AN INVESTIGATION OF ANTIOXIDANT AND ANTIDIABETIC EFFECT OF AQUEOUS LEAF EXTRACTS OF MUCUNA PRURIENS

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A thesis submitted in partial fulfilment of the requirements of the University of Brighton for the degree of Doctor of Philosophy

December 2016
Author’s Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed: Oke-Oghene Philomena Akpoveso

Dated: December 2016
Dedication

This thesis is dedicated to my parents Chief Chadwick and Grace Akpoveso and to everyone who made sacrifices for me to achieve this dream.
Acknowledgment

My unreserved and heartfelt gratitude to Jesus Christ my friend, personal Lord and Saviour, time and time again you have proven that you are alive and that your mercy endures forever.

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**Abbreviations**

GLP-1  
Glucagon like peptide -1

GIP  
Glucose-dependent insulinotropic peptide

T1D  
Type 1 Diabetes

T2D  
Type 2 Diabetes

WAT  
White adipose tissue

IGF-1  
Insulin-like growth factor-1

PET  
Positron emission tomography

IR  
Insulin receptor

IRS  
Insulin receptor substrate

PKCθ  
Protein Kinase C theta

DAG  
Diacylglycerol

ATP  
Adenosine triphosphate

TNF-α  
Tumour Necrosis alpha

JNK-1  
c-Jun N-terminal kinases

PI3-K  
Phosphatidylinositol 3-Kinase

ECB  
Endocannabinoid
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>CB1</td>
<td>Cannabinoid receptor 1</td>
</tr>
<tr>
<td>CB 2</td>
<td>Cannabinoid receptor 2</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling Protein</td>
</tr>
<tr>
<td>PPAR-λ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor- Kappa B</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation End products</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly ADP ribose Polymerase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin 1</td>
</tr>
<tr>
<td>DKD</td>
<td>Diabetic kidney disease</td>
</tr>
<tr>
<td>PON 1</td>
<td>Paraoxonase 1</td>
</tr>
<tr>
<td>NOX</td>
<td>Nicotinamide adenine dinucleotide phosphate-oxidase</td>
</tr>
<tr>
<td>DPP-4</td>
<td>Dipeptidyl peptidase-4</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>----------</td>
<td>-------------</td>
</tr>
<tr>
<td>AGIs</td>
<td>Alpha-glucosidase inhibitors</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>PGE</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PQ</td>
<td>Paraquat</td>
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<tr>
<td>2-NBDG</td>
<td>2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose</td>
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<td>6-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose</td>
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<tr>
<td>Tempol</td>
<td>4-Hydroxy-TEMPO</td>
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<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
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<td>PHT</td>
<td>Phloretin</td>
</tr>
<tr>
<td>PLDZ</td>
<td>Phloridzin</td>
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<tr>
<td>NRK-52E</td>
<td>Normal rat kidney proximal tubule epithelial cell line</td>
</tr>
<tr>
<td>3T3-L1</td>
<td>Murine pre-adipocyte cell line</td>
</tr>
<tr>
<td>MPLF</td>
<td><em>Mucuna pruriens</em> aqueous leaf extract</td>
</tr>
<tr>
<td>PMS</td>
<td>Phenazine Methosulfate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>MPMS</td>
<td>1-Methoxy-5-methylphenazinium methyl sulfate</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-Essential Amino acid</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin–angiotensin–aldosterone system</td>
</tr>
<tr>
<td>NCL</td>
<td>Neutralized chloroform fraction</td>
</tr>
<tr>
<td>CE</td>
<td>Crude extract</td>
</tr>
<tr>
<td>NDE</td>
<td>Neutralized Diethyl ether fraction</td>
</tr>
<tr>
<td>DE</td>
<td>Diethyl ether fraction</td>
</tr>
<tr>
<td>CL</td>
<td>Chloroform fraction</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>INTERCEDD</td>
<td>International Centre for Ethno-pharmacology and Drug Development</td>
</tr>
<tr>
<td>GRB</td>
<td>Growth factor bound protein</td>
</tr>
<tr>
<td>ATR</td>
<td>Angiotensin receptor</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular Filtration rate</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>PLZ</td>
<td>Phlorizin</td>
</tr>
<tr>
<td>MP</td>
<td><em>Mucuna pruriens</em></td>
</tr>
<tr>
<td>ET</td>
<td>Electron transfer</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulphide</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
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<td>---------------------------</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>PW</td>
<td>Pathway</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
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Abstract

Diabetes is currently a widespread global disease. As a result of the side effects of the current therapies, herbal plants may present alternative source of drugs for management of the disease. *Mucuna pruriens* is a plant that is traditionally used for diabetes and anaemia. There are experimental reports of the hypoglycaemic effect of the alcoholic extracts but the anti-diabetic effects of the aqueous extract has not been investigated. Therefore, the aim of this project was to investigate the potential anti-diabetic mechanisms of the aqueous extract of *Mucuna pruriens* leaves.

The leaf extract was prepared by decoction. The potential mechanisms of anti-diabetic effect of this extract was evaluated as follows: Antioxidant activity of the aqueous *Mucuna pruriens* leaf extract was investigated in reduced β-nicotinamide adenine dinucleotide (NADH)/phenazine methosulphate (PMS), and Xanthine /Xanthine oxidase superoxide generating systems.

In addition, the effect of aqueous *Mucuna pruriens* leaf extract against oxidative stress was measured as cytoprotective effect of the extract against paraquat induced oxidant injury in NRK-52E renal cells. Cytoprotective effect was measured as cell viability and cell death using 3-(4,5-Dimethylthiazol-2-Yl)-2,5 Diphenyltetrazolium Bromide (MTT) and Lactose dehydrogenase (LDH) assays respectively. Finally the effect of aqueous *Mucuna pruriens* leaf extract on glucose uptake was evaluated in NRK-52E renal cells and 3T3-L1 adipocytes.

The results revealed that aqueous *Mucuna pruriens* leaf extract had significant superoxide scavenging activity which increased from 21.35% to 99.8% in xanthine/xanthine oxidase and 36.15% to 62.4% in NADH/PMS superoxide generating systems at p<0.05. However, aqueous *Mucuna pruriens* leaf extract did not protect against paraquat induced oxidative stress.

Data from glucose uptake experiments showed that 1mg/ml of aqueous *Mucuna pruriens* leaf extract inhibited glucose uptake in NRK-52E renal by 35.5% compared to control at p<0.05. This effect was comparable to 1mM Phloridzin (a non-selective inhibitor of sodium glucose transporters).

Finally, 50 and 100μg/ml of both aqueous *Mucuna pruriens* leaf extract and its acid hydrolysed fractions prepared with liquid-liquid partitioning in diethyl ether, stimulated glucose uptake in 3T3-L1 adipocytes. Specifically, 50 and 100μg/ml aqueous *Mucuna pruriens* leaf extract stimulated glucose uptake be 57.06 and 86.24% respectively compared to negative control at p<0.05. Increase in glucose uptake was also observed in cells treated with diethyl ether acid hydrolysed fractions.

Taken together, the results show that aqueous the *Mucuna pruriens* leaf extract used in this study may exert anti-diabetic effects via antioxidant and glucose uptake modulatory mechanisms.
Chapter 1: Introduction
1.1 Statement of Problem

Diabetes is currently a wide spread disease across the globe. According to recent report in 2011, the number of people diagnosed with diabetes in the UK is up to 2.8 million (Holden et al., 2011). Globally, the number of diagnosed cases is estimated to rise to 552 million by 2030 (Whiting et al., 2011). In the same year diabetes cost the UK £23.7bn and it is projected that the cost of care for diabetic patients in the UK will increase to £39.8bn in 2035/2036 (Hex et al., 2012). Therefore the cost of care for diabetic patients is clearly an economic burden.

In 2010, it was estimated that the prevalence of diabetes was higher by approximately 4% in urban Sub Saharan Africa (Mbanya et al., 2010). This fact points to the possible role of life style and the incidence of diabetes. In fact, modification of diet and life style has been shown to reduce the risk of development of diabetes (Hu, 2011). Although there are drugs that target the control of plasma lipid and glucose, the prevalence of diabetic complications (Song, 2015) is evidence that further research is required to understand the causes of the disease and also to develop effective therapies for the prevention and management of the disease.

1.2 Normal Glucose Homeostasis

Control of plasma glucose is the overall aim of diabetes therapy. In normal conditions, after a meal the influx of glucose into the blood increases. Blood glucose is then adjusted to provide energy for normal functioning of the body and to avoid excessive exposure of organs to glucose thus preventing organ damage. Insulin enables the body to absorb and utilize excess glucose from the blood stream. The main process through which insulin promotes absorption of the glucose supply from the blood includes:
1. Increase in glucose uptake into effector organs such as skeletal Muscles and Adipocytes and Liver

2. Inhibition of glucose synthesis and glucose release from the Liver (inhibition of glucagon).

While the latter represents an organ specific effect of insulin action, the former involves a systemic response to insulin stimuli. Consequently, these effector organs share common signaling machinery for implementing insulin hypoglycaemic effects.

1.3 The Insulin signaling Pathway

The insulin signaling pathway can be broadly divided into activation and effector compartments. The principal point of the activation compartment is the insulin receptor.

1.4 Insulin receptor structure and Function

The insulin receptor can be described as a large cell surface glycoprotein that accumulates insulin at the site of action and on activation, initiates responses to insulin stimuli. The receptor is a disulfide-linked oligomer comprised of two α and two β subunits(Becker and Roth, 1990) (Fig.1.1). Activation of insulin receptor by insulin causes an autophosphorylation of the β subunits(Tatulian, 2015) and this causes a cascade of intracellular reactions that culminates in various cellular activities (Fig.1.2) such as regulation of protein and lipid metabolism, cell proliferation and glucose uptake.
Fig. 1.1: The basic structure of the insulin receptor
The two α-subunits on the outer membrane are disulfide-linked, and each α-chain is disulfide-linked to a β-subunit. A bound insulin molecule is shown as a hexagon, cross-linked to two α-chains. Upon activation, each β-subunit phosphorylates its counterpart depicted as small octagons with the letter P inside them. Diagram adapted from (Tatulian, 2015).
1.5 Insulin receptor function and Glucose uptake

The effector compartments of the insulin signaling pathway consist of various transducing proteins which co-ordinate insulin stimuli to achieve glycemic control. The main component of this compartment is the glucose transporters whose activity is central to glucose absorption from the blood. As mentioned earlier, activation of insulin receptor leads to increase of glucose transport into cells which results in reduction of blood glucose after digestion of a meal. Increase of glucose transport is primarily achieved by modulation of glucose transporters. In humans, glucose transporters are mainly classified into 2 namely: Sodium dependent glucose transporters (SGLT) and Glucose transporters (GLUT).

1.6 Glucose Transporters (GLUT)

GLUTs are encoded by the solute carrier (SLC) 2 genes and are members of the major facilitator superfamily(Fu et al., 2016). The GLUT proteins are comprised of ∼500 amino acid residues, possess a single N-linked oligosaccharide, and have 12 membrane-spanning domains. Fourteen GLUT proteins are expressed in humans(Fu et al., 2016). GLUTs are ubiquitously distributed in the human body and they catalyze facilitative diffusion of glucose down its concentration gradient(Mueckler and Thorens, 2013). In addition to glucose, some isoforms of the GLUT are involved in transport of Urate and myo-inositol(Thorens and Mueckler, 2010, Mueckler and Thorens, 2013). GLUT 1, 2, 3 and 4 isoforms are found in the liver, skeletal muscles, and adipocytes(Mueckler and Thorens, 2013). These transporters contribute to glucose homeostasis in the plasma. After the digestion and absorption of a meal, insulin is released in response to rising blood glucose levels. Glucose uptake also precedes glucose-stimulated insulin secretion.
In humans GLUT 1, 2, 3 are expressed in the pancreas (Coppieters et al., 2011). GLUT 2 is the most widely studied transporter and is generally known to be central to glucose-stimulated insulin secretion (Augustin, 2010). Influx of glucose through this transporter causes alteration of intracellular ATP and voltage levels, which initiates calcium dependent release of insulin into the blood stream (Schuit et al., 2001). In the skeletal muscles, adipocytes and liver, a rise in increasing insulin levels causes glucose transporters which usually reside intracellularly to redistribute to the plasma membrane, thus increasing glucose uptake and metabolism in these tissues (Fig 1.2) and thereby prevent chronic elevations in blood glucose levels (Augustin, 2010). In adipocytes for example, insulin binding to its receptor results in the dimerization and trans-phosphorylation of the receptor beta subunits (Fig.1.1), causing the activation of intrinsic tyrosine kinase activity leading to the recruitment and tyrosine phosphorylation of insulin receptor substrate-2 (IRS-2). The tyrosine phosphorylation of IRS-2 then binds to and activates PI3-kinase (Augustin, 2010). This eventually leads to phosphorylation of binding proteins that mediate, the effect of insulin on GLUT4 translocation (Fig.1.2) (Augustin, 2010). The inability of the glucose transporters to function as glucose sensors or to respond to insulin stimuli in the liver, skeletal muscles and adipocytes is known as insulin resistance (DeFronzo and Tripathy, 2009). This results in hyperglycaemia which is the hallmark of diabetes (Nathan et al., 2006). Insulin resistance will be further discussed in the later sections of this chapter.
I.7 Sodium Dependent Glucose transporters (SGLTs)

SGLTs are the second type of glucose transporters found in the kidneys, intestines, and heart (Wood and Trayhurn, 2003). They are encoded by the *SLC5A* genes (Wood and Trayhurn, 2003). Unlike the GLUT, they transport glucose against concentration gradient (Augustin, 2010). The major isoforms of the SGLTs involved with glucose transport are SGLT 1, 2. These isoforms are involved with absorption of glucose in the intestines and kidneys (Augustin, 2010). The third isoform SGLT3 is suggested to function as glucose sensor in the gut (Abdul-Ghani et al., 2011).

In the intestines SGLTs absorb digested food from the gut while in the kidneys they re-absorb filtered glucose back into the plasma. The latter activity of SGLTs in the kidneys implies that SGLTs in the kidneys contribute to keeping blood glucose levels at constant healthy limits (Mitrakou, 2011).
Fig. 1.2: The multiple intracellular effects of insulin. Activation of insulin receptor by binding of insulin causes binding and activation of insulin receptor substrates (IRS). There are 3 subtypes of IRS; IRS-1, IRS-2, and IRS-3. Each one transduces slightly different signals in response to insulin stimulation. IRS-1 binds growth factor bound protein-2 (Grb-2) and elicits cell growth and survival through activation of Mitogen-Activated Protein Kinase (MAPK) pathways (Skolnik et al., 1993). IRS-2, and IRS-3 activate phosphoinositide-3-kinase (PI3K). PI3K causes recruitment of other kinases and eventually the AKT/Protein kinase C zeta (PKCζ) activated by IRS-2 increase glucose uptake by causing translocation of Glucose transporter 4 (GLUT 4) (Govers, 2014). Overall, the effect of insulin results in increased influx of glucose from blood stream for anabolic processes such as lipid, protein synthesis and cell growth.
1.8 Pathophysiology of Diabetes

The onset of diabetes occurs to a large extent, as a result of impaired insulin function which is characterized by high fasting blood glucose levels. Apart from insulin deficit, an abnormally high blood glucose level is also associated with the deficiency of other hormones and peptides some of which include: glucagon, Amylin and Glucagon like peptide-1 (GLP-1), glucose-dependent insulino-tropic peptide (GIP), adrenaline, cortisol, and growth hormone and other incretins. The main hormones involved with controlling blood glucose levels are insulin and glucagon which are secreted in the pancreas. The other hormones and peptides mentioned above function to enhance insulin and glucagon effects either by synergistic mechanisms or by maintaining the health of the pancreas (Aronoff et al., 2004). Therefore, based on insulin function, diabetes can be classified as: Insulin dependent diabetes (Type 1) or non-insulin dependent diabetes (Type 2).

1.9 Pathophysiology of Type 1 Diabetes

5% of people diagnosed with diabetes have Type 1 diabetes or insulin dependent diabetes. Type 1 diabetes (T1D) is an autoimmune disease that results in damage of β insulin secreting cells in the pancreas leading to perturbation in glucose homeostasis. Immune reactivity to β-cells is thought to be triggered by infection of enteroviruses (Hyöty et al., 1995). Further studies have shown that the presence of immune-modulatory cytokines and hyperglycaemia can exacerbate destruction of β-cells by effector immune cells (Skowera et al., 2008, Van Belle et al., 2014). Based on the pathogenesis of T1D, current therapies for T1D compensate insulin deficiency by insulin supplementation. T1D may also be managed by non-insulin dependent approach
considering that adipose tissues are known to secrete adipokines that enhance insulin effect.

For instance, in experimental diabetic mice models, successful transplantation of brown adipose tissue stimulated regeneration of white adipose tissue (WAT) (Gunawardana and Piston, 2012). In the same study, blood glucose levels declined to normal range within 6 months. The authors showed that a possible mechanism was due to secretion of insulin growth factor-1 (IGF-1) an insulin receptor agonist (Gunawardana and Piston, 2012). Moreover, WAT also secrete adiponectin which suppresses glucagon activity leading to reduced hepatic glucose production in the liver (Combs and Marliss, 2014) and resultant low fasting blood glucose levels. Taken together, these studies show that T1D can be managed with non-insulin dependent therapies.

1.10 Pathophysiology of Type 2 Diabetes

As people tend to live longer, the incidence of age related diseases such as diabetes increases (Power et al., 2013). In contrast to T1D, T2D in the initial stages is not caused by insulin deficiency. Although the underlining pathology is not fully understood, T2D is characterized by disturbances in glucose and lipid metabolism due to aberrant signalling between the gut-brain and gut-adipose/skeletal tissue axis resulting in changes in food intake and energy expenditure, a loss of insulin sensitivity or insulin resistance, hyperglycaemia, dyslipidaemia and low grade inflammation (Fig. 1.3) (Schwartz et al., 2013). Among all of these indicators, Insulin resistance may be a central feature of T2D (DeFronzo and Tripathy, 2009).
Fig. 1.3: The current proposed two-system failure involved in development T2D. The development of T2D is suggested to develop due to failure of insulin to initiate glucose uptake in the muscles, liver and adipose tissue. This malfunction causes reduced secretion of hormones from adipose tissues which are responsible for triggering brain glucose control responses. Eventually, dysregulation of glucose metabolism and hyperlipidaemia (due to malfunctioning adipose tissues) causes β-cell failure. Therefore, defective insulin response from peripheral tissues and lack of compensatory response from the brain glucose control system in the hypothalamus results in T2D and related diseases (Schwartz et al., 2013).
1.11 Insulin resistance and T2D

The muscles and adipocytes play a major role in glucose homeostasis. Insulin resistance is known to occur mainly in adipose tissue, skeletal muscle, liver and brain. The main feature of insulin resistance is the inability of insulin to suppress hepatic glucose production and to stimulate glucose uptake in muscles and adipocytes, and this in turn causes hyperglycaemia, hyperinsulinaemia and dyslipidaemia. Consequently, insulin resistance within this context could be due to insulin signalling defect, accumulation of lipid metabolic intermediates within muscles and liver and glucose transporter defect. Therefore, the following will focus on insulin resistance in adipocytes and skeletal muscles.

1.12 Insulin resistance in skeletal muscles

A recent study in humans using dynamic positron emission tomography (PET) showed that skeletal muscle insulin resistance in T2D involves a severe impairment of glucose transport and also impaired entry of glucose into the glycolytic pathway (Goodpaster et al., 2014). The insulin signalling system, co-ordinates: glucose metabolism, lipid metabolism and glucose uptake in response to nutrient intake. Briefly, insulin receptor (IR) activation by insulin causes further activation of IR substrate (IRS) which transduce insulin effects by increasing glucose uptake, activating fatty acid synthesis, glucose metabolism and also cell growth, via different cellular pathways (Fig. 1.2).

Dyslipidaemia, as seen in obese states, is responsible for fatty acid induced disruption of insulin signalling that causes insulin resistance (Boden, 2011). Consequently, obesity is known to be the most common cause of insulin resistance, and it is the corresponding increase in obesity which is driving the rising incidence of T2D (Kahn et al., 2006). To
explain the underlning mechanism of fatty acid induced skeletal muscle resistance, it has been shown that fatty acids are known to inhibit IRS signalling by inhibiting IRS activation of phosphoinositide 3-kinase (PI3-Kinase) activity, a kinase which is central to mediating cellular responses to insulin. Fatty acid inhibition of this pathway was shown to impair glucose uptake, in spite of increased insulin concentrations(Yu et al., 2002). Furthermore, this mechanism for fatty acid induced insulin resistance was confirmed by a correlating study with muscles of diabetic and obese humans. According to their report, Szendroedi et al(Szendroedi et al., 2014) observed that increased fatty acid concentration caused increased formation of diacylglycerol (DAG) an intrinsic activator of Protein kinase CѲ (PKCѲ) and a subsequent serine phosphorylation of IRS subtype by the PKCѲ impaired glucose uptake in these muscles(Szendroedi et al., 2014). Therefore, it can be deduced that elevated fatty acid levels cause an increase in lipid metabolites such as DAG, which in turn activates PKCѲ leading to serine phosphorylation of IRS. Serine phosphorylation of IRS prevents it from activating PI3-Kinase (Fig 1.2) and causes impaired physiological response to insulin stimulation.

However, increased lipid intermediate metabolites do not have any effect on insulin sensitivity and mitochondrial activity in athletes(Goodpaster et al., 2001). The mitochondria are the converging point for intracellular response to insulin stimulus, as they synthesise ATP which is utilized for effector signalling and the overall cellular outcome. It is therefore possible that accumulation of lipid metabolites such as DAG and the ensuing insulin resistance may not be entirely due to elevated fatty acids, but also to mitochondrial dysfunction. A recent randomized clinical study by Phielix et al(Phielix et al., 2014) showed that reducing fatty acid levels improved insulin sensitivity but did not improve insulin stimulated mitochondrial function in T2D patients.
A possible explanation for this could be inferred from another study by Kelley et al. (Kelley et al., 2002a). According to this study, they observed reduced mitochondria size and activity in muscle biopsies obtained from T2D and obese patients with age difference > 10 years (Kelley et al., 2002a). A recent in vitro study revealed that saturated fatty acid reduced mitochondrial efficiency and downregulated insulin signalling proteins in skeletal muscles, but caused up regulation of electron transport chain proteins probably as an adaptive mechanism (Yang et al., 2012). These studies point to a fundamental mitochondrial malfunction that eventually contributes to insulin resistance. Within this context, it is evident that mitochondrial alteration in size and function in addition to the presence of lipid metabolites, contribute to the development of insulin resistance in skeletal muscles.

