EFFECTS OF SOME COMMONLY USED ANTIDIABETIC PLANT MATERIALS ON SODIUM-GLUCOSE COTRANSPORT THROUGH GASTROINTESTINAL AND RENAL TUBULAR ENDOTHELIUM IN DIABETIC MODEL RATS

This Thesis is submitted in partial fulfillment of the Degree of Doctor of Philosophy (PhD) under the Faculty of Postgraduate Medical Sciences & Research of the Dhaka University.

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DECLARATION

I hereby declare that the Thesis entitled **'EFFECTS OF SOME COMMONLY USED ANTIDIABETIC PLANT MATERIALS ON SODIUM-GLUCOSE COTRANSPORT THROUGH GASTROINTESTINAL AND RENAL TUBULAR ENDOTHELIUM IN DIABETIC MODEL RATS'** is my original research work carried out in the Department of Biochemistry and Cell Biology, Bangladesh Institute of Health Sciences (BIHS), Dhaka.

I also declare that I have not submitted this Thesis either in part or full or in any other form to any other University or Institution for any Degree or any other purpose.

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SUPERVISOR'S CERTIFICATE

This is to certify that the research work described in this Thesis entitled '**EFFECTS OF SOME COMMONLY USED ANTIDIABETIC PLANT MATERIALS ON SODIUM-GLUCOSE COTRANSPORT THROUGH GASTROINTESTINAL AND RENAL TUBULAR ENDOTHELIUM IN DIABETIC MODEL RATS**' by **Masfida Akhter**, is an original work and has been carried out under my supervision in the Department of Biochemistry & Cell Biology, Bangladesh Institute of Health Sciences (BIHS), Dhaka, for the degree of Doctor of Philosophy (PhD) under the Faculty of Postgraduate Medical Sciences and Research, Dhaka University.

No part of this thesis has previously been submitted to any Institute for any Degree.

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Dedicated

to

My Aunt SUFIA CHOWDHURY

&

Uncle LIAQUAT HUSSAIN CHOWDHURY

For their great Love and Inspiration

throughout my life

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ABSTRACT

Diabetes Mellitus (DM) is a major health problem all over the world and there is still need for more ideal therapeutic agents for the management of various types and subtypes of this disorder. Most of the diabetic subjects (90% on an average) suffer from type 2 Diabetes Mellitus (T2DM) followed by the Type 1 DM (T1DM) variety. It is now widely acknowledged that insulin deficiency (due to selective autoimmune damage of pancreatic B cells) is the major cause of T1DM; however, the etiopathogenesis of T2DM is much more complex with varying degrees of involvement of pancreatic B cell dysfunction and peripheral insulin resistance in different populations and subpopulations. Classical therapeutic approaches against DM are primarily based on ameliorating insulin secretary defect or insulin resistance by targeting the relevant cells and tissues in the body. Recently evidences has been generated on the important role of enhanced glucose absorption through gastrointestinal and renal tubular endothelium (mediated by sodium-glucose cotransporters, or SGLTs) in the pathophysiology of T2DM. Accordingly pharmacological research has already started to explore the potential use of the inhibitor of SGLT1 (for GI tract) and SGLT2 (renal tubule) in reducing glucose absorption/reabsorption in these organs. Plants are thought to be a major source of potential antidiabetic agents and evidence are present that many of the plant extracts exert their effects through inhibition of glucose absorption in the gut. The modern drug development initiatives around SGLTs were, in fact, driven by a plant derived compound, phlorizin. In spite of that, the effects of plant extracts on Na-mediated glucose transport have not yet been studied. A major obstacle in this case may be the nonavailability of appropriate animal models on which such plant based studies can be conducted using relatively easy and cost-effective techniques. Under this perspective, the present series of studies were undertaken to characterize the neonatal streptozotocin induced T2DM (nstzT2DM) model rats in terms of their glycemic and insulinemic as well as intestinal and renal glucose absorption/reabsorption (specially in relation to its association with Na) abnormalities. The effects of three commonly used antidiabetic plants (*Aegle marmelos, Trigonella feoenum-gracum* and *Ocimum sanctum*) on the above characterized parameters of the nstzT2DM rats were also tested in this study.

Collection of the plants and preparation of the study materials (powder or aqueous extracts) were done following standard botanical and chemical procedures. T2DM in the inbred Long-Evans rats (number varying in specific series) was induced by single intraperitoneal injection of streptozotocin (90mg/kg body weight) to 2 day old pups following procedures already standardized in BIRDEM-BIHS. Series of experiments were conducted after 3-6 months of induction of DM in the rats. Characterization of the pancreatic B cell dysfunction and insulin resistance in T2DM rats (on comparing with nondiabetic or ND rats) were done by homeostatic model assessment of pancreatic B cell secretory capacity (HOMA%B) and insulin sensitivity (HOMA%S) by using a HOMA calculator which used fasting serum glucose (measured by rat specific glucose-oxidase method) and insulin (by rat specific ELISA technique) values of the rats. Characterization of the Na-mediated glucose absorption in the duodenum and jejunum of T2DM rats (in parallel to ND rats) were done by a gut perfusion technique using Krebs-Ringer buffered media with varying concentrations of Na (or in its replacement, mannitol) and glucose in the solution. The renal functional status of the rats were

assessed by serum albumin and urinary creatinine as well as by the calculation of eGFR. Characterization of the urinary glucose excretion of the rats were done by measurement of serum and urinary glucose during OGTT experiments with serum and urine collected at 0, 60 and 120 min time points. Aqueous extracts of all the three plants (studied at doses at 0.01 and 1.0 g/ml in the medium) were tested on ND and T2DM rats for their effects on intestinal glucose absorption during gut perfusion and urinary glucose excretion during OGTT. The powder of the most potent plant in test series (in terms of its effects on Na-dependent glucose absorption) was further tested on T2DM rats with a chronic (30 days) oral feeding to explore its effects on glycemic and insulinemic as well as renal functional parameters.

The nstzT2DM rats were found to have significantly lower HOMA%B but not lower HOMA%S compared to the ND rats. Those rats also had a significantly higher percentage of glucose absorption ($p<0.019$) compared to the ND rats during gut perfusion. At 3.9 mmol/l glucose concentration the absorption was mainly Na-dependent, but at 15.6 mmol/l glucose it was Na-independent. The rats showed significant dyslipidemia (with higher serum TG and cholesterol values) and decreased renal function (with higher urinary albumin and lower eGFR) as compared to their ND counterparts. *A marmelos* aqueous extract showed a dose-dependent effect on Na-dependent glucose absorption at 3.9 mmol/l glucose concentration in the media, at higher concentration (15.6 mmol/l) of media glucose, the inhibition was not dose- dependent. The *T feoenum-gracum* aqueous extracts inhibited glucose absorption only at higher concentration of extract (1.0g/ml) and media glucose (15.6 mmol/l). The *O sanctum* extract did not have any effect on intestinal glucose absorption. The *A marmelos* aqueous extract showed significant stimulating effect on HOMA%B ($p<0.021$) and also on HOMA%S ($p<0.052$) in T2DM rats. It also lowered serum TG ($p<0.013$), serum LDL ($p<0.015$) and increased the eGFR ($p<0.011$) of these rats. None of the extracts had any significant effect on urinary glucose excretion during OGTT in the rats.

The present data lead to the following conclusions:

a. T2DM in nstz rats is associated mainly with pancreatic B cell dysfunction, not insulin resistance;
b. T2DM in nstz rats is associated with higher absorption of glucose from the intestinal lumen through both Na-dependent active (SGLT1 mediated) and Na-independent passive transport mechanisms;
c. Dyslipidemia and renal tubular abnormalities are already present in nstz rats, but hyperglycemia in these rats does not seem to be related to reabsorption of glucose (SGLT2 mediated) in the renal tubule;
d. *A marmelos* has beneficial effect on pancreatic B cell function in T2DM rats and the aqueous extract of the plant may inhibit glucose absorption in the intestine both involving sodium-glucose cotransport and passive transport mechanisms;
e. *T feoenum-gracum* may affect intestinal glucose absorption by affecting a passive transport process.

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BIHS	Bangladesh Institute of Health Sciences
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders
A M	*Aegle marmelos*
A marmelos	*Aegle marmelos*
AME	*Aegle marmelos* extract
AME0	*Aegle marmelos* extract (0 g/ml concentration)
AME0.1	*Aegle marmelos* extract (0.1 g/ml concentration)
AME1.0	*Aegle marmelos* extract (1.0 g/ml concentration)
α	Alpha
β	Beta
CAD	Coronary Artery Diseases
Chol	Cholesterol
CRP	C-Reactive Protein
CV	Cardiovascular
db/db	Diabetic/diabetic
DCCT	Diabetes Control and Complication Trial
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
eGFR	Estimated Glomerular Filtration Rate.
F	Fasting
FCPD	Fibrocalculus Pancreatic Diabetes
FFA	Free Fatty Acid

FGF	Fibroblast Groth Factor
GK	Glucokinase
g	Gram
HDL	High Density Lipoprotein
HK	Hexokinase
HLA	Human Leukocyte Antigen
HOMA %B	Homeostasis Model Assessment of β-Cell Function
HOMA %S	Homeostasis Model Assessment of Insulin Sensitivity
HOMA %IR	Homeostasis Model Assessment of Insulin Resistance (IR)
IDF	International Diabetic Foundation
IGF-1	Insulin like Growth Factor-1
IGFBP-1	Insulin like Growth Factor Binding Protein-1
IRS	Insulin Receptor Substrate
IRS-1	Insulin Receptor Substrate-1
ISI	Insulin Sensitivity Index
iv	Intra venous
ip	Intra-peritoneal
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
GDM	Gestational Diabetes Mellitus
GLUT	Glucose transporter
KK	Kuokundo
Kg	Kilogram
L	Liter
LDL	Low Density Lipoprotein
ml	Milliliter
mmol	Milimole
MODY	Maturity-Onset Diabetes of the Young
MRDM	Malnutrition Related diabetes Mellitus
MIT	Mitochondria

M	Mole
nmol	Nanomole
NDM	Non Diabetes Mellitus
NEFA	Non-essential fatty acid
n	Number
nstz	Neonatal Streptozotocin induced
nstzT2DM	Neonatal streptozotocin induced type 2 diabetes mellitus
n0-STZ	Neonatal 0 day Streptozotocin induced
n2-STZ	Neonatal 2 days Streptozotocin induced
n5-STZ	Neonatal 5 days Streptozotocin induced
OS	*Ocimum sanctum*
O sanctum	*Ocimum sanctum*
OSE0	*Ocimum sanctum* extract (0 g/ml concentration)
OSE0.1	*Ocimum sanctum* extract (0.1 g/ml concentration)
OSE1.0	*Ocimum sanctum* extract (1.0 g/ml concentration)
ob/ob	Obese/obese
PG	Plasma Glucose
pmol	Picomole
p	p value
ng	Nanogram
pgm	Picogram
SD	Standard Deviation
SPSS	Statistical Package for Social Sciences
STZ	Streptozotocin
SGPT	Serum glutamic-pyruvic transaminase
SCreat	Serum creatinine
sc	Sub-cutaneous
TNF	Tumor Necrosis Factor
Tg	Triglyceride
T2DM	Type 2 Diabetes Mellitus

TGF	*Trigonella foenum-graecum* extract (0 g/ml concentration)
T foenum-graecum	*Trigonella foenum-graecum*
TGFE0	*Tregonella foenum-graecum* extract (0 g/ml concentration)
TGFE0.1	*Tregonella foenum* extract (0.1 g/ml concentration)
TGFE1.0	*Tregonella foenum* extract (1.0 g/ml concentration)
TChol	Total Cholesterol
T1DM	Type 1 Diabetes Mellitus
UNPD	United Nations Population Division
UG	Urinary glucose
UCreat	Urinary creatinine
UAlb:UCreat	Urine Albumin–Urine Creatinine Ratio
µmol	Micromole
vs	Versus
WHO	World Health Organization
XOD	Xanthine Oxidase
ZDF	Zucker Diabetic Fatty
ZFR	Zucker Fatty Rats
%	Percentage
^{0}C	Degree Celsius
<	Less than
>	Greater than

Chapter I

Introduction

Introduction

BACKGROUND

Diabetes mellitus (DM) is now a major health burden all over the world and it is increasing at an epidemic rate particularly in developing countries (IDF Atlas 2015). With deeper understanding on the pathophysiology of the disorder substantial progress has been made in its therapeutic management; however, there are still a great need for more ideal agents in various types and subtypes of DM. It is now well recognized that DM is a syndrome with two major classes, namely Type 1 DM (TIDM) and Type 2 DM (T2DM), which have distinct etiopathological basis. T1DM has also various pheno- as well as genotypes and, particularly, T2DM is a much more complex entity with variable involvement of the central (pancreatic islets) and peripheral (mainly liver, adipocytes and muscle) tissues. Drug development, accordingly, has so far been targeted considering the morphological and/or functional abnormalities in these tissues and their specific cells (pancreatic B & A cells, hepatocytes, adipocytes and myocytes). These efforts have led to considerable advancement in this field resulting in the introduction of agents like insulin and its analogs, insulin secretagogues, insulin sensitizers and insulinomimetics. However, most of these agents suffer from considerable limitations and apart from the risks (like hypoglycemic attack and carcinogenicity), these agents still do not address many of the abnormalities in other tissues and cells which have now been recognized as important stakeholders in the development and progression of diabetes and its complications.

Most of the attention to explore the pathophysiology and management of DM, so far, have been focused on ameliorating the pancreatic B cell dysfunction and insulin resistance which are the two cardinal defects in all types of DM. While insulin secretory defect, due to pancreatic B cell damage, is fairly simple to be understood as the major defect in T1DM, the pathophysiology of T2DM is much more complex due to varying degrees of involvement of the two basic defects in individual populations

and subpopulations. Also, this heterogeneity extends up to family and individual levels as a consequence of interaction among genetic, dietary, and socio-cultural factors. Accordingly, effect of any pharmacological, dietary and life style intervention needs to be evaluated in terms of their effects on the glycemic levels as well as on their insulinemic status calculated in terms of insulin secretory capacity, insulin sensitivity and insulin resistance. Other morphological and functional abnormalities in T2DM like dyslipidemia, cardiovascular problems, and microvascular complications (nephropathy, retinopathy and neuropathy) are directly or indirectly related to insulin secretory dysfunction and insulin resistance in these subjects.

Gastrointestinal tract, due its role in glucose absorption, has long been recognized as a target for therapeutic and nutritional management through dampening of postprandial glucose peak (mainly by inhibition of carbohydrate breakdown and/or mechanical interference in the passage of glucose across GI endothelium). But, only during the last decade this 'nonclassical' tissue for diabetes has attracted extra attention due to its primary involvement in the pathophysiology of DM. More recently another 'nonclassical' tissue, ie renal tubular endothelium (mediating glucose reabsorption from urinary tract), has attracted similar attention. Both tissues have similar mechanism for the passage of glucose mediated by a sodium-glucose cotransport (SGLT) process (a secondary active transport) fuelled by the Na^+-K^+ pump. The cotransport process occurs at the apical region of the gut or renal endothelial cells and the Na^+-K^+ pump operates at their basolateral surfaces.

It has been found that the Na-Gl transporters vary in the enterocytes and renal tubular cells: in the gut the transport is mediated by a high capacity low affinity carrier (called sodium-glucose transporter or SGLTI) and in the renal tubule it is mediated by a low capacity high affinity carrier protein (termed as SGLT2). Inhibition of these two transporters have recently become a major focus in drug development against DM (John and White 2010). With SGLT1 the idea is to delay the absorption of glucose with inhibition of SGLT1 which may give specific therapeutic benefit avoiding the untoward side affects produced by the already marketed α-glucosidase inhibitors like

acarbose. With the inhibition of SGLT2 renal glucosuria is targeted as a mechanism to reduce blood glucose levels. A considerable progress has already been made in the exploration of molecules inhibiting SGLT1 and SGLT2 or both and some are in various phases of clinical trial. However, only a few drugs in this class have recently been introduced in the market for clinical use and long-term effects of these drugs are yet to be tested.

Plants have been used for centuries in the treatment of DM and these are now regarded as a major source of potential antidiabetic agents. Since almost all traditional antidiabetic plant preparations have been used orally it is probable that many of those exert their actions through a gut related mechanism. Studies conducted in BIRDEM-BIHS-BUHS in Dhaka also indicate that a number of plant preparations work through inhibition of glucose absorption in the gut (Rokeya *et al* 2011). Among those *T foenumgracum* (Ali *et al* 1995, Hannan *et al* 2003), *O sanctum* (Alamgir *et al* 2001, Hannan *et al* 2006) *P ovata* (Rokeya *et al* 1999), *A marmelos* (Akhter 2011) and *M paradisiaca* (Rokeya *et al* 1999) are commonly used in Bangladesh and other countries of the Indian subcontinent. The glucose absorption inhibitory action of these plants, however, have so far been attributed either to the mechanical action on guts (like affecting gastric emptying and GI motility) or inhibiting the enzymes (like disachharidases) involved in carbohydrate digestion and absorption. So far, as we know, nobody has yet explored these plants for their modulation of the sodium-dependent glucose transport mechanisms which may be a major target in controlling glucose absorption in the GI tract and renal tubules.

Apart from the fact this latest direction of pharmacological research may not be widely known, a major reason for the lack of studies in this area may be the no availability of easily implementable techniques suitable for plant studies. It is understood that animal studies will be of vital importance to explore these new areas; however, so far there is not a single animal model which mimics all the problems of T2DM. Neonatal streptozotocin induced nstzT2DM model rats have been widely used by the Dhaka group and it has some properties of human T2DM. Some of the

antidiabetic medicinal plants have already been studied in this model and few of those have been found to act through inhibition of glucose absorption in the gut. However, the nstzT2DM rats have not yet been characterized in terms of their intestinal glucose absorption patterns which are central for any pharmacological studies. In particular, Na-dependent absorption from gut lumen is vital to be explored in these rats which may give a fairly good idea about the effect of the plants or any other interventions on SGLT1 in the gut. Moreover, the effect of diabetes on urinary glucose absorption needs to be explored also as this will generate ideas about the effects of intervention materials on SGLT2 in the renal tubule.

RATIONALE

Bangladesh has presently an estimated 7.4 million diabetic subjects (IDF Atlas 2015) and an almost equal number of prediabetics. This is, increasingly, creating a huge healthcare and economic burden on the society and state. Most of the diabetic subjects in Bangladesh are deprived of modern treatment and a large number of them are still dependent on traditional medicine (mainly plants). It is thus our both social and scientific responsibility to study these plant materials for their efficacy and safety as well as their probable mechanism of action. In addition, study of these materials may lead to value addition (both as a crude drug or as a well characterized agent) with potential health as well as economic benefit. BMRG-BIRDEM-BIHS have earned regional and international recognition for its plant related research activities. BIHS, as a natural extension of BMRG-BIRDEM activities, have taken more advanced projects to go deeper into the plant related research. The present PhD work was a part of the overall program of BIHS and the findings of the study were conceived to generate more knowledge on the underlying mechanism of some plant materials which are commonly used in the management of DM.

HYPOTHESES

The present series of experiments were conducted with the following hypotheses:

a. The nstzT2DM model rats have reduced pancreatic B cell function and also impaired insulin action;
b. The nstzT2DM model rats have abnormalities in Na-dependent (ie SGLT-mediated) and/or Na-independent glucose absorption from the lumen of the proximal segments of the gastrointestinal tract;
c. The nstzT2DM model rats develop an abnormal pattern of urinary glucose excretion which is related to SGLT2 action in the renal tubule.
d. Three of the traditionally used medicinal plants *Aegle marmelos, Trigonella foenum-graecum* and *Ocimum sanctum* improves pancreatic B cell function and reduce insulin resistance in nstz T2DM model rats;
e. Three of the traditionally used medicinal plants - *Aegle marmelos, T foenum - graecum and Ocimum sanctum* ameliorate the abnormalities related to Na-dependent (ie SGLT1- mediated) intestinal glucose absorption in nstzT2DM rats;
f. The most promising plant within the previous three plants improves renal function in general and specifically it helps in combating the abnormalities related to urinary glucose excretion which is mediated through SGLT2 action in renal tubule.

OBJECTIVES

General Objectives

The general objective of the study was to investigate the effects of three commonly used antidiabetic plants (*Aegle marmelos, Trigonella foenum-graecum and Ocimum sanctum*) on sodium-dependent intestinal glucose absorption, urinary glucose excretion and some other biological parameters related to the etiopathogenesis and control of diabetes in T2DM model diabetic rats.

Specific Objectives

The specific objectives of the study were to:

1. Explore the effects of induction of diabetes on pancreatic B cell function and insulin sensitivity in neonatal streptozotocin (nstz) T2DM model rats;
2. Characterize the sodium-mediated glucose absorption from the gastrointestinal lumen of the nstzT2DM rats;
3. Explore the renal functional status as well as urinary glucose excretion in nstzT2DM rats;
4. Investigate the effects of three commonly used medicinal plants (*Aegle marmelos, Trigonella foenum-graecum and Ocimum sanctum*) on sodium-mediated gastrointestinal glucose absorption in nstzT2DM rats.
5. To investigate, in further details, the effect of the most promising plant (from among the three plants) on insulin secretory capacity, insulin sensitivity, gastrointestinal glucose absorption, renal function and urinary glucose excretion in nstzT2DM rats.

Chapter II

Review of Literature

Review of Literature

DIABETES MELLITUS

Diabetes mellitus (DM) is a major health problem all over the world. As per International Diabetes federation (IDF) there were about 445 million diabetic patients in the world in 2015 and it is estimated to be about 642 million by 2040 (IDF Atlas 2015). The low and middle income countries are facing the most rapid increase in the prevalence of DM (specially its type 2 variety) and its complications with about 80% of the global patients living within these countries. Bangladesh ranks 10th in the world in terms of the number of adult DM patients and it is estimated that it will rank 8th by 2040 (IDF Atlas 2015).

The psychosocial and economic consequence of this life-long disorder and its complications are devastating. Although clinically characterized by hyperglycemia, biochemically DM is a heterogeneous disorder with major varieties being Type1 (T1DM), T2DM and Gestational Diabetes Mellitus (GDM) (WHO 1999; ADA 2005; Roglic 2016). Also there are two subgroups of prediabetes namely Impaired Fasting Glucose (IFG) and Impaired Glucose Tolerance (IGT). Even these individual groups and subgroups are not homogeneous entities as they are developed by diverse genetic and environmental factors. Accordingly, DM is not a 'single disease', but a 'syndrome' constituted by a group of metabolic disorders characterized by hyperglycemia (WHO 2015).

