase proinsulin synthesis, improved insulin sensitivity and pancreatic β cell mass. We proposed that: the observed downward regulation of serum TNF-α level may shows retardation in the onset of diabetic complications.

In the present study, hyperglycaemia induced a significant downward regulation of serum IL-10 level in DM group compared to non-DM group. This finding is consistence with the work of Ahmed *et al.,* (2018) where he report the significant downward regulation in the IL-10 level, in diabetic rats. Furudoi *et al.* (2013) in another study carried out on diabetic rat demonstrated a downward regulation in the serum IL-10 level. Yaghini *et al.* (2011) in another study carried out on human subject, his result differ with our study, in which serum IL-10 level in T2D patients up regulated compared to apparently healthy control subject; this difference could be attributed to the differences in subject type used.

The downward regulation of IL-10 may contribute into exciting Th1 response and tissue damage (Furudoi *et al.* 2013). The hyper responsiveness of Th1 cytokines (TNF-α) observed in diabetic group lead to twisted Th2 cytokines (IL-10) response, downward regulation in Th2 cytokines may lead to inadequate production of antibodies and decrease sensitization of granulocytes (Bijjiga and Martino, 2013), consequently this effect may lead to higher susceptibility to viral and bacterial infection.

Besides, IL-10 concentrations were significantly up regulated in treated diabetic rats compared to untreated diabetic rats. These effects led us to suggest that the treatment with 200mg/kg/body weight of VPSBE hastens the switch from inflammatory to anti-inflammatory responses and dominance of Th2 on Th1.

In order to better appreciate the balance of cytokine production *in vivo*, we calculated the Th1/Th2 ratio. We observed that Th1/Th2 ratios expressed as TNF-α /IL-10 were shifted towards a proinflammatory Th1 phenotype in untreated diabetic rats, while these ratios were shifted towards IL-10, a Th2 cytokine, in diabetic rats treated with VPSBE.

These observations suggested that VPSBE promotes a Th2 anti-inflammatory phenotype *in vivo* by decreasing Th1 cytokines and increasing anti-inflammatory Th2 cytokines. Therefore, we can state that the antihyperglycemic effect of VPSBE may also pass through its decreased action on TNF-α and increased that of Th2 cytokines IL-10.

VPSBE produced its regulatory effects probably through inhibiting the production of TNF-α and blocking TNF-α-mediated inflammation. This effect may be directly mediated via prevention of activating extracellular signal-related kinase (ERK), c-Jun NH2-terminal kinase (JNK), c-Jun, and nuclear factor-κB (NF-κB), which are potent inducers of inflammatory gene expression and protein secretion or indirectly via stimulation of peroxisome proliferator-activated receptor-γ (PPARγ) activity, thereby antagonizing NF-κB or activator protein-1 (AP-1) transcriptional activation of inflammatory genes.

CHAPTER SIX

6.0 CONCLUSIONS, RECOMMENDATIONS AND LIMITATION

6.1 Conclusions

It is concluded from the findings of this study that:

1. VPSE contained abundant active natural compounds and considered very safe to use.
2. VPSBE reduced blood glucose level more effective than that of standard drugs metformin and stabilized food intake and increase body weight loss.
3. Hyperglycaemia induced upward regulation of proinflammatory cytokines and down regulated the expression of regulatory cytokines.
4. VPSBE down regulate the expression of inflammatory cytokines and up regulate the expression of regulatory cytokines

6.2 Recommendations

1. Persistent chronic inflammation and immune activation are potential risk factor that lead to diabetic complication even with effective control of hyperglycaemia, therefore more investigations on immune modulation should be carried out in order to expand the effect of VPSBE in the treatment of diabetes and it complication.
2. In view of the evidence of the potential effects of VPSBE as revealed in this study, there is still a need for further studies on the standardization and characterization of the extracts, isolating polyphenolics compounds in pure form so as to establish the exact bioactive component with immunomodulatory activity
3. Considering the complexity of diabetes, a clinical trial is highly recommended.

4. We discourage deforestation because the act will not only encourage desertification but rather make our sources to natural product become extinct.

6.3 Limitation of the Study

1. Financial constrain: this study lacks grant from any organization or individual and this limit the scope of the research especially in analyzing more cytokines in the study subject.

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