One of the ways insulin signalling is coupled to efficient oxidative phosphorylation is through the foxox head 1 (foxo 1), a transcription factor that is responsible for regulating haem components of the electron transport chain and gluconeogenesis in the liver (Cheng et al., 2009). The interference of IRS signalling led to hyperactivity of the foxo 1 and reduced expression of electron transport chain components, specifically, the complex III and IV which require haem for electron transfer (Cheng et al., 2009). Furthermore, knock down of Complex I leads to insulin resistance in skeletal muscle cell lines (Wijngaarden et al., 2014). Clearly, insulin affects the process of oxidative phosphorylation. Also, during short term fasting, foxo1 expression was increased in parallel with ketone bodies while PI3-kinase was decreased (Wijngaarden et al., 2014). In fact, mice over expressing foxo1 in their skeletal muscle have been shown to lack glycaemic control (Kamei et al., 2004). This indicates a
possible functional role for the foxo1 in maintaining energy homeostasis possibly by blunting mitochondrial efficiency in response to insulin stimulus in the skeletal muscle.

Using non-invasive techniques, Nisr and Affourtit (Nisr and Affourtit, 2014) observed that acute administration of insulin improves mitochondrial efficiency by preventing proton leakage; and this effect was blunted by palmitate, a saturated fatty acid. Therefore, a disruption in the electron transport chain by fatty acids perhaps through the foxo1 pathway could be the mechanism underlying mitochondria induced insulin resistance. Furthermore, 14 weeks of treatment of T2D patients with metformin, a drug which exerts its therapeutic effect by increasing mitochondrial function (Hardie et al., 2012), improved insulin sensitivity slightly in the treated patients (Kadoglou et al., 2010). However, more studies are required to unravel the mechanism of mitochondrial dysfunction in insulin resistance. In summary, impaired insulin signalling due to fatty acid or mitochondrial inefficiency, causes a poor response of skeletal muscle to insulin stimuli. Considering that the muscles are known to be responsible for 80% of whole body uptake of glucose (DeFronzo and Tripathy, 2009), an impaired response to insulin stimuli or insulin resistance, triggers the characteristic hyperglycaemia observed in T2D.
1.13 Insulin resistance and adipocyte dysfunction

As mentioned earlier, hyperglycaemia observed in T2D is also accompanied by dysregulation of lipid metabolism and subsequent hyperlipidaemia. Lipotoxicity due to hyperlipidaemia is known to induce β-cell damage eventually leading to reduced insulin secretion which is observed in later stages of T2D (Cnop et al., 2005). Therefore, compromised lipid metabolism can be viewed as a crucial factor in the progression of T2D. Adipose tissue is the primary endocrine organ for lipid storage and dysfunctional adipose tissue is implicated in deranged insulin signalling in skeletal muscle (Guilherme et al., 2008). According to results obtained from co-culture studies, differentiated fatty tissue from healthy donors caused diminished insulin signalling in skeletal muscle cells (which were also collected from healthy donors). The authors also reported that this effect was similar to skeletal muscle insulin resistance induced by the pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α) only at high concentrations (Dietze et al., 2002). In contrast, a recent study has shown that incubating rat skeletal muscle in adipocyte conditioned medium did not reduce insulin sensitivity, but increased pro-inflammatory markers within the skeletal muscles (Tishinsky et al., 2013). The difference in experimental design could explain the disparity in these results but the presence of inflammation as observed in the latter result can be interpreted as the early stages of skeletal muscle insulin resistance. Accordingly, inhibition of inflammatory mediating pathways in insulin resistance skeletal muscle restores insulin sensitivity in both cell lines and in obese mice (Meng et al., 2014). Therefore, it can be inferred from both studies that inflammation is a key factor in adipose tissue induced skeletal muscle insulin resistance. In fact Hirosumi et al. (Hirosumi et al., 2002), observed that obese mice lacking c-Jun N-terminal kinases -1 (JNK-1) expression, a pathway activated during
stress and inflammation, had reduced adipose tissue deposits, increased expression of activated of IRS-1 in their skeletal muscles and a corresponding better glycaemic control compared to the wild type.

Adipose tissues secrete various biologically active substances including pro-inflammatory cytokines which are called adipokines. Adipokines have several neuronal, anti-inflammatory, pro-inflammatory and metabolic effects. Examples include: leptin, adiponectin, apelin, angiotensinogen, resistin and TNF-α. In obesity related T2D, there is an interplay between adipokines and insulin that contributes to the progression of poor glycaemic control and pathogenesis of T2D and other T2D complications. For instance, visfatin/nicotinamide phosphoribosyltransferase, an adipokine from visceral adipose tissue, is known to have insulin mimetic effect (Fukuhara et al., 2005) but it is elevated in T2D (Chen et al., 2006). Although a recent study conducted in elderly patients argues that elevation of visfatin in T2D is more likely to be due to obesity and inflammation (McGee et al., 2011), there is evidence that high blood glucose stimulates a corresponding increase in visfatin levels (Haider et al., 2006). The relationship between visfatin and insulin resistance is not clear yet, however, a study has shown that increased expression of visfatin was related to increase in proteins that modulate inflammatory pathways and insulin resistance. Furthermore, treatment with insulin sensitizing drugs reduced visfatin levels in parallel with the modulatory inflammatory proteins (McGee et al., 2011). Therefore, within this context, it is obvious that hyperglycaemia, and the compensatory hyperinsulinaemia that occurs due to insulin resistance in obesity-related T2D, induces inflammatory responses and visfatin secretion from adipocytes which may further aggravate peripheral insulin resistance.
This delicate interplay between glycaemic control and the actions of adipokines in T2D is also exemplified by the metabolic effect of leptin. Leptin is one of the hormone/cytokine produced by adipocytes (Xie et al., 2008). In T2D, increased insulin levels due to insulin resistance have been shown to correlate with increase in leptin levels (Fischer et al., 2002). Also Coimbra et al. (Coimbra et al., 2014) suggested that increased leptin levels in T2D patients could be dependent on the duration of the disease. Leptin mediates the energy storage and metabolism function of the adipose tissue along the brain to adipose tissue axis. The feeding centres of the brain in the hypothalamus express receptors for insulin and leptin and these receptors mediate both insulin and leptin influence on satiety and energy expenditure (Schwartz et al., 2000). Morton et al. (Morton et al., 2005) observed that the IRS-PI3K pathway, which is a classical pathway for insulin signalling in the hypothalamus, was important for mediating leptin induced whole body insulin sensitivity in obese rats. Beyond central effects, leptin is also known to directly influence pathways that integrate nutrient sensing with cell growth and inflammation in the peripheral system (Maya-Monteiro and Bozza, 2008).

Adiponectin is another adipokine that is known to influence glucose homeostasis. There is evidence that suggests an inverse relationship between circulating levels of adiponectin and a lower risk of T2D independently of overall fat deposition and that adiponectin may present some advantage in both persons with and without insulin resistance (Li et al., 2009, Ahonen et al., 2012). It has also been proposed that adiponectin could have anti-inflammatory effects and could be a useful marker for predicting resolution of metabolic disturbances associated with obesity and T2D (Yamauchi and Kadowaki, 2008). The levels of adiponectin and other adipokines in
T2D disease state imply dysfunctional adipocyte function. Considering these effects of adipokines in T2D, adipocyte dysfunction in relation to altered adipokine levels contributes to feeding defects, poor glycaemic control and low grade inflammation observed in development of T2D.

1.14 Gut microbiota, adipocyte dysfunction and T2D

Recently, low grade inflammation and adipocyte dysfunction has been linked to the composition of gut microbiota. Indeed, it has been proposed by Membrez et al (Membrez et al., 2008) that the gut microbiota caused increased monosaccharide uptake from the digestive system and initiated host increase in hepatic production of lipids (triglycerides) associated with the development of insulin resistance in mice. The same study established that modulating gut microbiota with conventional antibiotics improved glucose tolerance, increased adiponectin levels and reduced inflammatory markers in treated mice (Membrez et al., 2008). Considering that adiponectin and its receptors increase fatty acid oxidation in peripheral tissues such as in skeletal muscles and improves insulin resistance (Yamauchi and Kadowaki, 2008), the influence of antibiotics on adiponectin levels observed by Membrez et al (Membrez et al., 2008), suggest an influence of gut microbiota on adipocyte function. Furthermore, ablation of mice gut host innate immunity led to altered gut microbiota population causing insulin resistance and increased adiposity. Insulin resistance was observed irrespective of dietary intake (Vijay-Kumar et al., 2010). A clinical study comparing non-diabetic and diabetic males revealed that T2D patients had higher Gram negative bacteria compared to non-diabetic males. Although the study did not specify if the patients were naïve or what type of treatment the patients were taking at the time of the experiment, their
results indicate that modified gut microbiota in T2D patients was independent of the age of the patients (Larsen et al., 2010).

Further evidence for the role of microbiota in development of T2D has been reported. In their study, Cani et al. (Cani et al., 2008, Cani et al., 2009) showed that obese mice and high fat diet feeding induced changes in gut microbiota and reduced expression of intestinal tight junction regulatory proteins in their intestines. This caused increased intestinal permeability and increased plasma lipopolysaccharide (LPS). Accordingly, the prevalence of LPS derived from gut Gram negative bacteria in the plasma is proposed to induce low grade inflammation (metabolic endotoxaemia) observed in T2D and related diseases (Geurts et al., 2013b). Moreover, gut microbiota affects energy balance by influencing the efficiency of calorie harvest from the diet, and how this harvested energy is used and stored (Fig1.4)(Tremaroli and Bäckhed, 2012).
Fig. 1.4: Effects of Diet induced modification of gut microbiota.

Gut microbiota results in increased appetite and availability of substrates such as Short fatty acids for lipogenesis and gluconeogenesis in the liver. The lipids synthesized are incorporated into adipose tissue causing abnormal expansion of adipose tissue, these in turn secrete adipokines that can reduce satiety in the brain, alter fatty acid oxidation in muscles and induce insulin resistance. Also, gut microbiome reduce GLP secretion and alter gut permeability. “Permeable” gut causes increase in plasma LPS and the ensuing inflammation and insulin resistance (Tremaroli and Bäckhed, 2012).
Bakhed et al. (Bäckhed et al., 2007) observed that germ free mice had increased ability to metabolise fatty acid and were resistant to diet induced obesity. This implies gut microbiota caused increased bioavailability of nutrients from the diet in the host. Studies by Turnbaugh et al. (Turnbaugh et al., 2006) denote that the obese microbiome has an increased capacity to harvest energy from the diet. They also observed that this trait was transmissible: colonization of germ-free mice with an ‘obese microbiota’ resulted in a significantly greater increase in total body fat than colonization with a ‘lean microbiota’. In humans, a recent comparative metagenomic analysis of the faecal samples of 171 diabetic patients and 174 healthy controls showed that samples from diabetic patients had lower abundance of butyrate-producing bacteria, such as *Faecalibacterium prausnitzii*, but greater abundance of opportunistic pathogens, including *Clostridium bolteae* and *Desulfovibrio* sp (Qin et al., 2012).

The endocannabinoid system has been acknowledged as one of the systems that act as a bridge between the gut microbiota and adipose tissue growth (Muccioli et al., 2010). This could be by means of the increase in systemic LPS and enhancement of inflammation (Muccioli et al., 2010). The endocannabinoid (ECB) system consists of cannabinoid (CB1 and CB2) receptors, endogenous ligands which are anandamide (*N*-arachidonoylthanolamide, AEA) and 2-arachidonoylglycerol (2-AG), and enzymes for ligand biosynthesis and inactivation which include *N*-acyltransferase and monoacylglyceride lipase respectively (Di Marzo et al., 2004).

Generally, the ECB has in recent times been identified as a significant modulatory system in the function of brain, endocrine, and immune tissues (Komorowski and Stepień, 2006). In the gut, the endocannabinoid system may influence intestinal barrier.
Koay et al. (Koay et al., 2014) showed that agonists of the CB-1 receptor induced autophagy via reduced expression of suppressor of cytokine signalling-3 (SOCS-3) which is involved in modulating autophagy in Caco-2 intestinal cell lines. Autophagy involves self-cell degradation of cellular contents that eventually leads to cell death. Although, the authors did not observe an eventual cell death after CB-1 activation yet within this context, it can be inferred that LPS from altered gut bacteria increases ECB tone in the intestines which in turn cause “leaky” intestinal barrier probably by modulating autophagy. This result of increased plasma LPS leads to low grade inflammation observed in T2D and related diseases. In addition, a deficiency in proglucagon-derived peptide (GLP-2) due to altered microbiota may also be responsible for permeable gut barrier in obese mice. In relation to ECB, affected gut barrier and increases plasma LPS and increased LPS has been shown to increase weight gain in a high fat diet fed to mice (Geurts et al., 2013a).

Furthermore, ECB tone is increased within the adipose tissue causing a raise in glucose uptake and increased fatty acid synthesis and accumulation, reduction in mitochondrial biogenesis and secretion of pro-inflammatory adipokines and increased insulin resistance in the skeletal muscles (Bäckhed et al., 2007, Silvestri et al., 2011, Silvestri and Di Marzo, 2013). Accordingly, inhibition of CB1 receptor has been shown to improve metabolism in fatty tissues (Cristino et al., 2014).
1.15 Mitochondrial Dysfunction, Reactive Oxygen Species and Development of T2D and Diabetic complications

In normal conditions, reactive oxygen species/nitrogen species (ROS/RNS) such as hydrogen peroxide, superoxide anions and nitric oxide are constitutively generated within cells as signalling molecules as well as effector molecules. A major process of ROS production is generation of superoxide by the mitochondrial electron transport chain. During normal metabolism, the breakdown of glucose begins in the cytoplasm, where glucose undergoes glycolysis.

During glycolysis, NADH and pyruvate are generated. NADH donates electrons to the mitochondrial electron transport chain, whereas pyruvate enters the TCA cycle and produces more NADH and FADH. Overall, NADH derived from both glucose oxidation and from the TCA cycle donates electrons to complex I of the electron transport chain while FADH donates its electrons to complex II. Complexes I and II then transfer the electrons to ubiquinone. Ubiquinone passes its electrons to complex III, cytochrome c, complex IV, and finally to molecular oxygen. As the electrons are transferred through the electron transport chain the energy is used to shuttle protons across the membrane. This creates a voltage across the inner and outer membrane of the mitochondrion and drives ATP synthesis. Generally, the process of electron transfer is not an efficient process and some of the electrons leak to molecular oxygen generating superoxide ion. This leakage may be important for regulation of cell activity since the ROS generated is used for cell signalling. Hence, the concentration of ROS/RNS is carefully regulated by endogenous free radical scavenging (i.e antioxidant) systems in order to achieve optimum cell function (Nordberg and Arnér, 2001, Rhee, 1999). Free radicals are also tightly regulated to prevent oxidative damage to cellular proteins and
cell death. However, under hyperglycaemic conditions, the number of substrates entering the TCA cycle is greatly increased and consequently the number of reducing equivalents donating electrons to the electron transport chain is also increased.

Once the electron transport chain reaches a threshold voltage across the membrane the excess electrons begin to accumulate at complex III. These electrons are then donated to molecular oxygen, which in turn result in an increase in mitochondrial superoxide production (fig. 1.5). The increase in ROS production overwhelms mitochondrial antioxidant system and ROS/RNS homeostasis is disrupted leading to: increased oxidized conditions within the cell, exaggerated responses or complete inhibition of cell response to stimuli, DNA damage and eventually cell death (fig. 1.5 and 1.6)(Mohora et al., 2007).

Excessive ROS contributes to the various pathological features of diabetes as discussed in the above sections. For example, increased glucose metabolites in the glycolytic pathway increase activate pro-oxidant enzymes such as NADPH oxidase and activate inflammatory response, a major contributing factor to insulin resistance, via NF-κB activation(Clark and Valente, 2004). Furthermore, in adipocytes, ROS has been implicated in lipid accumulation in adipocytes via antioxidant dependent mechanisms(Higuchi et al., 2012). In β-cells ROS production is a by-product of glucose stimulated insulin secretion(Edalat et al., 2015), however, increase in mitochondrial superoxide anion radical generation causes β-cell death(Barlow et al., 2015). In rats, the hypothalamic neurons responsible for nutrient sensing are known to produce excessive ROS due to high fat diet. Increased ROS production was connected to increased breakdown of glycogen and synthesis of glucose in the liver via over-activation of sympathetic nervous system resulting in high fasting blood glucose(Drougard et al.,
Therefore, uncontrolled ROS generation is a major contributor to the progression of T2D, because it contributes to inflammation, insulin sensitivity, adipocyte dysfunction, β-cell damage, improper functioning of feeding centres in the brain and imbalance of energy homeostasis.

Figure 1.5: ROS production during normal mitochondrial function.
Electrons are transferred from NADH through the complexes 1-4. The membrane potential generated during the transfer is used to generate ATP. ROS specifically superoxide anion radical is generated during electron transfer between the complexes via the activity of Q. UCP regulates membrane potential to reduce superoxide production. Cyt c=cytochrome c, UCP=Uncoupling proteins, Q = Coenzyme Q10 ubiquinol. Mn-SOD= Manganese Superoxide dismutase. Diagram adapted from (Brownlee, 2005).
Fig. 1.6: Over nutrition i.e. high fatty acid, glucose and amino acid in the blood, increase intracellular ROS production. ROS in turn “switch on” the expression of proteins in certain pathways and suppress others. This eventually culminates in metabolic syndrome and cancer. Diagrams adapted from (Rolo and Palmeira, 2006, Görlach et al., 2015) C= complex, TCA=Tricarboxylic acid, ETC=Electron Transport Chain, UCP=Uncoupling Proteins, GSH=Glutathione, GSSG=Glutathione disulphide, NOX=NADPH oxidase, SOD=Superoxide dismutase, PKC=Protein kinase C, PPAR=Peroxisome proliferator activated receptor, NF-kB= nuclear factor kappa-light-chain-enhancer of activated B cells.
1.5.1 Oxidative stress

Oxidative stress is the term used to indicate the imbalance between ROS production and the efficiency of cellular antioxidant systems. ROS can trigger signalling pathways and induce abnormal cell death in disease conditions as shown in Fig. 1.6. The different kinds of cell death associated with the progression of diabetes include: apoptosis and necrosis.

1.15.2 Oxidative stress induced apoptosis in the progression of Diabetes

Cells induce a type of cell death that involves a co-ordinated cascade of events that involve: 1. Shrinkage of the cell and display of markers at the cell membrane that signal for phagocytosis and; 2. Fragmentation of the DNA, a breakdown of the nuclear membrane and cytoskeleton(Elmore, 2007). ROS generation by the mitochondria is known to initiate apoptosis via activation of pro-apoptotic proteins called caspases(Circu and Aw, 2010).

In relation to diabetes, over nutrition and inflammation has been observed to induce β-cell death via apoptosis. Piro et al,(Piro et al., 2002) simulated over nutrition conditions by chronic exposure of β-cells to high glucose and free fatty acids. They observed that in these conditions there was an increase in apoptosis of β-cells which was prevented by Nicotinamide an antioxidant agent(Piro et al., 2002). In addition, inflammatory cytokines have been observed to induce ROS production and apoptosis in β-cells and over expression of MnSOD prevented inflammatory induced apoptosis(Wang et al., 2012). Considering that death of β-cells results in deficiency of insulin, oxidative stress induced apoptosis in these cells will exacerbate hyperglycaemia in diabetes.
Furthermore, high glucose induced ROS has been reported to induce apoptosis of components of the glomerular filtration barrier, thereby potentially accelerate the development of Diabetic kidney disease (Susztak et al., 2006). Taken together, oxidative stress induced apoptosis contributes to both progressions of diabetes and diabetic complications.

1.15.3 Oxidative stress induced Necrosis in the progression of Diabetes

Unlike apoptosis, when cells undergo necrosis the are usually swollen and the events leading up to cell death may not necessarily be co-ordinated (Golstein and Kroemer, 2007). Necrosis can occur due to decline in ATP levels, uncontrolled Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels, increase in ROS production, leading to membrane depolarization uncontrolled cell swelling, lysis of main cellular components and membrane and then cell death (Michiels, 2004, Golstein and Kroemer, 2007).

Consequently necrotic cell death causes the release substances that stimulate acute inflammatory response. Chen at al (Chen et al., 2007) report that inflammation and associated massive damage was reduced in mice with reduced ability to transmit inflammatory signals (Chen et al., 2007). In their report, Iyer et al (Iyer et al., 2009) found that viable mitochondria released from necrotic cells were responsible for necrotic induced inflammation (Iyer et al., 2009).

In relation to diabetes, hyperglycaemia has been observed to increase susceptibility to ulcer and tissue necrosis in the limb of diabetic rats. According to the authors, this could be due to hyperglycaemia induce oxidative stress (Lévigne et al., 2012). Moreover, a poor outcome has been linked to hyperglycaemia in patients admitted for stroke (Adams et al., 2007). Bearing in mind that both stroke and limb ulcers are characterized by
conditions of obstructed blood flow, inadequate oxygenation (ischaemia) and necrotic cell death, it can be inferred that hyperglycaemia induced cell necrosis worsens diabetic tissue injuries.

1.15.4 Oxidative stress and Chronic Diabetic Complications

Hyperglycaemic induced oxidative stress is also linked to the development of chronic complications of diabetes. This is because in T2D conditions, all the vital organs are exposed to a surplus of nutrients, especially the organs connected to the circulatory system, in fact, Type 2 diabetes is characterized by a two- to four-fold increased risk of cardiovascular disease (Laakso, 2011). ROS is produced in the presence of high concentrations of glucose via multiple mechanisms. These include oxidative phosphorylation, glucose auto-oxidation, and the Schiff reaction during glycation, PKC activation, methylglyoxal formation, and hexosamine metabolism.

According to Giacco and Brownlee (Giacco and Brownlee, 2010), mitochondria induced ROS generation is central to formation of substrates for ROS generation via the aforementioned mechanisms (see fig. 1.7). In their own view, mitochondria derived ROS causes irreversible DNA damage and triggers cell death by activating poly ADP ribose polymerase (PARP). PARP in combination with ROS inhibits Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This in turn causes glycolytic intermediates upstream of GAPDH to go into alternative pathways which in the process alter availability of the free radical scavenger glutathione through the polyol pathway and increase ROS generation by interacting with ROS receptors such as receptor of advanced glycation end products (RAGE). The flux of glycolytic intermediates to
alternative pathways also leads to activation of PKC and its downstream inflammatory pathways.

Finally, excess glucose is glycosylated and the end products cause transcription of inflammatory proteins. Consequently, chronic exposure of glucose to organs and tissues such as; the heart, micro and macro vascular vessels, the kidneys, the brain and cells responsible for immune response, cause them to deviate from their normal physiological activities especially in the endothelium (as explained in fig. 1.8). This leads to the onset and progression of diabetic complications. In effect, chronic diabetic complications are the additional diseases that arise due to diabetes of which there are many. These include mainly diabetic micro and macro vascular complications.
Fig: 1.7: Diagram showing how mitochondrial ROS generation is central to hyperglycaemic induced damage in micro-and macro-vascular complications. 

PW=Pathway. Diagram adapted from Giacco (Giacco and Brownlee, 2010).
1.15.5 Diabetic MicroVascular Complications

Diabetic microvascular disease is more likely to occur primarily in tissues where insulin activity is not necessary for glucose uptake (e.g., kidney, retina, and vascular endothelium). Therefore, these tissues are exposed to glucose levels that correlate very closely with blood glucose levels. The common component of microvascular vessels, which is usually the target for hyperglycaemic damage, is the endothelium.