The heterogeneous nature of DM poses a challenge to understand the etiopathogenesis of this complex disorder and, consequently, to find appropriate solutions for the management and prevention of the problem. Among the varieties, T2DM is the most prevalent type of DM worldwide (IDF Atlas 2015) and it poses a special challenge due to its greater complexity with varying degree of involvement of multiple organs in the body. It is now known that, in addition to pancreatic B cells, a number of cells including hepatocytes, adipocytes, myocytes, and renal and gastrointestinal epithelial cells contribute to the development of the disorder in a major way.

Even within the constraints, as mentioned above, a great degree of progress has been made in understanding the pathophysiology of different types of DM. Regarding T2DM, it is now understood that both defects of insulin secretion and insulin action contribute to the development of the disorder although the relative contribution of the defects vary in different population groups or even in individuals (ADA 2005; WHO 2016). The molecular basis of these two defects have also been explored to a large extent (Rhodes 2000; Katsilambros *et al* 2003; Accili 2001; Marchetti *et al* 2002; Kelley *et al* 2000) and many of the interaction between genetic and environmental factors are now well acknowledged. These understandings have contributed immensely in the development of therapeutic and environmental interventions against DM and its complications.

Type 1 Diabetes

Type 1 diabetes, defined by an absolute requirement for administration of exogenous insulin, results from the autoimmune destruction of the insulin-secreting pancreatic B cells. Type 1 diabetes is a severe form associated with ketosis in the untreated state. It arises most commonly in juveniles but occasionally in non-obese adults and elderly. It is a catabolic disorder in which circulating insulin is virtually absent with elevated level of plasma glucagon. Exogenous insulin is therefore required to reverse the catabolic state, prevent ketosis and reduce the elevated blood glucose level. It is thought to result from an infectious or toxic environmental-induced autoimmune disorder (Karam 1998). Autoimmunity has been proposed to be the main reason for B cell destruction associated with type1 diabetes (Eisenbarth 1986; Rossini *et al* 1993). The pathogenesis of type 1 diabetes is initiated by activation of monocytes by unidentified factors from islet tissues. With the production of IL-1 by macrophages, nitric oxide and free radicals can be induced resulting in the abolishment of glucose-induced insulin secretion in pancreatic B cells and ultimately B cell death (Sandler *et al* 1989; Dunger *et al* 1996; Hoorens *et al* 2001; Suk *et al* 2001). The release of auto-antigens due to destruction of B cells could further trigger the activation of T lymphocytes and the production of islet cell antibodies leading to a self-perpetuating

and self-limiting circuit of cytokine production (Nerup *et al* 1988). The secretion of tumor necrosis factor (TNF) by macrophages can also further enhance the effects of IL-1 on pancreatic B cells which is controlled by a gene in HLA regions (Nerup *et al* 1988). On the other hand, HLA-DR3, DR4, DR9, and HLA-DQ have been associated with susceptibility towards type 1 diabetes in various ethnic groups (Aparicio 1991; Baisch *et al* 1992; Ikegami *et al* 1992; Chuang *et al* 1995; Israel *et al* 1998). In addition, if one of a pair of identical twins has type 1 diabetes, the probability for the other to develop the condition is 20 - 30% (Abbas *et al* 1994; ADA 2002). Therefore, genetic factors are considered to be quite important in type 1 diabtes (ADA 2002; WHO 2002). Although the role of viral infections in inducing type 1 diabetes remains controversial, toxic chemicals with structural similarities to alloxan or streptozotocin, may contribute to pancreatic B cell demise and destruction in animal model of type 1 diabetes (Foulis *et al* 1997; Jorns *et al* 1997; Cheta 1998).

Type 2 Diabetes

Type 2 is characterized (ADA 2001) by a relative insulin deficiency due to predominantly an insulin secretory defect with insulin resistance. Type 2 diabetes represents a heterogenous group of disorders comprising milder forms of diabetes that occur predominantly in adults but occasionally in adolescents. Circulating exogenous insulin is sufficient to prevent ketoacidosis but is often either subnormal or relatively inadequate because of tissue insensitivity (Rodger 1991). Obesity, which generally results in an impaired insulin action, is a common risk factor for this type of diabetes, and most patients with type 2 diabetes are obese. Genetic factors also underlie the disease (Karam 1998). It does not appear to have a strong autoimmune component with the observation that general autoantibody titres are low or absent. However, recent studies also suggested that reduction of B cell mass due to unbalanced islet neogenesis and apoptosis might play an important role in type 2 diabetes apart from deficiency of insulin secretion (Zhu *et al* 1996; Butler *et al* 2003).

Complications of diabetes mellitus

Diabetes is a complex heterogeneous disease where multiple levels of abnormalities are present in various tissues. Defects of diabetes mellitus include long-term damage, dysfunction and failure of various organs. The major long-term complications of diabetes mellitus are macrovascular including Cerebrovascular Disease (atherosclerosis, myocardial infarction, congestive heart failure), Peripheral Artery Disease (hypertension) and microvascular diseases like Diabetic Retinopathy (Microaneurysms, visual disability, blindness, retinal detachment, central vision loss, retinal vessel leakage, subsequent macular edema), Diabetic Neuropathy (polydiabetic or monodiabetic neuropathy, autonomic neuropathy), Diabetic Nephropathy (microalbuminuria, albuminuria, thickening of glomerular basement membranes, glomerular and tubular sclerosis, renal failure). (Brazionis *et al* 2008; Toddcade 2008; Donnelly *et al* 2000).

BIOCHEMICAL ABNORMALITIES INVOLVED IN THE PATHOGENESIS OF DIABETES

Diabetes mellitus is a heterogeneous group of metabolic disorders. The major metabolic lesions associated with diabetes mellitus include defective insulin secretion and insulin sensitivity by peripheral tissue targets.

Defective insulin secretion in diabetes mellitus

Defective insulin secretion is a feature of type 2 diabetes that results from inadequate compensatory increase of B cell mass and impaired glucose-dependent insulin release (Rutter 2001; Kahn and Porte 1990; Leahy 1990; Flatt *et al* 1992). The ability of pancreatic B cells to synthesize, store, and release insulin in response to variations in circulating metabolite levels and intracellular glucose metabolism is regulated by changes in ATP/ADP ratios resulting in Ca^{2+} mobilization (Matschinsky *et al* 1996). Alterations of this sensing loop occur early in the pathogenesis of type 2 diabetes, but are initially compensated by an increase of B cell mass (Bonner 1994). In this respect, pancreatic B cells appear to differ from other terminally differentiated cell types by

retaining their ability to proliferate, as demonstrated in both physiological conditions (growth, gestation) and disease states (obesity, insulin resistance (Bonner 2000). In addition to presumptive proliferation of existing B cells, there is evidence for B cell neogenesis from undifferentiated progenitors, apparently arising from the epithelial lining of pancreatic ducts (Bendayan 1987; Bertelli *et al* 2001; Bonner 2000; Bouwens and Pipeleers 1998). The factors inducing B cell proliferation under normal or pathological conditions are largely unknown, although some evidence exists about the involvement of fibroblast growth factors (FGFs) (Hart *et al* 2000), hematopoietic growth factors (HGFs) (Garcia *et al* 2000) and placental lactogen (Vasavada 2000). Moreover, signaling by receptor tyrosine kinases has been implicated as a regulatory mechanism in both B cell proliferation (Rhodes 2000; Hugl *et al* 1998) and insulin release (Hart *et al* 2000; Khan *et al* 2001; Leibiger *et a,* 1998). In particular, insulin/insulin-like growth factor (IGF) signaling through insulin receptor substrate (IRS) and phosphoinositide 3-kinase (PI 3-kinase) appears to regulate several aspects of β cell function. Thus, ablation of the insulin/IGF receptor substrate IRS-2 impairs β cell proliferation (Kubota 2000), whereas ablation of $p70^{s6k1}$, an Akt substrate, is associated with a decrease in B cell size (Pende 2000). The mutations of insulin receptor (IR) (Kulkarni 1999a) or IRS-1 (Kulkarni 1999b) impair insulin synthesis and secretion mediated by PI 3-kinase–dependent pathways (Aspinwall 2000; Kulkarni 1999b). The signals regulating B cell proliferation and insulin secretion diverge downstream of PI 3-kinase and this strongly suggests that Akt is not the sole effector of PI 3-kinase. Nevertheless, the role of growth factor signaling through PI 3-kinase as related to insulin secretion remains poorly understood. It has been demonstrated that mice lacking IRS-1 develop defective insulin secretion, whereas mice lacking IRS-2 develop impaired β cell proliferation (Accili 2001). Recently it has been shown that β cells lacking IGF1R exhibit a profound decrease of insulin secretion in response to both glucose and arginine (Xuan *et al* 2002)

Insulin Resistance

Insulin resistance refers to the impairment of the physiological action of insulin ie blunting of insulin's action at circulating concentrations that are normally effective. Insulin resistance in fat cells results in hydrolysis of stored triglyceride, which elevates free fatty acids in the blood plasma. Insulin resistance in muscle reduces glucose uptake, whereas insulin resistance in liver reduces glucose storage, with both effects serving to elevate blood glucose. High plasma levels of insulin and glucose due to insulin resistance often lead to metabolic syndrome and type 2 DM. It was established that insulin resistance is an early feature in the natural history of type 2 diabetes. Three main steps are involved in the generation of insulin resistance- a) insulin binding to the cell membrane receptor b) insulin receptor phosphorylation and c) intracellular insulin signaling. It probably results from a genetically determined reduction in insulin sensitivity, compounded by exposure to the environmental factors, which further impair insulin action. During recent years it was established that impaired B cell function is the primary underlying possible defect (Katsilambros *et al* 2003).

Normally Insulin increases glucose uptake in cells by stimulating the translocation of the glucose transporter GLUT4 from intracellular sites to the cell surface. Up to 75% of insulin-dependent glucose disposal occurs in skeletal muscle, whereas adipose tissue accounts for only a small fraction (Klip and Paquet 1990). Despite this, mice with a knockout of the insulin receptor in muscle have normal glucose tolerance (Bruning, *et al* 1998*)*,whereas those with a knockout of the insulin-sensitive glucose transporter in fat have impaired glucose tolerance, apparently owing to insulin resistance being induced in muscle and liver (Abel *et al* 2001). Both obesity and lipoatrophy also cause insulin resistance and predisposition to type 2 diabetes, demonstrating that adipose tissue is crucial in regulating metabolism beyond its ability to take up glucose. Insulin resistance or deficiency results in profound dysregulation of these processes, and produces elevations in fasting and postprandial glucose and lipid levels. Both lean and especially obese type 2 diabetics are characterized by day-long elevations in the plasma free fatty acid concentration,

which fail to suppress normally following ingestion of a mixed meal or oral glucose load (Reaven *et al* 1988). Free fatty acids (FFA) are stored as triglycerides in adipocytes and serve as an important energy source during conditions of fasting. Insulin is a potent inhibitor of lipolysis, and restrains the release of FFA from the adipocyte by inhibiting the enzyme hormone sensitive lipase. In type 2 diabetics the ability of insulin to inhibit lipolysis (as reflected by impaired suppression of radioactive palmitate turnover) and reduce the plasma FFA concentration is markedly reduced (Jansson *et al* 1994). It is now recognized that chronically elevated plasma FFA concentrations can lead to insulin resistance in muscle and liver (McGarry *et al* 2002; Kelley *et al.*, 2000) and impair insulin secretion (McGarry *et al* 2002; Kashyap *et al* 2002; Carpentier *et al* 2000). Thus, elevated plasma FFA levels can cause/aggravate the three major pathogenic disturbances that are responsible for impaired glucose homeostasis in type 2 diabetic individuals and the time has arrived for the "triumvirate" (muscle, liver, beta cell) to be joined by the "fourth musketeer" (Reaven *et al* 1995) to form the "harmonious quartet" . In addition to FFA that circulate in plasma in increased amounts, type 2 diabetic and obese nondiabetic individuals have increased stores of triglycerides in muscle (Goodpaster *et al* 2000; Greco *et al* 2002) and liver (Ryysy *et al* 2000; Miyazaki *et al* 2002) and the increased fat content correlates closely with the presence of insulin resistance in these tissues. Triglycerides in liver and muscle are in a state of constant turnover and the metabolites (ie fatty acyl CoAs) of intracellular FFAs have been shown to impair insulin action in both liver and muscle (Prentki *et al* 1996). This sequence of events has been referred to as "lipotoxicity". (Unger *et al* 1995; McGarry *et al* 2002). Evidence also has accumulated to implicate "lipotoxicity" as an important cause of beta cell dysfunction (McGarry *et al* 2002)

INVOLVEMENT OF GASTROINTESTINAL TRACT IN THE ETIOPATHOGENESIS OF DM

Gastrointestinal tract, due to its role in glucose absorption, has long been recognized as a target for therapeutic and nutritional management through dampening of postprandial glucose peak (mainly by inhibition of carbohydrate breakdown). But only during the last decade this nonclassical tissue for diabetes has attracted extra attention duo to its primary involvement in the pathophysiology of DM.

Intestinal absorption of glucose is related in some manner to absorption of sodium. Studies both *in vitro* (Schultz and Zalusky1963; Schultz and Zalusky 1964) and *in vivo* (Schedl and Clifton 1963; Levinson and Schedl 1966; Malawar *et al* 1965 and Taylor *et al* 1967) have shown increased sodium transport during absorption of actively absorbed monosaccharides, even when the sugar studied was not metabolizable. In addition, active absorption of sugars had an absolute dependence on the presence of sodium on the mucosal side of the intestinal membrane when studied *in vitro* (Riklis and Quastel 1958; Csaky and Thale 1960; Bihler and Crane 1962 and Schultz *et al* 1966). Their observation is supported by studies *in vivo* which have shown a reduction in active sugar transport when sodium was not added to the luminal fluid (Csaky and Zollicoffer 1960). Absorption of a number of other actively transported substances amino acids (Csaky 1961; Rosenberg *et al* 1965), bile salts (Playoust and Isselbacher 1964), uracil (Csakay 1961), also depends on the presence of sodium. These observations have led to the formulation of two different concepts of the role of sodium in active transport of nonelectrolytes, one concept which suggests that the main effect of sodium is to participate in carrier –facilitated entrance to the cell (Crane 1965; Schutz and Zalusky 1964), and the other which postulates that the effect of sodium is within the cell and important in the production of the energy required for uphill transport (Csaky 1963). According to the later concept, if luminal substrate concentration is high enough, absorption will occur in a downhill direction by carrier mediated diffusion regardless of the sodium concentration in the intestinal lumen (Olsen and Ingelfinger 1968).

Proximal and distal small intestinal segments of the rats were perfused *in situ* at two different rates with isotonic solutions containing glucose in concentrations ranging from 25 to 600 mg/100ml. Absorption was measured as glucose disappearance rate from the lumen. Glucose absorption had not previously been studied at intraluminal concentrations above and below blood glucose. It was observed that absorption was more rapid from the proximal segment. In both segments absorption was independent of perfusion rate and of whether glucose was analyzed by counting ^{14}C or by the Somogyi method. The later finding suggests that of the unidirectional fluxes, flux out of the bowel is much greater than flux into the bowel. At the higher concentrations absorption rate continued to increase much more rapidly in the proximal than in the distal segment (Rider *et al* 1967).

Although it is generally agreed that active sugar absorption *in vitro* is absolutely dependent on the presence of sodium ions on the luminal side of the mucosa, previous *in vivo* studies in the ileum of rat, dog and man have shown that active glucose absorption is almost as rapid from a sodium-free mannitol solution as from a sodium chloride solution. Absorption of three actively transported sugars (glucose, galactose, and 3-O-methylglucose) having different apparent Km's, and of fructose (absorbed by a separate carrier-mediated process) were measured in the human ileum *in vivo*. The following observations were made: (1) Mannitol substitution for sodium results in only a slight reduction (23%) in the active absorption of glucose; (2) Magnesium substitution for sodium results in a greater depression (45%) of glucose absorption; (3) The apparent Km for glucose absorption is increased when sodium is replaced by magnesium, but the Vmax is not altered; (4) Magnesium does not depress glucose absorption or the apparent Km for glucose transport when sodium is present in the perfusing solution; (5) Neither sodium removal nor magnesium has any effect on fructose absorption; (6) Absorption of galactose and 3-O-methylglucose (low affinity sugars for the glucose carrier) is reduced by about 40 to 50% when mannitol replaces sodium, but magnesium substitution for mannitol in a sodium-free medium does not further depress absorption of these sugars. The following conclusions were suggested by these results: First, magnesium is more effective than mannitol in reducing sodium

concentration at the glucose transport site on the brush border. Second, luminal sodium ions have an important effect on active sugar absorption in the human small intestine *in vivo*, as they do *in vitro*. And, third, there is a component of active sugar absorption (about one-half) which appears to be independent of luminal sodium ions *in vivo* (Biederdorf *et al* 1975).

Active glucose absorption is thought to depend on a gradient of sodium ion concentration across the brush border membrane of intestinal epithelial cells. This concept is generally accepted, although its validity has never been adequately evaluated in the human small intestine *in vivo*. According to this hypothesis, the rate of glucose absorption should decrease markedly if the luminal sodium concentration is markedly reduced, and glucose absorption against a concentration gradient should cease entirely if luminal sodium is lower than intracellular sodium concentration. A series of experiments were not able to show an important role of intraluminal sodium concentration in the active absorption of glucose from the human, rat, and dog ileum *in vivo.* Specifically, glucose absorption was minimally reduced or not reduced at all when intraluminal sodium concentration was reduced from 140 to as low as 2.5 mEq/liter. The discrepancy, between their results and those of previous workers whose data suggest that removal of intraluminal sodium should markedly inhibit active glucose absorption is not entirely clear, but there are a number of differences in experimental design between most previous studies and their own. Although their data show that active glucose absorption proceeds at a near normal rate even when lumen sodium concentration is reduced below 3 mEq/liter, their result do not disprove the sodium gradient theory because of the theoretic possibility that the microclimate adjacent to the brush border has a high concentration of sodium even when luminal sodium concentration is markedly reduced. The validity of the sodium gradient hypothesis would appear to be critically dependent on such a microclimate (Saltzman *et al* 1972).

After intake and digestion, carbohydrates are absorbed in the small intestine of mammals by a two-step transport system. In the first step glucose is concentrated in the cells by a mechanism catalyzed by apically located sodium-dependent glucose

transporter SGLT1. This symporter uses the electrochemical gradient of two sodium ions to transport one glucose molecule (Hediger and Rhoads 1994). The second step is the release of the intracellular glucose into the interstitial space by a mechanism thought to occur by facilitated diffusion via the glucose transporter GLUT2 (Thoren 1996) located in the basolateral membrane (Stumpel *et al* 2001).

Intestinal glucose absorption is thought to be regulated by the Na^+ dependent glucose transporter-1 (SGLT1) at the apical membrane of the intestinal epithelia (Hediger and Rhoads 1994). It has been shown in the diabetic animal and humans that the capacity of the small intestine to absorb glucose increases at the brush border membrane vesicles (BBMVs). Due to the enhanced activity and abundance of SGLT1 (Fedorak *et al* 1991; Dyer *et al* 2002)

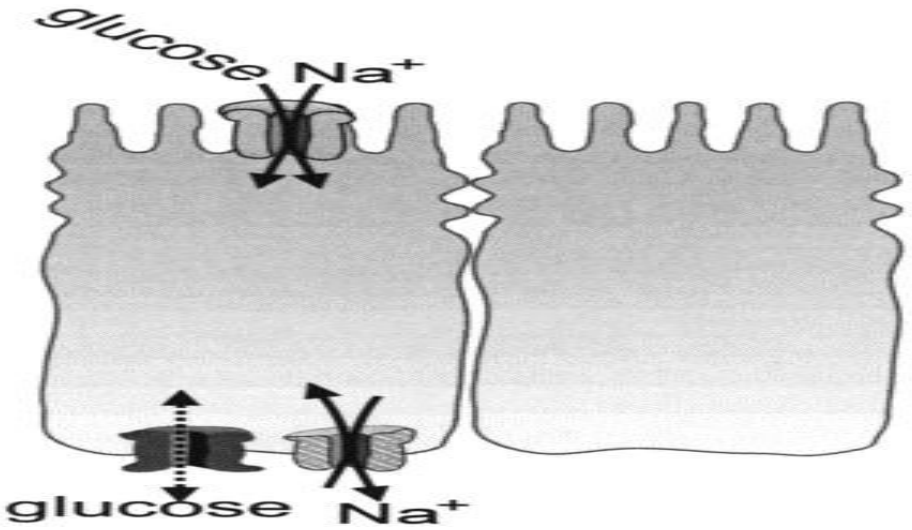

Figure1: Transcellular absorbtion of glucose. Glucose is taken up from the intestinal lumen by the brush border Na^+-glucose cotransporter via SGLT1. Glucose exits the cell via the basolateral glucose exchanger GLUT2. Na^+ traverses the basolateral membrane via ion transport channels, primarily the Na^+–K^+ ATPase. (Jerrold 2000)

Both of these elements are incorporated in current models on active transepithelial sugar transport. Only the apical membrane of the epithelial cell (termed brush border in the small intestine and in the renal proximal tubule) contains the SGLT. The sodium gradient across the brush border is generated by the sodium-potassium stimulated ATPase, a primary active ion transport ATPase which removes sodium from the cell interior in exchange to potassium ions. Thus, D-glucose can be accumulated in the epithelial cells uphill from the intestinal or renal tubular lumen

above the sugar concentration in the blood. The sugar leaves the cell along its concentration difference in a carrier-medicated, sodium-independent, passive movement (Wright *et al* 2007).

Sugar absorption in the human gut occurs in the first segments and there it has a high affinity and high velocity. As of the colon, sugar absorption ceases and sugar required for the intracellular metabolism enters the intestinal cells from the cell side exposed to the blood. SGLT1 is responsible for the active transport of glucose across the brush border membrane of the small intestine and also plays an important role in water absorption (Wright *et al* 2004).

Diabetes mellitus in rats has been shown to be associated with enhanced intestinal absorption of glucose (Aulsenbroock 1965; Caspary *et al* 1972; Crane 1961; Dubois *et al* 1969; Flores *et al* 1968; Laszt *et al* 1946, Leese *et al* 1971; Olson *et al* 1970 and Sols 1948), but the kinetic basis for this observation is disputed. An experimental study concluded that (1) the greater unidirectional flux rate of glucose in DM is due to a greater maximal transport rate and permeability co-efficient, rather than due to changes in the properties of the glucose carrier itself, and (2) these difference may be obscured by variations in unstirred water layer (Thomson 1981).

The effect of streptozotocin-induced diabetes (7 day duration) in rats on D-glucose uptake *in vivo*, the unidirectional uptake of D-glucose and L-proline *in vitro*, the passive uptake of L-glucose *in vitro* and the potential difference across the brush-border membrane has been studied. Diabetes resulted in an increased carrier-mediated glucose uptake both *in vivo* and *in vitro* and a stimulation of L-proline uptake at a concentration of the amino acid (0.025 mM) at which uptake was largely Na^+ dependent. Diabetes was without effect on uptake using a proline concentration of 50 mM at which transport was predominantly Na^+ independent. A marked hyperpolarization of the brush-border membrane and an enhanced passive glucose uptake were also evident during diabetes. They concluded that the stimulation of glucose uptake *in vivo* in diabetic intestine involves events at the brush-border membrane. The mechanisms include an increased surface area for uptake and an enhanced transmembrane electrical gradient. The latter

will have a major effect on the transport of other substrates when the uptake pathway is primarily Na^+ dependent (Debnam *et al* 1988).

In a recent experiment, the physiological role of Na-D glucose cotransporter SGLT1 in small intestine and kidney Sglt1 (-/-) mice were characterized phenotypically, compared with wild type. The authors indicate that SGLT1 is 1) pivotal for intestinal mass absorption of D-glucose, 2) triggers the glucose-induced secretion of GIP and GLP-1, and 3) triggers the upregulation of GLUT2 (Gorboulev *et al* 2012).

In recent years, the idea has evolved that affecting glucose absorption in the intestine and/or the glucose reabsorption in the kidney might be a possible way to control the sugar level. Therefore, initially inhibitors of sugar absorption have been developed which inhibit the hydrolysis of sucrose and lactose by disaccharidases in the intestinal lumen. Examples that have been successfully introduced in the market are acarbose voglibose and miglitol (Asano 2003; deMelo *et al* 2006). As the molecular understanding of the sugar transport progress, inhibitors of the transport molecule itself have been synthesized, some of which are currently undergoing preclinical and clinical testing (Castaneda and Kinne 2005; Kinne *et al* 2007).

ANIMAL MODELS OF HUMAN DIABETES

In spite of the appreciable progress in the basic, clinical and public health areas of DM, the burden of the disorder is steadily growing (IDF 2017) and the need for improved therapeutic as well as lifestyle interventions, based on scientific evidence, is still a priority.

A major obstacle to obtain optimum progress in diabetes research is to get appropriate animal models for individual types and subtypes of DM. It is well known that animal models have played key roles in exploring the etiopathogenesis and clinical characterization of human diseases and, also in designing appropriate therapeutic and other interventions against these problems (Shafrir 2003; Mc Neil 1999). There are no natural animal equivalent for human diabetes and, thus, attempts have been made to induce diabetes in animals mimicking the defects of various types and subtypes of DM. All of these strategies suffer from one or more limitations. In particular, a very

few of the models simulate T2DM which is the overwhelming prevalent type of DM all over the world. Accordingly, research involving appropriate type 2 models become difficult and frequently data from T1DM models are extrapolated to the T2DM variety. This occurs more commonly for diabetes research in resource constrained situations where availability of appropriate models is highly limited.

Among the animals, rats have been widely used in diabetes research and chemical methods using alloxan or streptozotocin injection to adult rats have been the method of choice to induce DM. Alloxan, a uric acid derivative, acts by selectively destroying pancreatic beta cells leading to insulin deficiency, hyperglycemia and ketosis (Rerup *et al* 1970). Alloxan induced diabetic animal models also show signs of glucosuria, hyperlipidemia, polyphagia, polydypsia and various complications of uncontrolled diabetes like neuropathy, cardiomyopathy, retinopathy and others. But researchers have found alloxan disadvantageous as the percentage incidence of diabetes is quite variable and is not proportionately related to increasing doses of ALX (Battell *et al* 1990). Now a days Streptozotocin (STZ) is preferably used for induction of diabetes in laboratory animals instead of alloxan.

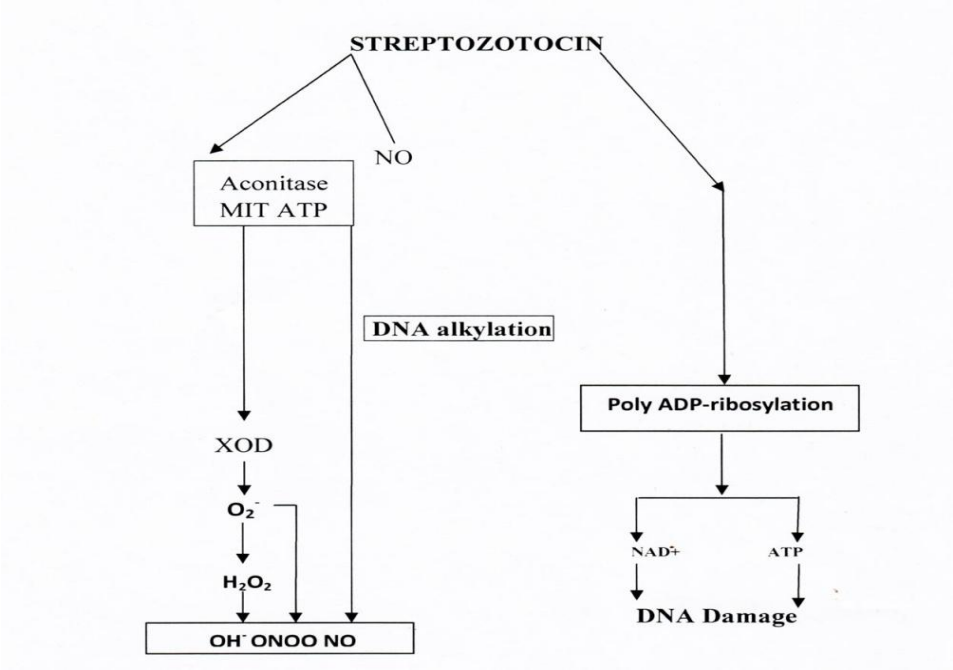

Figure 2: Mechanism of streptozotocin (STZ) induced toxic events in B cells of rat pancreas MIT (Mitochondria), XOD (Xanthine oxidase)

Streptozotocin is an antibiotic derived from *Streptomyces achromogenes* and structurally it is a glucosamine derivative of nitrosourea. It causes hyperglycemia mainly by its direct cytotoxic action on the pancreatic beta cells (Ozturk *et al* 1996; Junod *et al* 1967). Streptozotocin enters the B cell via a glucose transporter (GLUT2) and causes damage of DNA. DNA damage induces activation of poly ADP-ribosylation, a process that is more important for the diabetogenicity of streptozotocin than DNA damage itself. Furthermore, streptozotocin liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage. As a result of the streptozotocin action, B cells go through a process of destruction by necrosis (Szkudelski 2001). STZ is preferred because it has more specific B cell cytotoxicity; but the sensitivity of this agent varies with species, strain, sex and nutritional state and there are batch differences in activity (Okamato 1981). Thus diabetes produced by the high dose injection of ALX/ STZ to animals (adult or neonatal) result mainly from reduction in beta cell mass and consequent insulin deficiency and most of the developed diabetic animal models represent Type 1 DM varieties. As the percentages of type 2 diabetic subjects are rapidly increasing worldwide, it is a priority to find out the complications of T2DM and the way to manage these disorders. For this reason, induction of type 2 diabetic model is essential which can demonstrate most of the complications of T2DM.

The major strategies to develop animal models for T2DM include genetic manipulation, chemical induction, surgery, nutritional intervention and combination of two or more of these techniques (Table 1).

Table 1: Major approaches for development of T2 animal models for diabetes mellitus (Roy *et al* 2017)

Model category	Obese	Non Obese
Spontaneous or genetically derived diabetic animals	Ob/ob mouse, Db/db mouse, Kk mouse, Kk Ay mouse, NZO mouse, TSOD mouse, M16 mouse, Zucker fatty rat,ZDF rat, SHR/LA-cp rat, OLETEF rat, Obese rhesus monkey	Cohen diabetic rat GK rat Tori rat Non obese C57BL/6 ALS/Lt mouse
Diet/nutrition induced diabetic animals	Sand rat C57/ BL 6J mouse Spiny mouse	-
Chemically induced diabetic animals	GTG treated obese mouse	Low dose ALX or STZ adult rats, mice etc. Neonatal STZ rat (n-STZ).
Surgical diabetic animals	VMH lesioned diateryobese diabetic rat	Partial pancreatectomized animals eg dog, primate, pig and rats.
Transgenic / Knockout diabetic animals	B receptor knockout mice Uncoupling protein(UCPI) knockout mouse	Transgenic or knockout mice involving genes of insulin and insulin receptor & its components of downstream insulin signaling eg IRS-1,IRS-2,GLUT-4,PTP-1B & others. PPAR-Y tissue specific knockout mouse. Glucokinase or GLUT 2 gene knockout mice. Human islet amyloid polypeptide overexpressed rat (HIP rat).

KK; Kuo Kondo, KK/Ay; yellow kk obese, VMH; ventomedial hypothalamus, ZDF; zucker diabetic fatty, NZO; New Zealand obese, TSOD; Tsumara Suzuki obese diabetic, OLETEF; Otuska Long Evans Tokushima fatty, GTG; gold thio glucose, ALX; Alloxan, STZ; Streptozotocin, GLUT; glucose transporter.

Neonatal streptozotocin diabetic rats

Unlike the injection of single high dose of STZ, which can produce type 1 diabetes in adult rats, STZ when injected neonatally or immediately after birth, rats develop type 2 diabetes in the adult age. Single injection of STZ at the dose range of 80-100 mg /kg of STZ (iv or ip or sc) to one or two or five day old Wistar or Sprague-Dawley neonatal rats has been reported to produce type 2 diabetic conditions (Portha *et al* 1994). The neonatal STZ rats are considered to be better tools for the elucidation of the mechanisms associated with regeneration of the beta cells, the functional exhaustion of the beta cells and the emergence of defects in insulin action (Fernandez-Alvarez *et al* 2004). Some investigators have also developed neonatal type 2 diabetic models by injecting ALX (200 mg/kg, ip) to male neonatal rats at age of 2, 4 or 6 day after birth (Kodama *et al* 1993) and found to be much useful for the investigation of long-term complication of type 2 diabetes. Thus diabetes produced by the injection of ALX/ STZ to animals (adult or neonatal) result mainly from reduction in beta cell mass and consequent insulin deficiency. However, some investigators have attempted to replicate the disease process that naturally occurs in human beings from the progression of insulin resistance to type 2 diabetes in outbred animals. It has been achieved either by injecting chemicals (STZ) into animals which are genetically insulin resistant in its background (*eg*, SHR, ZFR). Reaven or by combination of diet [high fat (HFD) or high fructose diet] plus STZ treatment. Feeding of above special diets produces hyperinsulinemia and insulin resistance initially followed by treatment with STZ that causes the beta cell damage and frank hyperglycemia in the presence of almost absolute normal insulin circulating concentrations in nongenetic, outbred animals such as rats (Reed *et al* 2000; Zhang *et al* 2003) and mice (Manchem *et al* 2001).

Insulin secretory defects in nstz model rats

Quantitative and qualitative defects in insulin secretion in Type 2 diabetes mellitus: Type 2 diabetic subjects display more subtle changes in the dynamics of insulin secretion, such as blunting of the first phase insulin secretion and disruption of the

insulin secretory pulses (Matthews 1991). Adult n0-STZ rats are characterized by a low insulin release *in vivo* in response to glucose or amino acids (Okamato 1981). In the insulin secretion studies of the 10-16 week-old n0-STZ rats, there was a complete loss of B cell sensitivity to glucose (Portha *et al* 1979). The insulin pulse amplitude was also affected by mild to moderate B cell damage induced by STZ (Weir *et al* 1981; Bonnier *et al* 1981). The impairment of glucose-induced insulin release in n-STZ rat is clearly related to a defect in oxidative glycolysis. This leads to a severe decrease in the mitochondrial oxidative catabolism of glucose-derived pyruvate. It coincides with a lower ATP/ADP ratio in simulated islets and their subsequent alteration of ionic events rightly coupled to the fuel function of the hexose in the islet cells (Bonnier *et al* 1981). It has been found that the nstz rats exhibited an increased amylin-insulin molar ratio. This has been identified as a major component of amyloid deposits in the pancreatic islets of patients with Type 2 diabetes mellitus (Polonsky *et al* 1988; Hellerstorm *et al* 1976). It has been reported that after the n0-STZ injection, from the postnatal Day 4 onwards, signs of regeneration are apparent, in that numerous insulin positive cells are found throughout the acinar parenchyma and within the duct epithelium, but in 4-month-old animals the regeneration process was incomplete (Bonnier-Weir *et al* 1981; Okamato 1981)

Insulin resistance in nstz model rats

There are evidences that a severe reduction in the B cells obtained from subjects with Type 2 diabetes mellitus or animals after STZ injection is associated with no severe insulin resistance (Kergoat and Portha 1985). It is found that the induction of insulin resistance in an individual with reasonably normal islet function leads to modest elevation of the plasma glucose level, whereas in an individual with impaired islet cell functions it leads to hyperglycemia (Daniel 1991). In contrast to the above findings, in 8-week-old n0-STZ female rats, it was shown that hepatic glucose production measured in the basal state was higher in the diabetes mellitus models than in the controls, despite similar peripheral insulin levels in both groups (Portha *et al* 1974).

PLANT MATERIALS IN THE MANAGEMENT OF DIABETES

Plants are considered to be an attractive source for alternate agents against DM as they are known to have multiple therapeutic properties like antiulcer, antidiabetic, antihyperlipidaemic, antioxidant, anticancer, antimicrobial, radioprotective, anti-inflammatory, antipyretic, analgesic and antispermatogenic effects (Badam *et al* 2002; Jagetia *et al* 2005; Arul *et al* 2005; Kamalakkanan *et al* 2005; Rajadurai *et al* 2005; Rani *et al* 2004). Plants are sources of multiple compounds with nutritional and medicinal value, and WHO has recognized the importance of natural products in the prevention and management of diseases. The plant kingdom has become a target for multinational drug companies and research institutes for the discovery of new biologically active compounds and potential drugs (Evans 1996). The World Health Organization has recommended, especially in developing countries, the initiation of programes designed to use medicinal plants more effectively in the traditional health care system (WHO 1978). The resolution of the 31st World Health Organization Assembly requested a complete inventory, and a thorough evaluation of the efficacy, safety and standardization of medicinal plants for the treatment of diabetes (Farnsworth 1980).

The ethnobotanical information reports about 800 plants that may have antidiabetic activities. Many herbs, species and other plant materials had since been described for the treatment of diabetes, but scientific studies with these materials are limited. In the present series of study attempts have been made to scientifically evaluate the mechanism of action of a commonly used antidiabetic medicinal plants *Aegle marmelos, Trigonella foenum-graecum* and *Ocimum sanctum.*

DEVELOPMENT OF PLANT BASED DRUGS TARGETING INTESTINAL AND RENAL GLUCOSE ABSORPTION

In line with the understanding of the pathophysiology of DM drug development, has, so far, been mainly targeted considering the morphological and/or functional abnormalities in target tissues and their specific cells (pancreatic B & A cells, hepatocytes, adipocytes and myocytes).These efforts have led to considerable

advancement in this field resulting in the introduction of agents like insulin and its analogs, insulin secretagogues, insulin sensitizers and insulinomimetics.

However, most of these agents suffer from considerable limitations and apart from the risks (like hypoglycemic attack and carcinogenicity), these agents still do not address many of the abnormalities in other tissues and cells which have now been recognized as important stakeholders in the development and progression of diabetes and its complications.

Gastrointestinal tract, due its role in glucose absorption, has long been recognized as a target for therapeutic and nutritional management through dampening of postprandial glucose peak (mainly by inhibition of carbohydrate breakdown and/or mechanical interference in the passage of glucose by GI endothelium). But, only during the last decade this 'nonclassical' tissue for diabetes has attracted extra attention due to its primary involvement in the pathophysiology of DM. More recently another 'nonclassical' tissue, ie renal tubular endothelium (mediating glucose reabsorption from urinary tract), has attracted similar attention. Both tissues have similar mechanism for the passage of glucose mediated by a sodium-glucose cotransport (SGLT) mechanism (a secondary active transport) fuelled by the Na^+-K^+ pump. The cotransport process occurs at the apical region of the gut endothelial cells and the Na^+-K^+ pump operates at their basolateral surfaces.

It has been found that the Na-Gl transporters vary in the enterocytes and renal tubular cells: in the gut the transport is mediated by a high capacity low affinity carrier (called sodium-glucose transporterl or SGLTI) and in the renal tubule it is mediated by a low capacity high affinity carrier protein (termed as SGLT2). Inhibition of these two transporters have recently become a major focus in drug development against DM (John and White 2010). With SGLT1 the idea is to delay the absorption of glucose with inhibition of SGLT1 which may give specific therapeutic benefit avoiding the untoward side affects produced by the already marketed α-glucosidase inhibitors like acarbose. With the inhibition of SGLT2 renal glucosuria is targeted as a mechanism to reduce blood glucose levels. A considerable progress has already

been made in the exploration of molecules inhibiting SGLT1 and SGLT2 or both and some are in various phases of clinical trial. However, no drug in this class have yet been introduced in the market for clinical use.

Plants have been used for centuries in the treatment of DM and these are now regarded as a major source of potential antidiabetic agents. Since almost all traditional antidiabetic plant preparations have been used orally it is probable that many of those exert their action through a gut related mechanism. Studies conducted in BIRDEM-BIHS-BUHS also indicate that a number of plant preparations work through inhibition of glucose absorption in the gut (Rokeya *et al* 2011). Among those *T foenum-gracum* (Ali *et al* 1995, Hannan *et al* 2003), *O sanctum* (Alamgir *et al* 2001, Hannan *et al* 2006) *P ovata* (Rokeya *et al* 1999), *A marmelos* (Akhter 2011) and *M paraisiaca* (Rokeya *et al*, 1999) are commonly used in Bangladesh and other countries of the Indian subcontinent. The glucose inhibit absorption inhibitory action of these plants, however, have so far been attributed either to the mechanical action on guts (like affecting gastric emptying and GI motility) or inhibiting the enzymes (like disachharidases) involved in carbohydrate digestion and absorption. So far as we know, nobody has yet explored plants for modulation of SGLTs which may be a major target in controlling glucose absorption in the GI tract and renal tubules. In the above perspective the present series of studies have been designed with the following.

Antidiabetic properties of *Aegle marmelos*

Aegle marmelos (Family Rutaceae; Bael in Bengali) is widely used in Indian Ayurvedic medicine for the treatment of diabetes mellitus (Kamalakkanan *et al* 2003). Leaf extract produced anti-hyperglycemic activity in alloxan diabetic rats along with decreased cholesterol and blood urea (Ponnachan *et al* 1993). In diabetic rats leaf extract exhibited insulin like activity (Paulose *et al* 1993). Aqueous leaf extract has been shown to improve the functional state of pancreatic cells in streptozotocin induced diabetic rats (Das *et al* 1996). Aqueous leaf extract (250 & 500mg/kg, orally) produced hypoglycemic effect and increased plasma insulin level of STZ-diabetic rats. LD50 (lethal dose) observed greater than 10.0g/kg at oral administration to rats

(Sharma *et al* 1996). Anti-hyperglycemic activity of leaf extract (250mg/kg, orally) in glucose fed hyperglycemic rats was absorved (Sachdewa *et al* 2001). Aqueous extract of *Aegle marmelos* leaves, was evaluated for hypoglycemic and antioxidant effect by Upadhya *et al* (2004), by using alloxon induced diabetes in male albino rats. They proposed AML may be useful in the long-term management of diabetes. Kuttan & Sabu (2004) studied leaf extract of *A Marmelos* on alloxan induced diabetes and reported that the extract was enough capable to reduce oxidative stress by scavenging lipid peroxidation and enhancing certain antioxidant levels which causes lowering of elevated blood glucose level. Beside of all above cited works, Hema & Lalithakumari (1999) documented its hypoglycemic action along with other pharmacological actions on molecular level. Devi *et al* (2010) suggests that the combined extract of *C. auriculata* and *A marmelos* synergetic hypoglycemic effect revealed by increased serum insulin levels, decreased serum lipid levels and they therefore attributed the therapeutic value of the extract. Arumugan *et al* (2006) suggested that the leaf contain antidiabetic active principles, which would reduce the sugar level in STZ-diabetic rabbits experimented in rabbit. *Aegle marmelos* leaf extract has been reported to regenerate damaged pancreatic beta cells in diabetic rats (Das *et al* 1996) and increased the activities of peroxidase in the liver tissues of isoproterenol treated rats (Rajadurai *et al* 2005). An aqueous decoction of the leaves has been shown to possess a significant hypoglycemic effect (Karunanayeke *et al* 1984). *Aegle marmelos* leaf extract was found to be a potential antioxidant drug, which reduces the blood sugar level in alloxan induced diabetic rats (Sabu and Ramadasan 2004). It was found to be as effective as insulin in the restoration of blood glucose and body weight to normal levels on hyperglycemic state (Seema *et al* 1996). Aqueous leaf extract significantly controlled blood glucose, urea, body weight, liver glycogen and serum cholesterol. Showed histopathological alterations in the pancreatic, liver and the kidney tissues indicating the potential of hypoglycemic nature of the extract. The methanolic leaf extract elucidated it as an effective agent which can be used for hypoglycemic and antioxidant activity.

Antidiabetic properties of *Trigonella foenum-graecum*

Trigonilla foenum-graecum (fenugreek) is native to the area from the Eastern Mediterranean to Central Asia and Ethiopia, and is much cultivated in India, Pakistan and China (Morton 1990). It has a long history of medical uses in Ayurvedic and Chinese medicine, and has been used for numerous indications, including labour induction, digestion and as a general tonic to improve metabolism and health (Basch *et al* 2003). In India, the seeds of fenugreek are commonly consumed by people suffering from diabetes. An antidiabetic effect of fenugreek seeds has been demonstrated in experimentally induced diabetes in dogs, rats and mice (Ribes *et al* 1986; Amin *et al* 1987; Swanston-Flatt *et al* 1989; Grover *et al* 2002). It has also been reported to exert antihyperglycaemic effects in human subjects with both type 1 and type 2 diabetes (Riyad *et al* 1988; Sharma *et al* 1990; Madar & Shomer 1998).

An active component of fenugreek seeds has been found to be associated with a defatted fraction, rich in fiber containing steroidal saponins and proteins (Valette *et al* 1984). A novel amino acid derivative, 4-hydroxyisoleucine, also extracted from fenugreek seeds, has been shown to stimulate glucose-dependent insulin release from isolated rat and human islets (Sauvaire *et al* 1998). It has been reported also that fenugreek acts by delaying glucose absorption and enhancing its utilization (Raghuram *et al* 1994). Effects of fenugreek on glucose uptake and utilization have been noted in peripheral tissues (Sharma 1986) and an antioxidant effect has also been described (Ravikumar & Anuradha 1999).