1.15.6 Oxidative stress, Endothelial Dysfunction and Diabetic Vascular Complications

The vascular endothelium is an active component of the vasculature. It forms the lining of the lumen of vascular vessels that regulates other components of the vasculature (e.g., vascular smooth muscle), vascular tone, growth, division, migration, and inflammation (see fig. 1.8). The endothelium carries out most of its function by balancing the physiological factors and cytokines that elicit these effects. Disruption of the balance of endothelial derived factors causes endothelial dysfunction which is the underlining base for microvascular diseases. High glucose and increased metabolites of glucose in T2D causes alterations in endothelium function. For example, in physiological conditions, insulin is known to induce nitric oxide synthesis which causes relaxation of blood vessels and controls blood pressure.

High glucose induces insulin resistance in endothelial cells by inhibiting nitric oxide production and increases expression of proteins that regulate inflammatory pathways (De Nigris et al., 2015). Indeed, reduced nitric oxide synthesis has been observed in T2D patients with microvascular complications (Tessari et al., 2010). On the contrary, endothelin-1, a vascular constrictor also produced in the endothelium is known to be
elevated in patients with T2D and was suggested as a marker for vascular
disease(Takahashi et al., 1990). Endothelin-1 has been shown to cause increase in
resting phase blood pressure in nitric oxide synthase knockout mice that over express
endothelin-1(Vignon-Zellweger et al., 2014). In relation to diabetes, a randomized
clinical trial showed that inhibition of ET-1 receptors with the ET-1 receptor antagonist,
bosentan, caused endothelium dependent improvement in microvascular
dilation(Rafnsson et al., 2012).

Therefore, in principle, diabetes causes an inequality in vasoactive compounds
synthesized in the endothelium, culminating in endothelial dysfunction. Generally,
endothelial dysfunction has been associated with microvascular complications such as;
diabetic nephropathy, diabetic retinopathy and neuropathy. The following will focus
mainly on diabetic nephropathy and retinopathy.
The endothelium releases endothelium derived factors which have various effects on vasculature. For example, nitric oxide influences vascular tone and blood flow to tissues and controls inflammation. The endothelium also expresses interleukins and chemo-attractants which control inflammation. Prostacyclin affects clot formation by preventing platelet aggregation. The diagram is adopted from Sena et al. (Sena et al., 2013).
1.15.7 Diabetic Nephropathy

The kidneys contribute significantly to metabolism and blood pressure in the peripheral vascular system, by means of glucose reabsorption and control of blood pressure. Consequently, diabetic nephropathy or diabetic kidney disease (DKD) is a result of malfunction in metabolism and haemostasis. Indeed, the major determinants of DKD are hypertension and hyperglycaemia. Current treatment for slowing down the progression of DKD therefore involves use of anti-hypertensive drugs in addition to tight glycaemic control. Experimentally, streptozotocin induced diabetic rats were observed to have improved renal function when co-treated with sildenafil, a potent vasodilator, and glimepiride, an insulin secretagogue (Tripathi et al., 2016). Vascular endothelium, as already mentioned, controls vascular blood pressure through NO synthesis. The role of endothelial damage in the development of DKD was observed in mice lacking the ability to synthesise NO. Mice resistant to Adriamycin, a toxicant used to induce kidney damage by ROS production, were observed to become susceptible to kidney damage if they lacked the ability to synthesize NO. These mice experienced proteinuria which is typical of DKD (Sun et al., 2013). This study highlights the importance of endothelium in the kidney filtration barrier and its contribution to the development of DKD.

Hyperglycaemic injury to the glomerular endothelium erodes the charged components of the endothelium layer which is important for proper function of the kidney filtration barrier. Recently, clinical trials in T2D showed a strong correlation between microalbuminuria, the current marker for DKD, and damage to glomerular endothelium (Weil et al., 2012). Further studies are still required to determine how the glomerular endothelium affects the other components of the kidney filtration barrier.
such as the podocytes. This will be useful for elucidating target pathways that would slow down the progression of the disease. Similarly, proteinuria due to damaged filtration barrier is pivotal to inducing damage to the proximal tubules. In accordance with this view, albumin was observed to induce tubular cell inflammation and apoptosis via ROS production due to mitochondrial dysfunction (Zhuang et al., 2015). Also in the proximal tubules, albumin is also known to cause epithelial-mesenchymal transformation [EMT] (Ibrini et al., 2012) which is the hallmark of renal fibrosis. ROS production is also implicated in EMT. Albumin conjugated to glucose or glycated albumin (a mimic of hyperglycaemia and proteinuria in kidneys) induced EMT in rat proximal tubules by increasing the activity of pro-oxidant enzymes (Qi et al., 2015).

Furthermore, Astragaloside IV, an antioxidant derived from *Astragalus membranaceus* has been shown to inhibit EMT via decrease in activity of pro-oxidant enzyme NADPH oxidase (NOX) (Qi et al., 2014).

In summary, the development of DKD involves among other things, high glucose induced damage to the glomerular endothelium (fig. 1.5), and other components of the kidney filtration barrier via ROS dependent mechanisms which results in leakage of albumin into the proximal tubules. Albumin in turn causes inflammatory responses, EMT and cell apoptosis via oxidative stress in proximal tubules leading to renal fibrosis and acceleration of DKD (Tang and Lai, 2012).
1.15.8 Diabetic Retinopathy

Diabetic retinopathy (DR) is a major microvascular complication of diabetes. It occurs in patients with T2D and proliferative DR is one of the principal causes of blindness in diabetic adults of working age and is responsible for deterioration in quality of life (Rodriguez-Poncelas et al., 2015). DR is usually classified as either non proliferative and proliferative DR. The clinically evident vascular injuries that occur in non-proliferative DR include venous bleeding and loops, blood vessel closure, tissue ischaemia (Wilkinson-Berka et al., 2013), while, abnormal endothelial proliferation/angiogenesis is a fundamental pathology in proliferative DR. In fact, drugs that aim to inhibit angiogenesis have been explored as treatment for proliferative DR (Osaadon et al., 2014). Recently, Yun et al (Yun et al., 2013) measured endothelial dysfunction using flow mediated dilatation as an index for measuring NO bioavailability and endothelial function. In their study, they observed that DR correlates with degree of endothelial dysfunction in patients with T2D. ROS production may also play a role in progression of retinopathy. A recent clinical trial showed that antioxidant supplementation may improve circulating ROS and retinal thickness in patients with non-proliferative DR (Domanico et al., 2015). Increase in NOX pro-oxidant enzyme is also implicated in angiogenesis and may contribute to the development proliferative DR.

Clearly, poor glycaemic control and damage to the endothelium are key factors in the development of microvascular diseases. More importantly, hyperglycaemia induced oxidative stress plays a significant role in endothelial dysfunction and is a key contributor to microvascular diseases.
The different types of diabetic cardiovascular diseases include coronary heart disease, myocardial infarction, ischaemic heart disease, and other coronary artery disease. The underlining vascular damage that occurs in all of these conditions is the development of atherosclerosis. Unlike microvascular diabetic complications, there is increasing evidence that hyperlipidaemia is pivotal to accelerating atherosclerosis. Among the processes that foster the development of atherosclerosis is inflammation. Both hyperglycaemia and hyperlipidaemia may contribute to inflammation in atherosclerosis, however, high plasma lipids more than glucose enhances inflammatory markers in vascular endothelium (Horvath et al., 2015). Furthermore, a recent study has shown that a combination of lipid lowering drugs reduced inflammatory markers and increased NO bioavailability. This suggests that controlling lipid plasma levels can improve endothelial function and stall progression of atherosclerotic lesions (Zhang et al., 2014). The liver synthesizes and excretes cholesterol and other fatty acids. Thus, it is pivotal to the control of plasma lipid levels.

Oxidative stress is also implicated in the development of diabetic macrovascular complications. Xu et al (Xu et al., 2015a) reported that reducing oxidative stress in the liver by increasing the availability of methionine, an antioxidant amino acid, reduced atherosclerotic lesions in mice. They suggested that this was due to reduced inflammation and improved lipid metabolism. The accumulation and oxidation of low density lipids in arterial walls is one of the causes of hardening of the arteries i.e. atherosclerosis (Berliner et al., 1995). In addition, expression of adhesive molecules in the vascular endothelium which encourage migration of macrophages and generation of foam cells via ROS producing macrophages all contribute to plaque formation leading to
hardening of arterial walls (Singh et al., 2002). In agreement with this, antioxidant activity of paraoxonase 1 (PON1) through hydrolysis of lipid peroxides prevents the formation of plaques. Indeed, PON1 was found to be positively correlated with myocardial flow reserve, in a small group of T2D patients (Kacerovsky, 2011). However, the authors did not observe a link between PON1 and endothelium function. Although, in non-diabetic hypertensive men with a certain polymorphism of PON1 (which improved its antioxidant activity) was found to correlate with improves insulin sensitivity and endothelial function (Dunet et al., 2011). Further studies are required to determine if endothelial function is directly related to PON1 antioxidant activity in T2D patients (Dell'Omo et al., 2014). Notwithstanding, increase in pro-oxidant thioredoxin interacting protein was observed to correspond with inflammation, high post prandial glycaemia and thickness of artery in naïve T2D patients (Zhao et al., 2015). In the light of the evidences discussed above, progression of atherosclerosis and eventual development of macrovascular diseases is associated with hyperglycaemia induced ROS production and to a larger extent, hyperlipidaemia.

1.16 Antioxidants and Diabetes

Several endogenous antioxidant and free radical scavenging systems exist to maintain intracellular redox state for optimum cell function and prevent ROS induced cellular /organ damage. Endogenous antioxidant systems are broadly classified into Enzymatic and Non-Enzymatic antioxidants. Some of these endogenous systems are discussed below:
1.16.1 Enzymatic Antioxidants

The key endogenous enzymatic antioxidants are Superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase.

1.16.2 Superoxide dismutase:

Superoxide dismutase is an enzyme that is widely distributed around the body. SOD catalyzes the dismutation of superoxide to hydrogen peroxide (Rahman, 2007). Hydrogen peroxide produced can be further transformed to hydroxyl radicals in the presence of metals. The hydroxyl radical has is very reactive to cellular components. As a result, it causes destruction of the adjacent cells. SOD has three isoforms. The most abundant one is copper-zinc containing enzymes and they are found in the cytoplasm while manganese SOD is found within the mitochondria. The copper-Zinc SOD, the third type is present both intra and extracellurally. SOD is the first line of defence against mitochondrial generated ROS because it scavenges superoxide ion which is produced during oxidative phosphorylation (Fig. 1.4). The role of SOD in the development of diabetes is reported in genetic modified mice lacking expression of SOD; these were observed to have reduced β-cell insulin secretion and decreased β-cell volume and glucose intolerance (Muscogiuri et al., 2013). It can be inferred from this study that SOD can potentially limit the damaging effects of hyperglycaemia induced ROS production. Conversely, intensive insulin therapy can increase the SOD levels in diabetic patients (Wiryana, 2009). This inverse relationship of SOD and hyperglycaemia implies that SOD can be potentially used for prevention of ROS damage in diabetes. Indeed, administering extracellular SOD to diabetic rats prevented the progression of diabetic induced kidney damage via antioxidant mechanisms (Kuo et al., 2015).
1.16.3 Catalase

Catalase is an antioxidant enzyme that acts as a catalyst for the conversion of hydrogen peroxide to oxygen and water. It protects the cells from the damaging effect of hydrogen peroxide that is present intracellularly. In T2D patients, plasma catalase activity is observed to be reduced and genetic modifications in catalase has been suggested as a risk factor for development of T2D (Góth, 2008). Furthermore, overexpression of Catalase in the mitochondria of insulin producing cells protected the cells from ROS induced cell death by pro-inflammatory cytokines (Gurgul et al., 2004). Taken together Catalase may play a role in both development and progression of T2D.

1.16.3 Glutathione peroxidase

Glutathione peroxidase (GPx) is an enzyme that causes the reduction of ROS to reduce their harmful effects. There are several forms of they include GPx -1, 2, 3, 4 (Lubos et al., 2011). These enzymes are key players in preventing increased levels of oxidative stress. GPx-1 is the primary antioxidant enzyme involved with preventing the accumulation of intracellular hydrogen peroxide; it converts hydrogen peroxide to water (Lubos et al., 2011). Suppressed levels of GPx activity have been observed in T2D patients compared to normal patients (Goyal et al., 2011). Furthermore, it has been observed that genetic variation in GPx-1 may contribute to the development of T2D in South Indians (Ramprasath et al., 2012). Collectively, the studies indicate that GPx may play a significant role in the development of T2D.
1.17. Non-Enzymatic Antioxidants

Examples of the members of this group of antioxidants include Vitamins C and E, Glutathione.

1.17.1 Vitamin C, E and Glutathione

Vitamin C is a water-soluble free radical scavenger while, Vitamin E is a lipid soluble free radical scavenger found in the cell membranes (Nimse and Pal, 2015). Glutathione (GSH) is an intracellular thiol that acts as both free radical scavenger, and also serves to regenerate Vitamin E (Hakki Kalkan and Suher, 2013). In a 20 yrs. follow up study, Vitamin C intake, together with fruits and vegetables was found lower among incident cases of T2DM (Feskens et al., 1995). On the contrary, in a 23 yrs follow up cohort study, no association was observed between intake of vitamin C and risk of developing T2D, while Vitamin E was shown to reduce the risk of T2D (Montonen et al., 2004). However, in a recent study by Rafighi et al (Rafighi et al., 2013), supplementation of Vitamin C and E and Glutathione in T2D patients already on oral anti-diabetic medication caused significant reduction in HbA1C (a marker for hyperglycaemia) (Rafighi et al., 2013).

Moreover, a reduced synthesis of Glutathione has been observed to be involved with T2D development (Kalkan and Suher, 2013). Taken together, non-enzymatic can influence the progression of diabetes be useful for management of the disease and prevent ROS induced organ damage. Yet, antioxidant therapy has not yielded much success for management of T2D. In addition, supplementation with Vitamins have not shown any promise for reducing ROS organ induced damage (Golbidi et al., 2011). This could probably be a result of bioavailability of the antioxidants at the site of ROS (Paganini et al., 1997) and also, increased toxicity of higher concentrations of
currently available antioxidants. An increased understanding of ROS induction and the signalling pathways they trigger or inhibit and their effect on glucose metabolism may help in implementing antioxidant therapy for T2D management.

In conclusion, poor glycaemic control in T2D develops due to a defective insulin function as a result of diverse metabolic aberrations such as insulin resistance in skeletal muscle, adipose tissue dysfunction, neuronal defects, gut microbiota induced inflammation adiposity and β-cell damage. Furthermore, poor glycaemic control causes oxidative stress and organ damage which eventually causes development of diabetic micro and macrovascular complications.

Therefore, ideal therapies should aim to restore normal glycaemic control by improving the various metabolic processes directly or indirectly associated with glucose metabolism. Moreover, aiming to avoid fluctuations in glucose blood levels via tight postprandial blood glucose control will reduce risk of T2D induced vascular diseases(Shukla et al., 2015).
1.18 Current and Future therapies for T2D

The pharmacological consequences of the different processes involved in the development of T2D have informed the design of the current therapies and continues to inform future drug designs. The actions of current and potential therapies for diabetes aim to improve glycemic control via multiple mechanisms. Generally, the different ant-diabetic drugs function by:

- Enhancing optimum β-cell function and targeting gut digestion enzymes
- Harnessing gut microbiota for optimum energy homeostasis.
- Enhancing neuro-endocrine signaling for glucose metabolism.
- Promoting efficient control of post prandial blood glucose levels by, e.g. By improving glucose uptake/ insulin resistance in skeletal muscles, and by reducing glucose re-absorption in the kidneys.

1.18.1 Enhancing optimum β-cell function and targeting gut digestion enzymes

Insulin secretion is the primary function of the β-cell therefore; supplementing β-cell function with insulin analogues is one of the current strategies for diabetic therapy. These drugs are designed to augment β-cell output as β-cell function declines during the progression of diabetes(Horton, 2009). They function as insulin with slight difference in pharmacokinetics and pharmacodynamics(Grunberger, 2014). There are rapid-acting (prandial) analogs such as Lispro, Aspart, or Glulisine insulin and long (basal) acting such as Glargine, Detemir(Grunberger, 2014). The side effects of Insulin analogues are: the risk of hypoglycemia, weight gain and the pain of injection.
Sulphonylureas and the Meglitinides are another group of drugs directly targeted at enhancing insulin secretion function of the β-cell. These drugs bind to Sulphonylurea receptors on ATP dependent potassium channels. By binding these receptors they inhibit these channels and elicit calcium dependent insulin secretion in β-cells (Cannon et al., 2015, Norman and Rabasseda, 2001). Examples of Sulphonylureas include, Gliclazide and Tolbutamide, while, examples of the Meglitinides include Repaglinide and Nateglinide (Richard and Raskin, 2011).

The β-cell role in glucose homeostasis is physiologically enhanced by incretins. The main incretins are Glucagon like peptide-1 (GLP-1) and Glucose dependent insulinotropic polypeptide (GIP). GLP-1 has been the most exploited for T2D therapy and examples of GLP-1 mimetics include Exenatide and Liraglutide (Ahrén and Schmitz, 2004). They enhance insulin function by stimulating insulin release and reducing glucagon release (Ahrén and Schmitz, 2004). GLP-2, a different isoform of GLP, may also improve gut function by improving gut permeability and by reducing LPS induced inflammation from gut microbiota (Cani et al., 2009).

In addition, certain drugs can inhibit the breakdown of GLP-1, thus prolong the duration of GLP-1 bioavailability. Dipeptidyl peptidase 4 (DPP-4) inhibitors or Gliptins such as Alogliptin and Saxagliptin prevent breakdown of GLP-1 and prolong its activity (Ahrén and Schmitz, 2004). The side effects of GLP-1 include nausea, vomiting, and pancreatitis (George and Joseph, 2014). Considering that the use of GLP-1, Gliptins, Sulphonylureas and Meglitinides is limited to the function β-cells. In absence of functioning β-cells which occurs as T2D progresses or in T1D, these groups of drugs may not be effective.

Inhibition of enzymes involved in the hydrolysis of carbohydrates such as α-amylase and α-glucosidase are being employed as a therapeutic approach for
controlling postprandial hyperglycaemia since they reduce net glucose delivery to the blood after food consumption. α-Glucosidase inhibitors (AGIs; Acarbose, Miglitol, Voglibose) are used in the treatment of patients with T2D. AGIs interrupt the absorption of carbohydrates from the small intestine and thus have a lowering effect on postprandial blood glucose and insulin levels, without affecting lipid levels (Van De Laar et al., 2005). One of the drawbacks of AGIs is they are associated with gastrointestinal disorders such as flatulence, abdominal distention, diarrhoea and nausea (Weng et al., 2015).

1.18.2 Harnessing gut microbiota for optimum energy homeostasis.

As mentioned earlier, gut microbiota in the colon, converts starch and fibre that are resistant to intestinal enzymes to short chain fatty acids (SCFAs). The rate and amount of SCFA production is dependent on the species and amounts of microflora present in the colon, the food source and the gut transit time (Eckburg et al., 2005). The firmicutes and bacteroidetes are the most abundant bacteria in the gut and may be key players for the production of SCFAs in the gut (Fernandes et al., 2014). The amount of bacteroidetes in the gut has been negatively correlated with increase in body mass (Fernandes et al., 2014, Fava et al., 2013). Accordingly, fermentation of food by colonic bacteria and total amount of SCFAs was observed to be different in obese and lean human subjects (Fernandes et al., 2014). Therefore, production of SCFAs by gut microbiota may be linked to adiposity (Rahat-Rozenbloom et al., 2014). There are three well known SCFAs. One of them is acetate which is the main SCFA produced in the colon, and after absorption it has been shown to increase cholesterol synthesis. In contrast, propionate another metabolite of the colon microbiota, is used for glucose synthesis, and was observed to inhibit cholesterol synthesis while butyrate the third one, is used for
synthesis of triglycerols in the liver (Wong et al., 2006). Recently, SCFAs have been shown to activate certain G-protein receptors called GPR43 that may have metabolic effects (Kimura et al., 2014). These receptors may link gut microbiota to energy metabolism and hence, may be useful targets for T2D therapy. In addition, use of diets which include indigestible carbohydrates as supplements to modulate gut microbiota may also be a useful strategy for management of the disease (Fujimura et al., 2010).

1.18.3 Enhancing neuro-endocrine signaling for glucose metabolism

Based on the observation that the low levels of dopamine in certain areas of the hypothalamus in animals during the winter season is similar to insulin resistance states in T2D patients, the use of the dopamine agonist, Bromocriptine, has been approved for treatment of insulin resistance in both T2D and insulin related diseases. It is possible that use of dopamine agonists would also prevent or delay progression of microvascular complications such as hypertension and diabetic nephropathy, since increasing intrarenal dopamine levels have shown some benefits in reducing the progression of the DKD (Zhang et al., 2012). Accordingly, recent clinical trials have shown that use of dopamine agonist is well tolerated in patients with moderate renal insufficiency. However, long term effects of dopamine on prolactin levels and mental health could be seen as potential side effects (Diepenbroek et al., 2013, DeFronzo, 2011). GLP-1 mimetics may also have neuronal effects that reduce weight gain and appetite if they cross the blood brain barrier (Kahn et al., 2014). Targeting ROS induction in pro-opiomelanocortin (POMC) neurons; neurons responsible for energy regulation and satiety in the hypothalamus, could be useful for future management of diabetes (Diano et al., 2011).
1.18.4 Promoting efficient control of post prandial blood glucose levels by e.g. by improving glucose uptake/insulin resistance in skeletal muscles and/or reducing glucose re-absorption in the kidneys.

- **Drugs that improve insulin glucose uptake/insulin resistance**

On the other hand, the Thiazolidinediones increase insulin sensitivity in the peripheral tissues. They are primarily peroxisome proliferator-activated receptor (PPAR) agonist. The proposed hypoglycaemic mechanism for PPARs includes reduction in inflammation, increase in adipocyte differentiation, increase in fatty acid oxidation and increase in GLUT expression. This implies that they would improve insulin sensitivity and glucose uptake. They may not improve the lipid profile of the patients (Staels and Fruchart, 2005, Fowler, 2007). The Thiazolidinediones, e.g. Rosiglitazone, belong to this group of drugs. The side effect of these drugs include; weight gain, hepatotoxicity, increased risk of bone fracture and heart failure (Rizos et al., 2009). The new drug Aleglitazar, which is an agonist for both receptor subtypes of the PPAR, may also be useful for preventing cardiovascular complications of diabetes (Cavender and Lincoff, 2010).