Antidiabetic properties of *Ocimum sanctum*

Ocimum sanctum Linn (Labiatae), commonly known as ‘Holy basil’ is a herbaceous plant found throughout the Southern Asian region. It grows wild in India but is also widely cultivated for food in many homes and temple gardens due to its religious significance. *O. sanctum* has a long history of medicinal use and was mentioned in Charak Samhita, the ancient textbook of Ayurveda. The leaf of *O. sanctum* has been reported to contain bioactive phytochemicals such as saponin, flavonoids,

triterpenoids, and tannins (Jaggi *et al* 2003). Specific bioactive compounds previously isolated from *O sanctum* leaf are presented in (Table 1) (Kelm *et al* 2000; Hakkim *et al* 2007; Rahman *et al* 2011). The use of *O sanctum* leaves in conditions, including catarrhal bronchitis, bronchial asthma, dysentery, dyspepsia, skin diseases, chronic fever, haemorrhage, helminthiasis and ring worms have been reported (Wagner *et al* 1994, Warier *et al* 1995). Fresh leaves of basil taken together with black pepper are used as a prophylactic treatment for malaria (Dastur *et al* 1962). Extracts of *O sanctum* leaves have been shown to exert hypoglycaemic effects in various experimental animals (Chattopadhyay *et al* 1993; Rai *et al* 1997). Ethanol extracts reduced blood glucose in normal, glucose-fed hyperglycaemic and streptozotocin-diabetic rats (Chattopadhyay *et al* 1993). A diet containing *O sanctum* leaf powder fed to diabetic rats for 1 month also significantly decreased fasting blood glucose (Rai *et al* 1997). Ethanol extracts of *O sanctum* have also been reported to exhibit acute and chronic beneficial effects in alloxan-induced diabetes in rats (Vats *et al* 2002). Finally, in a randomized, placebo-controlled, clinical trial, leaf extract of *O sanctum* caused a significant decrease in fasting and post-prandial glucose (Agrawal *et al* 1996). These observations plus the evidence that compounds isolated from ethanol extracts of *O sanctum* exert antioxidant effects (Kelm *et al* 2000), illustrate the value of further studies to elucidate the antidiabetic actions of this plant.

Chapter III

Materials and Methods

Materials and Methods

PLACE OF THE STUDY

The study was conducted in the Dept of Biochemistry and Cell Biology, Bangladesh Institute of Health Sciences, Under the Faculty of faculty of Postgraduate Medical Sciences & Research, University of Dhaka, Bangladesh.

STUDY PERIOD

The study was carried out during the period of September 2013 to February 2018.

EXPERIMENTAL DESIGN

This was an interventional (experimental) study and conducted as per design described in the flow diagram.

There were few steps in the design:

a. The experimental plants *Aegle marmelos, Trigonella foenum-graecum, Ocimum sanctum* were collected following the standard botanical procedures and materials (powder/aqueous extracts) were prepared by standard chemical procedures;

b. T2DM model rats were developed by nstz method;

c. Characterization of the ND and T2DM rats were done; and

d. Intervention studies were conducted with selected plant materials on specific group of rats.

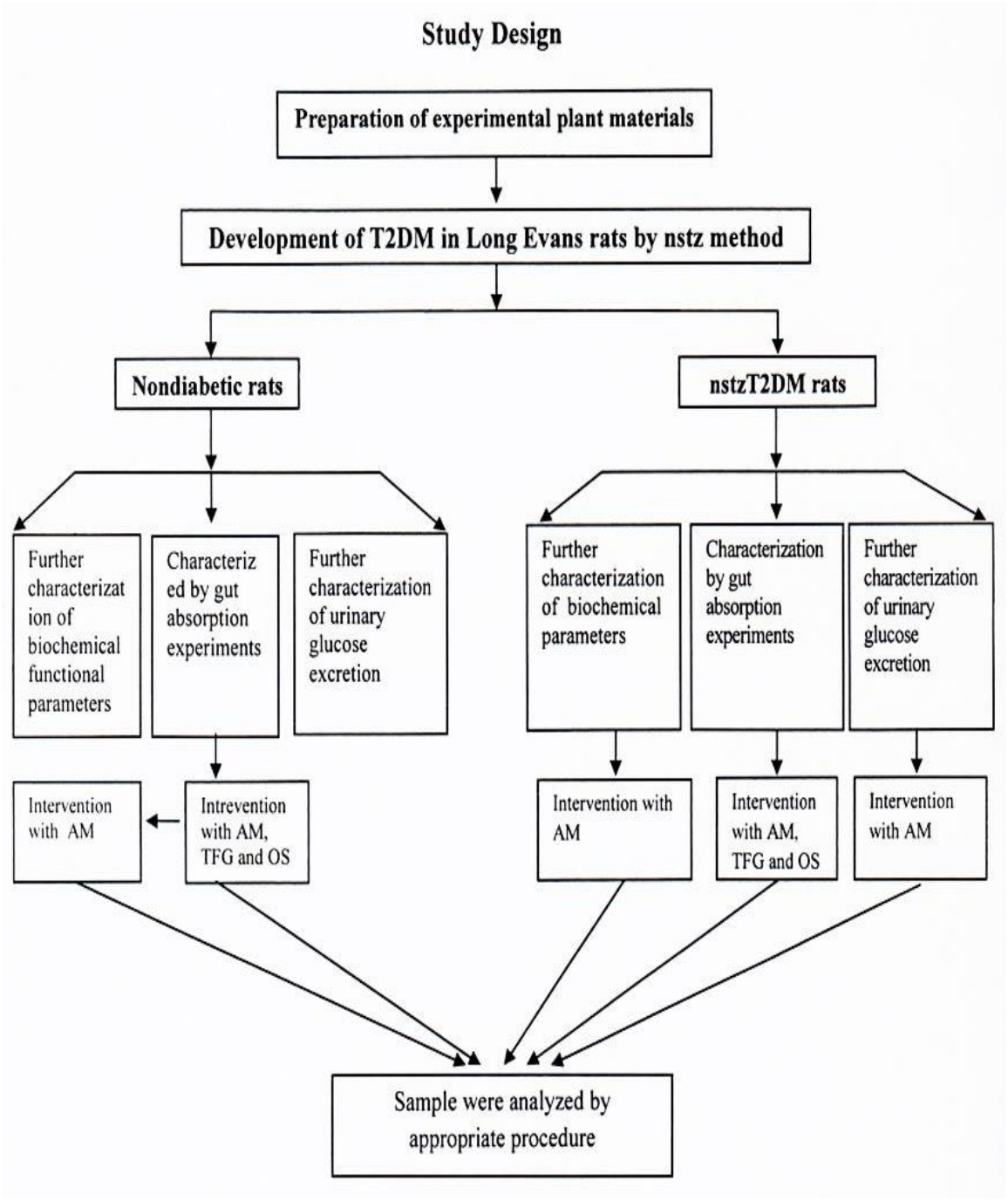

Figure 3: Flow diagram of major experimental approaches in the study AM, *Aegle marmelos;* TFG, *Trigonella foenum-graecum* and OS, *Ocimum sanctum*

CHEMICALS AND REAGENTS

Reagents and chemicals of analytical grade were used in this study. The chemicals and reagents used in the study are as follows:

-Deionized water used throughout the study was prepared by using a water purification plant (Purite, Oxon UK).

-Streptozotocin (STZ), Phenobarbital sodium salt ($C_{12}H_{11}N_2O_3Na$), Sodium chloride (NaCl), Sodium bicarbonate ($NaHCO_{3)}$ and Calcium chloride ($CaCl_2.2H_2O$) were procured from SIGMA Chemical Company Ltd, Germany.

-Potassium chloride (KCl) from FISONS Chemical Company Ltd. UK, Dextrose Anhydrous ($C_6H_{12}O_6$) and D (-) Mannitol Pure, $C_6H_{14}O_6$ were purchased from MERCK, India.

-Sodium dihydrogen phosphate dihydrate ($NaH_2PO_{4.}2H_2O$) purchased from Guangdong Guanghua Chemical Factory Co. Ltd., China.

-Glucose Reagent Kit, Serum Triglyceride Reagent Kit, Serum Cholesterol Reagent, Serum HDL Cholesterol Reagent, Serum Creatinine Reagent kit and Serum GPT Reagent were procured from Randox Laboratories Ltd., UK.

-Urine Albumin Reagent Kits were procured from Siemens Healthcare Diagnostics Inc., USA.

-Rat Insulin ELISA Kits were procured from Crystal Chem Inc, USA.

EQUIPMENT AND SOFTWARES

-Grinding of plant material were done by cyclo grinder machine (India), electric blender (Shahara, India).

-Plant extracts were dried with a Freeze Dryer (Ilshin BioBase Co. Ltd, Korea).

-Plant extracts were concentrated from water solution with the help of a Rotary evaporator (EYELA SB 1100, Japan),

-Glucose from urine sample was measured by a semi-autoanalyzer, Humalyzer, 3000 Human, Humber, Germany.

-Serum sample from rat tail tip was measured with the help of a portable pocket glucometer (Quick Check, Taiwan).

-Serum Insulin was measured by ELISA reader Multiskan Fc (Thermo Scientific Co Ltd., UK)

-Glucose from gut perfusate and serum sample and other biochemical parameters [serum serum triglyceride (TG), serum cholesterol (TChol), serum glutamate pyruvate transaminase (SGPT), serum creatinine (SCreat), urine creatinine (UCreat) and urine albumine (UAlb)] were measured by Dade Behring, Dimension, RXL, Clinical chemistry auto analyzer, Siemens, Health Care, Inc; USA.

-Estimated glomerular filtration rate (eGFR) was calculated by an App named Pocket GFR Calculator (Cockkroft-Gault Formula).

-All Homeostasis model assessments (HOMA%B, HOMA%S, HOMA%IR) were performed using software named HOMA2 Calculator v2.2.3 software**.**

PREPARATION OF THE PLANT MATERIALS

Materials from three plants were used for this study. These are unripe fruit pulp powder and aqueous extract of *Aegle marmelos* (L.) (Family: Rutaceae, Wood apple, Bael in Bengali), leaves of *Ocimum sanctum* (Family: Labiatae, Holy basil, Tulsi in Bengali), seeds of *Trigonella Foenum-graecum* L. (Family: Fabaceae, Fenugreek, Methi in Bengali).

Collection of plant materials: The *A marmelos* fruits were collected from the village Bhanga, Faridpur district. Dried seed of *Trigonella foenum-graecum* was collected from a local market (‘Karwan Bazar’ whole sale market, Tezgaon) of Dhaka and *Ocimum sanctum* leaf was collected from a village named Mithapukur, Gaibandha district.

Figure 4: *Aegle marmelos* Fruits

Aegle marmelos

Preparation of *A marmelos* fruit powder: Fruit pulp of unripe *A marmelos* was dried in the sunlight for 7 to 10 days, coarsely powdered by a cyclo grinder machine and stored in room temperature until the isolation of aqueous extract.

Preparation of aqueous extract from *A marmelos L.* Fruit: To prepare the *Aegle marmelos* fruit pulp extract (AME), the fruit pulp of 100 g dry weight, was suspended in 500 ml of distilled water in a beaker. This suspension was vigorously swirled with a spoon. The beaker was covered with aluminum foil and placed on a magnetic stirrer with a magnate inside the beaker. The stirrer was set at 600 rotations per minute and the suspension was stirred this way for 8 hours. The suspension was then sieved into another beaker and the solid part was again suspended in 100 ml of distilled water and again sieved after stirring for one hour. Combined liquid portion was taken in a round bottom flask and evaporated and concentrated in rotary evaporator under a temperature ranging from 38^0C at 60 rotations per minute. It was concentrated till the liquid was changed into a viscous pest. This pest was freeze-dried under vacuum to yield a dry solid extract. The dry sample was stored in a glass bottle at -25^0C temperature (Devi *et al* 2010).

Figure 5: Preparation of *A marmelos* fruit aqueous extract

Ocimum sanctum

Preparation of *Ocimum sanctum* L. leaf powder: Leaf of *O Sanctum* was dried in the sunlight for 4-5 days, coarsely powdered by hand and stored in room temperature until the isolation of aqueous extract.

Figure 6: Plant of *Ocimum sanctum*

Preparation of aqueous extract from *O sanctum L.* leaf powder: To prepare the *Ocimum sanctum* leaf extract (OSE), the leaf powder, 200 g dry *we*ight, was suspended in 2700 ml of distilled water in a beaker. This suspension was vigorously swirled with a spoon. The beaker was covered with aluminum foil and placed on a magnetic stirrer with a magnate inside the beaker. The stirrer was set at 600 rotations per minute and the suspension was stirred this way for 8 hours. The suspension was then sieved and liquid portion was taken in a round bottom flask and evaporated and concentrated in rotary evaporator under a temperature ranging from 38^0C at 60 rotations per minute. It was concentrated till the liquid was changed into a viscous pest. This pest was freeze-dried under vacuum to yield a dry solid extract. 33.19 g water extract was collected from 200g leaf powder. The dry sample was stored in a glass bottle at -25^0C temperature (Devi *et al.* 2010).

Trigonella foenum-graecum

Preparation of *Trigonella foenum-graecum L.* seed powder: Sun dried *Trigonella foenum-graecum* seed was powdered by electric blender stored in room temperature until the isolation of aqueous extract.

Figure 7: Seeds of *Trigonella foenum-graecum*

Preparation of aqueous extract from *Trigonella foenum-graecum L.* seed extract: The *Trigonella foenum-graecum seed* powder 500 g dry weight, was suspended in 1000 ml of distilled water repeatedly for 3 days in a beaker (after in refrigerator (4-8^0c) This suspension was vigorously swirled with a spoon. The beaker was covered with aluminum foil and placed on a magnetic stirrer with a magnate inside the beaker. The stirrer was set at 600 rotations per minute and the suspension was stirred this way for 8 hours. The suspension was then sieved and liquid portion was taken in a round bottom flask and evaporated and concentrated in rotary evaporator under a temperature ranging from 38^0C at 60 rotations per minute. It was concentrated till the liquid was changed into a viscous pest. This pest was freeze-dried under vacuum to yield a dry solid extract. 169 g aqueous extract was collected from 500g seed powder. The dry sample was stored in a glass bottle at -25^0C temperature (Devi *et al*. 2010).

EXPERIMENTAL ANIMALS

The experiments were carried out on adult Long-Evans rats of both sexes, bred at BIHS animal house and maintained at a constant room temperature of 22±5^0C with humidity of 40-70% and the natural 12 hours day-night cycle. The rats were fed on a standard laboratory pellet diet and water supplied ad *libitum*, except during the day of experimental procedures.

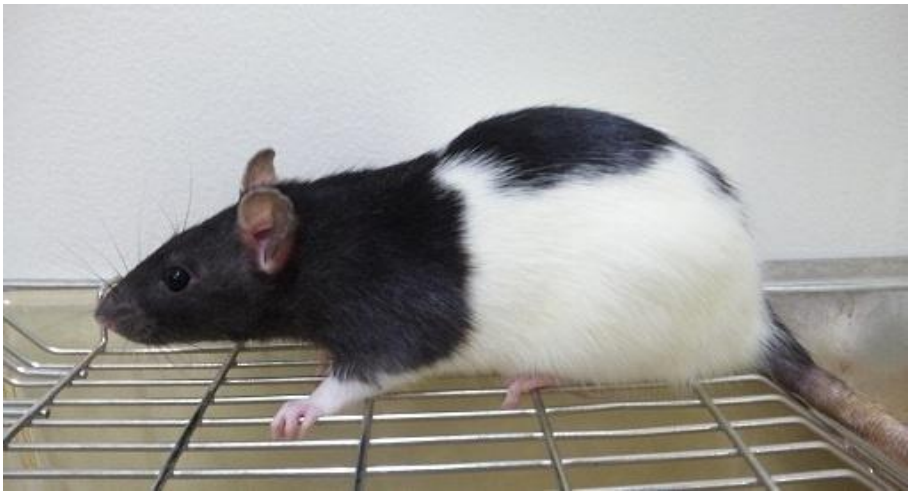

Figure 8 : Experimental Animal

Induction of T2 Diabetes Mellitus

Diabetes in the nstzT2DM rat models was induced by a single intraperitoneal injection of streptozotocin (90mg/ kg body weight, dissolved in 0.1 M cytrate buffer, pH 4.5) to the 48 hours old pups (Bonner-weir *et al 1981).* Experiments were carried out 3 months after streptozotocin injection and rats having blood glucose level 7.5 - 12 mmol/l at fasting condition were taken as diabetic. The animal models were kept at a constant room temperature of 22 ± 5^0C with humidity of 40-70 % and the natural 12 hours day-night cycle. The rats were fed on a standard laboratory pellet diet and water supplied *ad libitum.*

Figure 9: Induction of type 2 diabetes mellitus (T2DM) by injecting streptozotocin (90mg/kg bw) to 48 hours old pups of Long-Evans rats

EXPERIMENTAL PROCEDURES

Feeding of experimental substances

The experimental animals were fed with various types of solutions of glucose, fruit pulp powder of *A marmelos* and extracts of 3 medicinal plantS (AME, TFGE, OSE) during the study period with the help of a metallic feeding tube.

Sample collection Procedures

The animals, fasted for 12-14 hrs, were anesthetized with phenobarbital sodium (50mg/kg bw) and samples were collected from rats by following the procedures as mentioned below:

Collection of blood from tail tip: Tail of the anesthetized rat was kept in the water (at $38\text{-}40^0$C) for 45 seconds; the blood sample was then collected by amputation of the tail tip.

Collection of blood by heart puncture: During the sacrificing of rats after 30 days, extract feeding, they were anesthetized and after opening the abdomen blood was collected from the heart directly by syringe. Serum samples were collected by standard methods of centrifugation and stored at -25^{0}C until the estimation of biochemical parameters.

Collection of Overnight urine sample: Rats were fasted for overnight in a special cage and urine stored in the container in the early morning was collected by syringe.

Collection of urine from urinary bladder: Abdomen of the anesthetized rat was opened and urine from the urinary bladder was collected with the help of insulin syringe.

Figure 10: Collection of blood by heart puncture.

Figure 11: Blood Collection from tail tip

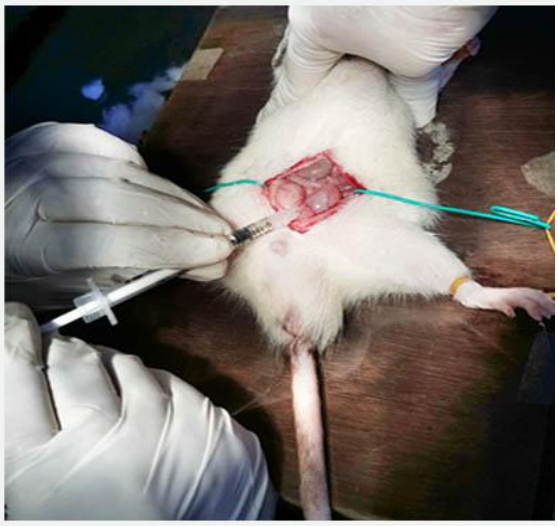

Figure 12: Collection of urine from urinary bladder.

Figure 13: Cages for overnight urine collection

Collection of perfusate from perfused gut segments (duodenum and jejunum): Krebs's ringer buffer (KRB), solution supplemented with different concentrations of sodium and glucose (expressed as molar solution), with or without plant extracts, was passed through the upper part of the duodenum (just after stomach) with the help of cannula (22G). The perfusate was collected through a catheter fixed at the end of the duodenum. Similarly various media were passed through the first 20 cm of jejunum (starting just after 25 cm of duodenum) with the help of cannula (22G). Again the perfusate was collected, using a fraction collector, from a catheter fixed at the end of the 20 cm of jejunum. A wash out period of 4-6 minutes was used during changing of media type in all the experiments. The system was set at a constant temperature of 37 ^{0}C and the perfusion rate was 0.5 ml/min in all cases. The perfusion time was 2min/sample; thus 5 samples in 10 minutes were collected by a fraction collector. The washout period before each solution was 4-6 minutes.

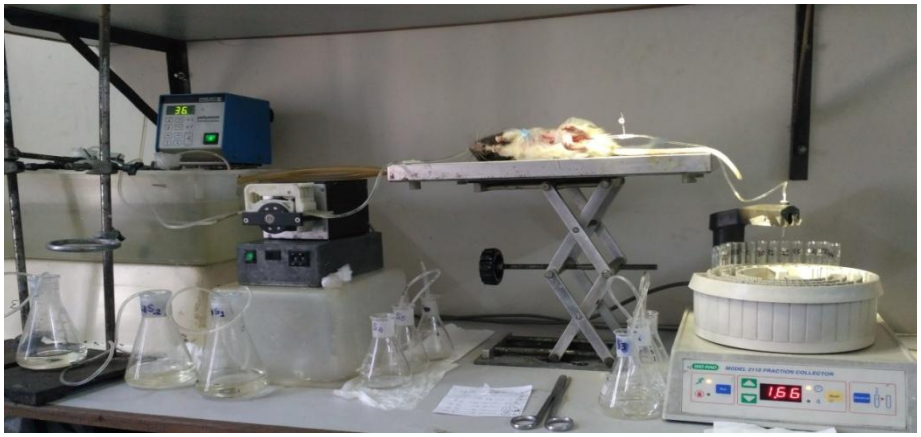

Figure 14: Collection of perfusates from perfused gut segments (duodenum and jejunum).

Perfusion media

The perfusion media were buffered by Krebs's Ringer Buffer (KRB), (Annexure- **XIV**). Experiments were conducted with the following perfusion media;

1. Preparation of Gut perfusion media Na constant (126mmol/l) varying glucose concentration

Na constant (126 mmol/l) and varying glucose concentration 3.9, 7.8, 12.5,15.6, 31.25, 62.5, 125, 250, 500, 1000, 1500, 2000, 2500, 3000, 3500 mmol/l in KRB solution.

2. ***Preparation of Gut perfusion media Na constant at (500mmol/l) varying glucose concentration***

In some series Sodium chloride (NaCl) was kept at a higher concentration (500mmol/l) and glucose concentration varied at higher levels (1500, 2000, 2500, 3000, 3500 mmol/l in water solvent)

3. ***Perfusion media replacing sodium concentration with Mannitol***

In experiments for Na-dependence of glucose absorption during gut perfusion NaCl in KRB (126 mmol/l) was replaced by equivalent concentration of D(-)Mannitol pure.

4. ***Perfusion media with plant extracts AME, TFGE, OSE (Na constant at 126 mmol/l)***

In experiments to explore the effect of the plant materials on intestinal glucose absorption respective plant extracts were used at a concentration of 0.01 and 1.0g/ml of the perfusion media.