The Biguanides consist currently of only Metformin. The core mechanism of action for metformin is the change of the energy metabolic rate of the body. Metformin exerts its principal, hypoglycaemic effect by inhibiting hepatic gluconeogenesis and opposing the action of glucagon. The inhibition of mitochondrial complex I (Hardie et al., 2012) results reduced ATP synthesis and eventually defective cyclic adenosine monophosphate (cAMP) and protein kinase A signalling in response to glucagon (Miller et al., 2013). Moreover, the stimulation of 5′-AMP-activated protein kinase, which modulates lipid metabolism, is an additional mechanism for the glucose-lowering effect of metformin.
Overall, Metformin induces insulin sensitivity and improves glucose uptake in cells. The side effects of Metformin are proposed to be as a result of its inhibitory effect on mitochondrial function. Impeding the ETC causes accumulation of pyruvate and hence an increase in lactic acid production (Piel et al., 2015). Mitochondrial toxicity of metformin occurs at high concentrations. Therefore, Metformin propensity for lactic acidosis in T2D demands for cautious use of the drug in T2D patients with renal complications (Inzucchi et al., 2014).

- **Drugs that inhibit Glucose Re-absorption in Kidneys and in the Intestine**

The kidney filters 180 litres of plasma per day and serves to maintain osmotic and pH balance by re-absorbing water, sodium, chloride and bicarbonate and secreting hydrogen ions and potassium produced by ingested foodstuffs. Also, the kidney is capable of gluconeogenesis, thus alongside the liver, plays a crucial role in glucose homeostasis especially during fasting thereby helping to maintain normal fasting plasma glucose (FPG) levels (~5.6 mmol/l) (Mitrakou, 2011).

In contrast to the liver, the entry of glucose in the kidney is not dependent on insulin, although the release of glucose from the kidney is inhibited by insulin (Gerich, 2010, Mitrakou, 2011). Besides gluconeogenesis, the kidney impacts plasma glucose levels via the process of glucose re-absorption. Glucose re-absorption from filtered plasma occurs in the proximal tubules via sodium dependent glucose transporters (SGLTs) (fig.1.9). The SGLTs are of two main subtypes: SGLT1 and SGLT2. SGLT2 is a low-affinity, high-capacity glucose transport protein that reabsorbs 90% of filtered glucose, while the high-affinity, low-capacity SGLT1 transporter reabsorbs the remaining 10% (Vallon, 2011).
SGLTs are also found in the intestine and therefore are desirable targets for non-insulin dependent control of post prandial blood glucose via control of glucose absorption from ingested food (Hasan et al., 2014). In diabetic conditions, the kidneys increase expression of SGLT2 and unlike in the liver, increased glucose ingestion increases kidney gluconeogenesis even in diabetic patients (Meyer et al., 1998, Hasan et al., 2014).

Consequently, the hyperglycaemic state is maintained, and continuous high blood glucose exacerbates insulin resistance and causes impaired β-cell function and death due to glucotoxicity (Mather and Pollock, 2011). The initial SGLT inhibitor was Phlorizin (fig: 1.10), discovered from the bark of the apple tree and is abundant in the *Malus* spp. (White, 2010). It has been used as a template for developing the recent drugs that have been approved for T2D therapy.

From the structural modifications that have evolved in SGLT2 specificity it appears the glucosidal molecule in the structure is important interaction with SGLT2. However, an open middle ring in the backbone may still be very vital for any form of glucose transporter interaction. In fact, hydrolysis of Phlorizin yields Phloretin (fig. 1.10) which is known to inhibit GLUT transporters (Zheng et al., 2012). Clinical trials of SGLT2 inhibitors have shown that they reduce fasting blood glucose as monotherapy and as add-on therapy to other anti-diabetic drugs. SGLT2 inhibitors may also have the additional benefit of causing weight loss and reduction in blood pressure. Recently, due to their additional benefit of inhibiting glucose absorption in the intestine, clinical trials on drugs that inhibit both SGLT1 and 2 such as Satogliflozin and LX4211 are underway. However, SGLT2 specific inhibitors are contra-indicated in patients with moderate to
severe renal insufficiencies. They might also increase urinary tract infections and may cause cancer of the bladder (Hasan et al., 2014, White, 2010, Oliva and Bakris, 2014, Volino et al., 2014). In addition, the approved SGLT drugs Canagliflozin, have been reported to predispose patients to ketoacidosis. The mechanisms may involve increase in glucagon secretion, inhibiting the excretion of ketoacidosis, reduced insulin dosage which is common practice when SGLT2 inhibitors are used in combination therapy (Taylor et al., 2015).

In summary, most drugs in line for treatment of T2D and the ones already in use all have potential toxic effects. Therefore, the search for safer drugs will continue. Medicinal plants and natural products present a mining pool for screening for potential lead molecules. In addition, including these plants in diet may be a better approach since medicinal plants contain diverse medicinal compounds that would exert less specific effects and therefore, reduce chances of toxicity.
Fig. 1.9: Proximal tubules and characteristic Sodium dependent glucose transporters 1 and 2 (SGLT 1 and 2). SGLT are involved in re-absorption of 100% of the plasma glucose filtered by the kidneys. Phlorizin (fig. 1.9), is a natural compound that exerts its anti-diabetic effect by inhibiting both SGLT1 and SGLT2 transporters found in the kidneys and intestine. This non-insulin dependent mechanism of action serves as an alternative approach to glycaemic control in T2D.
Fig 1.10: Structures of Phlorizin (an O-glycoside), Phloretin (aglycone of Phlorizin) and synthetic SGLT2 specific inhibitors. Synthetic SGLT2 inhibitors are c-glycosides. This modification confers resistance to digestive enzymes and open middle ring may be important for interacting with both glucose transporters.
1.19 Role of Medicinal Plants in the Management of Diabetes in Developing Countries.

Ageing follows the process of progressive loss of physiological functions and increased risk of developing incapacitating disorders, including chronic inflammation which as discussed already, is a key component of T2D (Donath and Shoelson, 2011). In addition, 12.1 million people have been diagnosed with diabetes in sub-Saharan Africa. This number may increase to 23.9 million by 2030 (Hall et al., 2011). According to a study by Hall et al. (Hall et al., 2011), the percentage of diabetic complications such as microalbuminuria in this region, ranges from 10%-83% (Hall et al., 2011). According to the authors, the wide variation in prevalence of microalbuminuria could be a function of the difference in urban development across the sub-Saharan African region (Hall et al., 2011). Consequently, the current therapies and appropriate healthy lifestyle to prevent occurrences of diabetes and its complications are not affordable. Therefore there is a need for affordable remedies for the management of diabetes in sub-Saharan African regions in order to reduce the risk occurrence of the disease, especially in the light of the current rapid urbanization of the continent.

The use of herbal medicine for management of diseases is a common practice in Africa, because it is cheap and accessible and has anecdotal relevance. Basic and clinical research on the mechanisms and application of African remedies will enable the appropriate and safe use of these herbs, with the additional benefit of reduced risk of development and probably effective management of diseases such as diabetes.

Several plants have been studied for their anti-diabetic effects. Examples of such plants are: Allium Sativum (garlic) has been reported to contain sulphur containing
metabolites (Allicin and S-allyl cysteine sulfoxide) are known to stimulate insulin secretion from pancreatic β-cells (Khan et al., 2012).

*Camellia sinensis* (Green tea) contains catechins that possess hypoglycaemic potentials (Haidari et al., 2013). Recently, an arabinogalactan (a polysaccharide) has also been isolated from Green tea and has been identified to stimulate insulin secretion through the same pathways GLP-1 exerts its insulin stimulatory effect (Wang et al., 2015, Doyle and Egan, 2007). Clinical studies with *Nigella sativa* showed that application of this plant as an adjuvant in T2D patients already receiving treatment was effective for controlling hyperglycaemia (Kaatabi et al., 2015). In the light of these clinical studies herbal medicines do have great potential as adjunct therapy or dietary interventions for management of diabetes.

Furthermore, some clinical studies have shown that diet modification and exercise are beneficial to diabetic patients. For example, diets rich in fruits and vegetables tend to reduce the chances of diabetic patients for developing diabetes associated heart disease (Evert et al., 2014, Andrews et al., 2011). The potential of fruits as dietary intervention was shown by a recent study in healthy volunteers which reported that unripe apples improved post-prandial glycaemia and increased urinary glucose excretion after oral glucose tolerance test (Makarova et al., 2015). Also, in a pilot study consuming fruits and vegetables before having carbohydrate improved postprandial glucose levels in T2D patients (Shukla et al., 2015). Fruits, vegetables and herbal plants all have in common, chemicals that enhance metabolism and the functions of organs in the body (Kennedy and Wightman, 2011). These compounds are known as secondary metabolites.
1.20 Secondary Metabolites and their health benefits

Secondary metabolites are organic chemicals generated by plants which enable them to adapt to their environment (Wink, 2003). Secondary metabolites are known to have diverse pharmacological activities. For example; *Momordica charantia* (bitter melon), a herbal plant used traditionally for management of diabetes, is known to contain polyphenols (Nerurkar et al., 2010, Islam et al., 2011), a class of secondary metabolite synthesized for protection against infections in plants. Consistent with this, epidemiological studies have repeatedly shown an inverse relationship between the risk of chronic human diseases and the intake of diet with high polyphenolic content (Pandey and Rizvi, 2009).

Examples of classes of various plant metabolites include: alkaloids, polyphenols, saponins, and iridoids. The general structures of these compounds and their pharmacological activities are shown in the table overleaf.
<table>
<thead>
<tr>
<th>NAME</th>
<th>BASIC STRUCTURE</th>
<th>SOME KNOWN PHARMACOLOGICAL ACTIVITIES</th>
<th>COMMON PLANT SOURCES</th>
<th>REF.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iridoids and Seco-iridoids</td>
<td><img src="#" alt="Iridoid Structure" /></td>
<td>Anti-inflammatory, Anti-cancer, Neuroprotective and Anti-antioxidant.</td>
<td>Rubiaceae, Lamiaceae, Gentianaceae, Pyrolaceae, Oleaceae, Cornaceae, Scrophulariaceae,</td>
<td>(Georgiev et al., 2013)</td>
</tr>
</tbody>
</table>

**Table 1.1:** The table above shows some common phytochemical compounds and their pharmacological activities. *Plant secondary metabolites possess more than one biological activity and they can be found in more than one Genus.*
### Table 1.1 continued: The table above shows some common phytochemical compounds and their pharmacological activities.

<table>
<thead>
<tr>
<th>NAME</th>
<th>BASIC STRUCTURE</th>
<th>SOME KNOWN PHARMACOLOGICAL ACTIVITIES</th>
<th>COMMON PLANT SOURCES</th>
<th>REF.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyphenols and phenolic acids</td>
<td><img src="image" alt="Basic structure of Phenolic acids" /></td>
<td>Antioxidant, Antidiabetic, Neuroprotection.</td>
<td>Abundant in most fruits and vegetables. For example: Solanaceae, Vitaceae, Sapotaceae, Myrtaceae, Moraceae, Anacardaceae, Rhamnaceae, Sapindaceae, Malvaceae</td>
<td>(Pandey and Rizvi, 2009, Scalbert et al., 2005)</td>
</tr>
</tbody>
</table>

*Table 1.1 continued: The table above shows some common phytochemical compounds and their pharmacological activities. Plant secondary metabolites possess more than one biological activity and they can be found in more than one Genus.*
<table>
<thead>
<tr>
<th>NAME</th>
<th>BASIC/EXAMPLE OF STRUCTURE</th>
<th>SOME KNOWN PHARMACOLOGICAL ACTIVITIES</th>
<th>COMMON PLANT SOURCES</th>
<th>REF.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponins (Triterpenoids steroids and steroidal glycoalkaloids)</td>
<td><img src="image" alt="Image of Saponins" /></td>
<td>Antioxidant, Anti-cancer</td>
<td>Agavaceae, Alliaceae, Asparagaceae, Convallariaceae, Dioscoreaceae</td>
<td>(Moses et al., 2014)</td>
</tr>
<tr>
<td>Alkaloids</td>
<td><img src="image" alt="Image of Morphine" /></td>
<td>Anti-tuissive, Anti-malaria, Anti-hypertensive, Analgesics.</td>
<td>Widely distributed in both plants and insects.</td>
<td>(Block, 1999)</td>
</tr>
</tbody>
</table>

Table 1.1 continued: The table above shows some common phytochemical compounds and their pharmacological activities. Plant secondary metabolites possess more than one biological activity and they can be found in more than one Genus.
Most secondary metabolites are not restricted to a particular family in the plant kingdom. Therefore, different families can have the same class of compounds. In addition, the different plant families tend to have different combinations of secondary metabolites. This could be the reason for their different efficacy and use in management of diseases. In view of the multiple effects of plant secondary metabolites as shown in the table above, medicinal plants represents a large repository for mining lead compounds that could be modified for drug use.

Essentially, it will be helpful to introduce herbal plants, into regular diet, since these therapeutic compounds can be easily accessed in this form. In other words, they can serve as both food and medicines. This approach is more likely to improve the outcome of patients that use them since their non-specific effects reduce the chances of toxicity which is observed with synthetic drugs. In the case of T2D and related metabolic disorders, diet intervention with medicinal plants can prevent the occurrences of diet related diseases, as well as reduce the socioeconomic burden of managing diabetes in regions affected by the diseases. Notwithstanding, in order to validate the use of herbal plants, the efficacy and safety of these medicinal plants need to be evaluated.
1.21 Review on Pharmacological Activity of *Mucuna pruriens*

*Mucuna pruriens* (L) Fabaceae (MP), the plant of focus for this study, is a common medicinal plant used for the management of several diseases including diabetes.

Fig. 1.11: A picture of *Mucuna pruriens* shoot with its characteristic velvet flowers.

*Mucuna pruriens* is an annual plant that grows as a weed among food crops. The thin crawling shoots show it is adapted for climbing other plants. The picture was taken during collection of the leaf samples in October. The presence of flowers shows that leaves were collected prior to the time for seeds to mature. Camera Details: Samsung Galaxy Pocket mobile 2 Mega Pixels. Date: 15th Nov 2012.
**Mucuna pruriens** (Common Name: velvet bean) belongs to the *Fabaceae* family of the plant kingdom. They are also known as the pea family. Plants belonging to this family are dicotyledonous flowering plants. The common phytochemicals associated with this family are Protobberine alkaloids, which are known to bind deoxyribonucleic acids (DNA) (Kumar, 2015), and steroidal saponins, which are known to have diuretic effects (Diniz et al., 2012).

### 1.22 Biological activity of *Mucuna pruriens* seeds

*Mucuna pruriens* (MP) produces seeds annually. The seeds are known to contain carbohydrates, proteins and anti-nutritional compounds such as tannins (Tavares et al., 2015). The seeds are used traditionally as prophylaxis for snake bite (Fung et al., 2014). The precise mechanism for this protective effect is not known, however, gpMuc is a glycoprotein with close resemblance to Kunitz-type trypsin inhibitor family which was recently isolated from MP seeds (Scirè et al., 2011). The presence of Kunitz-type trypsin inhibitor could explain its anti-venom properties as proteins belonging to this family inhibit protein degrading enzymes.

In addition, Kunitz-type trypsin inhibitors are useful anti-inflammatory agents and may also have the ability to inhibit tumour growth (Zhu et al., 2011). MP seeds may also provide protection from snake venom by delaying cardio-respiratory failure induced by snake venom (Fung et al., 2012). However, pre-treatment with the seed extract followed by snake venom challenge was observed to cause up regulation of genes related to immune response, energy metabolism and muscle contraction of the cardiac tissues of rats (Fung et al., 2014). Specifically, MP seed extract caused up-regulation of pyruvate dehydrogenase kinase gene expression (Fung et al., 2014), which causes a metabolic
switch from phosphorylative oxidation to glycolysis. Considering that certain snake venoms disrupt mitochondrial membrane potential (Park et al., 2009), MP seed extract could exert protective effect against snake venom on the heart by providing alternative energy by increasing glycolysis.

Based on the effect of MP on cardiac muscles; this study provides insight into other applications for the seed extracts. For instance, MP seed extract was shown to increase expression of metallothionein. This could be beneficial in high glucose induced ROS damage in cardiomyocytes. Indeed, metallothionein is an antioxidant protein and it has been shown to ameliorate ROS induced myocardial dysfunction (Ye et al., 2003).

Furthermore, the seeds contain levodopa and are thus known to be effective for management of Parkinson's disease (Manyam et al., 2004). In addition, the presence of levodopa in the seeds could have additional therapeutic effects, by reducing development of DKD, since increased bioavailability of dopamine in kidneys, has been shown to delay the progression of the disease in rats (Zhang et al., 2012). Potential effects of MP on the reproductive system have also been observed. These studies revealed that MP seeds may improve erectile dysfunction, fertility and sexual behaviour in non-diabetic and diabetic conditions (Suresh et al., 2009, Suresh and Prakash, 2011, Suresh and Prakash, 2012). Anti-diabetic oligocyclitols which are known to mimic insulin signal transduction have been also isolated from MP seeds (Donati et al., 2005).

Therefore, more studies are required to understand the potential benefits of the use of MP seeds as a crude drug for management of diseases and to provide leads for future drug molecules.

However, toxicity due to Levodopa content has been observed for consumption of Mucuna species (spp.) (Tse et al., 2013). The degradation of L-dopa contents of MP seeds
have been observed to degrade into damaging quinones and ROS (Pulikkalpura et al., 2015), the significance of these findings are yet to be fully studied. The seeds have also been observed to increase bilirubin levels thereby inducing mild cholestasis in male rats (Chukwudi et al., 2011). Therefore, further studies are required to ascertain the safety of the seeds in clinical studies.

1.23 Bioactivity of *Mucuna pruriens* leaves and roots

MP leaves are also used traditionally for anaemia and several other diseases (Obioma et al., 2014b). Most studies with MP leaf extracts indicate effective hypoglycaemic activity when leaves are extracted in non-polar solvents. Ethanolic extracts of the leaves made by maceration in 70% ethanol for 72 hours reduced plasma lipid levels and glucose levels in diabetic rats comparable to metformin (Eze et al., 2012). The investigators also observed the regeneration of pancreatic tissues in the diabetic rats used for their study (Eze et al., 2012). Metformin is known to improve dyslipidaemia by inhibiting fatty acid synthesis through the activation of adenosine monophosphate activated kinase (AMPK). The AMPK pathway is also responsible for glucose uptake into skeletal muscles (Hardie et al., 2012) (see Fig. 1.9). It is possible that the MP extracts induced the hypoglycaemic and hypo-lipidaemic effects observed through the AMPK pathway. However, further studies are required to confirm this.

Furthermore, MP ethanolic leaf extract has been observed to reduce aminotransferase enzymes which increases substrate of the glucose metabolic pathway that are usually used for fatty acid synthesis (Alo et al., 2012). Thus, modulation of AMPK pathway could be a mechanism of action of the hypolipidaemic and hypoglycaemic effects of MP. Another study with the chloroform fraction of the alcoholic leaf extract made by soxhlet
extraction in 95% ethanol indicated hypoglycaemic and hypo-lipidaemic activity comparable to Glibenclamide (a known anti-diabetic drug) which could be due to the presence of alkaloids and glycosides (Murugan and Reddy, 2009). Yet, the anti-diabetic effects of the aqueous extract have not been studied.

General phytochemical screening using methods of Harbone (Harborne, 1973) and Trease et al (Trease GE, 1983), of the water and alcoholic extract of MP leaves revealed the presence of flavonoids, saponins, and cardiac glycosides (Agbafor and Nwachukwu, 2011). Although, specific compounds where not identified, these are traits of most plants used for management of diabetes and diabetic complications (Singh et al., 2013). The presence of glycosides suggests flavonoids, coumarins or saponins; all of which are known to possess antioxidant activity. Moreover, by a similar extraction process, MP alcoholic leaf extract was observed to have anti-ulcer activity in ethanol induced stomach ulcer in rats. MP alcoholic leaf extract preserved the gastric mucosal layer by increasing endogenous antioxidant activity and mucus secretion, and also via relaxation of gastric walls (Golbabapour et al., 2013). The relaxation of gastrointestinal walls and consequent inhibition of peristalsis is characteristic of flavonoids (Gharzouli and Holzer, 2004, Amira et al., 2008). An increase in mucus secretion is partially mediated by increase in prostaglandin E2 (PGE2). PGE2 causes vasodilatation in blood vessels and is also known to prevent apoptosis in cardiomyocytes by activating growth signal pathways (Frias et al., 2008). Although, upregulation of PGE2 may be deleterious in certain conditions such as progression of diabetic kidney disease (Quilley et al., 2011) it is important to note that the phytochemical components of the ethanolic leaf extract could also influence growth of specialized cells and influence metabolic processes in vivo. These properties could be useful for preserving β-islet cells (which are often
degraded by hyperglycaemia) and improve metabolic disorder which are common features of diabetes.

MP leaves have been tested for protective effects against liver damage. In order to evaluate protective effect against alcohol and anti-tuberculosis drugs induced liver damage, MP leaves were extracted in a mixture of alcohol and water. In this study MP hydroethanolic leaf extract was suggested to reduce plasma markers for liver damage by improving activity of antioxidant enzymes (Obogwu et al., 2014). The authors also observed that hydroethanolic extract of the leaves prolonged sleep induced by hexobarbitone and this was suggested to be an inhibitory effect on cytochrome p450 enzyme activity. A potential inhibitory effect on cytochrome p450 enzyme activity suggests a toxic effect of the hydroethanolic extract of the leaves. Inhibition of cytochrome p450 enzyme can cause negative drug interactions with drugs dependent on this enzyme for deactivation. Conversely, prodrugs that require this enzyme for activation may also lose effectiveness. A similar interaction is known for grape fruit (Sweeney and Bromilow, 2006).

However, using the same method of extraction and the same doses, Champatisingh et al (Champatisingh et al., 2011) observed dopaminergic effects of MP leaves in cataleptic rat models. They also observed that MP leaf extracts reduced seizure in epileptic rat models. This suggests that MP leaves may also have effects on the nervous system.

The aqueous extract of MP leaves have also been reported to improve haemoglobin and packed cell volume in rats (Obioma et al., 2014b). However, the aqueous extract was also observed to increase aspartate transaminase (AST) and alanine transaminase (ALT) and some trace elements of copper and iron in rats that consumed the
extract (Obioma et al., 2014a). This suggest that the aqueous extract of MP leaves can induce hepatocellular liver damage similar to acetaminophen (Yang et al., 2014b).

A comparison between antioxidant activity of alcoholic and water MP extracts revealed that the alcoholic extract had a better antioxidant activity (Agbafor and Nwachukwu, 2011). But due to stearic hindrance, the structure of the phenolic compound contained in the plant extracts could affect the efficiency of reactions in the Electron Transfer (ET) model that was used by the investigators for evaluating the antioxidant activity of MP leaf extracts in water and alcoholic solvents (Apak et al., 2013). Moreover, the investigators compared in vitro antioxidant methods to in vivo antioxidant methods to draw their conclusion. However, the ET based in vitro method used in this study does not necessarily correlate with results derived from in vivo studies (López-Alarcón and Denicola, 2013), however, their results did confirm antioxidant activity of MP leaf extracts reported by other studies.