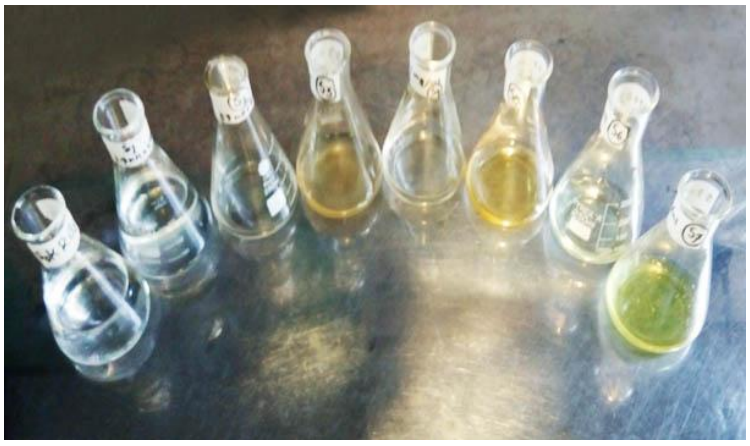

Figure 15: Solutions of different concentration with plant extracts AME, TFGE, OSE.

CHARACTERIZATION EXPERIMENTS

Characterization of nstzT2DM model rats for various biochemical parameters

The nstzT2DM model rats were characterized (by comparing with their ND counterparts) in terms of their glycemic, insulinemic, lipidemic, renal function, glucose absorption status through gut segments (duodenum and jejunum), urinary excretion status of glucose, creatinine and albumin excretion through urine.

This experiments were carried out with 14 nondiabetic and 11 nstzT2DM rats. Rats were kept in a specially designed individual cage and overnight fasting urine sample was collected in the morning. No food or water was supplied during the study period at night. The fasting blood sample was collected from all the anesthetized ND and T2DM rats. Urinary creatinine, urinary albumin, serum glucose, serum insulin, serum triglycerides, serum cholesterol, serum HDL, serum LDL, serum GPT, serum creatinine, were measured by standard methods.

Gut Perfusion Techniques for Intestinal Glucose Absorption study

General

An intestinal perfusion technique (Swintosky & Pogonowskawala 1982) was used to study the intestinal absorption of glucose in rats. The rats were fasted for (12-15hrs) and anesthetized with sodium phenobarbital (50g/kg). This experiment was carried out on both ND and nstzT2DM model rats. Age was matched (4 months). Gut perfusion experiments were performed separately on two different segments of the small intestine Duodenum and Jejunum. Twenty (20) centimeters from the starting point were used for perfusion both in case of duodenum and jejunum.

During the gut perfusion, concentrations of glucose ranging from 3.9mmol/l to 3500mmol/l were used. Aqueous extract of 3 medicinal plants (AME, TFGE, OSE) were also used at various concentrations (0.01g/ml and 1g/ml extract in 3.9 mmol/l and 15.6 mmol/l glucose solution in KRB). The sodium chloride (NaCl) concentration in the perfusion media were ether 126 mmol/l or 500mmol/l. The experiments were divided into different series. There was a washout period before each glucose solution

that passed through gut segment one after another. The results were expressed as percentage (%) of absorbed glucose, calculated from the amount of glucose in the perfusate before and after the perfusion (see the derived method).

Characterization of duodenal and jejunal glucose absorption in ND and nstzT2DM rats

Experiments with water having various concentration of glucose

In some experiments deionized water, instead of Krebs buffer, was used as the basic perfusion media. Then jejunum of nondiabetic rats were perfused with these solution and the perfusates were collected through fraction collector.

The solution used in these experiment was prepared with glucose (1500, 2000, 2500, 3000, 3500 mmol/l) and sodium (500 mmol/l) in water. Then jejunum of nondiabetic rats were perfused with these solution and the perfusates were collected through fraction collector.

Experiments with KRB buffered medium having various glucose solutions

These series were carried out with 5 nondiabetic and 12 diabetic rats. The solution used in these experiments was prepared with glucose and KRB solution. Sodium Chloride (NaCl) in KRB's solution was not manipulated, it was constant at 126mmol/l. Nondiabetic rats were perfused with (12.5, 62.5, 125, 250, 500, 1000mmol/l) and T2DM rats perfused with (3.9, 7.81, 12.5, 15.6, 31.25, 62.5, 125, 250, 500mmol/l) glucose solution. There was a washout period before each glucose solution that passed through gut segment one after another.

Experiments with 3.9 and 15.6 mmol/l glucose in KRB buffer within duodenum and jejunum

These series was carried out with 18 nondiabetic rats. Experiments of this series were conducted on both segment of gastrointestinal tract - duodenum (6 rats) and jejunum (12 rats). The solution of these series was prepared with 3.9 and 15.6mmol glucose in Kreb's solution. NaCl concentration was 126mmol/l in kreb's solution. Solution was passed as (Na126+Gl 3.9) vs (Mean126+Gl 3.9) and (Na126+Gl 15.6) vs.

(Mean126+Gl 15.6). In some solution the NaCl concentration was replaced by equimolar Mannitol. There was a washout period before each glucose solution that passed through gut segment one after another.

Renal glucose excretion during OGTT experiments

The experiments in this series were carried out on 10 ND, 10 nstzT2DM rats aged 4 months. All the animals, fasted for 12-14 hrs, were anesthetized with phenobarbital sodium and the blood sample was collected from the tail tip at 0 min time point. The rats were then fed with glucose (dextrose anhydrous 2.5g/kg bw in 20 ml water). Urine sample was collected at 0, 60, and 120 minute time points directly from the urinary bladder by 1 ml syringe (used for giving insulin). Blood sample was collected simultaneously from the tail tip and glucose level was measured by a portable glucometer on the spot but the urine sample was preserved at -25^0c for future glucose analysis.

INTERVENTION STUDIES

Effects of *A marmelos, T foenum-graecum* and *O sanctum* on glucose absorption through jejunum of nstzT2DM rats

The experiments of this series were conducted with all T2DM rats (n=18). Aqueous extract of 3 medicinal plants (*Aegle marmelos, Ocimum sanctum, Trigonella foenum-gracum*) was perfused simultaneously through the gut segment (jejunum). The three plant extracts were perfused one after another in the same experiment.

Effect of one month feeding of *A marmelos* powder on nstzT2DM model rats

After characterization the rats were fed with *A marmelos* extract (2.5g/kg body weight in 10 ml water) by a metallic tube twice daily for 30 days simultaneously with regular diet. At the end of the experiment on 31 day the overnight urine sample was collected by the same manner as 0 day (previously described). Blood sample was collected by puncturing the heart directly. Urinary glucose, urinary albumin, serum glucose, serum insulin, serum triglycerides, serum cholesterol, serum HDL, serum LDL, serum GPT and serum creatinine were measured by standard methods.

Effects of *A marmelos, T foenum-graecum* and *O sanctum* on glucose absorption through jejunum of nondiabetic and nstzT2DM rats

The number of ND rats (all ND) used in this series were 10 for *A marmelos* (AM), 9 for *T foenum-graecum* (TFG) and 10 for *O sanctum* (OS). For nstzT2DM group the number of rats were 6-10. The glucose solution in the media varied between 3.9 and 15.6 mmol/l and for every extract two concentrations (0.01 mg/ml and 1.0 mg/ml) were used. NaCl concentration 126mmol/l was constant all through the experiment. There was a washout period of 4 to 6 before switching to any alternate solution.

Effects of *Aegle marmelos* fruit pulp extract on urinary glucose excretion in nstzT2DM rats models

Ten nstzT2DM rats fed aqueous extract of *Aegle marmelos* (AME). All the animals fasted for 12-14 hrs were anesthetized with phenobarbital sodium and the blood sample was collected from the tail tip at 0 min time point. The control rats were fed with glucose (Dextrose anhydrous 2.5g/kg bw in 20 ml water) and the extract treated rats were fed with *Aegle marmelos* aqueous extract simultaneously with glucose (Dextrose anhydrous 2.5 g/kg bw + 2.5g/bw extract in 20 ml of water). Urine sample was collected at 0, 60, and 120 minutes time points by insulin syringe from the urinary bladder directly. The blood sample was collected simultaneously from the tail tips with anesthesia. The serum sample was separated through centrifugation process and all the samples (glucose and urine) were preserved at -25^0c for future glucose analysis.

BIOCHEMICAL ANALYSIS

Serum /Whole blood

- Glucose was measured by Glucometer Glucose Oxidase (GOD-PAP) method (Quick Check, Taiwan) only during rapid checking of whole blood from tail tips.
- In most of the experiments of glucose was measured by Glucose Oxidase (GOD-PAP) method (Randox Laboratories Ltd., UK). (Annexure-1)
- Serum insulin was estimated by the Rat Insulin ELISA (Enzyme Linked Immunosorbant Assay) kit of Crystal Chem Inc. USA). (Annexure-VI)

- Serum triglyceride was measured by enzymatic-colorimetric (GPO-PAP) method in the automated analyzer, using reagents of Randox Laboratories Ltd., (UK). (Annexure-III)
- Serum cholesterol was measured by enzymatic endpoint (Cholesterol Oxidase/ Peroxidase) method (Randox Laboratories Ltd., UK) (Annexure-II)
- Serum HDL cholesterol was measured by enzymatic colorimetric (cholesterol CHOD-PAP) method (Randox Laboratories Ltd., UK).(Annexure-V)
- Serum Creatinine was measured by colorimetric method method (Randox Laboratories Ltd., UK).(Annexure-VIII)
- Serum GPT was measured by colorimetric method method (Randox Laboratories Ltd., UK). (Annexure-IX)

Urine:

- Glucose was measured by Glucose Oxidase (GOD-PAP) method (Randox Laboratories Ltd., UK).(Annexure-I)
- Urine creatinine was measured by colorimetric method method (Randox Laboratories Ltd., UK). (Annexure-VIII)
- Urinary albumin was measured by Particle-enhanced turbidimetric inhibition immunoassay (PETINIA) method, USA. (Annexure- VII)

Derived parameters:

- Insulin secretion (HOMA B) was calculated by Homeostasis Model Assessment (HOMA) method using the HOMA2 Calculator v2.2.3 software. (Annexure-X)
- Insulin sensitivity (HOMA S) was calculated by Homeostasis Model Assessment (HOMA) method. In this study HOMA2 Calculator v2.2.3 software. (Annexure-XI)
- Insulin resistance (HOMA IR) was calculated by Homeostasis Model Assessment (HOMA) method. In this study HOMA2 Calculator v2.2.3 software was used. (Annexure-XII)

- Serum LDL cholesterol was determined by standard formula using values of serum total cholesterol, triglyceride and HDL-cholesterol. (Annexure- IV)
- eGFR was calculated by Cockcroft and Gault formula (CG) containing Andrid Apps(eGFR calculator). (Annexure-XIII)
- All ratios were calculated by using traditional mathematical rules.

STATISTICAL ANALYSIS

The data from all assayed biochemical parameters and derived parameters were expressed by using Median (range) and Mean±SD as appropriate significances of differences between two groups, time points or dose were compared by Mann-Whitney U test, paired t test or unpaired t test, as appropriate. The correlations were determined by Spearman's or Pearson's correlation analysis, as appropriate.

Chapter IV

Results

Results

Effect of diabetes on glycemic status, insulinemic status, insulin secretory capacity, insulin sensitivity and insulin resistance in nstzT2DM rats 3-6 months after induction of diabetes

The glycemic and insulinemic status of nondiabetic (ND) and T2DM rats are shown in table 2. The FSG was significantly higher in T2DM as compared to the ND group [FSG, Median (Range), mmol/l, ND vs T2DM, 6.72(5.70-9.40) vs 5.70(3.90-6.60), $p<0.001$]. HOMA%B was significantly higher in ND as compared to the T2DM rats [HOMA B, 167.60(39.7-367.10) vs 122.9(13.8-170.20), $p<0.008$]. No significant difference between the two groups were found regarding Fasting serum insulin (FSI), HOMA%S and HOMA%IR.

Table 2: Effect of diabetes on glycemic status, insulinemic status, insulin secretory capacity, insulin sensitivity and insulin resistance in T2DM rats 3-6 months after induction of diabetes

Parameter	ND (n=14)	T2DM (n=11)	u/p value
FSG(mmol/l)	5.70(3.90-6.60)	6.72(5.70-9.40)	18.5/0.001
FSI (ng/ml)	1.39(0.87-2.22)	1.16(0.70-2.06)	60/0.352
HOMA%B	167.60(39.7-367.10)	122.9(13.8-170.20)	23/0.008
HOMA%S	33.25(18.40-62.40)	34.50(19.20-96.80)	56.5/0.559
HOMA%IR	3.02(1.60-5.43)	2.90(1.03-5.21)	56.5/0.559

Data were expressed as Median (Range). Comparison between the ND and T2DM groups were made by Mann-Whitney U test. FSG, Fasting serum glucose; FSI, Fasting serum insulin; HOMA%B, Insulin secretory capacity; HOMA %S, Insulin sensitivity; HOMA%IR, Insulin resistance.

Lipidemic status of nstz induced T2DM model rats 3-6 months after induction of DM

No significant difference between the ND and T2DM rats was found in terms of TG, TChol, and LDL values (Table 3).

Table 3: Lipidemic status of nstz induced T2DM model rats 3-6 months after induction of DM

Parameter	ND (n=14)	T2DM (n=11)	u/p value
TG (mg/dl)	83.50(36.05-101.70)	79.150(65.95-172.60)	62.5/0.877
T Chol (mg/dl)	74.05(59.90-91.55)	78.40(71.5-118.70)	48/0.112
LDL (mg/dl)	144.36(112.62-184.40)	153.08(145.28-177.66)	10/0.308

Data were expressed as Median (Range). Comparison between the ND and nstz T2DM groups were made by Mann-Whitney U test. TG, Triglyceride; T Chol, Total Cholesterol; LDL, Low Density Lipoprotein

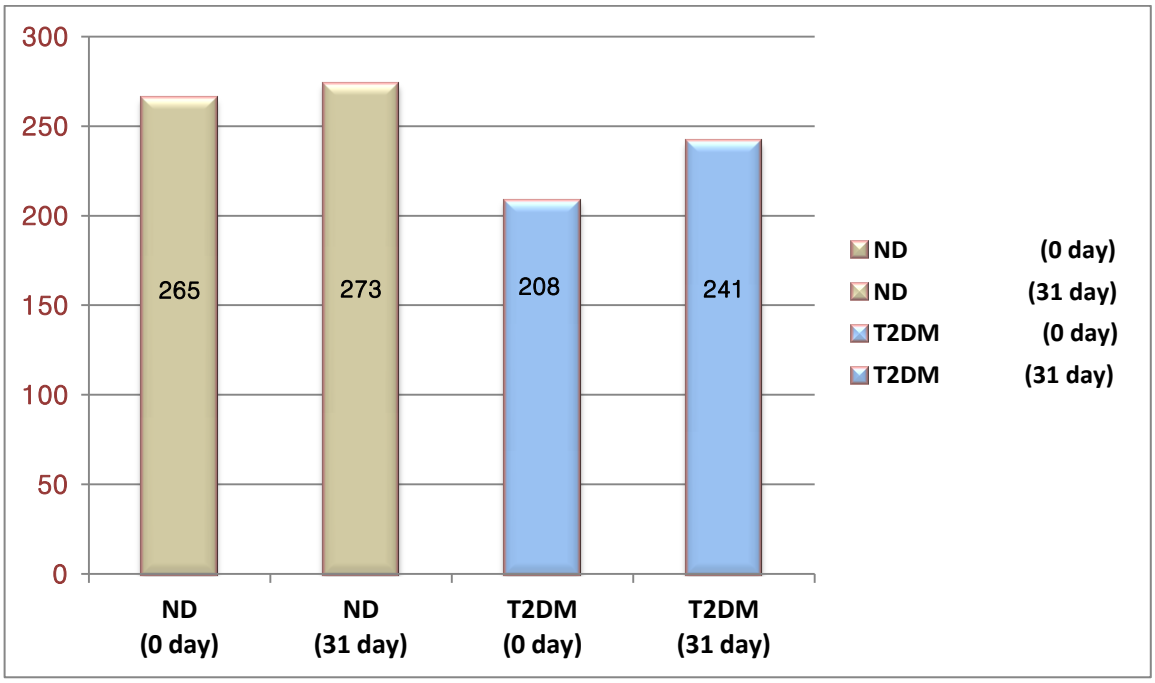

Figure 16: Effect of 30 days oral feeding of *A marmelos* powder on body weight of ND and nstz rats

Renal functional status of nstz T2DM model rats 3-6 months after induction

Table 4 shows the renal status of nondiabetic and T2DM rats. A significantly higher value was found in the nstzT2DM group regarding urinary albumin [UAlb, mg/l, ND vs T2DM, 5.90(0.70-17) vs 25.8(10.6-102.80) ($p<0.006$) and [UAlb:Ucreat ratio, ND vs T2DM, 6.56(0.39-12.81) vs 27.94(21.87-34.0) ($p<0.040$)]. No significant differences between two groups was found regarding serum creatinine (SCreat) and urinary creatinine (UCreat). The estimated glomerular filtration rate (eGFR) was significantly higher in the ND as compared to the T2DM rats [eGFR, ml/min, ND vs T2DM, 0.69(0.58-1.12) vs 0.58(0.41-0.92), $p<0.007$].

Table 4: Renal functional status of nstz T2DM model rats 3-6 months after induction

Parameter	ND (n=14)	T2DM (n=11)	u/p value
SCreat (mg/dl)	0.65 (0.40-0.90)	0.70 (0.40 -0.90)	75/0.911
UCreat (mg/dl)	95.86 (46.04-2.61)	101.65 (63.64-170.58)	67/0.861
UAlb (mg/l)	5.90 (0.70-17)	25.8 (10.6-102.80)	3/0.006
UAlb :UCreat	6.51 (0.39-12.81)	27.94 (21.87-34.0)	0.001/0.040
eGFR (ml/min)	0.69 (0.58-1.12)	0.580 (0.41-0.92)	28/0.007

Data were expressed as Median (Range). Comparison between the ND and T2DM groups were made by Mann-Whitney U test. SCreat, serum creatinine; UCreat, urinary creatinine; UAlb:UCreat, Urinary Albumin–Urinary Creatinine Ratio; eGFR, Estimated Glomerular Filtration Rate.

Comparison of jejunal absorption of glucose (%) between nondiabetic and nstz T2DM rats with various concentrations of glucose in the perfusion media

Table 5 shows the jejunal absorption of glucose (%) in ND and T2DM rats with various concentrations of glucose in the perfusion media. Jejunal absorption of glucose with 3.9 mmol/l concentration was significantly higher in T2DM as compared to the ND rats [Glucose absorption, ND vs T2DM, 21.13(13.51-50.85) vs 38.48(12.80-45.14), $p<0.019$)]. No significant difference between the two groups were found in case perfusion media with higher level of glucose concentration, 15.5, 62.5, 125, 250, 500 and 1000 mmol/l concentrations.

Table 5: Comparison of jejunal absorption of glucose (%) between ND and T2DM rats with various concentrations of glucose in the perfusion media

Group of rat	Glucose concentration in the media						
	3.9	**15.6**	**62.5**	**125**	**250**	**500**	**1000**
ND	21.13 (13.51-50.85)	16.34 (1.99-48.27)	59.04 (30.88-79.52)	44 (10.40-62.88)	32.80 (2.40-46.88)	34.72 (12.80-42.72)	36.48 (6.72-52.64)
T2DM	38.48 (12.80-45.14)	18.16 (14.37-31.52)	42.09 (9.04-77.60)	33.65 (27.04-65.44)	29.92 (25.12-64.80)	27.67 (18.72-31.84)	18.40 (13.40-35.20)
u/p	72/0.019	97/0.504	17/0.169	15/1	14/0.85	11/0.465	7/0.144

Data were expressed as Median(Range). Comparison between the two groups were done by Mann-Whitney U test. ND, Nondiabetic; T2DM, Type 2 diabetes mellitus.

Absorption of glucose (%) from the duodenum and jejunum of nondiabetic rats in response to 3.9 and 15.6 mmol/l glucose in the perfusion medium

Table 6 shows the absorption of glucose (%) from the duodenum and jejunum of nondiabetic rats in response to 3.9 and 15.6 mmol/l glucose in the perfusion medium. The percent absorption of glucose with 3.9 mmol/l concentration shows significantly higher value as compared to 15.6 mmol/l concentration in both duodenum [Glucose, Median (Range),%, 33.33(25.10-49.44) vs 10.67(10.83-39.22), p=0.045] and jejunum [Glucose, Median (Range), %, Jejunum 3.9 mmol vs. 15.6mmol, 21.13 (16.28-39.27) vs 15.99(9.96-30.74), p<0.004]. There was a trend (p=0.07) of higher percentage of glucose absorption from duodenum as compared to that from jejunum.

Table 6: Absorption of glucose (%) from the duodenum and jejunum of nondiabetic rats in response to 3.9 and 15.6 mmol/l glucose in the perfusion medium.

Glucose conc	Duodenum	Jejunum	u/p
3.9 n	33.33(25.10-49.44) 5	21.13(16.28-39.27) 8	9/0.072
15.6 n	21.67(10.83-39.22) 6	15.99(9.96-30.74) 12	19/0.079
u/p	4/0.045	15/0.004	

Data were expressed as Median (Range). Differences in glucose absorption between duodenum and jejunum as well as between 3.9 mmol/l vs. 15.6 mmol/l are compared by Mann-Whitney U test.

Na-dependent and Na-independent absorption (%) in duodenum at 3.9 and 15.6 mmol/l glucose concentration in the perfusion medium

Table 7 shows the Na-dependent and Na-independent of glucose absorption (%) in duodenum at 3.9 and 15.6 mmol/l glucose concentrations in the perfusion medium. Na-dependent absorption was significantly higher compared to Na-independent absorption at 3.9 mmol/l glucose concentration [Duodenal glucose absorption Median(Range), %, 22.98(9.92-34.28) vs 18.82(7.91-19.69), p=0.042]. The pattern, however, was reverse in case of 15.6 mmol/l glucose in the medium [4.59 (2.21-25.30) vs 13.92 (8.62-18.98), p=0.021] .

Table 7: Na-dependent and Na-independent % absorption in duodenum at 3.9 and 15.6 mmol/l glucose concentration in the perfusion medium.

Na-dependence(n=6)	3.9 mmol/l	15.6 mmol/l	u/p
Na-dependent n	22.98(9.92-34.28) 5	4.59(2.21-25.30) 6	5.00/0.035
Na-independent n	18.82(7.91-19.69) 6	13.92(8.62-18.98) 6	12.00/0.337
u/p	15/0.042	11/0.021	-

Data were expressed as Median (Range). Difference in glucose absorption between Na-dependent and Na-independent as well as between 3.9 mmol/l vs. 15.6 mmol/l were compared by Mann-Whitney U test.

Na-dependent and Na-independent glucose absorption (%) in jejunum at 3.9 mmol/l & 15.6 mmol/l glucose concentration in the perfusion medium.

Table 8 shows the Na-dependent and Na-independent % absorption of glucose in jejunum at 3.9 mmol/l & 15.6 mmol/l glucose concentration in the perfusion medium. Like duodenum, Na-dependent jejunal glucose absorption was higher compared to Na-independent absorption only at 3.9 mmol/l glucose concentration [jejunal glucose absorption, Median (Range), %, 24.31(0.36-29.33) vs 15.8(9.28-36.49), p= 0.047]. In case of 15.6 mmol/l, however, there was again reverse trend [4.24(0.36-21.73) vs 20.84(9.01-18.00), p=0.04]

Table 8: Na-dependent and Na-independent glucose absorption (%) in jejunum at 3.9 mmol/l & 15.6 mmol/l glucose concentration in the perfusion medium.

Na-dependent (n=10)	3.9 mmol/l	15.6 mmol/l	u/p value
Na-dependent n	24.31(0.36 - 29.33) 7	4.24 (0.36 - 21.73) 9	20.00/0.03
Na-independent n	15.8(9.28 - 36.49) 10	20.84(9.01 - 58.58) 11	57.00/0.620
u/p value	16.00/0.047	7.00/0.001	-

Data were expressed as Median (Range). Difference in glucose absorption between Na-dependent and Na-independent mechanism as well as between 3.9 mmol/l & 15.6 mmol/l glucose concentration in jejunum were compared by Mann-Whitney U test.

Comparison of the ratios between Na-independent/Na-dependent glucose absorption in the duodenum and jejunum of ND rats at 3.9 and 15.6 mmol/l glucose concentration in the perfusion medium

Table 9 compares the ratio between Na-independent and Na-dependent glucose absorption in the duodenum and jejunum of ND rats at 3.9 mmol/l and 15.6 mmol/l glucose concentration in the perfusion medium. In both duodenum and jejunum the ratio between the Na-independent to Na-dependent glucose absorption was significantly lower at 3.9 mmol/l glucose as compared to 15.6 mmol/l concentration. At 15.6 mmol/l the alternate trend was found.

Table 9: Comparison of the ratios between Na-independent/Na-dependent glucose absorption in the duodenum and jejunum of ND rats at 3.9 and 15.6 mmol/l glucose concentration in the perfusion medium

Segment (n=5)	**3.9**	**15.6**	**u/p**
Duodenum	0.82(0.31-1.74)	3.03(1.56-6.30)	12.00/0.024
Jejunum	0.65(0.32-7.72)	4.90(3.60-7.35)	14.00/0.018
u/p	7.00/0.08	23.00/0.223	-

Data were expressed as Median (Range). Difference in glucose absorption between duodenum and jejunum as well as 3.9 mmol/l vs 15.6 mmol/l are compared by Mann-Whitney U test.

Effects of *A marmelos, T foenum-graecum* and *O sanctum* aqueous extracts on glucose absorption (%) in the jejunum of nondiabetic rats at perfusion media of 3.9 mmol/l glucose concentration

Table 10 shows the effects of *A marmelos, T foenum-graecum* and *O sanctum* aqueous extracts on glucose absorption (%) in the jejunum of nondiabetic rats at perfusion media glucose concentration of 3.9 mmol/l. Jejunal absorption with *A marmrlos* plant extract showed a dose-dependent effect on reduction of jejunal glucose absorption (%, Median (Range), 24.8 (14.7-50.9) for AME0, 22.0(3.5-33.4) for AME 0.01 and 16.3(2.9-29.6) for AME 1.0 g/ml; p=0.046 for 0 vs 0.01, p= 0.015 for 0 vs 1.0 and p = 0.05 for 0.01 vs 1.0). The *T foenum-gracum* extract also showed a significant effect on the reduction of glucose absorption; however, the effect was significant only at a higher concentration [jejunal glucose absorption, %, 26.03(14.77-35.44) for TGFE0 vs 15.0(12.5-35.2), for TFG 1.0, p=0.041)].

Table 10: Effects of *A marmelos, T foenum-graecum* and *O sanctum* aqueous extracts on glucose absorption (%) in the jejunum of nondiabetic rats at perfusion media of 3.9 mmol/l

Plant	Extract concentration			u/p value		
	0 (g/ml)	**0.01 (g/ml)**	**1.0 (g/ml)**	**0 vs 0.01**	**0 vs 1.0**	**0.01 vs 1.0**
A marmelos n	24. 81(14.69-50.85) 9	21.95(3.5-33.93) 10	16.32(2.86-29.56) 8	40/0.046	17/0.015	29/0.05
T. foenum-graecum n	26.03(14.77-35.44) 7	17.85(9.4-36.67) 8	15(12.5-35.24) 5	22/0.487	8/0.041	14/0.380
O. sanctum n	17.12(13.51-39.33) 8	22.73(12.3-9.67) 9	20.09(4.88-33.79) 10	36/1	37/0.790	37/0.514
u/p value						
AME vs TFGE	-	29/0.328	19/0.883	-	-	-
TFGE vs OSE	-	29/0.501	22/0.713	-	-	-
OSE vs AME	-	39/0.624	31/0.424	-	-	-

Data were expressed as Median (Range). Comparisons of glucose absorption (%) between two plants as well as between two extract concentrations were made by Mann-Whitney U test.

Effects of *A marmelos, T foenum-graecum and O sanctum* extracts on jejunal glucose absorption (%) in T2DM rats with 3.9 mmol/l glucose in the medium

Table 11 shows the effects of *A marmelos, T foenum-graecum and O sanctum* extracts on jejunal glucose absorption (%) in T2DM rats with 3.9 mmol/l glucose in the media. A dose-dependent reduction in jejunal glucose absorption was found in case of the *A marmelos* extract [jejunal glucose absorption, %, Median(Range), 38.5(12.8-45.1) for AME 0 g/ml, 25.9(10.3-42.0) for AME 0.01 g/ml, and 20.1(15.4-40.4) for AME 1g/ml; p=0.046 for 0 vs 0.01, p>0.015 for 0 vs 1.0 and p= 0.041 for 0.01 vs 1.0 g/ml groups]. In case of *T. foenum-graecum* extracts, a significant reduction in jejunal glucose absorption was found only in case of higher extract concentration [38.5(12.8-45.1) for TFGE 0g/ml and 15.4(6.7-32.1) for TFG 1.0 g/ml; p<0.048]. The *O sanctum* extract showed no significant effect on jejunal glucose absorption.

Table 11: Effects of *A marmelos, T foenum-graecum and O sanctum* extracts on jejunal glucose absorption (%) in T2DM rats with 3.9 mmol/l glucose in the medium

Plant	Extract concentration			*u/p value*		
	0 (g/ml)	0.01 g/ml	1.0 g/ml	0 vs 0.01	0 vs 1.0	0.01 vs 1.0
A marmelos n	38.48(12.8-45.14) 9	2.85(10.3-42.0) 9	20.1(15.4-40.4) 9	15.5/0.046	11/0.015	11/0.041
T. foenum-graecum n	38.5(12.8-45.14) 9	26.97(8.6-45.9) 4	15.4(6.7-32.09) 3	13/0.440	4/0.048	4/0.480
O. sanctum n	38.48(12.8-45.14) 9	25.5(1.4-49.61) 2	28.3(21.40-41.7) 5	9/1.000	14/0.257	5/1.000
u/p value						
AM vsTFG	-	9.00/0.806	4.500/0.368	-	-	-
TFG vs OS	-	4.000/1.000	4.000/0.297	-	-	-
OS vs AM	-	5.000/1.000	7.000/0.251	-	-	-

Data were expressed as Median (Range). Differences among *Aegle marmelos, Trigonella Foenum-graecum* and *Ocimum sanctum* aqueous extracts on % glucose absorption at various concentrations (0.01, 1.0 g/ml) in the jejunum of T2DM rats at perfusion media glucose concentration of 3.9 mmol/l are compared by Mann-Whitney U test.

Effects of various concentrations (0.01 g/ml and 1.0 g/ml) of *A marmelos* on glucose absorption in the jejunum of nondiabetic rats at 3.9 and 15.6 mmol/l of glucose concentration in the perfusion media.

Table 12 shows the effects of various concentrations (0.01 g/ml and 1.0 g/ml) of *A marmelos* on glucose absorption in the jejunum of nondiabetic rats at 3.9 and 15.6 mmol/l of glucose concentration in the perfusion media. At 3.9 mmol/l glucose concentration in the media there was a dose-dependent reduction jejunal glucose absorption in response to the *A marmelos* extract [jejunal glucose absorption, %, Median(Range), 26.8(14.7-50.9) for AME0, 22.0(3.5-33.9) for AME 0.01 and 16.3(2.9-29.6) for AME 1.0 g/ml; p=0.035 for 0 vs 0.01; p=0.012 for 0 vs 1.0; and p=0.043 for 0.01 vs 1.0 g/ml.

Table 12: Effects of various concentrations (0.01g/ml and 1.0g/ml) of *A marmelos* on glucose absorption in the jejunum of nondiabetic rats at 3.9 and 15.6 mmol/l of glucose concentration in the perfusion media.

Glucose conc	Extract concentration			u/p value		
	0 g/ml	0.01 g/ml	1.0g/ml	0 vs 0.01	0 vs 1.0	0.01 vs 1.0
3.9	26.83(14.69-50.85)	21.95(3.50-33.93)	16.32(2.86-29.56)	40/0.035	17/0.012	29/0.043
15.6	21.29(15.49-46.29)	10.73(1.94-43.84)	9.86(4.63-7.50)	11/0.003	4/0.001	42/0.806
u/p value	44/0.935	20/0.023	25/0.290	-	-	-

Data were expressed as Median (Range). Difference in glucose absorption between 3.9 mmol/l vs 15.6 mmol/l and with different concentration (0.01 and 1.0g/ml) of *Aegle marmelos* extract were compared by Mann-Whitney U test.

Effect of 30 days feeding of *A marmelos* extracts on glycemic & insulinemic status, insulin secretory capacity, insulin sensitivity and insulin resistance in ND and T2DM rats

Table 13 shows the glycemic status of ND and T2DM rats. Fasting serum glucose (FSG) was significantly higher in the T2DM rats at 0 day compared to the ND rats, [Glucose, Mean ± SD, mmol/l, T2DM vs ND, 6.83±1.06 vs 5.48±0.86, $p<0.002$]. FSI was significantly lower in the T2DM rats compared to the ND rats at the 31 day [Insulin, Mean±SD, ng/ml, T2DM vs ND, (1.08 ±0.92 vs 2.26±1.62) ($p<0.032$)]. B cell secretory capacity (HOMA%B) at 0 day and 31 day ware significantly higher in ND rats compared to T2DM rats [HOMA%B, Mean±SD, %, T2DM vs ND, (100.69±49.49 vs 183.8±85.13), $p<0.010$]. Insulin sensitivity, HOMA%S in T2DM rats was significantly higher in T2DM rats compared to ND at 31 day[HOMA%S, Mean±SD, %, T2DM vs ND, (63.10±30.38 vs 39.33±19.3) ($p<0.052$)] .

Table 13: Effect of 30 days feeding of *A marmelos* extracts on glycemic & insulinemic status, insulin secretory capacity, insulin sensitivity and insulin resistance in ND and T2DM rats

Parameter	0 Day	31 Day	t/p value
FSG (mg/dl)			
ND (n=14)	5.483±0.86	5.65±1.08	-0.445/0.660
T2DM (n=11)	6.83±1.06	6.28±1.22	1.130/0.272
t/p	-3.512/0.002	-1.376/0.182	
FSI (ng/ml)			
ND (n=14)	1.46±0.42	2.26±1.62	-1.789/0.085
T2DM (n=11)	1.31±0.43	1.08±0.92	0.759/0.457
t/p	0.874/0.391	2.296/0.032	
HOMA%B			
ND (n=12)	183.8±85.13	187.35±97.65	-0.091/0.928
T2DM (n=11)	100.69±49.49	104.97±33.20	-0.235/0.817
t/p	2.826/0.010	2.526/0.021	
HOMA%S			
ND (n=10)	33.94±12.59	39.33±19.3	-0.789/0.440
T2DM (n=10)	41.78±23.16	63.01±30.38	-1.811/0.086
t/p	-1.021/0.319	-2.081/0.052	
HOMA%IR			
ND (n=12)	3.33±1.22	3.28±1.88	0.081/0.936
T2DM (n=10)	2.99±1.35	2.14±1.45	1.402/0.177
t/p	0.621/0.541	1.511/0.148	

Data were expressed as Mean±SD. Comparison between 0 day vs. 31 day values were done by paired t test and that between ND and T2DM rats were done by unpaired t-test.

Effect of 30 days feeding of *A marmelos* fruit pulp powder on lipidemic status of ND and T2DM rats

Table 14 shows the effects of 30 days feeding of *A marmelos* fruit pulp powder on lipidemic status of ND and T2DM rats.

TG decreased significantly at the end point compared to the baseline both in ND and T2DM rats [TG, Mean±SD, mg/dl, 0 day vs 31 day, ND (79.42±16.65 vs 58.40±11.73), p>0.001 and T2DM (94.54±35.79 vs 61.23±17.91), p>0.013].

The Cholesterol level was significantly higher in T2DM rats both in 0 day and 31 day compared to their counter parts ND, [Chol, Mean±SD, mg/dl, ND vs T2DM, 0 day (75.22±8.47 vs 85.02±14.91), 31 day (77.14±10.06 vs 87.35±4.86), p<0.003].

The LDL level of 31 day was significantly higher in T2DM compared to ND rats, [LDL, Mean±SD, mg/dl, ND vs T2DM, 31 day, (140.96±26.23 vs 172.22±32.92), p<0.015]. No significant changes in HDL values were found regarding 0 day vs 31 day.

Table 14: Effect of 30 days feeding of *A marmelos* fruit pulp powder on lipidemic status of ND and T2DM rats

Parameter	0 Day	31 Day	t/p value
TG (mg/dl)			
ND (n=13)	79.42±16.65	58.40±11.73	3.720/0.001
T2DM (n=10)	94.54±35.79	61.23±17.91	2.738/0.013
t/p	-1.352/0.191	-0.464/0.647	
Cholesterol (mg/dl)			
ND (n=14)	75.22±8.47	77.14±10.06	-0.546/0.590
T2DM (n=11)	85.02±14.91	87.35±4.86	-0.493/0.627
t/p	-2.076/0.049	-3.336/0.003	
LDL (mg/dl)			
ND (n=8)	151.35±23.37	140.96±26.23	0.958/0.352
T2DM (n=4)	157.28±14.21	172.22±32.95	-1.223/0.244
t/p	-0.544/0.599	-2.644/0.015	

Data were expressed as Mean±SD. Comparison between 0 day vs 31 day values were done by paired t-test and that between ND and T2DM rats done by unpaired t-test.

Effect of 30 days feeding of *A marmelos* fruit pulp powder on renal functional status of ND and T2DM rats

Table 15 shows the effects of 30 days feeding of *A marmelos* fruit pulp powder on renal and hepatic status of ND and T2DM rats, no changes were found in fasting serum creatinine but there was significant decreased value in ND rats at 31 day value compared to 0 day.

A significantly decreased value was found at 31 day regarding fasting urinary creatinine, [UCreat, Mean±SD, mg/dl, ND vs T2DM, 31 day (229.54±27.43 vs 146.53±81.90), $p<0.037$]. A significantly increased value was found in ND rats in eGFR compared to T2DM rats, [eGFR, Mean±SD, ml/min, ND vs T2DM, 0 day (0.765±0.17 vs 0.59±0.15).

Table 15: Effect of 30 days feeding of *A marmelos* fruit pulp powder on renal functional status of ND and T2DM rats

Parameter	0 Day	31 Day	t/p value
SCreat (mg/dl)			
ND (n=14)	0.67±0.15	0.746±0.14	-1.357/0.186
T2DM (n=11)	0.67±0.13	0.70±0.12	-0.525/0.605
t/p	-0.023/0.982	0.913/0.371	
UCreat (mg/dl)			
ND (n=14)	111.46±56.69	229.54±27.43	-4.811/0.001
T2DM (n=10)	100.37±31.64	146.53±81.90	-1.635/0.123
t/p	0.611/0.548	2.522/0.037	
eGFR			
ND (n=14)	0.765±0.17	0.705±0.16	0.973/0.339
T2DM (n=11)	0.59±0.15	0.66±0.17	-1.082/0.292
t/p	2.774/0.011	0.606/0.550	

Data were expressed as Mean±SD. Comparison between 0 day vs 31 day values of renal functional status before and after feeding *A marmelos* fruit pulp powder was made by paired t test. $p<0.05$ is considered as statistically significant.

Serum glucose, urinary glucose and urinary vs serum glucose ratio at different time points during OGTT in ND and T2DM model rats 3 months after induction of diabetes

Table 16 shows the serum glucose, urinary glucose and urinary vs serum glucose ratios at different time point during OGTT in ND and T2DM model rats 3.5 months after induction at different time points. Urinary glucose excretion at 0 min was higher in T2DM compared to ND rats, but the differences not significant. The urinary glucose at 60 min and 120 min after OGTT load were significantly higher in T2DM compared to ND rats, [UG, Median(Range), mg/dl, ND vs T2DM, 60 min, 0.47 (0.13-4.42) vs 33(0.41-56.52) ($p>0.001$) and 120 min, 0.66(0.32-1.52) vs 37.9(24.34-56.52) ($p>0.025$)].

S glucose at 0 min, 60 min and 120 min was significantly higher in T2DM group, as compared to the ND group NDM, [SG, mmol/l, T2DM vs ND, 0min, 4.70(3.90-7.30) vs 6.25(5.50-10.10) ($p>0.001$), 60 min 8.9(7.40-12.90) vs 20.05(12.60-28.10) ($p<0.001$), 120min, 9.1(7-21.70) vs 21.3(15.30-24.70) ($p>0.003$)].

The UG/SG ratio were also significantly higher at baseline (0 min) and 60 min in T2DM compared to ND rats, [UG: SG), ND vs T2DM, 0 min, 0.04(0.02-0.37) vs 0.11(0.04-0.30) ($p>0.037$); 60 min, 0.04(0.03-0.50) vs 2.28(0.04-4.53) ($p<0.001$)].The corresponding ratio at 120 min was higher but the difference was not significant.

Table 16: Serum glucose, urinary glucose and urinary vs serum glucose ratio at different time points during OGTT in ND and T2DM model rats 3 months after induction of diabetes

Group	UG0	UG 60	UG 120	SG0	SG60	SG120	UG0:SG0	UG60:SG60	UG120: SG120
ND (n=13)	0. 18(0.11-1.73)	0.47(0.13-4.42)	0.66 (0.32-1.52)	4.70(3.90-7.30)	8.9(7.40 -12.90)	9.1(7-21.70)	0.04(0.02-0.37)	0.04(0.03-0.50)	0.00(0.0-0.2)
T2DM (n=10)	0.53(0.13 -1.92)	33(0.41-56.52)	37.9(24. 34-56.52)	16.25 (5.50-20.10)	20.05(12.60-28.10)	21.3(15. 30-24.70)	0.11(0.04-0.30)	2.28(0.04-4.53)	0.92(0.0-5.5)
u/p value	36/0.072	9/0.001	0.0/0.025	8/0.001	2/0.001	8/0.003	31/0.037	7.5/0.001	40/0.068

Data were expressed as Median (Range). Differences in values between the two groups were evaluated by Mann- Whitney U test. UG0, Urinary glucose at 0 min; UG60, Urinary glucose at 60 min; UG120, Urinary glucose at120 min. SG0, Serum glucose at 0 min; SG60,Serum glucose at 60 min; SG120,Serum glucose at120 min.

Effect of *A marmelos* on serum glucose level of T2DM rats at different time points after oral glucose load aqueous extract

Table 17 shows the effects of *A marmelos* on aqueous extract serum glucose level of T2DM rats at different time points after oral glucose load. The extract was found to have significant hypoglycemic/antihyperglycemic effect ($p=0.047$ to 0.30) at all the time points during OGTT.

Table 17: Effect of *A marmelos* aqueous extract on serum glucose level of T2DM rats at different time points after oral glucose load along with extract

Group	SG0	SG 60	SG120
T2DM(n=10)	16.25(5.50-20.10)	20.05(12.60-28.10)	21.30(15.30-24.70)
T2DME(n=7)	10.20(5.60-13.40)	16.60(10.30-28.20)	13.90(6.20-25.70)
u/p value	26/0.035	24/0.047	16/0.03

Data were expressed as Median (Range). Differences of values between the two groups were evaluated by Mann-Whitney u test. T2DME, T2DM rats fed with plant extract at a dose of 0.01g/ml. SG0, Serum glucose at 0 min, SG60, Serum glucose at 60 min, SG120,Serum glucose at120 min.

Effect of *A marmelos* aqueous extract on urinary glucose excretion of T2DM rats at different time points after oral glucose load

Table 18 shows the effects of *A marmelos* aqueous extract on urinary glucose excretion level of T2DM rats at different time points after oral glucose load. No significant difference between the extract and control group was found at any time point.

Table 18: Effect of *A marmelos* aqueous extract on urinary glucose excretion of T2DM rats at different time points after oral glucose load along with extract

Group	UG0	UG60	UG120
T2DM(n=10)	0.54(0.13-1.92)	33(0.41-56.52)	37.9(24.34-56.52)
T2DME(n=7)	0.58(0.12-0.95)	34.40(0.29-54.20)	13(0.69-44.60)
u/p value	33/0.84 5	28/0.495	6/0.062

Data were expressed as Median (Range). Differences of values between the two groups were evaluated by Mann-Whitney u test. T2DME, T2DM rats fed with plant extract at a dose of 0.01g/ml. UG0, Urinary glucose at 0 min, UG60, Urinary glucose at 60 min, UG120, Urinary glucose at120 min.

Effect of *A marmelos* aqueous extract on the ratios between urinary glucose and serum glucose levels of T2DM rats at different time points after oral glucose load

Table 19 shows the effects of *A marmelos* aqueous extract (0.01g/ml) on serum glucose and urinary glucose ratio of T2DM rats at different time points after oral glucose load No significant difference between the Extract and Control groups was found at any time point.

Table 19: Effect of *A marmelos* aqueous extract on the ratios between urinary glucose and serum glucose levels of T2DM rats at different time points after oral glucose

Group	UG0:SG0	UG60:SG60	UG120:SG120
T2DM(n=10)	0.11(0.04-0.30)	2.28(0.04-4.53)	0.92(0.0-5.5)
T2DME(n=7)	0.08(0.03-0.21)	1.83(0.03-6.04)	1.71(0.2-10.6)
u/p value	27.5/0.462	30/0.626	26/0.374

Data were expressed as Median (Range). Differences of values between the two groups were evaluated by Mann-Whitney u test. T2DME, T2DM rats fed with plant extract at a dose of 0.01g/ml. UG0, Urinary glucose at 0 min, UG60, Urinary glucose at 60 min, UG120, Urinary glucose at120 min. SG0, Serum glucose at 0 min, SG60,Serum glucose at 60 min, SG120,Serum glucose at120 min

Correlation between urinary and serum glucose in ND, T2DM and *A marmelos* extracted T2DM (T2DME) rats at various time points

On Spearman's correlation analysis, no significant correlation between urinary and serum glucose was found in any of the three groups at any time point (Table 20)

Table 20: Correlation between urinary and serum glucose in ND, T2DM and *A marmelos* extracted T2DM (T2DME) rats at various time points

Time point	Groups		
	NDM (r/p)	T2DM (r/p)	T2DME (r/p)
0 min	-0.405/0.170	0.855/0.002	-0.107/0.819
60 min	0.417/0.178	0.389/0.266	0.214/0.645
120 min	-0.500/0.667	-0.200/0.747	0.071/0.879

Correlation analysis was done by Speareman's correlation test. T2DME, T2DM rats fed with plant extract at a dose of 0.01g/ml.