Recently the roots of MP were found to contain three new isoflavonones and known pterocarpans which have close resemblance to isoflavonoids (Dendup et al., 2014). Also, this study reported the inhibitory effect of these polyphenols found in the roots of MP on α-Glucosidase activity; however, this inhibitory effect was two-fold less than the conventional drug, acarbose. This inhibitory effect on α-Glucosidase activity suggests antidiabetic activity of the roots. They also observed that phytochemical constituent of roots varied with seasonal changes. Considering that anecdotal or traditional uses of the leaf often involve slight heating of the leaves in water after hand washing or soaking the leaves in reasonable amounts of consumable alcohol, it will be enlightening to know what beneficial effects of tea made from MP leaves can be observed within the context
of T2D. This would give a scientific basis for regular consumption of the leaves as a beverage.

Fig. 1.12: Effects of activation of AMPK pathway on glucose and lipid metabolism. AMPK is activated in response to reduction of intracellular ATP levels. Reduction of intracellular ATP levels in diabetic conditions can occur due to oxidative stress induced mitochondrial damage. AMPK inhibits ATP consuming cellular processes such as lipid synthesis, gluconeogenesis and glycogen synthesis in fat, muscle and liver tissues. Increase in glucose and lipid catabolism causes corresponding increase in glucose uptake, which is useful for improving insulin resistance and reducing hyperlipidaemia in T2D (Hardie et al., 2012, Towler and Hardie, 2007).
1.24 Hypothesis

In the light of the above discussions the following hypothesis was drawn:

- Considering the reports on the anti-diabetic effect of MP leaf extracts made in organic solvents, it is plausible to propose that MP aqueous leaf extract could also have anti-diabetic effects.

- Furthermore, some of the current anti-diabetic agents improve glycaemic control by modulating glucose uptake in the kidneys, gut, skeletal muscles, adipocytes and liver. If MP aqueous leaf would have anti-diabetic effects it may exert its effect through similar mechanisms.

- On the basis of reported anti-oxidant activity of an MP aqueous leaf extract in cell free and \textit{in vivo} experiments, MP aqueous leaf extract could also scavenge ROS in oxidative stress related physiological systems.
1.25. Aims and Objectives

**Aim:** In view of the hypotheses listed above, the aim of this project was to investigate the potential anti-diabetic effects of MPLE. The potential anti-diabetic effect of MPLE was studied by investigating the potential mechanisms through which MPLE may reduce hyperglycaemia and oxidative stress.

**Objectives:** The potential anti-diabetic mechanisms of MPLE were investigated by carrying out the following studies listed below:

1. Evaluation of MPLE antioxidant activity in cell free models and in oxidative stress cell models using in NRK-52E cell lines.
2. Investigation of MPLE inhibitory effect on glucose uptake in NRK-52E cell lines.
3. Examination of insulin mimetic effect in 3T3-L1 adipocytes.

1.26  *In vitro* models used for Evaluating Anti-diabetic Effects

On the basis of glucose digestive pathway, laboratory experiments to test for hypoglycaemic effects tend to evaluate; inhibition of glucose digestive enzymes in the gut, effect on pancreatic cell health and glucose and lipid metabolism in the liver, insulin mimetic effect on both skeletal muscles and adipose cells, and more recently, inhibition of glucose uptake in kidney cells. For the purpose of this study, the following discussion will focus on established kidney and adipocyte cell lines used for *in vitro* study of anti-diabetic effects.
1.27.1 Normal rat epithelial kidney (NRK-52E) cell lines

The NRK-52E cell lines are derived from rat kidney proximal tubules. They have a characteristic flat polyhedral shape and cobblestone morphology with distinct nuclei and nucleoli like the parent cell line (De Larco and Todaro, 1978). NRK-52E grown on culture plastic for six days were shown have many microvilli and apical-basal polarity (Fan et al., 1999). This is a characteristic of the brush border found in the proximal tubule in vivo. They are also known to express C-type natriuretic peptide; a peptide also found in human proximal tubules (Dean et al., 1994) which may be involved with renal sodium handling and could serve as a marker for progression of chronic kidney disease (CKD) (Sangaralingham et al., 2011). Furthermore, NRK-52E cell lines are known to express low activity of organic anion transporters which are known to be dependent on sodium ions (Lash et al., 2007). Moreover, NRK-52E cell lines express collagen similar to proximal tubules in vivo. Collagen forms extracellular matrix during the progression of diabetic nephropathy, thus the cell lines are a good model for studying EMT in diabetic kidney disease (Creely et al., 1992).

Within this context, NRK-52E cell lines still retain some vital structural and functional characteristics of proximal tubules in vivo. Considering that glucose transport is dependent on sodium ions, they could also be used as a model for glucose transport studies. In relation to oxidative stress, NRK-52E cell lines have been reported to portray oxidative stress in both hypoxic/reperfusion conditions, ischaemic reperfusion conditions, and pro-oxidant substances (i.e. hydrogen peroxide) similar to other cell lines (Sáenz-Morales et al., 2006). They have also been shown to be sensitive to mitochondrial toxicants. In the light of this, NRK-52E cell lines are suitable for studies regarding metabolic induced oxidative stress within the kidney and glucose uptake.
1.27.2 Mouse pre-adipocyte (3T3-L1) cell lines

Pre-adipose cell lines 3T3-L1 cell line was isolated from Swiss 3T3 cells derived from a mouse embryo, and are morphologically analogous to fibroblasts and in growth arrest in cell culture conditions they can differentiate into mature adipocyte given appropriate conditions (Green and Kehinde, 1976). A recent study of the phenotype of 3T3-L1 cells revealed that these cell lines express characteristics typical of both white and brown adipocytes (Morrison and McGee, 2015). 3T3-L1 cells were reported to express glucose transporter (GLUT) isoforms 1 and 2. While the pre-adipocytes expressed glut 1 after differentiation, they were observed to express both GLUT 1 and 2. The latter is known to be highly expressed in the heart and skeletal muscles both of which respond to insulin stimulated glucose uptake (Kaestner et al., 1989). Moreover, as 3T3-L1 pre-adipocytes differentiate into mature adipocytes, they express increased numbers caveolae, which are specialized plasma membrane structures involved in receptor-mediated uptake processes and vesicular trafficking (Lafontan, 2012).

Moreover, Bogan et al (Bogan et al., 2001) demonstrated that 3T3-L1 before differentiation and throughout differentiation contain intracellular compartments from which GLUT4 is quickly mobilized in response to insulin. Considering that adipocytes respond to insulin stimulated glucose uptake in vivo, this implies that 3T3-L1 pre-adipocytes after differentiation are capable of responding to insulin stimulated glucose uptake. Therefore, they are a suitable model that can be used to evaluate the potential insulin mimetic effect of aqueous Mucuna pruriens aqueous leaf extract (MPLE) on glucose uptake.
Chapter 2: Materials and Methods
2.1. Collection and Storage of MP Leaves

MP is a tropical plant that grows in Africa, Asia and some parts of America. The leaves used for this study, were collected from different farms in Aku, Ezinifite, Anambra state in the south eastern part of Nigeria, West Africa. The leaves were then wrapped in pre-heated Colocasia esculenta leaves to prevent them from decaying as recommended by the farmers who assisted with collecting the leaves. The wrapped leaves were put in a bag and transported by courier to the University of Brighton, United Kingdom. The leaves were then removed from their stalk and air dried in a room for 3 days. Gloves were worn while separating leaves from stalk to prevent pruritus (the plant is known to cause itching on direct contact with skin). Then, approximately equal amounts of the crisp dried leaves were packaged and sealed in transparent small cellophane bags and labelled with different batch numbers. The labelled bags were stored at -80°C for preservation.

2.2. Identification of MP

The identification of the plant was done in the south eastern part of Nigeria West Africa. Briefly, samples of the leaves, seed and shoot of the MP were collected from the same region as mentioned above. The samples were identified at the International Center for Ethno-pharmacology and Drug Development, Enugu State, Nigeria, West Africa. The specimen identification number is: INTERCEDD-16018.
2.3. Preparation of MPLE

The leaves were powdered by carefully pouring of a small amount of liquid nitrogen to a batch of leaves in a ceramic mortar and crushing the frozen leaves with a pestle in a fume cupboard. For local uses, the leaves are usually extracted in alcoholic drinks or hand washed and boiled for few minutes before drinking. For laboratory purposes, 2.5g of powdered leaves was prepared in a water bath. The heating temperature of the water bath was set at 85°C. The leaves were soaked in 37.5ml of distilled water and put in the water bath for 15min (crude drug: water ratio 1:15) (Handa SS, 2008). The extract was filtered to give a final volume of 11-13ml. 4.5ml of the aqueous extract was dispensed into small bottles of known weight and freeze dried. These bottles were weighed again after freeze drying, and the weight of the extract in each bottle was determined. The small bottles containing freeze dried extracts were stored at -80°C. It was observed that when the extract was stored at 4°C, the activity of the extract may have been affected. Each small bottle was then regenerated in 1 ml of appropriate solvent depending on the experiment to give a known concentration of MPLE in mg/ml.
2.4. Sterilization of the plant extract

For cell culture experiments, dried extracts were regenerated in the appropriate medium and filtered into sterile 20ml tubes with 0.22µm filters, under sterile conditions.

2.5 Method to evaluate antioxidant activity of aqueous MPLE in cell free/chemical assays.

In order to investigate antioxidant activity of MPLE in cell free systems, established xanthine/xanthine oxidase and PMS/NADH superoxide anion radical generating systems were used. The procedures for the tests used are detailed in the section below:

- **2.5.1 Materials:**

**Chemicals:** Nitroblue tetrazolium (NBT), β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH), Phenazine methosulfate (PMS), Phosphate Buffer saline (PBS) tablets, Xanthine Oxidase from bovine milk, Xanthine, 4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl, (TEMPOL) where ordered from Sigma Aldrich (Poole, Dorset, UK). Distilled water, Monosodium phosphate and disodium phosphate ordered from Fisher scientific (East Grinstead, East Sussex, UK).

**Lab Ware:** Nunc microwell 96F plates, Corning centrifuge tubes, sterile syringes and 0.22µm filters were supplied by Fisher Scientific.

**Apparatus:** HT synergy Biotek reader (Highland Park, Illinois, USA). Pure flex lab (High Wycombe, Buckinghamshire, UK).
2.5.2 Protocol for Evaluating Antioxidant Activity of MPLE using Xanthine Oxidase\Xanthine Superoxide anion radical Generating System.

The assay of MPLE in xanthine/xanthine oxidase system was performed as described by Behera et al (Behera et al., 2003) with slight modifications. Briefly, 100µl of solution containing 0.4mM xanthine, 0.24mM NBT (the indicator) and 10µl each of 0.4mg, 1.2mg and 6.4mg of MPLE or 10µl 0.17mg and 1.7mg Tempol (a recognized superoxide scavenger) in PBS solution, was dispensed into separate wells in a 96 well plate.

Negative controls containing equivalent volumes of PBS were included in the experiments and they served as control with 0% scavenging activity. 100µl of 0.049units/ml of xanthine oxidase was added to each of these wells to start the reaction. The final concentrations of both MPLE and Tempol were: 0.019mg/ml, 0.057mg/ml, 0.3mg/ml MPLE and 0.0081mg/ml and 0.081mg/ml Tempol. The absorbance of reduced NBT was measured at 560nm (Behera et al., 2003).

The absorbance reading was taken at intervals of 2mins for total of 20mins. The degree of NBT reduced by the superoxide generated in the system was indicative of superoxide scavenging ability. Blank wells containing PBS solutions, the test extract and NBT only were included in the same 96 well plate during the experiments to cross check for possible interaction of the extract with NBT. The pH and temperature conditions for this experiment were maintained at 7.4 and 37°C, respectively.
% superoxide scavenging activity was the index for antioxidant activity and it was calculated (after removing absorbance of blank for each concentration) as:

\[ \% \text{NBT inhibition} = 100 - \frac{\text{Change in absorbance of MPLE or Tempol at 20min}}{\text{change in absorbance of control at 20 min}} \times 100 \]

2.5.4 Protocol for Evaluating Antioxidant Activity of MPLE using the NADH/PMS Superoxide anion radical Generating System.

Superoxide anion radical was generated in 96 well plates using a method modified from Ewing and Janero with slight modifications (Ewing and Janero, 1995). Briefly, 1mM NBT, 3mM NADH, 0.4mg/ml, 1.2mg/ml, and 6.4mg/ml of MPLE or 0.17mg/ml and 1.7mg/ml Tempol were made up in 0.1M phosphate buffer (pH 7.8). Then, a combination of 30µl each of 1mM NBT and 3mM NADH was dispensed into disparate wells in a 96 well plate.

Subsequently, 30µl of each of 0.4mg/ml, 1.2mg/ml, 6.4mg/ml concentrations of MPLE or 30µl of Tempol 0.17mg or 1.7mg was added to the wells (already containing the NBT and NADH). Each of these wells was then made up in the appropriate volume of 0.1M phosphate buffer (pH 7.8) in order to achieve equal volumes in each well.

Controls containing equivalent volumes of PBS were included in in the experiments and they served as standard for 0% scavenging activity. The reaction mixture was then incubated for 2 min. The absorbance of this mixture was measured at 560nm and used as blank values. Then the reaction was started by adding 30µl of 0.3mM PMS dissolved in 0.1M phosphate buffer (pH 7.8). The final volume in each well was 250µl. The corresponding final concentrations for MPLE were 0.048mg/ml, 0.144mg/ml, 0.77mg/ml. The corresponding final concentrations for Tempol were 0.02mg/ml and 0.2mg/ml. The absorbance at 560nm was measured at intervals of 1 min for a total of 2
min, using a Biotek plate reader. The degree of reduced NBT by the superoxide generated in the system was indicative of superoxide scavenging ability. The pH and temperature conditions for this experiment were maintained at 7.8 and 25°C, respectively.

% superoxide scavenging activity was the index for antioxidant activity and was calculated (after removing absorbance of blank for each concentration) as:

\[
\text{% NBT inhibition} = 100 \times \frac{\text{Control absorbance} - \text{MPLE or Tempol absorbance}}{\text{Control Absorbance}}
\]
2.6 Method for investigating Antioxidant activities of MPLE in NRK-52E cell line

To determine if MP aqueous leaf extract could also scavenge ROS produced intracellularly, MPLE was evaluated for antioxidant activity against paraquat induced oxidative stress in NRK-52E renal cells.

#### 2.6.1 Materials

**Cell line:** NRK-52E cell line (CRL 1571) was supplied by American Type Culture Collection (ATCC) (Porton Down, Wiltshire, UK).

**Chemicals:** Low glucose Dulbecco’s Modified Eagle Medium (DMEM), Trypsin/EDTA (0.05%/0.02% in Phosphate buffered Saline), Penicillin/Streptomycin (100X), Fetal Bovine Serum (FBS), Non-essential amino acids (NEAA) were supplied by GE Healthcare PAA (Amersham, Buckinghamshire, UK). β-Nicotinamide adenine dinucleotide (β-NAD), 1-Methoxy-5-methylphenazinium methyl sulfate (MPMS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), Triton X-100, Trizma hydrochloride buffer solution (Tris-HCl buffer), 1M, pH 8.0, L-Lactate, Dimethyl sulfoxide (DMSO) were supplied by Sigma Aldrich (Poole, Dorset, UK).

**Lab ware:** 10ml and 25ml serological pipette, Nunc multidish 24, TC flask 80cm² were supplied from Thermo Fisher Scientific.

**Apparatus:** Biochrom Asys UVM 340 micro plate reader (Cambridge, UK). Except otherwise stated, EscoClass II basic cell culture hood (Changi South Street, Singapore) was used for all cell culture procedures.
2.6.2 Cell culture Methods

Generally, NRK-52E cell lines were grown in TC flask 80cm² and maintained in a Heraus cell incubator at 37°C and 5% carbon dioxide (CO₂). The procedure for seeding cell lines was done in class II basic cell culture hood under sterile conditions. The growth medium for growing NRK-52E cells constituted of 500ml DMEM, 5% FBS, 1% NEAA, 1% pen/strep (100x). Confluent flasks were passaged in a 1:4 split using the following procedure: Flasks were emptied of growth medium. 10ml of warm trypsin/EDTA was used to rinse off excess medium.

The trypsin and excess growth medium was drained of the flask using sterile pastures connected to a vacuum pump. Another 10ml of trypsin/EDTA was added to the flasks. The flasks were then incubated for 5min or longer until the cells detached. Detached cells in the trypsin/EDTA were poured into sterile corning centrifuge tubes. Trypsin/EDTA was neutralized with 10ml of warm growth medium. Subsequently, the cell suspension was spun in centrifuge at 500G for 5min. Tubes containing spun cells were drained of medium in the hood. The residual cell pellets were re-suspended in 20ml of growth medium. Suspension was mixed gently to obtain a homogenous mixture. The homogenous cell suspension was diluted in 100ml (final volume) growth medium to achieve cell concentration of approximately 50,000 cells/ml. 1ml of this cell solution was dispensed into three 24 well plates. The remaining solution was poured into a TC 80cm² flask. The plates and the flasks were kept in the Heraus incubator. Cell medium was replenished the next day and then every 48hr. Plates were grown for 6 days and serum starved overnight before experiments.
2.6.3 Toxicity Studies of MPLE on NRK-52E cell line

Prior to antioxidant experiments, toxicity studies were performed to determine suitable concentrations for antioxidant studies in NRK-52E cell line. The potential cytotoxic effect of two concentrations that covered the range of concentrations used in the cell free assays was evaluated.

Briefly, the cells seeded in the 24 well plates were serum starved overnight before commencing toxicity studies. After serum starvation, the cells where incubated with 0.1mg/ml and 1mg/ml MPLE for 24hr in cell culture medium modified for experimental purposes. This experimental medium constituted of 500ml DMEM, 1% FBS, 1% NEAA, 1% pen/strep (100X). Untreated cell or 0mg/ml where designated as control group for this experiments.

2.6.4 Cell viability Assay

The MTT is metabolized by both mitochondrial and other reductases to insoluble formazan crystals(Liu et al., 1997). The amount of formazan present is proportional to the number of viable cells present in the well/plate(Mosmann, 1983). Therefore it used to evaluate general cell viability.

In this study, the procedure for the cell viability assay with MTT was as follows: after 24hr incubation, the MTT assay was used to measure cell viability. The MTT salt was dissolved in experimental medium to a concentration of 0.2mg/ml. The contents of the plates were emptied before adding the MTT solution. 500µl of the MTT solution was dispensed into each well. Cells were incubated at 37ºC and 5% CO₂, for 45min.
After incubation, MTT solution was removed; formazan crystals were then dissolved with 125µl DMSO. Two 50µl aliquots of dissolved formazan crystal solution were dispensed into 96 well plates from each well in order to obtain replicate values for each well. The absorbance of formazan was read at 540 nm using the Asys micro plate reader.

Cell viability was calculated as:

Mean absorbance obtained from treated cells/ Mean absorbance obtained from untreated cells *100
2.6.5 Lactose Dehydrogenase Assay

In addition to cell viability assay, cell death was also measured. The Lactate dehydrogenase (LDH) enzyme is released from cells when the cell membranes are disrupted. It is used to measure necrotic cells. The procedure for measuring LDH release was performed according to the method described by Abe and Matsuki (Abe and Matsuki, 2000).

Briefly, 10 ml of LDH solution as prepared by dissolving 25mg L-lactate and β-NAD 25mg, 2.5mg of MTT, 0.34mg MPMS in 9ml Tris-HCl. 1ml of Triton-X100 was added to the mixture to make it up to 10ml. The solution was wrapped with foil paper to avoid light oxidation. 125µl of Triton X-100 was then added to 1 well of untreated cells 1hr before the end of each experiment.

Next, two 50µl aliquots of the supernatant were removed from each well in the 24 well plates and transferred to 96 well plates. 50µl of the LDH solution was then dispensed into the each well containing the supernatant from the 24 well plates. The 96 well plates were then incubated at 37°C in the incubator for 15 minutes. The absorbance of the plates were measured at 540nm using the 96 well plate Asys microplate reader.

Using the cells treated with 125µl of Triton X-100 as positive controls (i.e. 100% necrosis) the % necrosis was calculated as:

Mean absorbance obtained from treated cells/ Mean absorbance obtained from cells treated with Triton X  100
2.6.6 Investigation of antioxidant activity of MPLE against oxidant injury induced by Paraquat

After toxicity studies, MPLE was evaluated for antioxidant activity against paraquat induced oxidative stress in NRK-52E renal cells using the following experimental study designs described below:

**Co-incubation studies:** For co-incubation experiments, 24 well plates were divided into 4 groups comprising of 6 wells per group. Cells in each group were then co-incubated with MPLE (0.075mg/ml, 0.1mg/ml and 0.3mg/ml) and paraquat (1.0, 1.5, 2.0, 2.5 or 3.0mM) for 24 hr. In separate wells culture medium was added to obtain untreated control of Tempol (1mM or 0.17mg/ml) to obtain positive control. Cell viability and LDH release assay described in section 2.6.4 and 2.6.5 respectively were used to measure cytoprotective effect of MPLE against paraquat toxicity after 24 hr.

**Pre-incubation studies:** For pre-incubation experiments, the exposure of the cells to paraquat was varied in order to achieve acute and sub-acute models of oxidative stress. For the acute model, 24 well plates were divided into 4 groups comprising 6 wells per group. Cells in each group were then pre-incubated with MPLE (0.075mg/ml, 0.1mg/ml and 0.3mg/ml) for 24 hr. In separate wells culture medium was added to obtain untreated control. After 24 hr, the wells where then emptied and the medium was replaced with paraquat (0, 1.0, 1.5, 2.0, 2.5 and 3.0mM) for each of the 4 groups. The cells were the incubated for 24 hr. Cell viability and LDH release were assayed as described in section 2.6.4 and 2.6.5 for as indices for protection against paraquat induced oxidant injury.
Investigating superoxide scavenging activity in NRK-52E cell lines:

To further investigate superoxide scavenging activity in cell lines, the NBT method was used as described by Scheid et al (Scheid et al., 1996) with slight modifications. Cells were pre-treated with MPLE for 24 hrs as described in section 3.4.2 and incubated for 1hr with cell culture medium as rinsing step to avoid interaction of MPLE with NBT (Bruggisser et al., 2002). Afterwards, sub-acute oxidant injury was induced by incubating the cells with 10mM paraquat for 3hr at 37°C (Shibata et al., 2010). The dishes were emptied of paraquat contents, and were incubated with NBT 25µg/ml (final concentrations) for 8 hr.