Chapter V

Discussion and Conclusions

Discussion and Conclusions

The insulin secretory defect and insulin resistance of the nstzT2DM rats have been characterized through the present series of works. Also the Na-independent and Na-dependent absorption of glucose from the duodenum and jejunum of these rats have been explored. The renal function (along with renal tubular reabsorption of glucose) of these model rats has also been assessed in this study. Finally the effect of the aqueous extracts of three traditionally used medicinal plants (*Aegle marmelos, Trigonella foenumgraecum* and *Ocimun sanctum*) on insulinemic status and intestinal glucose absorption have been investigated in relation to its Na-dependence and also the effects of *A marmelos* on renal tubular glucose handling has been explored.

a. Insulin secretory defect and insulin resistance in nstzT2DM model rats

Defects in insulin secretion and insulin sensitivity are the hallmarks of T2DM and both of these defects are usually present (although in variable proportions) in diabetic patients (WHO 2002). In the present study the nstz rats show only insulin secretory defect as evident from the significant lower HOMA%B, but no significant difference in HOMA%S as compared to the ND rats (table2). Thus these rats express only a partial pathophysiological mechanism in comparison to human T2DM. The findings confirm to our earlier reports (Mudi *et al* 2017) on insulin secretion and sensitivity in these rats; however, it contradicts to the data reported by (Roy 2017) who claims that both defects are present in these rats.

The present findings raise an argument whether the nstz rats can be claimed to represent as models of T2DM since the characteristic defects are only partially expressed. A more traditional argument can claim these rats even as models of the T1DM variety since deficient insulin is a more typical characteristic of this type of DM. In response to these objections it can be argued that the blood glucose levels of these rats show a more characteristic T2DM pattern than that of T1DM. In stz-induced T1DM rats the fasting serum glucose (FSG) levels became substantially high at the range of 9 to 12 mmo/l and the postprandial serum glucose (PSG) becomes

much higher (Ali *et al* 1993). FSG in the present experimental nstz rats are only marginally higher than those of the nondiabetic rats (table2) and the PSG values are not raised as much as the T1DM rats. Even after this counterargument is should be noted that nstz rats may not represent the main bulk of human T2DM patients. However, there are special groups with Maturity Onset Diabetes of the Young (MODY) who show such type of isolated insulin secretory defect (ADA 2005).

In Bangladesh there are reports about a special group of young diabetic subjects who show only insulin secretory defect without insulin resistance (Zinnat *et al* 2007; Al Mahmood *et al* 2007 and Roy *et al* 2017). Also the isolated Impaired Fasting Glucose (IFG) subjects in Bangladesh have previously been shown to have only insulin secretory defects (Roy *et al,* 2017 and Sheffin *et al* 2013). Accordingly, there may be the possibility that nstz model rats more closely mimic the pathophysiological abnormalities of these specific groups of dysglycemic subjects. Further works are required to clarify these assumptions.

b. Na-dependent and Na-independent absorption of glucose through the small intestine of nondiabetic rats

It is now well recognized that Na-Gl cotransport, mediated by SGLT1, is a major mechanism in the absorption of dietary glucose from the small intestine (Inagaki 2012). It is also known that glucose, being an electrically neutral molecule, is also absorbed through the gut by simple diffusion (a Na-independent process) in spite of its relatively large size (John & White 2010). The proportional contribution of these mechanisms in rats, however, have not yet been reported and also it has not been investigated whether the proportions vary in duodenal and jejunal segments on the small intestine.

Data from the present study indicate that the proportion of Na-dependent vs Na-independent glucose absorption critically depends on the concentration of glucose in the gut lumen (with no significant difference between duodenum and jejunum). At levels of glucose in the perfusion medium, below that in the serum, a large percentage (mean around 40%) of the sugar is absorbed, whereas at level higher than serum the

percentage falls to about 25 %. It is reasonable to assume that glucose absorption at low luminal concentration is mainly mediated by the active transport process mediated by SGLT1; on the other hand the passive absorption process by diffusion will take more dominant role as the luminal concentration of glucose increases. It can also be postulated that the passive diffusion process for a large molecule like glucose is slower one compared to its faster active transport counterpart. The significant difference in the rate of glucose absorption at lower (3.9 mmol/l) and higher (15.6 mmol/l) glucose concentration can thus be explained by the fact that Na-Gl cotransport is the predominant mechanism at lower luminal glucose levels and passive diffusion is the predominant mechanism at higher glucose levels for glucose absorption in the small intestine.

c. Na-dependent and Na-independent absorption of glucose through the small intestine in nstz rats

A remarkable feature of the nstz rats, as revealed through the present perfusion experiments, is the significant increase in the rate of glucose absorption as compared to the nondiabetic controls (table5). It needs to be carefully noted that the higher rate of glucose absorption in the gut of the nstz rats occur only in case of lower levels of luminal glucose, but not at higher levels. This may signify an abnormality of the active transport process (mediated by SGLT1) in the T2DM rats. There are reports on higher rate of glucose absorption in human diabetes and therapeutic and nutritional interventions may be targeted to reduce this rate.

Lower levels of luminal glucose are, in fact, are more physiological levels as the postprandial brush border glucose concentration in humans have been shown to be around 10 mmol/l in an earlier report.

d. Renal function in nstz rats

The renal system is getting increasing attention in diabetes research not only due to the fact that nephropathy is one of the major microvascular complications of DM (ADA 2002), but also due to the importance of renal tubular handling of glucose (mediated by Na-Gl cotransport) in the maintenance of euglycemia itself. To the best

of our knowledge renal function have not been explored before in nstz rats. The present data exclude the presence of clinical nephropathy (as evidenced by no significant difference in serum creatinine from nondiabetic controls) in nstz rats; however, significantly lower eGFR values (table 4) indicate an already compromised renal function in these rats. In human diabetes the incipient nephropathy stage is characterized by microalbuminuria (or raised ACR). In the present study ACR was raised in some of the rats, but statistical analysis was not possible due to low number.

e. Urinary glucose excretion in nstz rats

In experiments on urinary glucose excretion during OGTT there was no significant difference in the nstz rats compared to their ND counterparts. In human diabetes renal excretion of glucose has been shown to increase due to increased glomerular filtration as well as reduced activity of the Na-Gl cotransporter located in the renal tubule (John & White 2010). It seems that the renal glucose handling is still not affected in nSTZ rats although the overall function already starts to be compromised as indicated by reduced eGFR.

f. Effect of Extracts of *A marmelos, T foenum-gracum* and *O sanctum* on biological parameters of nstz rats

On feeding of the extract for 30 days there was a significant fall of FSG in nstz rats in response to *A marmelos* and *T foenum-gracum.* Previous studies on these plants has reported hypoglycemic/antihyperglycemic effect of *A marmelos* (Rokeya *et al* 2011, Akhter *et al* 2012) and *T foenum-gracum (*Ali *et al* 1995, Rokeya *et al* 2011*)*in nstzT2DM rats. Thus the present data conform to these findings. There are also reports regarding the hypoglycemic/antihyperglycemic effects of *O sanctum* in T1DM model rats and in human subjects (Hannan *et al* 2006); however, the plants has not yet been studied in this particular model. In the present study no hypo-or anti-hyperglycemic effect of the plant was observed.

Among the 3 plants only *A marmelos* showed lowering effect on serum triglyceride levels of the nstz rats. Such effects have previously been described by Rokeya (2011).

g. Effect of aqueous extracts of *A marmelos, T foenum-gracum* and *O sanctum* on intestinal glucose absorption in nstz rats.

A major objectives of the present studies was to explore the effect of the three hypoglycemia/ antihyperglycemic plants on SGLT1 activity with Na-dependent glucose absorption as a marker. From the present findings *A marmelos* seems to have a pharmacological effect an the process as evidenced by the graded inhibition of glucose absorption in a dose-dependent manner. On the other hand *T foenumgracum* has a reducing effect on jejunal glucose absorption only at higher does (1mg/ml) in the perfusion medium. This indicate that *T foenumgracum* probably has a mechanical effect on the process which inhibit the absorption. The inhibition of glucose absorption, possibly by mechanical means has been reported before and it has been shown to be mediated by soluble dietary fiber, which are galactomannan in nature (Ali *et al* 1995, Hannan *et al* 2003).

h. Effect of *A marmelos* fruit extract on renal excretion of glucose after one month feeding of the extracts

During the oral administration of *A marmelos* powder to the nstz rats for 30 days the baseline and endpoint urinary glucose values indicate that the plant material does not have any significant effect on the renal tubular handling of glucose. Since the tubular reabsorption of glucose is medicated by SGLT2 the data, indirectly, indicate a lack of effect of the plant material on this transport mechanism. Along with the urinary glucose excretion the effect of the plant on serum creatinine, which is a well accepted indication of renal function, was assessed. Nephropathy is one of the major microvascular complications of T2DM and *A marmelos* has been reported to improve renal function (Mudi *et al* 2017). In the present study the plant did not affect serum creatinine; however, when the effect was evaluated on eGFR a significant beneficial effect of the plant was observed which confirm the findings of the previous study by (Mudi *et al* 2017).

i. Effect of *A marmelos* fruit extracts on renal glucose excretion during OGTT

In the present study, a significant effect of *A marmelos* extract was found on renal excretion of glucose. Increasing glucose excretion through renal pathway is now important target for antidiabetic development and SGLT2 inhibitors agents are not present *in A marmelos.*

Conclusions

Data from the present series of works lead to the following conclusions:

a. T2DM in nstz rats is associated mainly with pancreatic B cell dysfunction, not insulin resistance;
b. T2DM in nstz rats is associated with higher absorption of glucose from the intestinal lumen through both Na-dependent active (SGLT1 mediated) and Na-independent passive transport mechanisms;
c. Dyslipidemia and renal tubular abnormalities are already present in nstz rats, but hyperglycemia in these rats does not seem to be related to reabsorption of glucose (SGLT2 mediated) in the renal tubule;
d. *A marmelos* has beneficial effect on pancreatic B cell function in T2DM rats and the aqueous extract of the plant may inhibit glucose absorption in the intestine both involving sodium-glucose cotransport and passive transport mechanisms;
e. *T feoenum-gracum* may affect intestinal glucose absorption by affecting a passive transport process.

Chapter VI

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Chapter VII

Appendices

Appendices

Appendix (I): Glucose was measured by Glucose Oxidase (GOD-PAP) method (Randox Laboratories Ltd., UK).

Principle of the assay:

The Aldehyde group of p-D-glucose is oxidized by glucose oxidase to produce gluconic acid and hydrogen peroxide. Hydrogen peroxide is further broken down to water and oxygen in the presence of peroxidase and in presence of an oxygen acceptor (i.e. phenol) to produce a colored compound. The reaction of GOD-POD reagent with glucose produces 4-aminophenazone, a red colored compound.

Reaction Principle:

$$\text{Glucose} + 0_2 + H_20 \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2$$

$$2H_20_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{POD}} \text{quinoneimine} + 4H_2O$$

Sample:

Rat serum (fasting)

Buffer:

Phosphate Buffer	: 0.1mol/l, pH 7.0
Phenol	: 11 mmol/l

GOD-PAP Reagent:

4-aminophenazone	: 0.77 mmol/l
Glucose oxidase	: ≥1.5 kU/l
Peroxidase	: ≥1.5 kU/l

Standard:

Glucose	: 5.55 mmol/l (100 mg/dl)

Procedure:

While pipetting into the wells, the first two wells were kept blank and the 7 standard glucose solutions (5µl) of each concentration were pipetted in the next 7 wells of the micro-plate with duplicates. The serum samples (5µl) were pipette in the remaining micro-wells of the plate, each of them were pipeted twice. GOD-PAP reagent (250µl) was next added in all the wells. The plate was then incubated in Labsystem siEMS Shaker incubator for proper dilution for 15minutes at 37°C and using the Ultra micro- plate ELISA Reader (Bio-TekELx 808 USA), the absorbance of the samples were read.

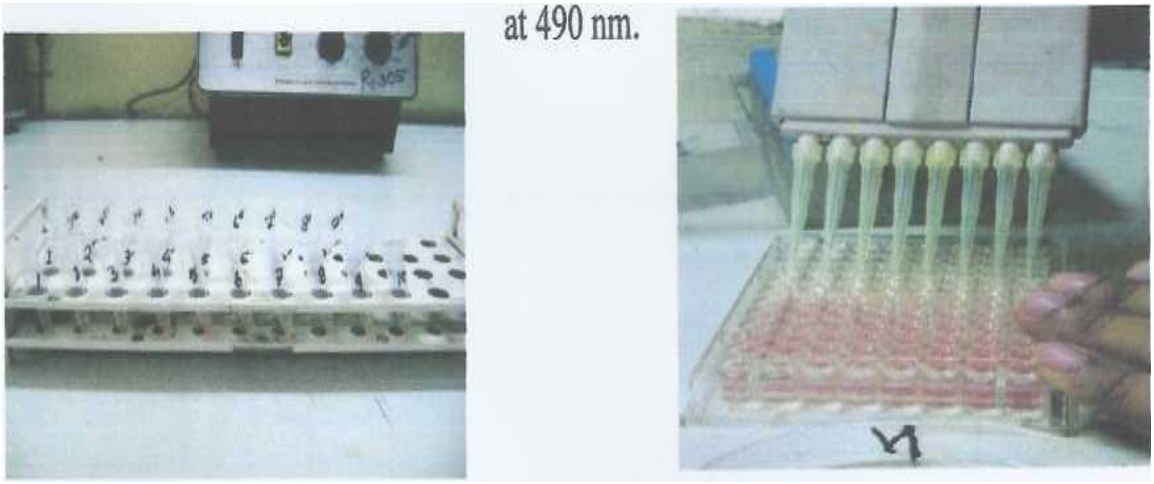

Figure ***:*** *Serum samples of rat and GOD-PAP reagent being added to all the samples for determination of glucose.*

Two parallel experiments were carried out for each sample. Thus a calibration curve was obtained for the absorbance vs. concentration of the standard solutions against a reagent blank. Based on the calibration curve, the unknown concentrations of glucose in the serum sample were measured maintaining the same mixing and incubation conditions as for the standard solutions. The standard curve was drawn parallel on every experiment day.

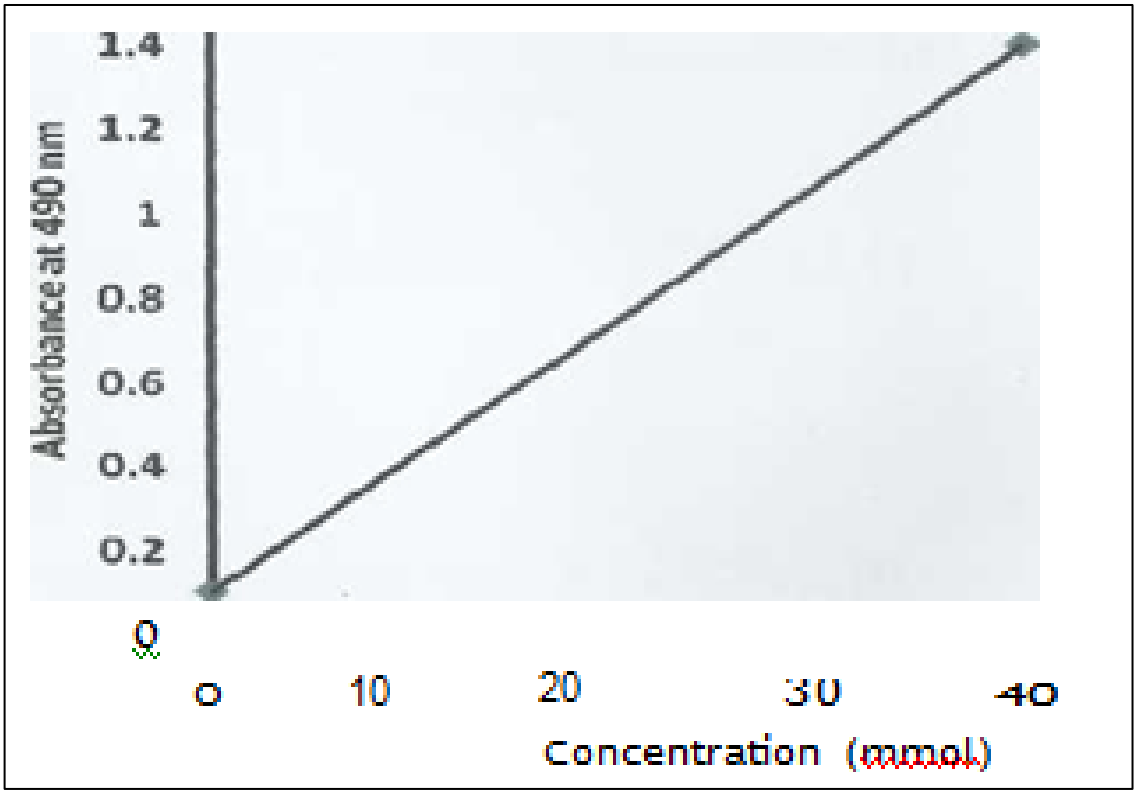

Figure: *Standard curve for glucose*

Appendix (II): Serum cholesterol was measured by enzymatic endpoint (Cholesterol Oxidase/ Peroxidase) method (Randox Laboratories Ltd., UK).

Principle of the assay:

The Cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine was formed from hydrogen peroxide and 4aminoantipyrine in the presence of phenol and peroxidase.

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O2 \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimmine} + 4H_2O$$

Reagent:

4-Aminoantipyrine	: .30mmol/l
Phenol	: 6mmol/l
Peroxidase	: >0.5U/ml
Cholesterol esterase	: >0.15U/ml
Cholesterol oxidase	: >0.1U/ml
Pipes Buffer	: 80mmol/l; pH 6.8

Materials:

Micro-centrifuge tube
Centrifuge
Micropipettes and pipettes
Disposable tips
Automatic Analyzer

Procedure:

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the Automatic Analyzer. 5µl sample and 200µl reagent were mixed and incubated at 37°C for 5 minutes within the Auto Analyzer. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result:

Concentration of cholesterol in sample was calculated by using software program with the following formula and expressed in mg/dl.

$$\text{Cholesterol concentration (mg/dl)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{Concentration of standard}$$

Appendix (III): Serum triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in the automatic analyzer, using reagents of Randox Laboratories Ltd., UK.

Principle of the assay:

Sample triglycerides incubated with a lipoprotein lipase liberate glycerol and fatty acids. Glycerol is converted to glycerol-3-phosphate by glycerol kinase and ATP. Glycerol-3- phosphate oxidase (GPO) oxidizes glycerol-3-phosphate into dihydroxy acetone phosphate and H_20_2. In the presence of peroxidase, hydrogen peroxide oxidizes the chromogen-4-aminoantipyrine and 4-chlorophenol to a violet colored complex. The quinone formed is proportional to the amount of triglycerides present in the sample. The principle is based on the following reaction system.

Reaction Principle:

$$\text{Triglyceride} + H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty acid}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-3- phosphate} + \text{ADP}$$

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

$$2H_2O_2 + \text{4-aminophenaone} + \text{4-chlorophenol} \xrightarrow{\text{Peroxide}} \text{Quinoneimine} + HCl + 4\,H_2O$$

Reagent:

Pipe Buffer	: 40 mmol/l, pH -7.6
4-chlorophenol	: 5.5 mmol/l
Megnesium ions	: 17.5 mmol/l
ATP	: 1 mmol/l
Lipase	: > 150 U/ml
Glycerol-3-phosphate oxidase	: 1.5U/ml
Peroxidase	: 0.5U/ml

Materials:

Micropipettes

Disposable tips

Auto analyzer

Procedure:

Serum and reagents was taken in specific cup. They were arranged serially. Then ID number for test was entered in the analyzer. Five 5µl sample and 200µl reagent was mixed and incubated at 37°C for 5 minutes within the cell. Reading was taken at 500 nm.

Calculation of result:

Triglyceride concentration was calculated by following formula;

$$\text{Triglyceride concentration (mg/dl)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{Concentration of standard}$$

Appendix (IV): Serum LDL cholesterol was determined by standard formula. Serum LDL-cholesterol was determined from the values of serum total cholesterol, triglyceride and HDL-cholesterol by using the following equation:

Calculation of result:

Serum LDL cholesterol = Serum total cholesterol – (HDL + Triglyceride / 5)

Appendix (V): Serum HDL cholesterol was measured by enzymatic colorimetric (cholesterol CHOD-PAP) method (Randox Laboratories Ltd., UK).

Principle of the assay:

High density lipoproteins are assayed, after precipitation of LDL and very low density lipoproteins (VLDL) with PEG (Polyethylene glycol) 6000, measuring their content of cholesterol or phospholipids. This measurement seems to be more reliable than high density proteins one.

Reagent:

Composition of precipitating reagent: Solution of PEG 6000 at 14.5%; preservatives and surfactant.

Procedure:

50 L of serum and 100 L of precipitating reagent were pippeted in to a centrifugation tube. The mixture was mixed well and centrifuged at 4000 rpm for 10 minutes. After centrifugation 5L of supernatant was taken and further procedure was followed by the procedure of cholesterol estimation.

The result was multiplied by 3 (The dilution factor of the sample).

Calculation of result:

$$\text{Serum HDL (mg/dl)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \text{ x Concentration of standard x dilution factor}$$

Appendix (VI): Serum insulin was estimated by the Rat Insulin ELISA (Enzyme Linked Immuno Sorbant Assay) kit of Crystal Chem Inc. USA.

Principle of the assay:

- First reaction: Insulin in the sample is simultaneously bound to the mouse anti-insulin monoclonal antibody coated on a micro-plate well and the anti-insulin antibody of the guinea pig serum added to each well. Consequently the mouse anti-insulin monoclonal antibody/insulin/guinea pig anti-insulin antibody complex is immobilized on the micro-plate well.
- Washing: Unbound materials are removed.
- Second reaction: Horseradish peroxidase (POD)-conjugated anti-guinea pig antibody is bound to the guinea pig anti-insulin antibody of the complex immobilized on the micro-plate well.
- Washing: Excess POD-conjugate is removed.

- Enzyme reaction: The bound POD conjugate on the micro-plate well is detected by o-phenylenediamine substrate solution.
- Measurement of the absorbance
- Evaluation of the results: The insulin concentration is determined by the standard curve obtained by plotting the absorbance vs. corresponding concentration of rat insulin standard.

Rat insulin ELISA Kit: Catalog # 90010

Mark	Description	Amount
A	Antibody-coated Micro-plate (One pack contains 6x8 well modules, i.e., 48 wells in each pack)	2 packs
B	Rat Insulin Standard, Lyophilized	2ng per vial (for 200µl).
C	Guinea pig Anti-insulin, Lyophilized Serum	2 vial (6ml each)
D	Anti-Guinea pig Antibody Enzyme Conjugate, Lyophilized: 2 vials (for 6ml each).	2vial (6ml each)
E	Enzyme Substrate (TMB) solution	1 bottle (13 ml).
F	Enzyme Reaction Stopping Solution (1N Sulfuric acid):	1 bottle (13 ml).
G	Sample Diluents	1 bottle (30 ml).
H	Wash Buffer Stock solution (20 × concentrate)	1 bottle (50 ml).
I	Frame for affixing the micro-plate well module	1 piece.
J	Plastic micro-plate cover.	1 piece

Materials:

Micropipettes

Disposable tips

Volumetric pipettes

Volumetric cylinders

Distilled water (or de ionized water)

Polypropylene test tubes

Tube racks

Vortex mixer

Aspirator for washing procedure

Micro-plate reader (Measuring wave length: 450 nm, Subtracting wave length: 630nm)

Preparation of reagents:

1. Antibody-coated Micro-plate: The micro-plate well modules were taken out from the sealed foil pouch after the pouch had been equilibrated to room temperature. Although some wells were appeared slightly white material in the well, it did not affect the performance of the assay. The micro-plate was used immediately after opening the pouch.
2. Rat insulin stock solution: Rat Insulin Standard, Lyophilized was reconstituted with the careful addition of 200µl of sample diluents. The vial was inverted gently until the contents dissolved completely. This stock solution contains 10ng/ml rat insulin.
3. Guinea pig Anti-insulin: Required volume of Guinea pig Anti-insulin, Lyophilized Serum was prepared by mixing 1 vial Guinea pig Anti-insulin, Lyophilized Serum (Marked C) with 6 ml of the sample diluents (Marked G). It was mixed well to provide the homogenous solution. Guinea pig Anti-insulin was used immediately just after reconstitution.
4. Anti-Guinea pig Antibody Enzyme Conjugate: Prepared the needed volume of Anti-Guinea pig Antibody Enzyme Conjugate solution by mixing 1 vial of Anti-Guinea pig antibody enzyme conjugate, lyophilized (Marked D) with 6 ml of Diluent (Marked G). It was mixed well to provide the homogenous solution. Anti-Guinea pig Antibody Enzyme Conjugate was used immediately just after reconstitution.

5. Enzyme Substrate Solution: (Enzyme Substrate Solution (Marked E) was provided as a ready–to-use preparation. Once the bottle is opened, the enzymes substrate solution is stable for one week at 2-8 C. It was protected from the exposure of light.
6. Enzymes reaction stop solution (1N Sulfuric acid): The enzymes reaction stop solution (Marked F) was provided as a ready –to-use preparation.
7. Sample Diluent: The sample Diluent (Marked G) as a ready to use preparation. Once the bottle is opened, the sample diluent is stable for one week at 2-6 °C.
8. Wash Buffer: Washing buffer stock solution (Marked H) should be brought to 1 lit with distilled or deionized water in a volumetric flask. It was mixed well to provide the homogenous solution. This washing buffer is stable for one month at 2-8^{o}C.

Sample: Rat serum (fasting)

The assay procedure for assaying the serum sample:

Preparation of the working standards of rat insulin:

- Pipetting of 50µl of sample Diluent (Marked G) and 50µl of Rat insulin stock solution (10ng/ml) into a polypropylene micro-tubes were labeled 5ng/ml, and then mixed thoroughly.
- 50µl of sample diluent was dispensed in to five polypropylene micro-tubes labeled as 0.156, 0.313, 0.625, 1.250 and 2.5 ng/ml respectively.
- Pipette of 50µl of 5ng/ml standard into 2.5ng/ml microtube, and it was mixed thoroughly.
- 50µl of 2.5 ng/ml standard was transferred into 1.25-ng/ml tube, and it was mixed thoroughly.
- This dilution was repeated successively on remaining microtubes.

- Dispense 50µl of sample diluent into one polypropylene microtube labeled 0 ng/ml.
- Working insulin standard should be prepared shortly before use and discarded after use.

Preparation of working rat standard

	Insulin concentration (ng/ml)							
	10	5	2.5	1.25	0.625	0. 313	0.156	0
RISS∗ (µl)	200	50						
SD∗∗ (µl)		50	50					
			50	50	50	50	50	50
				50	50	50	50	
Total	200	100	100	100	100	100	100	50

RISS∗: Rat insulin stock solution (10ng/ml)

SD∗∗: Sample diluent

Note: The working insulin standards were prepared using the polypropylene test tube because polypropylene test tube shows the minimum adsorption of insulin. Working insulin standards was discarded after use.

Assay procedure:

First reaction;

1. Antibody-coated microplate well module was taken out from the sealed foil pouch after the pouch has been equibrated to room temperature. Then it was set in a fixing frame.
2. Each well was dispense 95 µl of guinea pig anti-insulin (reconstituted by sample diluent)
3. Pipette 5µl samples (or 0 0.156 0. 313 0.625 1.25 2.5 5 10 ng/ml working rat insulin standard) into the wells.
4. The micro-plate was covered with a plastic micro-plate cover, and stand overnight (19 hours) at 4^{o}C.

Assay procedure:

Second reaction;

1. The well contents were aspirated and washed three times with 300µl washing buffer. After the last wash, remaining solution was removed by inverting the plate on the clean paper towel.
2. Dispensed 100µl of Anti-Guinea pig Antibody Enzyme Conjugate.
3. The micro-plate was covered with a plate cover and incubated for 1 hour at room temperature.

Assay procedure: Third reaction;

1. The well contents were aspirated and washed five times with 300µl washing buffer. After the last wash, remaining solution was removed by inverting the plate on the clean paper towel.
2. Immediately, 100µl of Enzyme substrate solution was dispensed and react for 30 minute at room temperature.
3. During enzyme reaction, exposuring of the micro-plate from light was avoided.

4. Provide 100µl of Enzyme Reaction Stopping Solution to stop enzyme reaction.
5. The absorbance was measured by plate reader within 30 minute
6. The absorbance was measured by using wave length: 450nm.
7. Insulin concentration was calculated from the standard curve.

Determination of the insulin concentration:

1. Mean absorbance of each set of duplicate standards or samples was determined.

(Note: If the individual absorbance values differ by greater than 20% from the corresponding mean value, the re-examination is recommended. The mean absorbance of 0ng/ml standard should be less than 0.1).

2. On a semi-log section paper, the insulin standard was constructed by plotting the mean absorbance value for each standard on Y-axis versus the corresponding standards rat insulin concentration on the X-axis.

Note: A standard curve was generated for each day.

3. The insulin concentration of the sample was determined by the standard curve using the mean absorbance value of each sample.

Appendix (VII): Urin microalbumin was measured by Particle-enhanced turbidimetric inhibition immunoassay (PETINIA) method, using flex of Siemens, Dimention RXL ,USA

Principle of procedure:

The microalbumin (MALB) method is based on a Particle-enhanced turbidimetric inhibition immunoassay (PETINIA) adapted to the Dimention Clinical Chemistry system which allows direct quantitation of albumin in urine samples. The MALB flex reagent cartage contains a particle reagent (PR) consisting of synthetic particles with human bound to surface. Aggrergation of these particles are formed when a monoclonal antibody (Ab) to human albumin in introduced. Albumin present in the sample competes with the particle for the antibody. Thereby decreasing the rate of aggregation. Hence, the rate of aggregation is inversely proportional to the

concentration of albumin (ALB) in the sample. The rete of aggregation measured using biometric turbidimetric reading at 340 and 700 nm.

Reaction Principle:

PR + Ab+ ALB (sample) ⟶ PR + Ab + ALB (sample) (scatters light at 340 nm)

Reagent ingredient:

- Particle Reagent
- Microbial inhibitors
- NaOH
- Antibody to human albumin
- Buffer
- Microbial inhibitors

Appendix (VIII): Urine and Serum creatinine was measured by colorimetric method (Randox Laboratories Ltd., UK).

Principle:

Creatinine in alkaline solution reacts with picric acid to form a colored complex. The amount of the complex formed is directly proportional to the creatinine concentration.

Reagent:

CAL. Standard : Supplied ready to use

R1a. Picric Acid : 35mmo/l

R1b. Sodium Hydroxid : 0.32mol/l

Preparation of working reagent:

Mix equal volumes of solution R1a and R1b. This working reagent is stable for 3 days at 15 to 25 ^{0} C.

Sample: Rat serum

Procedure:

Wavelength : 490-510nm

Cuvette : 1 cm light path

Incubation Temperature : 37^0C

Measurement : Against air blank

Pipette in to test tubes: Semi Micro

	Standard	**Sample**
Working reagent	1.0 ml	1.0 ml
Standard solution	0.1 ml	-
Sample	-	0.1 ml

Mix well and after 30 seconds read the absorbance A_1 of the standard and sample. Exactly 2 minutes later, read absorbance A_2 of standard and sample.

Calculation of result:

$A_2 - A_1 = \Delta A_{sample}$ or $\Delta A_{standard}$

$$\text{Serum creatinine (µmol/l)} = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times \text{Standard conc. (µmol/l)}$$

Appendix (IX): Serum GPT was measured by colorimetric method (Randox Laboratories Ltd., UK).

Principle:

In the presence of alanine, pyruvate is formed by the action of ALT. This in turn is converted to lactate by the enzyme lactate dehydrogenase (LDH) in the presence of NADH, which is simultaneously converted to NAD and this is monitored at 340nm. As the action of LDH is inhibited by increasing lactate concentration which actually favours the reverse reaction and formation of pyruvate, the assay is monitored by following the initial rate of NAD formation over 3 minutes.

Reaction principle:

The enzyme Alanine Aminotransferase (ALT/GPT) reacts primarily with Lalanine and L-glutamate, but will also use aminobutyrate, ornithine or aspartate instead of alanine.

$$\alpha\text{ - oxoglutarate + L-alanine + pyruvate} \xrightarrow{\text{GPT}} \text{L- glutamate + pyruvate}$$

The liver is an important site for this metabolic reaction. Increased levels of ALT are associated with hepatitis and other liver disorders associated with hepatic necrosis as in cirrhosis, carcinoma and obstructive jaundice. Elevated levels occur in circulatory failure and extensive trauma. Expected values are up to 30U/l.

Reagent:

R 1- Buffer	: Ready for use
Phosphate buffer	: 100 mmol/l, pH 7.4
L- alanine	: 200 mmol/l
α – oxoglutarate	: 2.0 mmol/l
R 2- 2,4- dinitrophenylhydrazine	: 2.0 mmol/l
Sodium hydroxide Solution	: 0.4mol/l

Sample: Rat serum

Procedure:

Wavelength	: Hg 546 nm
Cuvette	: 1 cm light path
Incubation Temperature	: 37^0C
Measurement	: Against reagent blank

Pipette in to test tubes:

	Reagent Blank	Sample
Sample	-	0.1 ml
Solution R1	0.5 ml	0.5 ml
Distilled water	0.1 ml	-

Mix well and incubate for exactly 30 minute at 37^0C by using incubator. After incubation, Mix 0.5 ml solution R2 in each test tube and allow standing for exactly 20 minute at 25^0 C. Finally, mix 5.0 ml sodium hydroxide in each test tube.

Calculation of result:

Read the absorbance of sample (A sample) against the reagent blank after 5 minutes and calculate the activity of ALT in the serum from the following table;

Absorbance	U/I	Absorbance	U/I	Absorbance	U/I
0.025	4	0.200	34	0.375	67
0.050	8	0.225	39	0.400	72
0.075	12	0.250	43	0.425	77
0.100	17	0.275	48	0.450	83
0.125	21	0.300	52	0.475	88
0.150	25	0.325	57	0.500	94
0.175	29	0.350	62	0.525	96

Appendix (X): Insulin secretion (HOMA B) was calculated by Homeostasis Model Assessment (HOMA) method.

In this study HOMA2 Calculator v2.2.3 software was used. The Homeostasis Model Assessment (HOMA) estimates steady state beta cell function (%B) as percentages of a normal reference population. These measures correspond well, but are not necessarily equivalent, to non-steady state estimates of beta cell function and insulin sensitivity derived from stimulatory models such as the hyperinsulinaemic clamp, the hyperglycaemic clamp, the intravenous glucose tolerance test (acute insulin response, minimal model), and the oral glucose tolerance test (0-30 delta I/G).

In 2004, the **HOMA Calculator** was released. This provides quick and easy access to the HOMA2 model for researchers who wish to use model-derived estimates of %B and %S, rather than linear approximations.

As HOMA is a steady state model, only clinically realistic values that would be seen in a fasting subject should be used. These are:

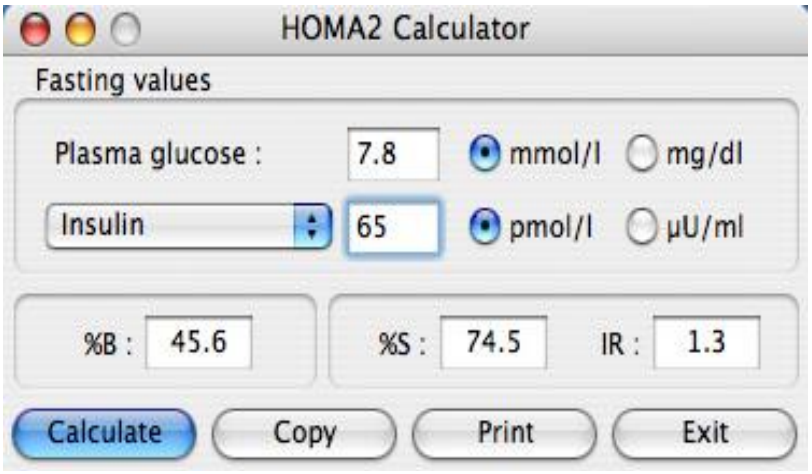

Figure: HOMA2 Calculator v2.2.3

Set up:

Run the "HOMA2 calculator Excel setup' application before first using this sheet. Click the "Enable macros" button when opening the sheet.

HOMA2 Calculator functions:

The functions available in this worksheet are;

HOMA2_Insulin (Glu.Ins)

HOMA2_Specific Insulin (Glu, Specins)

HOMA2_C- peptide (Glu, C Pep)

Each function takes two parameters are shown and return an array containing the calculated values of % and IR.

HOMA2 Calculator ranges and units:

Acceptable steady state glucose and peptide input values are:

Glucose : 3.0 to 25.0 mmol/L

Insulin : 20 to 400 pmol/L

Specific insulin : 20 to 300 pmol/L

C-peptide : 0.2 to 3.5 mmol/

Glucose and peptide values measured in other units must be converted to the units above before use.

Appendix (XI): Insulin sensitivity (HOMA S) was calculated by Homeostasis Model Assessment (HOMA) method. In this study HOMA2 Calculator v2.2.3 software was used.

The Homeostasis Model Assessment (HOMA) estimates insulin sensitivity (%S), as percentages of a normal reference population. These measures correspond well, but are not necessarily equivalent, to non-steady state estimates of beta cell function and insulin sensitivity derived from stimulatory models such as the hyperinsulinaemic clamp, the hyperglycaemic clamp, the intravenous glucose tolerance test (acute insulin response, minimal model), and the oral glucose tolerance test (0-30 delta I/G). In 2004, the **HOMA Calculator** was released. This provides quick and easy access to the HOMA2 model for researchers who wish to use model-derived estimates of %B and %S, rather than linear approximations.

As HOMA is a steady state model, only clinically realistic values that would be seen in a fasting subject should be used. These are:

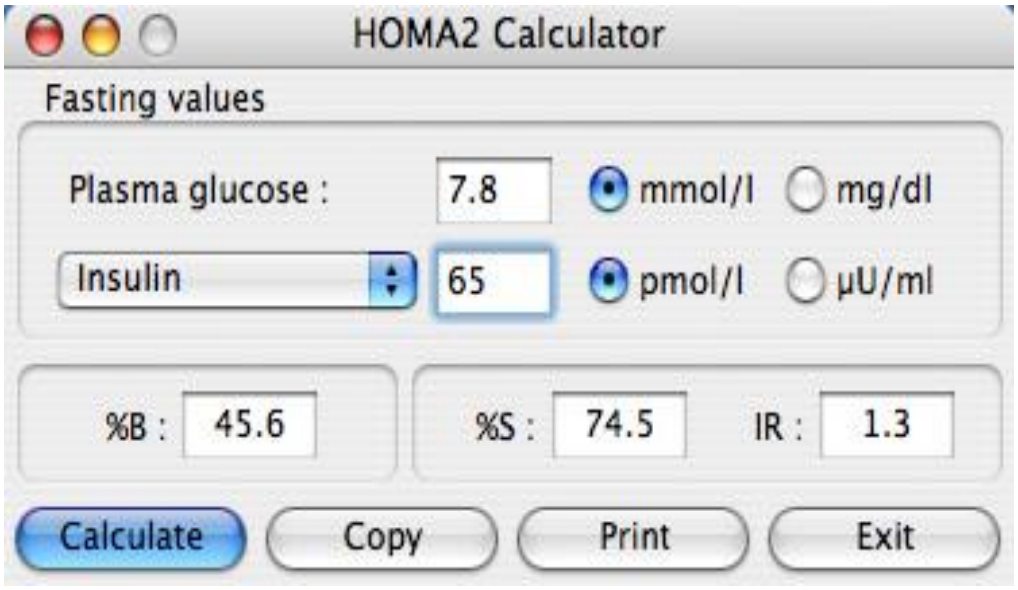

Figure: HOMA2 Calculator v2.2.3

Set up:

Run the "HOMA2 calculator Excel setup' application before first using this sheet. Click the "Enable macros" button when opening the sheet.

HOMA2 Calculator functions:

The functions available in this worksheet are;

HOMA2_Insulin (Glu.Ins)

HOMA2_Specific Insulin (Glu, Specins)

HOMA2_C- peptide (Glu, C Pep)

Each function takes two parameters are shown and return an array containing the calculated values of % and IR.

HOMA2 Calculator ranges and units:

Acceptable steady state glucose and peptide input values are:

Glucose : 3.0 to 25.0 mmol/L

Insulin : 20 to 400 pmol/L

Specific insulin : 20 to 300 pmol/L

C-peptide : 0.2 to 3.5 mmol/

Appendix (XII): Insulin resistance (HOMA IR) was calculated by Homeostasis Model Assessment (HOMA) method.

In this study HOMA2 Calculator v2.2.3 software was used. As HOMA is a steady state model, only clinically realistic values that would be seen in a fasting subject should be used. These are:

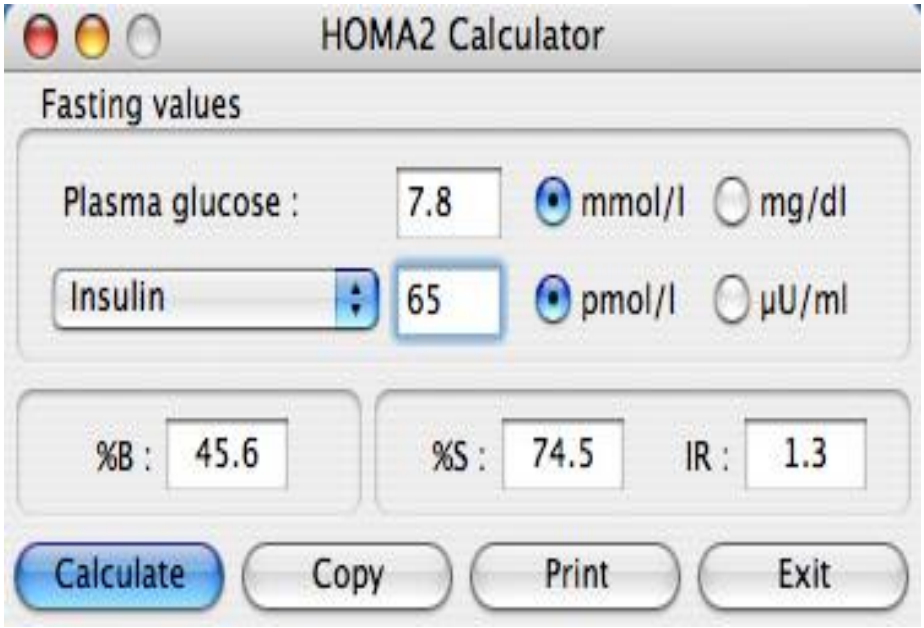

Figure: HOMA2 Calculator v2.2.3

Set up:

Run the "HOMA2 calculator Excel setup' application before first using this sheet. Click the "Enable macros" button when opening the sheet.

HOMA2 Calculator functions:

The functions available in this worksheet are;

HOMA2_Insulin (Glu.Ins)

HOMA2_Specific Insulin (Glu, Specins)

HOMA2_C- peptide (Glu, C Pep)

Each function takes two parameters are shown and return an array containing the calculated values of % and IR.

HOMA2 Calculator ranges and units:

Acceptable steady state glucose and peptide input values are:

Glucose : 3.0 to 25.0 mmol/L

Insulin : 20 to 400 pmol/L

Specific insulin : 20 to 300 pmol/L

C-peptide : 0.2 to 3.5 mmol/

Appendix (XIII): eGFR was calculated by Cockcroft and Gault formula (CG) containing Andrid Apps (Pocket GFR Calculator)

The Cockcroft and Gault formula (CG) was developed in 1973 using data from 249 men with creatinine clearance (CCr) from approximately 30 to 130 mL/m^2. It is not adjusted for body surface area. CCr is expressed in milliliters per minute, age in years, weight in kilograms:

$$CCr=\{((140-age) \times weight)/(72\ SCr)\} \times (0.85 \text{ if female})$$

Appendix (XIV): Krebs's Ringer Buffer (KRB) solution (Perfusion media) was prepared by the following method.

For preparing 1liter KRB solution the following amount of solutes in the table below were mixed with deionized water (as much as needed to make ultimately 1 liter volume of solution).

Name of the chemical	Amount used in KRBs
1. Sodium chloride (NaCl)	7.37g
2. Potassium Chloride (KCl)	0.20g
3. Calcium Chloride ($CaCl_2.2H_2O$)	1.02g
4. Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	0.065g
5. Sodium bicarbonate ($NaHCO_3$)	0.6g