After 8 hr, the cells were emptied of the medium containing NBT. The reduction of NBT was stopped by adding 1ml of 70% (v/v) methanol. Unreduced NBT was removed by washing twice with 1ml of 100% methanol. The wells were then air dried and crystals of NBT were dissolved with 250µl of 2mM Potassium hydroxide and DMSO (1:1.167) solution. The plate was then centrifuged for 4min at 1500rpm in order to completely dissolve the crystals. The absorbance of reduced NBT was measured at 690nm. The readings are shown as arbitrary units after normalizing with absorbance of untreated cells (Scheid et al., 1996).
2.7 Method for evaluating glucose uptake in NRK-52E cell line

In order to determine potential mechanism for anti-diabetic effect of MPLE, the effect of MPLE on glucose uptake in NRK-52E cells was investigated. The methods used for this investigation is detailed in the following section below:

**Materials**

**Chemicals:** Sodium chloride (NaCl), Potassium chloride (KCl), Magnesium sulphate (MgSO4), Calcium chloride (CaCl2), Potassium dihydrogen orthophosphate (KH2PO4), Ethanol 95% and HEPES were purchased from Fisher Scientific. Choline chloride, Trizma, Phlorizin (Phloretin-2’-O-glucoside) hydrate, Phloridzin (Phloretin 2’-β-D-glucoside) dihydrate, Phloretin (β-(4-Hydroxyphenyl)-2,4,6-trihydroxypropiophenone).2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG), Dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich, and Deionized water.

**Lab ware:** 24 well Nunc plates, pipettes, tips, Eppendorfs, volumetric flasks, measuring cylinders were supplied by Fisher Scientific.

**Apparatus:** HT synergy Biotek reader.
2.7.1 Buffers

All the salts used to make the buffers including the buffers where made up in distilled water.

**Incubation Buffer:** The incubation buffer was a sodium (Na+) buffer. The Na+ buffer contained 10ml of 1400mM NaCl, 50mM KCl, 25mM CaCl₂, 10mM MgSO₄, 10mM KH₂PO₄ and 100mM HEPES. This mixture was then adjusted to pH 7.4 using 2.5mM Tris (Blodgett et al., 2011). The solution was then made up to 100ml with distilled water (total volume) in a measuring cylinder.

**Wash Buffer:** The wash buffer was a Choline buffer. The buffer was made in the same way as the incubation buffer. However, the Na+ was replaced with an equal concentration of Choline chloride.

**Lysis Buffer:** The lysis buffer consisted of 10ml each of 400mM KCl and 200mM Tris (adjusted to pH 7.4 with HCl) and 1g of sodium deoxycholate. The mixture was made up to 100ml with distilled in a measuring cylinder.

**Phosphate Buffered Saline (PBS):** PBS was made by dissolving PBS tablets in 100ml of distilled water.

2.7.2 Dissolution of standards

**Phlorizin:** A stock solution of Phlorizin (1250mM) was made by gradually dissolving 100mg of Phlorizin hydrate (Mw=454.42g/mol) in 176.0μl of DMSO. The solution was homogenized by using ultra sound sonicator until it was completely dissolved. The solution was stored at -20°C. Before the start of the experiment, 20μl of this stock
solution was diluted in 25ml of incubation buffer to obtain final concentration of 1mM Phlorizin.

**Phloridzin:** A stock solution of Phloridzin (1250mM) was prepared by dissolving 295.3mg of Phloridzin (Mw= 472.44 g/mol) in 500µl of DMSO. The solution was homogenized by using ultra sound sonicator until it was completely dissolved. Aliquots of this solution were stored at -200C for long term storage and at 40C for short-term storage.

**Phloretin:** A stock solution of Phloretin (Mw= 274.44g/mol) was prepared by dissolving 21.45mg in 125µl of Ethanol to obtain 625mM concentration. This solution was prepared freshly before the start of the experiment to avoid oxidation.

**2-NBDG:** 5mg of NBDG fluorescent glucose was dissolved in 5ml of Na+ buffer or choline buffer2 to obtain a concentration of 2.92mM stock concentration.

### 2.7.3 Experimental Design 1:

Two different experimental procedure for inhibition of glucose uptake in NRK-52E cell lines where adopted for this study. The first design was a slight modification of the method described by Blodgett et al(Blodgett et al., 2011). The modifications are detailed in the sections below:

#### 2.7.4 Cell culture

NRK-52E cell line was seeded as detailed in section 2.6.2. The cells were grown for 3 days after confluence and medium was changed every 48 hr throughout the growth period. The cells were incubated in serum free medium the night before the experiment to ensure serum starvation time for 18 hr.
2.7.5 Experimental protocol for inhibition of glucose uptake in NRK-52E cell lines

Serum starved cells were incubated with Na+ buffer for 1 hr in order to deprive the cells of glucose. Subsequently, the cells were incubated for stipulated times with standard / the plant extract and 2NBDG in separate wells (in 500 µl final volume). After incubation period, the contents of each well was aspirated and discarded. The cells were then washed once with 1000 µl of choline buffer. After washing, the cells were incubated with cold lysis buffer in the dark for 15 min. The plate of lysed cells was then spun in the centrifuge at 24000 g for 10 min. Finally fluorescence of glucose was measured. The fluorescence was read at 480 nM excitation and 520 nM emission wavelength (gain=50) using fluorescence plate reader. Three readings for each well were taken at 2 min intervals for 5 mins. The results were expressed as the average of fluorescent units obtained.

2.7.6 Experimental Design 2:

The second design was a slight modification of the method described by Maeda et al (Maeda et al., 2013). The modifications are detailed in the sections below.

2.7.7 Cell culture

NRK-52E cell line was seeded as detailed in section 2.6.2. The cells were grown for 8 days after confluence and medium was changed every 48 hr throughout the growth period. The cells were incubated in serum free medium the night before the experiment to ensure serum starvation time for a maximum of 18 hr.
2.7.8 Experimental protocol for measurement of glucose uptake in NRK-52E cell lines

In the serum free incubating medium, 0.4µl Phloretin was added to obtain a final concentration of 0.25mM. After 30 min, Phloridzin was added to separate designated wells to obtain final concentration 1.0mM and 0.1mM. Also, MPLE was added to separate designated wells to obtain 1mg/ml final concentration. The cells were then further pre-incubated for 1hr. Following this incubation period, 2-NBDG (dissolved in Na+ buffer or choline buffer) was added to the cells to obtain 400µM final concentration. Then the cells were further incubated for 90 min. After incubation period, the contents of each well were aspirated and discarded. Subsequently, 500µl of PBS was dispensed into each well and glucose in the cell was measured as fluorescence.

The fluorescence was read at 480nm excitation and 520nm wavelength (gain=50) using a fluorescence plate reader. Three readings for each well were taken at 2 min intervals for 5 min. The results are expressed as the average of fluorescent units.
2.8 Method for evaluating the effect of MPLE on glucose uptake in 3T3-L1 adipocytes

Finally the potential of MPLE to stimulate glucose uptake in differentiated 3T3-L1 was performed in order to propose an alternative mechanism for the potential anti-diabetic effect of MPLE.

➢ 2.8.1 Materials

Cell line: 3T3-L1 cell line (CRL 1658) was ordered from American Type Culture Collection (ATCC) (Porton Down, Wiltshire, UK).

Lab ware: T-25 flasks, white coated 96 well plates and plastic tips were supplied by Fischer Scientific, clamps, 250ml round bottom flasks, 10 ml round bottom flasks and rubber bung.

Chemicals: Diethyl ether, chloroform, hydrochloric acid (HCl), Sodium hydroxide (NaOH), Dimethyl sulfoxide (DMSO), Methanol and de-ionized distilled water were ordered from Fisher Scientific. Dulbecco’s Modified Eagle’s medium (DMEM, high glucose), Dulbecco’s phosphate buffered saline (DPBS), fetal calf serum (FBS), penicillin and streptomycin solution, Trypsin/EDTA, Dexamethasone, Troglitazone, Insulin and ascorbic acid were ordered from Sigma. 6-(6-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-Deoxyglucose)(6-NBDG) was purchased from Life Technologies (California, USA).

Apparatus: Hot plate (Medline Scientific LTD, UK), Biotek synergy HT plate reader (Highland Park, USA), Heraus incubator (Thermo Scientific), Lieberg condenser (Fischer Scientific), multi-channel and single-channel pipettes.
2.8.1 Extraction Procedure

In addition to the crude extraction detailed in section, acid hydrolysis of the aqueous crude extract was made. The extract treatment was performed according to a procedure described by Careri et al (Careri et al., 2000) with slight modifications. A larger amount of the crude extract was prepared by using a scaled increase in the ratio of leaves in mass: water ratio from the original amounts mentioned in section 2.3. Extract (50ml) was mixed with methanol, 12.5ml of 12M hydrochloric acid and 169g of ascorbic acid, as antioxidant, to obtain a mixture consisting of 1.5 M HCl in a methanol–water (extract) solution (50:50, v/v) containing 1500 mg/l ascorbic acid. This mixture was mixed and refluxed for 1hr and the heating mantle was set at 85°C.

After reflux, the mixture was allowed to cool and kept away from light. After that, the mixture was separated to two fractions in 1:1 ratio of diethyl ether and the final volume of the mixture after reflux. Methanol was removed from the aqueous fraction by rotary evaporation at low pressures and 30°C maximum temperature. After that, the aqueous fraction was neutralized with 1mM sodium hydroxide to pH 6.4. This neutralized fraction was labelled neutralized diethyl ether fraction (NDE), freeze dried and stored at -20°C. The non-aqueous fraction labelled diethyl ether fraction (DE) was washed with 5ml of water (3 times) and was dispensed into small round bottom flasks.

The weight each of these flasks was taken before dispensing the diethyl ether fraction in them. The diethyl ether was then removed by rotary evaporation set at low pressure and temperature. A similar hydrolysis was done 2hrs and the heating mantle was set at 85°C. The mixture was then separated in an equal volume of chloroform. Methanol was removed from the aqueous fraction by rotary evaporation at low pressures and 30°C maximum temperature. Thereafter, the aqueous fraction was neutralized with 1mM
sodium hydroxide to pH 6.4. This neutralized fraction was freeze dried, labelled neutralized chloroform fraction (NCL) and stored at -20°C. The non-aqueous fraction labelled chloroform fraction (CL) was washed with 5ml of water 3 times and was dispensed into small round bottom flasks. The weight each of these flasks was taken before dispensing the chloroform fraction in them. The chloroform was then removed by rotary evaporation set at low pressure and temperature. The crude extract CE was prepared as already mentioned in section 2.3.

2.8.2 Cell culture

The cells were obtained from ATCC at passage number 13. 3T3-L1 pre-adipocytes were maintained in growth medium in T-25 flasks. The growth medium was high glucose DMEM with L-Glutamine supplemented with 10% FBS and 50 units/mL penicillin and 50μg/mL streptomycin. The flasks were incubated at 37°C and 5% CO₂. When the cells were 80% confluent they were sub cultured into 96 well plates for experiments.
2.8.3 Differentiation procedure

3T3-L1 pre-adipocytes were seeded in 96 well plates at 3*10^4 cells per well. The cells were maintained in growth medium until they were confluent. 48hrs after confluence (day 0), the growth medium was changed to differentiating medium. The differentiation medium contained 40μl of 0.01M Troglitazone, 10μl of 1000X insulin, 10μl of 0.25mM Dexamethasone in 10ml of high glucose DMEM supplemented with 10% FCS. The final concentrations were 40μM Troglitazone, 1μg/ml insulin, and 0.25μM Dexamethasone. This was done to induce differentiation. Subsequently, after every 2 days, the medium was changed to DMEM containing 10% FCS and 1μg/ml insulin. The cells were used fully differentiated between 8-12 days after the induction of differentiation (Vishwanath et al., 2013, Jung et al., 2011).

2.8.4 Procedure for glucose uptake assay in 3T3-L1

Differentiated cells were changed to serum free medium for 3hrs. The crude extracts and the acid hydrolysed fractions were regenerated in DMSO. After 3hrs of serum starvation, 50μg/ml and 100μg/ml of each of the crude extract, and acid hydrolysed fractions were added to the designated wells in duplicates in serum free DMEM. The final concentrations of DMSO for the extracts were < 0.05%. An equivalent amount of DMSO was added to the negative control cells.

The cells were pre-incubated with the extract for 4 hr at 37°C and 5% CO₂. 30 min to the end of this pre-incubation period, 15ng/ml insulin was added to wells designated for insulin. At the end of the pre-incubation period, the media was aspirated from the wells and replaced with serum free media containing 400μM 6-NBDG, dissolved in DPBS to enhance solubility. The cells were further incubated for 60min at 37°C and 5% CO₂.
Finally, the medium was removed and washed twice with DPBS. Glucose uptake was measured as fluorescence at excitation 485/20nm and emission at 528/20nm using a fluorescent micro plate reader (gain=35).

2.9 Statistical Analysis and Data Presentation

All experiments were performed on at least three different occasions (n=3). All data is expressed as mean value and standard deviation. Data analyses was performed on both Microsoft excel (2010) and Graph Pad prism 5. The graphs for the data were plotted on Graph Pad Prism 5 and statistical significance was determined at p value <0.05. Statistical analysis was done using, the Analysis of variance (ANOVA) followed by Bonferoni’s post-test for multiple comparisons or Dunnett’s test for multiple comparisons to one control.
Chapter 3: Antioxidant activities of aqueous *Mucuna pruriens* leaf extract
3.1 Introduction

“An antioxidant is a substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate” (Halliwell, 1990). Endogenous anti-oxidants such as superoxide dismutase (SOD), breakdown free radicals generated during intracellular processes and in doing so, they prevent reactive species induced cellular damage or oxidative stress. Natural occurring compounds from plant species are known to reduce free radicals and therefore they are proposed to also possess antioxidant abilities. There are several tests used to assay antioxidant capacity of natural compounds. These tests can be broadly classified as electron transfer based or hydrogen atom transfer based assay. Although these tests do not necessarily predict in vivo antioxidant activity, they yield valuable information on the antioxidant capacity of extracts and food substances (Apak et al., 2013).

The onset of diabetes and diabetic complications is associated with oxidative stress. Oxidative stress leads to cellular damage that occurs as a consequence of inefficient antioxidant systems and/or due to excessive ROS generation from various cellular processes (Giacco and Brownlee, 2010). In particular, the mitochondria can be a major site for ROS production and oxidative stress because of the abundance of redox reactions that occur during the production of ATP. In pancreatic β-cells for example, dysfunctional mitochondria induce oxidative stress. This has been linked to β-cell failure and improving antioxidant systems has been shown to restore insulin secretory function of β-cells which in turn was beneficial for glycaemic control in diabetic mice (Yagishita et al., 2014, Lu et al., 2010). Furthermore, pathways that increase mitochondrial antioxidant enzymes improved insulin sensitivity in skeletal muscle cells.
In diabetic kidney disease, ROS production contributes to the key changes that are hallmarks for the progression of the disease. These include; inflammation in the glomeruli and proximal tubules, increased formation of extracellular matrix in the glomerulus and interstitial fibrosis of the proximal tubules (Vallon, 2011). Considering that ROS are major contributors to the development and progression of T2D and associated complications, further research for mitochondrial targeted antioxidants may be a useful therapeutic approach for the management of diabetes and diabetic complications.

In attempt to simulate metabolic oxidative stress conditions, paraquat was used for this study. Paraquat is a poisonous herbicide that has been used in several antioxidant studies. The mechanism of paraquat toxicity is not clearly defined, however, paraquat toxicity has been associated with increased ROS production due to inhibition of mitochondrial electron transport at complexes I and III (Cochemé and Murphy, 2008, Castello et al., 2007). This closely mimics mitochondrial damage caused by nutrient overload (James et al., 2012).

In the light of this, paraquat induced oxidant injury is a useful model for studying effects of antioxidants on the mitochondria. The natural compounds contained in most types of herbal green tea are known to possess antioxidant activity and improve mitochondrial function (Ramirez-Sanchez et al., 2014). In relation to diabetes, tea consumption was observed to prevent diabetes induced organ damage and improve antioxidant status in rats (Nunes et al., 2015, Hininger-Favier et al., 2009). Moreover, consumption of medicinal plants in the form of tea may reduce the risk of T2D in humans (Yang et al.,
Experimental studies have shown anti-diabetic and antioxidant effects of *Mucuna pruriens* leaf extracts in animals (Murugan and Reddy, 2009, Agbafor and Nwachukwu, 2011). Traditionally, the leaves are preferably prepared in aqueous solution and consumed in order to treat various diseases including diabetes (Murugan and Reddy, 2009). However, the anti-diabetic effects of the aqueous extracts and the mechanisms that may underlie its potential anti-diabetic effect remain unknown. In view of the role of ROS production in T2D and diabetic complications, the effects of the aqueous leaf extract on oxidative stress could provide scientific basis for consuming the leaves in the form of a tea. Specifically, ROS contributes to the progression of diabetic kidney disease and evaluating MPLE antioxidant activity in oxidative stress models in renal cells would suggest additional reno-protective benefits if it is consumed in the form of a tea by diabetic patients. Therefore, the aim was to evaluate the antioxidant activity of MPLE in cell free models and the protective effect of MPLE against oxidative stress using paraquat as oxidative stress inducer in NRK-52E cell lines.
3.2 Results

Antioxidant activity of MPLE was evaluated in cell free systems using Xanthine/Xanthine Oxidase superoxide generating system and in NADH/PMS superoxide generating systems. The methods for superoxide generation in both Xanthine/Xanthine Oxidase and NADH/PMS systems are detailed in sections 2.5.3 and 2.5.4.

In cell lines the antioxidant activity of MPLE was evaluated in NRK-52E cells and paraquat to induce oxidative stress. The methods for evaluating the antioxidant activity of MPLE in NRK-52E are detailed in section 2.6.

3.2.1 Antioxidant activity of MPLE in the Xanthine/Xanthine Oxidase superoxide generating system

In the xanthine/xanthine oxidase superoxide generating system, MPLE showed significant increase in antioxidant activity as the concentration increased from 0.019 to 0.3mg/ml at p<0.05 (Fig. 3.1). The average antioxidant activity for MPLE concentrations was as follows: 0.19mg/ml was approximately 21.25%, 0.057mg/ml was approx. 86% while 0.3mg/ml was approx. 99.8%. Tempol, which was used as the positive control, also showed a significant concentration-dependent increase in antioxidant activity at p<0.05 (Fig 3.2). When the highest and the lowest concentrations of both MPLE and Tempol were compared, no significant difference was observed between 0.3mg/ml MPLE and 0.081 mg/ml Tempol (Fig. 3.3). However, the lowest concentration of 0.091mg/ml MPLE showed significantly less antioxidant activity compared to the lowest concentration of Tempol (0.0081mg/ml) at p<0.05.
3.2.2 Antioxidant activity of MPLE in the NADH/PMS superoxide generating system

A concentration dependent antioxidant activity was also observed for MPLE in the NADH/PMS system (Fig. 3.4) and this activity was significant at p<0.05. The average antioxidant activity for MPLE concentrations in this system was as follows: no antioxidant activity was observed 0.048mg/ml, 0.144mg/ml was approx. 36% while 0.77mg/ml was approx. 62.4%. Again, Tempol was also used as a positive control and only showed a significant antioxidant activity at 0.2mg/ml at p<0.05 (Fig. 3.5). When the highest and the lowest concentrations of MPLE and Tempol were compared, MPLE showed a slightly better antioxidant activity than Tempol in the NADH/PMS superoxide generating system, i.e. 0.144mg/ml MPLE showed significantly higher antioxidant activity compared to 0.20mg/ml Tempol at p<0.05 (Fig. 3.6).

3.2.3 Antioxidant activity of MPLE in NRK-52E cells against paraquat induced oxidative stress

Prior to antioxidant activity studies, a toxicity study was carried out to determine suitable concentrations for antioxidant studies. NRK-52E cells were incubated with 0.1 and 1mg/ml MPLE for 24hr after which cell viability and LDH release assays were used to measure cytotoxic effect of MPLE. The methods for measuring cell viability and LDH release are detailed in sections 2.6.4 and 2.6.5. The toxicity studies showed that 24hr treatment of NRK-52E cells with 0.1 and 1mg/ml MPLE did not reduce cell viability of NRK-52E cell lines (Fig. 3.7). In addition, no significant LDH release was observed after treating NRK-52E cells with 0.1 and 1mg/ml MPLE at p<0.05 (Fig. 3.8).
3.2.4 Effect of co-incubating NRK-52E cells with MPLE and paraquat

Initially the ability of MPLE to protect against paraquat induced oxidative stress in NRK-52E cells was evaluated by co-incubating MPLE and paraquat at different concentrations. The method used is detailed in section 2.6.6.

From the data obtained, 24hr incubation of NRK-52E cells with paraquat caused a general decline in cell viability. Significant reduction in cell viability was observed within the range of 1-2mM paraquat at p<0.05 (fig. 3.9). No further significant reductions in cell viability was observed beyond 2mM paraquat (probably due to total loss of cell viability). However, the cells co-incubated with Tempol 1mM for 24 hr were protected from reduction in cell viability at 1.5mM paraquat. This effect was significant at p<0.05 (Fig. 3.9). In contrast, MPLE had no significant effect against paraquat induced cell death (Fig. 3.9).

In accordance with cell viability results, paraquat induced significant LDH release with the range of 1-3mM. Co-incubation of cells with 1mM Tempol significantly prevented LDH release in cells incubated with 1.0 and 1.5mM paraquat (p<0.05). However, co-incubation of the cells with the concentrations of MPLE stated did not prevent paraquat induced LDH release (Fig. 3.10).

3.2.5 Effect of pre-incubating NRK-52E cells with MPLE 24hr before inducing oxidant injury with paraquat

Antioxidant activity in biological systems is not limited to free radical scavenging. Phytochemical antioxidants may modulate antioxidant pathways in order to exert antioxidant activity(Erlank et al., 2011). In line with this, NRK-52E cells were pre-
incubated with increasing concentrations of MPLE 24hr before inducing oxidative stress with paraquat. The procedure for this experiment is detailed in section 2.6.6.

From the obtained results (Fig. 3.11), paraquat significantly reduced cell viability in a concentration-dependent manner between 1.5 and 2mM at p<0.05. No further significant reductions in cell viability was observed beyond 2mM paraquat (probably due to total loss of cell viability). Pre-treatment of NRK-52E cells with MPLE for 24hr did not prevent paraquat induced oxidant injury in NRK-52E cells between 2 and 3mM paraquat. Pre-treatment of NRK-52E cells with 0.075mg/ml MPLE for 24hr slightly prevented reduction of cell viability. This effect was significant at p<0.05.

In contrast, no significant effects in LDH release were observed between PQ only and MPLE groups after 24hr pre-incubation with gradient doses of MPLE (Fig.3.12). Therefore with reference to Fig. 3.11, it is possible that the effect of 0.075mg/ml MPLE on cell viability may not be an antioxidant effect. Further tests were therefore required to determine the effects of MPLE within the context of this experimental model.

To investigate further, the mechanism of the observed effect on cell viability, the sub-acute model was designed and the NBT assay was used to determine if the protection of cell viability was due to a slight reduction in ROS production within the cells. The procedure for this experiment is detailed in section 2.6.6.

In contrast to slight antioxidant activity observed in Fig 3.11, the data in Fig 3.13 suggests that 24hr pre-incubation with MPLE elicits a paradoxical pro-oxidant activity within the cells. Pre-incubating NRK-52E rat renal cell lines with doses within the range of concentrations, which showed antioxidant activity (0.01-0.3mg/ml MPLE) in the cell free system, caused NRK-52E cell lines to be more sensitive to oxidative stress induced
by paraquat. The results revealed that 0.075mg/ml increased oxidative stress by approximately 57.67% compared to cells treated with 10mM paraquat only, 0.01mg/ml increased oxidative stress by approximately 76.6% compared to cells treated with 10mM paraquat only, while 0.3mg/ml increased oxidative stress by approximately 91.9% compared to cells treated with 10mM paraquat only.

When the cells were pretreated with 0.1 and 0.3mg/ml MPLE, oxidative stress in these cells in the absence of 10mM paraquat was not significantly different to control cells and cells treated with 10mM paraquat. In cells pretreated with 0.075mg/ml MPLE oxidative stress was lesser than in 10mM paraquat but was not significantly different compared to control cells. This implies that 0.1 and 0.3mg/ml MPLE may cause slight but insignificant oxidative stress while 0.075mg/ml MPLE has the least prooxidant activity.

To establish pro-oxidant activity, cells were pre-treated with MPLE for 24hr before inducing oxidative stress. In summary, the results show that cells pre-treated with MPLE had significantly higher ROS production (p<0.05). Therefore, the concentrations of MPLE tested potentiate ROS production and oxidative stress induced by paraquat. This effect was not concentration dependent.
Fig 3.1: Antioxidant activity of MPLE in xanthine/xanthine oxidase superoxide anion radical generating system. * Shows significant difference to 0.00mg at p<0.05 (one way ANOVA, Dunnett’s multiple comparison test). Data represents mean ± S.D of six replicates (n=6).
Fig 3.2: Antioxidant activity of MPLE in xanthine/xanthine oxidase superoxide anion radical generating system. * Shows significant difference to 0.0mg/ml at p<0.05 (one way ANOVA). Data represents mean ± S.D of six replicates (n=6).
Fig 3.3: Antioxidant activity of MPLE in xanthine/xanthine oxidase superoxide anion radical generating system. * Shows significant difference to both 0.08 and 0.0081 mg/ml Tempol, at p<0.05 (one way ANOVA followed by Bonferroni post hoc test). No significance was observed between 0.081 mg/ml Tempol and 0.3 mg/ml MPLE. Data represents mean± S.D of six replicates (n=6).
Fig 3.4: Antioxidant activity of MPLE in NADH/PMS superoxide anion radical generating system. * Shows significant difference to 0.0mg at p<0.05 (one way ANOVA, followed by Dunnett's multiple comparison test). Data represents mean± S.D of six replicates (n=6).

Fig 3.5: Antioxidant activity of Tempol in NADH/PMS superoxide anion radical generating system. * Shows significant difference to 0.0mg/ml at p<0.05 (one way ANOVA, followed by Dunnett's multiple comparison test). Data represents mean± S.D of six replicates (n=6).
Fig 3.6: Comparison of antioxidant activity of MPLE and Tempol in NADH/PMS superoxide anion radical generating system. * Shows significant difference to 0.0mg at p<0.05. # shows significance to 0.02mg Tempol (one way ANOVA followed by Bonferroni post hoc test). Data represents mean±S.D of six replicates (n=6).
Fig 3.7: Effect of MPLE on cell viability of NRK-52E cell lines. No significant difference was observed between 0.0mg/ml and MPLE concentrations. Significance was measured at p<0.05 (One way ANOVA). Data represents mean±S.D of eight replicates (n=8)
**Fig 3.8: Effect MPLE on cell membrane of NRK-52E cell lines.** *Represents significant difference to positive control (100% LDH release) when compared with 0.0mg/ml and both MPLE concentrations tested. Significance was measured at p<0.05 (one way ANOVA, followed by Dunnett’s multiple comparison test). Data represents mean±S.D of eight replicates (n=8).*
Fig: 3.9: Cell viability of NRK-52E cell lines after co-incubation with gradient doses of paraquat (PQ) and gradient concentrations of MPLE for 24 hrs. * Shows significance at p<0.05 compared to 1.5mM PQ only. # Shows significance at p<0.05 compared to 0.00mM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc test. The data represents mean±S.D of nine replicates (n=9).
Fig: 3.10: Necrosis measured as lactate dehydrogenase (LDH) release from NRK-52E cell lines after 24hr co-treatment with PQ and gradient doses of MPLE. * Shows significant difference to 1.0 and 1.5mM paraquat at p<0.05. # shows significant difference to 0.00mM at p<0.05. Statistical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc test. The data represents Mean±S.D of nine replicates (n=9).
Fig: 3.11: The cell lines were pre-treated for 24 hr with increasing concentrations of MPLE before inducing paraquat oxidant injury. # depicts significance compared to 0.00mM, while, * represents significance compared to 1.5mM paraquat. Statistical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc tests at p value <0.05. The data represents mean ± S.D of eight replicates (n=8).
Fig: 3.12: Necrosis measured as lactate dehydrogenase (LDH) release from NRK-52E cell lines. No significance is observed between MPLE treated groups and paraquat treated groups (one way ANOVA). Significance was derived at $p < 0.05$ when compared to $0.00\text{mM}$. The data represents mean $\pm$ S.D of 8 replicates ($n=8$).
Fig. 3.13: Pro-oxidant effect of MPLE. * Shows significant oxidative stress (measured as superoxide anion radical generation) compared to control cells. # Shows significant oxidative stress compared to cells treated with 10mM PQ only. Significance was determined at p <0.05 (Statistical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc test). The data represents mean±S.D of eight replicates (n=8).
3.3 Discussion

ROS production can activate pathways that regulate inflammation, apoptosis and cell proliferation. These cellular processes are associated with insulin resistance, death of β cells, and the abnormal cell growth which occurs in proximal tubules during the early stages of DKD (Vallon, 2011). In the kidneys, increased ROS generation can also alter blood perfusion of proximal tubules and limit their oxygen supply (Sedeek et al., 2013b). The proximal tubules are susceptible to damage due to low oxygen levels in diabetic conditions because they express more glucose transporters which are dependent on ATP (Vallon, 2011). The ensuing hypoxic conditions causes an increased electron leakage from the ETC. This results in increased ROS production which initiates abnormal growth, inflammation and deposition of extracellular matrix aggravating the development of DKD (Vallon, 2011).

Furthermore, the kidneys play a role in the development of hypertension that is a common complication of T2D. Hyperglycaemia-induced ROS has been reported to upregulate a system of kidney proteins that function to co-ordinate blood pressure by increasing salt and water retention (i.e. the Renin Angiotensin Aldosterone System - RAAS) and exacerbate hypertension (Vallon, 2011). Over expression of the ROS scavenger catalase prevents the development of hypertension and renal injury (Abdo et al., 2014). Furthermore, some studies have shown that diabetes-induced overactivation of the RAAS is blunted by the superoxide dismutase mimetic, Tempol (Brand et al., 2014).

In this study MPLE was evaluated for superoxide dismutase activity in cell free assays and antioxidant activity in cell based experiments using NRK-52E renal cell lines. This would suggest its potential benefits for management of T2D and diabetic complications such as DKD.
Antioxidant activity in the Xanthine/Xanthine Oxidase and NADH/PMS superoxide generating systems: Xanthine Oxidase (XO) is a key enzyme involved with breakdown of nucleotides in humans. ROS derived from XO is used for a wide range of physiological activities including: pro-inflammatory responses, cell proliferation, iron absorption from intestines and antibacterial activity (Battelli et al., 2014). In relation to diabetic complications, Liu et al (Liu et al., 2015) observed an increased activity of XO and increased deposits of uric acid (the by-product of XO reactions) in the kidneys of diabetic rats. In the light of this, XO is a good therapeutic target for diabetes and diabetic complications (although MPLE did not inhibit XO activity). Moreover, ROS produced by XO react with endogenous metals via the Haber Weiss reaction to produce hydroxyl radical. This highly reactive species worsen oxidative stress and cause more damage in tissues. From the data, it can be inferred that MPLE antioxidant activity is less than the antioxidant activity of Tempol.

Generally, in clinically relevant conditions (i.e. between oxygen tensions of 1 and 21% and at physiological pH) XO may generate predominantly hydrogen peroxide and less superoxide ion (Kelley et al., 2010). Considering that the pH conditions for this experiment was 7.4, it can be inferred that the constituents in MPLE have a non-selective reactivity with ROS. Moreover, MPLE has been reported to show antioxidant capacity in DPPH free radical system. Also, in the same study, MPLE was reported to protect against tetrachloride oxidative stress induced liver damage (Agbafor and Nwachukwu, 2011). In comparison to this study, the free radicals generated in the XO system are not as stable as the DPPH radical. Consequently, ROS generated in these systems have shorter lives compared to the DPPH radical. Also these assays employ the superoxide anion radical which is physiologically relevant. Moreover, the half lives of ROS in biological systems are relatively short, the ROS generating systems employed in this study closely mimic
physiological conditions. Furthermore, the chemical antioxidant assays used in this study are categorized as hydrogen atom transfer (HAT) assays because unlike the electron transfer assays, the HAT assays measure the ability of the antioxidant to quickly react with free radicals (Apak et al., 2013). For example, in the XO system used for this study, the test samples and the marker (NBT in this study) compete for the generated radical.

In essence, increased colourization due to oxidized NBT indicates reduced antioxidant activity of the test extract. This principle also applies to the NADH/PMS system. Although in contrast to the XO generating system, the NADH/PMS system generates solely superoxide ion at a slightly alkaline pH. Also, the rate of NBT reduction is quicker than the XO system and as a result the time limit for observing the reaction in this system was very brief (2 min).

A possible explanation could be that an alkaline environment increases the antioxidant activity of MPLE. The crude MPLE is slightly acidic (pH 3.0). The acidic constituents the MPLE extract could become dissociated and readily donate hydrogen protons to the free radicals, generated in this system. Moreover, general phytochemical screening of MPLE was shown to have tannins, saponins, flavonoids, anthraquinones, terpenoids, and cardiac glycosides (Agbafor and Nwachukwu, 2011). The presence of these phytochemicals could be the reason for the antioxidant activity observed in this study. It is important to note that no reaction between the extract and NBT or PMS solution was observed during the time frame of both cell free anti-oxidant assays. Therefore, it is not likely that the use of NBT or PMS in this system interfered with the assay.

In summary, MPLE showed good antioxidant activity in cell free ROS generating system comparable to the recognized antioxidant Tempol at high concentrations.
Antioxidant activity of MPL×E against paraquat induced oxidative stress in NRK-52E cells

Toxicity studies: Toxicity studies revealed that MPL×E is not toxic between 0.1 and 1mg/ml concentrations. It is not clear if the extract may induce apoptosis. Further studies are required to confirm this.

Co-incubation studies: Paraquat increases ROS production in the mitochondria by disrupting electron transfer within during oxidative phosphorylation (Castello et al., 2007). Excessive ROS production in the mitochondria causes reduced ATP synthesis which results in cell death (Hartley et al., 1994). As a result of lack of ATP synthesis and reduction of intracellular ATP, paraquat causes cell death. Moreover, the ROS generated by paraquat reacts indiscriminately with intracellular lipids and proteins. This causes disruption of cell membrane structure and cell necrosis (Castello et al., 2007). Previous studies have shown that Tempol can protect against oxidative stress when co-incubated with paraquat (Samai et al., 2007). To investigate if superoxide scavenging activity may also occur within cells, MPL×E was co-incubated with paraquat for 24hr. However, there was no significant difference between paraquat only group and the concentrations of MPL×E that was tested. This implies that the co-incubation studies did not show any protective effects (fig. 3.10 and 3.11).

Moreover, since the concentrations used in the cell based assays were comparable to the final concentrations used in cell free ROS generating systems, it was speculated that MPL×E may exert antioxidant activity via other mechanisms. Furthermore, certain phytochemicals e.g. polyphenols have the ability to bind serum proteins. Aqueous extract of MP leaves has been reported to contain polyphenols (Agbafor and Nwachukwu, 2011). Bearing in mind that the cell culture medium used for the experiments contained serum, such protein-protein interactions could have occurred with the extract and reduced the bioavailability of the extracts.
within the cells (Hara et al., 2012, Li and Hao, 2015). In essence from the data obtained, MPLE did not have a direct ROS scavenging effect in cell based assays when co-incubated with paraquat. This is in contrast to what was observed in cell free superoxide anion radical generating systems.

*Pre-incubation studies:* In the pre-incubation studies, MPLE has shown a slightly protective effect at a low concentration (0.075mg/ml) by preserving cell viability after subsequent exposure to paraquat toxicity. The other concentrations did not show any effect on cell viability. Furthermore, MPLE did not protect against LDH release.

To further investigate the reason for the effect of MPLE (0.075mg/ml) on cell viability, it was assumed that antioxidant activity was more subtle and would be better observed if oxidative stress was milder and did not lead to cell necrosis. Therefore, a method by Shibata et al (Shibata et al., 2010) was used to induce short term oxidative stress.

However, from the results obtained, cells that were pretreated with 0.075, 0.1 and 0.3mg/ml MPLE were observed to show increased in superoxide anion radical generation compared to cells treated with 10mM paraquat and this effect was not significant. However, cells pretreated with 0.075mg/ml MPLE showed less superoxide anion radical generation compared to cells treated with 10mM paraquat.

Furthermore, a general increase in superoxide anion radical generation was observed when cells were pretreated with increasing concentrations of MPLE and subsequently incubated with paraquat. The data suggests that MPLE enhances pro-oxidant activity of paraquat. This pro-oxidant effect was not concentration dependent. The concentrations used in this pre-incubation study fall within the range of concentrations used for cell free antioxidant assays. Specifically, in the xanthine/xanthine oxidase superoxide anion radical generating system, 0.3mg/ml MPLE showed the highest antioxidant activity that was comparable to 0.081mg/ml Tempol.
Contrary to antioxidant effect, in the cell based assay 0.3mg/ml MPLE showed significantly increased paraquat pro-oxidant activity in NRK-52E cell lines. Similarly, 0.075mg/ml MPLE was the least pro-oxidant concentration in the cell based assay and this concentration also falls withing the range of concentrations with lower anti-oxidant activity in the cell free antioxidant assays.

In general, the data suggest that MPLE can act both as antioxidant and as pro-oxidant. Within this context, one explanation for the pro-oxidant effect of MPLE could be that, pre-treatment with MPLE causes ROS generation that inturn increase activity of uncoupling proteins (UCP). Increase in UCP activity by transporting protons across mitochondria inner membrane and simultaneously preventing ATP production simultaneously causes decrease in mitochondrial membrane potential. This prevents mitochondrial membrane potential collapse (Fig.3.10). Studies with paraquat have shown that mitochondrial uncouplers do not prevent ROS production inspite of their effect on mitochondrial membrane potential (Castello et al., 2007, Cochemé and Murphy, 2008). Therefore, the slight increase in cell viability observed by 0.075mg/ml without a corresponding effect on cell lysis could have the following explanation: the least pro-oxidant dose (0.075mg/ml MPLE), enhanced cell viability significantly by preventing mitochondrial membrane collapse as a result of increased expression or activity of UCP. However, higher doses cause severe pro-oxidant activity and aggravate paraquat damage in the cells.

The implication of this is that MPLE may exert antioxidant effect that will further exacerbate oxidative stress damage in hyperglyceamic conditions. In the kidneys for instance, in order to maintain ATP production, increased UCP expression causes an increase in oxygen consumption. By default, the kidneys do not compensate for increased oxygen demand with increased oxygen supply. Consequently, increased
oxygen consumption usually creates tissue hypoxia which triggers inflammation and fibrosis of the kidneys in diabetic conditions (Hansell et al., 2013).

Fig. 3.14: Proposed mechanism of action of MPLE. ATP synthase in the coupled state uses the energy from moving protons across the mitochondria inner membrane to make ATP. In the uncoupled state, Uncoupling Proteins (UCP) transport protons across the mitochondria inner membrane without synthesis of ATP. This dissipates membrane potential difference which occurs due to accumulation of electrons in the matrix in the case of complex I and III inhibition. UCPs are activated by increased ROS. The lowest dose of MPLE may have enhanced cell viability (fig. 3.7) by inducing slight prooxidant activity (Fig. 3.7) and caused increase in UCP expression or activity. Diagram adapted from: people.edue.duk/~sc9/pics/figure1.jpg.
Furthermore, pro-oxidant activity may involve upregulating pro-oxidant enzymes that regulate autophagy (Qiao et al., 2015). Moreover, polyphenols induce autophagy via ROS dependent inactivation of the mammalian target of Rapamycin (mTOR protein complex responsible for cell growth and nutrient synthesis), and triggers cell autophagy in cancer cells (Hasima and Ozpolat, 2014). A major function of autophagy is the removal of damaged mitochondria, dysfunctional autophagy has also recently been implicated in islet dysfunction (Jung and Lee, 2010) and maybe actively related to development of DKD. Xu et al. (Xu et al., 2015b) showed that increasing autophagy via stimulation of mTOR, prevented high glucose induced lipid accumulation and epithelial mesenchymal transformation in human kidney (HK) cell line. Although, this study has not provided any evidence that MPLE may influence autophagy for reno-protection in diabetic kidney disease (Ding and Choi, 2015), it is clear that MPLE has pro-oxidant activity.

Plant secondary metabolites can act both as anti-oxidant or pro-oxidant depending on the environmental conditions. Cell culture media containing transition metals and sodium pyruvate and sodium bi-carbonate encourage oxidation of polyphenols (Halliwell, 2008, Odiatou et al., 2013). Plant derived polyphenols which are recognized for antidiabetic effects and antioxidant activity, exert mild pro-oxidant activity that upregulate pathways responsible for increased expression of antioxidant proteins (Erlank et al., 2011). The up regulation of antioxidant proteins result in cytoprotection against oxidant injury (de Roos and Duthie, 2015).

Furthermore, antioxidant/pro-oxidant activity of polyphenols could depend on duration of treatment. A recent study with catechin rich oil palm leaf extract on streptozotocin induced diabetic rats, improved antioxidant status of the rats treated with the extracts when high doses where used in short term treatments (Varatharajan et
On the contrary, long term treatment caused worsening of anti-oxidant status (Varatharajan et al., 2013). Within this context and based on the assumption that MPLE may contain polyphenols (similar to the ones found in the MP roots) (Dendup et al., 2014), MPLE may have shown antioxidant activity if pre-treatment time was reduced to about 6-12 hrs, just enough to stimulate an upregulation of anti-oxidant system or if lower doses were employed for the study. This could be investigated in future studies.

**Limitations of the study:** The MTT assay is not exclusively a measure of mitochondrial function since it may not localize in the mitochondria and can be metabolised in the cytosol by other enzymes e.g isocitric dehydrogenase (Stockert et al., 2012). However, it is dependent on metabolic activity of the cells (Liu et al., 1997, van Tonder et al., 2015). Therefore, the data suggests that MPLE may have antioxidant effects against oxidative stress by enhancing cellular metabolic activities. Other methods for measuring apoptosis and mitochondrial membrane potential can be used to evaluate the effect of MPLE on the mitochondria. These methods would give a better picture since they measure directly the activity of the mitochondria.

Considering that the crude extract is digested after consumption, the effects of the extract may be more pronounced with its metabolites. In addition, the extracts contain several components that may have antagonistic effects. These could be the reasons why the observed effects were not completely protective.

Plant extracts, phytoestrogens and antioxidants are known to react with formazan salts. According to Bruggisser et al. (Bruggisser et al., 2002), rinsing cells before incubating with MTT may reduce interference. For the pre-incubation experiments the cells were pre-incubated with extracts, after which they were rinsed with culture...
medium. Then paraquat induced oxidant injury was performed by incubating with paraquat for 24hr in the absence of the extracts. The MTT assay was then carried out after inducing oxidant injury. On this basis, it is not likely that there was interference with the MTT assay by the plant extracts because 24hr incubation of the cells with the paraquat only after pretreatment can serve as a rinsing step. Moreover, the volume of the extracts used were very small volumes (7.14-28.5µl/ml). In summary, the data suggests that although MPLE scavenges ROS in cell free conditions and may slightly improve cell viability during oxidative stress but it does not scavenge ROS generated within cell lines.

ROS is central to the development of diabetes and diabetic complications. Pre-incubation studies with MPLE have shown that it may exert some beneficial effect on mitochondrial dysfunction through enhancing metabolism of cells, although, it is not clear if this is a direct effect on mitochondria. However, further studies are required to determine the specific effects that MPLE may have on the cellular metabolism especially on mitochondria function. On the contrary, the observed pro-oxidant activity of MPLE was similar to the activity of green tea extracts. Green tea (Camellia sinensis, Theaceae) extracts are rich in catechins. Tea catechins increased ROS production, reduced mitochondrial membrane potential and antioxidant levels in isolated hepatocytes after 1hr pre-treatment (Lambert and Elias, 2010, Galati et al., 2006). Interestingly, Camellia sinensis has shown benefits for T2D management (Al-Attar and Zari, 2010). Therefore, based on this similar behaviour, MPLE may be useful for managing T2D. However, further studies are required to investigate its phenolic content in more detail.
3.4 Conclusion

The study in this chapter reveals that MPLE has both antioxidant and pro-oxidant properties. Further studies are therefore required to determine the potential benefits of MPLE for diabetes and diabetic complications.
Chapter 4: Glucose uptake inhibitory effect of Aqueous *Mucuna pruriens* leaf extract
4.1 Introduction

Intensive glucose control in management of T2D is proven to reduce the risk of developing cardiovascular disease. However, the effect of glucose lowering drugs engaged in T2D therapy is largely dependent on endogenous insulin. For instance, the glitazones generally improve insulin sensitivity (Hauner, 2002) and the sulphonylureas stimulate insulin secretion. In the case of T2D progression and in the late stages of the T2D disease β-cell failure occurs and it becomes difficult to achieve tight glycaemic control with the use of most of the conventional anti-diabetic drugs. Moreover, exogenous insulin and insulin secretagogues may induce hypoglycaemia and weight gain, both of which contribute to the risk of developing cardiovascular disease. Therefore, controlling glucose levels via mechanisms that are independent of insulin may be a better way to achieve optimum blood glucose controls in T2D patients especially for patients that have become insulin dependent and non-responsive to insulin dependent therapies (Kelley et al., 2002b). Moreover, this approach will improve the risk of developing cardiovascular and microvascular complications in T2D patients.

The sodium glucose transporters (SGLT) belong to a family of the solute carrier gene series, which consist also of the facilitative glucose transporters (GLUT). The SGLT consist of three isoforms SGLT 1, 2 and 3. While SGLT 3 is known to play a sensory role in carbohydrate digestions by potentiating GLP-1 release in the intestines (Lee et al., 2015), SGLT 1 and 2 are known to be involved in glucose transport in the heart, kidney and intestine. Due to their major role in glucose transport in the kidneys and in the intestine, which is largely insulin independent, SGLT 1 and 2 have become major targets for drugs that control hyperglycaemia without involving insulin signalling pathways.
However, SGLT are known to aggravate the effect of high plasma glucose on several organs of the body. For instance, a significant feature of diabetes is an increase in kidney sizes specifically, an increase in glomerulus and the length of the proximal tubules (Vallon, 2011). This growth could be the reason for the increased expression of SGLT and parallel increase in kidney glucose and sodium retention observed at the onset of diabetes. Similarly, the pro-inflammatory and fibrotic factor, transforming growth factor-β (TGF-β), was observed to cause an increase in SGLT 2 expression in human proximal tubules in cell culture (Panchapakesan et al., 2013). Considering that TGF-β is induced in hyperglycaemic conditions, this could imply that high glucose causes a feedforward cycle by stimulating oxidative stress induced inflammatory and fibrotic pathways which further increase glucose reabsorption. Moreover, increased salt retention at the proximal tubule leads to low ion delivery at the distal tubules in the nephron. When this dilute urine is delivered at the distal tubule, it triggers tubuloglomerular feedback that causes an initial increase in glomerular filtration. Indeed glomerular hyperfiltration and increased kidney size has been shown to be characteristic of diabetic patients and also in diabetic animals (Christiansen et al., 1981, Maric-Bilkan, 2013, Vallon et al., 2013). SGLT inhibition was observed to prevent this increase in glomerular hyperfiltration in obese mice (Vallon et al., 2013).

Also, inflammation in proximal tubules is a major contributor to the progression of DKD. Hyperglycaemia is known to elicit inflammation in proximal tubules. By increasing the ROS production via increased activity of NOX hyperglycaemia induces albuminuria and renal fibrosis (Sedeek et al., 2013a). Treatment of obese rats with the conventional anti-inflammatory paracetamol reduced NOX expression and reduced renal fibrosis and inflammation (Wang et al., 2013). Although SGLT inhibition may not influence NOX activity and fibrosis in the kidneys (Balteau et al., 2011) inhibition of SGLT...
in cardiac myocytes counteracted high glucose induced NOX activation and ROS production (Ishibashi et al., 2016). Furthermore, SGLT inhibition was observed to blunt high glucose induced inflammation and apoptosis in normal primary human proximal tubular cells (Ishibashi et al., 2016). Besides, chronic (4 weeks) and acute administration of SGLT 2 specific inhibitor Empagliflozin to T2D patients changed metabolism of the patients by inducing glycosuria, causing a switch from glucose to lipid oxidation. This in turn improved insulin sensitivity and consequently improved glycaemic control (Ferrannini et al., 2014). In the light of the above evidence, targeting SGLT may have multiple benefits for management of both diabetes and DKD.

In addition, recent studies have demonstrated a beneficial effect of SGLT 2 inhibitors on pancreatic β-cells. Experimental studies in mice revealed that db/db obese mice lacking SGLT 2 had increased plasma insulin levels and β-cell function when infused with glucose (Jurczak et al., 2011). In clinical studies SGLT 2 inhibition was also observed to improve β-cell function (Ferrannini et al., 2014). However, Ferrannini et al. (Ferrannini et al., 2014) observed an increase in endogenous glucose production in T2D patients after acute and (4-weeks) treatment with Dapagliflozin. The mechanism of this effect could be explained by another study which reported Dapagliflozin, an SGLT 2 specific inhibitor, had the ability to stimulate glucagon secretion from α-pancreatic cells (Bonner et al., 2015). The physiological role of glucagon is to initiate gluconeogenesis.

Although, this may counteract hypoglycaemic effect of SGLT inhibition, glucagon has been proposed to have beneficial effects in glucose metabolism. For instance, overexpression of glucagon receptor gene in β-cells enhanced glucose-stimulated insulin release in vitro and also significantly expanded β-cell volume. It also resulted in slightly improved hyperglycaemia and impaired glucose tolerance in mice fed high fat
diet (Gelling et al., 2009). Therefore, SGLT 2 specific inhibitors may also improve glycaemic control by improving pancreatic β-cell function as a glucagon secretagogue, in addition to their effect on urinary glucose excretion.

Furthermore, the effect of SGLT 2 inhibition has been studied in a 2 year clinical study in T2D patients of the older age ranged within 55-80 years. This group of patients are more likely to represent a population with reduced β-cell function. These patients when treated with Canagliflozin (combined with other anti-diabetic drugs) showed dose dependent reduction in glycated haemoglobin (HbA1c) compared to the placebo group (Bode et al., 2015). This study indicates that SGLT inhibition in combination with other hypoglycaemic drugs have a good long term glycaemic control effect in older people. In addition, the authors also reported reduction in body weight and blood pressure. However, in contrast to studies in mice (Vallon et al., 2013), SGLT 2 inhibition did not affect GFR in T2D patients during this study (Bode et al., 2015). However, considering that, strict glycaemic control and low blood pressure significantly decreases the risk of developing and the progression of DKD in both T1D and T2D patients, SGLT 2 inhibitors present good alternative to achieve tight blood glucose control (Chan and Tang, 2015). Moreover, SGLT 2 specific inhibitor was reported to reduce arterial stiffness in T1D patients (without complications) and reduce the rate of death from cardiovascular events in T2D (Zinman et al., 2015). This suggests that targeting SGLT could also prevent the onset of macrovascular complications in diabetic patients (Cherney et al., 2014). Overall, targeting SGLT for treatment of diabetes is beneficial for β-cell function, micro and macrovascular function and glycaemic control. Therefore, it presents a reasonable and effective alternative to insulin dependent therapies.
In the same vein, the natural occurring glucose transporter inhibitors, from which the current synthetic SGLT inhibitors are derived from, have also been reported to show beneficial effects for diabetic complications. Diabetic db/db mice treated with the non-selective SGLT inhibitor Phlorizin (derived from apple tree Malus spp.) prevented damage to mice aorta (Shen et al., 2012). In the same way, other medicinal plants could contain chemicals that have similar protective effect against diabetic induced vascular complications. For example, genistein an isoflavone common to flowering plants of the leguminous family Fabaceae (Kim et al., 2014) has been shown to reduce blood pressure and prevent inflammation and changes to the renal structure of hypertensive rats fed with fructose (Palanisamy and Venkataraman, 2013).

MP belongs to this family of plants and is used traditionally for treatment of diabetes (Grover et al., 2001). The aqueous extract of the seeds has been shown to reduce plasma blood glucose levels, increase urine volume and prevent rise in urinary albumin levels in streptozotocin diabetic rats (Grover et al., 2001). Equally, the ethanolic extract of the leaves have shown hypoglycaemic and lipid normalizing effect probably through preserving β-cells from damage in alloxan induced damage. Within this context, MP plant parts may contain similar class of compounds to Phlorizin with the potential to inhibit SGLTs. Therefore, it may be potentially useful for management of T2D. Since SGLT inhibition is a non-insulin dependent mechanism for plasma glucose control, MP leaves may have insulin independent mechanisms for reducing blood glucose. However, the exact mechanism of action of extracts from the leaves remains unknown. Furthermore, the effect of the leaf extract on the kidneys in vivo has not been studied. Therefore, the aim of this part of the study was to evaluate the effects of aqueous MP leaf extract (the common form of ingestion) on glucose uptake in NRK-52E renal cell
lines in order to propose an insulin independent mechanism of action for its anti-diabetic effect.

4.2 Results

The effect of MPLE on glucose uptake was performed using two different experimental designs. Both designs are described in section 2.7. The first method as described by Blodgett (Blodgett et al., 2011). This method was slightly modified and then used to determine the most suitable concentration of 2-NBDG for measuring glucose uptake in NRK-52E cells. After, 1hr incubation of NRK-52E cell lines with gradient concentrations of 2-NBDG, the cells showed an increase in 2-NBDG uptake with increasing concentrations. This parallel increase was significant when the cells where incubated with 400 and 500µM of 2-NBDG (Fig. 4.1) at p<0.05. Although at 500µM 2-NBDG concentration, the cells showed a large variance in glucose uptake. Therefore, the optimum concentration for the assay was chosen to be 400µM.

In order to optimize the duration of incubation with 2-NBDG the incubation time was increased to 1hr 30min. No further base line experiments were run. The data showed that increasing the duration of incubation with 2-NBDG increased glucose uptake in the cells (Fig. 4.2). In the same experiment, Phlorizin (PLZ) a standard nonspecific sodium dependent glucose transporter (SGLT) was used to validate the experimental design.

The data showed that there was no significant difference between the cells incubated with 2-NBDG only and the cells incubated with 2-NBDG and PLZ 100µM. Furthermore, 1mg/ml MPLE was simultaneously evaluated for potential glucose uptake inhibition in NRK-52E cells. There was also no significant difference between cells
treated with MPLE and 2-NBDG (Fig. 4.2). Therefore, it was deduced that the NRK-52E cells where not sensitive without transfection.

On this basis, the second method was developed to improve the sensitivity of the cells without transfecting the cells with SGLT genes. The details for the second experimental design are detailed in section 2.7.6. After adjusting for the growth duration of the cell and Na⁺ in the assay medium, the data in fig. 4.3, showed that PLDZ (standard non-selective SGLT inhibitor), reduced glucose uptake in a significant dose dependent manner at p<0.05. Specifically, 1mM PLDZ reduced glucose by 36.5% and 0.1mM PLDZ reduced glucose uptake by 26.7% compared to control cells. Also 0.25mM PHT (a standard GLUT inhibitor) showed significant inhibitory effect on glucose uptake at p<0.05. Also, 1mg/ml MPLE caused 35.5% reduction of glucose uptake which was significant at p<0.05.

Further experiments were carried out with and without Na⁺. The details of the experiment are also described in section 2.7.6. Dissolution of NBDG in choline buffer was intended to simulate conditions of SGLT inactivity (Goto et al., 2012). From the data shown in fig. 4.4, MPLE did not show a dose dependent inhibitory effect on glucose uptake in NRK-52E cells, in both Na⁺ and Na⁺ absent conditions. The effect of MPLE on glucose uptake is different to what is observed in Fig. 4.3. In particular, a higher concentration of MPLE (2mg/ml) is required to elicit glucose uptake inhibition of about 15% compared to control cells. Furthermore, this effect is only observed in the presence of Na⁺. However, the effect was not statistically significant. In the absence of Na⁺, MPLE has no effect on glucose absorption in NRK-52E cells.
Similarly, there was no effect of Phloridzin in the absence of Na⁺ (Fig. 4.5). Incubation of cells with 1mM Phloridzin also caused slight inhibition of glucose uptake in the presence of Na⁺ (Fig. 4.5). However, this activity was also not significant. In this case an increase in N numbers from 6 to 9 could have improved the data obtained from this experiment.

Fig 4.1: Glucose uptake in NRK-52E cell lines. NRK-52E cell lines were exposed to increasing concentrations of 2-NBDG for 1 hr. * Shows significant glucose uptake at p<0.05 vs 0.00µM (control). The analysis was done using one way ANOVA followed by Dunnett’s multiple comparison test. Data represents mean± S.D of three replicates (n=3).
Fig. 4.2: Effect of MPLE and Phlorizin on NRK-52E cell line using Blodgett et al. (Blodgett et al., 2011) method with slight modifications. # Shows significant difference compared to blank. Significance measured at p<0.05 (One-way ANOVA) followed by Dunnett's multiple comparison test. Data represents mean±S.D of 11 replicates (n=11).
Fig 4.3: Effect of MPLE on glucose uptake in NRK-52E cell lines using Maeda et al. (Maeda et al., 2013) method with slight modifications. * Shows significant difference compared to 2-NBDG only. # shows significance compared to the blank measured at p<0.05 (one way ANOVA followed by Dunnett’s multiple comparison test). Data represents mean±S.D of 9 replicates (n=9).
Fig. 4.4: Effect of different concentrations of MPLE on glucose uptake in NRK-52E cells incubated with/without Na+. The data represents mean±S.D. of 6 replicates (N=6).
Fig 4.5: Effect of 0.1 and 1 mM concentrations of Phloridzin on glucose uptake in NRK-2E cells with or without Na+. The data represents mean±S.D. of 6 replicates (N=6).
4.3 Discussion

Natural products such as catechin containing green tea have reported to have inhibitory effect on glucose transport in the gastrointestinal system (Kobayashi et al., 2000). A recent work by Schulze (Schulze, 2014) detected two stilbene flavonoids found in grapevine extract that could inhibit SGLT1, albeit they had a lesser potency compared to phlorizin \textit{in vitro} (Schulze, 2014). The aim of this study was to evaluate the ability of MPLE to interact with glucose transporters using NRK-52E cell lines as model system. The results show that MPLE interacts with glucose transporters and may exert anti-diabetic activity via this mechanism.

The methods used in this chapter were adapted from two different already established methods that are used for evaluating SGLT inhibitory activity. Both methods use the 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG), as a marker for glucose uptake. Initially, the use of 2-NBDG as an alternative to radioactive labelled glucose for measuring glucose uptake in cell lines using flow cytometry was established by Zou et al. (Zou et al., 2005). Subsequently, Blodgett et al. (Blodgett et al., 2011) in their method observed that the uptake of 2-NBDG in transfected COS-7 cells, primary mouse and porcine proximal tubule cell line, was similar to the conventional radioactive α-methyl-D-glucopyranoside (AMG). In contrast, Chang et al. (Chang et al., 2013) showed that in COS-7 cell lines expressing human SGLT (hSGLT), 1-NBDG was more sensitive to SGLT inhibition and therefore might be a better analogue compared to the 2-NBDG for screening potential SGLT inhibitor candidates.
Notwithstanding, Kanwal et al. (Kanwal et al., 2012) observed that inhibition of 2-NBDG uptake in kidney cells expressing hSGLT was sensitive to phlorizin. Huan et al. (Huan et al., 2013) also developed Chinese hamster ovary (CHO) cell line that express hSGLT to bypass transfection step. Their data using 2-NBDG as glucose marker indicated that CHO cell line stably expressing hSGLT can serve as cell based screening system for SGLT inhibitory assay. Taken together, these results suggest that 2-NBDG is a sensitive dye for measuring SGLT mediated glucose uptake in cell lines. Besides it is a safer marker for glucose uptake compared to the use of AMG.

Blodgett et al. (Blodgett et al., 2011) in their method used the LLC-PK1 which is a porcine derived cell line. The LLC-PK1 cell line is known to have certain characteristics similar to in vivo proximal tubules. In relation to glucose transport, the LLC-PK1 cell line is known to express mRNA of SGLT1 in increasing glucose concentrations (Ohta et al., 1990). There is also evidence that they carry out apical membrane transport (Rabito, 1981). Furthermore, LLC-PK1 is known to have very high transepithelial membrane resistance (TER). They are also known to express proteins involved in formation of tight junctions (Prozialeck et al., 2006). Therefore, it is a suitable model for cellular transport studies since it possess the required restrictive barrier function and transporter activity for such studies.

Unlike the Blodgett method, glucose uptake was investigated in this study by using the NRK-52E rat cell line. This cell line has been shown to have the ability to carry out glucose transport by both apical and basal membrane, although glucose transport through the basolateral membrane was higher compared to transport on the brush border membrane (Lash et al., 2002). Also, NRK-52E cell line was observed to have high activity on sodium/potassium ATP dependent channels (Lash et al., 2002). This implies that NRK-52E cell line have very active GLUT and actively engage in Na+ transport. It
also suggests that they possess SGLTs although their SGLTs may not be as active as GLUT. This could probably be due to the growth conditions of the cell lines. The stage of growth can affect the up-regulation of SGLT in LLC-PK1 cell line (Tarloff, 2004) and this might be the case for NRK-52E cell line. Coincidentally, the glucose uptake experiments carried out by Lash et al. (Lash et al., 2002) with NRK-52E cell line were done after they reached confluence.

Indeed, there are other similarities between these cell lines. They both do not express claudin-2 which may be necessary for the leaky phenotype of the proximal tubule in vivo (Prozialeck et al., 2006). They are also known to not express some organic anions transporters (Nakanishi et al., 2011). There are differences between the LLC-PK1 and NRK-52E cells. For example, NRK-52E cell line has been observed to express to a lesser extent, similar tight junctions as the LLC-PK1 cell line. Accordingly, they were also observed to have a lesser TER compared to LLC-PK1 cell line (Wilmes and Jennings, 2014, Prozialeck et al., 2006).

Contrary to this study, a recent study observed even lower expression of tight junction proteins and TER after 3 days of confluence (Limonciel et al., 2012). The difference in the both studies may be the seeding density since in the study that observed tight junctions and TER in NRK-52E cell line a higher number of cells was used. Moreover, the authors compared the TER of NRK-52E cell lines to another cell line that had been grown for a longer period of time. In this human proximal tubule cell line they observed a high TER after 2 weeks of confluence. According to the authors, 14 days post confluence was allowed for the cells to attain a state of complete quiescence i.e. matured non-mitotic cells (Limonciel et al., 2012).

Within this context, NRK-52E cell line may not have very restrictive intercellular barriers. However, with the right seeding density and growth conditions, their barriers
can be improved and they can be implored in transport studies. In consonance with this assumption, another study has observed that the expression of genes involved in differentiated proximal tubule function, brush border formation and transport as well as formation of domes, which are indicative of salt and water absorptive capacity are increased during epithelial cell line maturation (Aschauer et al., 2013). Furthermore cell response in glucose uptake experiments can be improved by growing cells in low glucose medium and doing glucose transport assay in buffers with high Na⁺ concentrations (Tarloff, 2004). Therefore, this study explored different growth conditions and assay medium in order to optimize glucose transport in NRK-52E cell line.

The first experimental design explores glucose uptake in NRK-52E cell line by varying growth conditions and increasing 2-NBDG concentrations; the cells were used for the assay after 3 days of reaching confluence. NRK-52E cells showed dose dependent increase in 2-NBDG glucose uptake. Glucose uptake was significant and less variable at 400μM 2-NBDG. Also, modifying the time of exposure increased intracellular uptake of 2-NBDG in the NRK-52E cell line. In order to test for SGLT inhibition, both MPLE and Phlorizin were evaluated for their effect on glucose uptake. Although, Phlorizin showed inhibitory effect on glucose uptake after several repeats, the data suggests that the cells were not very sensitive to SGLT inhibition by 100μM Phlorizin contrary to reports about this concentration in literature (Blodgett et al., 2011, Huan et al., 2013).

This could be explained by the study of Limonciel et al (Limonciel et al., 2012) that reported low TER and low expression of tight junctions in NRK-52E cell lines even after 3 days of confluence. Low TER and low tight junctions could facilitate loss of absorbed NBDG during the washing step that is described during the method used and therefore result in low fluorescence measurements after cell lysis. Actually, skipping the
washing step did improve the read outs during the development of the assay (data not included). Based on this observation it was inferred that the NRK-52E cell lines may not be suitable for application of the method described by Blodgett et al. (Blodgett et al., 2011). Therefore, a second method was developed.

The second method was developed by a modification of Maeda et al. (Maeda et al., 2013). The growth time was adjusted. Considering that an extended time of growth could improve cell tight junctions and improve formation of domes, the cell lines were grown for additional 8 days after confluence. The maximum time for cell growth was two weeks. Furthermore, Maeda et al. (Maeda et al., 2013) carried out their assay in the Hanks buffered salt solution (HBSS) which contains 8.0 g/L of NaCl. Although they used transfected human proximal tubules grown in relatively low glucose medium (7.2mM), the use of high NaCl buffer theoretically would improve the cell response (Tarloff, 2004). Also, glucose uptake experiments can be carried out directly in cell culture medium. For example, glucose uptake experiments by Lash et al. (Lash et al., 2002) were carried out in NRK-52E cell lines by introducing AMG in the culture medium.

Therefore in this study, the culture medium was used in the second method. However, the medium was modified by introducing the 2-NDBG at 400μM final concentration after dissolving it in the Krebs-Ringer modified buffer described in the Blodgett’s method (pH.4). This adjustment was done in order to increase the overall concentrations of NaCl. The total NaCl levels after introducing the 2-NBDG in the culture medium during the assay was approximately 7.5 g/l (which is similar to the HBSS). The percentage of the buffer in the culture medium during the assay was approximately 12% of the total volume in each well.

Furthermore, before the experiments, the cells were serum starved to stimulate differentiation and growth arrest. In addition, with the view that glucose concentration
levels are likely to fall below the initial 5mM concentrations in incubation medium due to metabolic activity of the cells. In order to avoid glucose competitive inhibition of 2-NBDG uptake into the cells, the 2-NBDG was dissolved in the glucose free Na⁺ buffer and the assay was carried out in the same incubation medium contained in the wells after the time for serum starvation had elapsed. Therefore, the modifications above were aimed at: improving the restrictive barriers of the NRK-52E cell lines used in the assay in order to improve unidirectional transport; increasing NaCl concentrations to enhance Na⁺ dependent glucose uptake; increase glucose uptake in the cell by carrying out the assay in pH stable and approximately glucose free medium. The washing step was avoided which is similar to the method by Maeda et al (Maeda et al., 2013). This was done to improve the final fluorescent read outs. Also, in view of the solubility problems encountered with the standards pre-incubation times were included in order to allow for adequate interaction of both the standards and the plant extract with the transporters.

The morphology of the cells was observed throughout the growth period and no change in cobblestone morphology was observed before or during the experiments. DMSO and ethanol were used to dissolve the standards used in this study. The final percentages in the medium for both solvents during the assay was <0.005% and <0.05% respectively (Blodgett et al., 2011). According to literature, these percentages of DMSO and ethanol do not interfere with the assay in other kidney cell lines (Blodgett et al., 2011). The caveat for these adjustments may be an increase in osmotic pressure due to the high percentage of the buffer in the medium.

The data (Fig. 4.3) suggests that these modifications improved glucose uptake in NRK-52E cell line. The glucose uptake in control cells (NBDG only), as indicated by the average fluorescence, increased from approximately 1300 (fig. 4.2) to approximately
4000 (fig 4.3). Furthermore, sensitivity SGLT inhibition as indicated by the effect of phlorizin is greater compared to the first method. In the first method 100μM phlorizin (PLZ) caused approximately 14% inhibition of glucose uptake (fig. 4.2) compared to approximately 34% inhibition by 100μM phloridzin (PLDZ). In addition, SGLT inhibition in the second method was observed to be significantly dose dependent (p<0.05). Inhibition of non Na⁺ dependent glucose uptake as indicated by phloretin (PHT) was observed on two different occasions out of the three separate experiments. The solubility of this compound could have been the reason for this variation as the compound was seen to form precipitates when it was added to the culture medium.

Secondly, the 2-NBDG was dissolved in a Na⁺ buffer and the Na⁺ content could have affected the overall uptake of the dye in spite of GLUT inhibition by PHT. In other words, if 2NBDG was dissolved in a choline buffer (i.e. Na⁺ free buffer) instead of Na⁺ buffer, the variation observed with PHT may be have been reduced. Therefore, the data shows for the first time that this is a suitable method for assay of glucose uptake inhibition in NRK-52E cell line.

Rahmoune et al (Rahmoune et al., 2005) made a critical finding about glucose transporters in the kidneys of diabetic patients. From the urine of healthy and diabetic patients for the first time they isolated exfoliates of the kidney proximal tubules. In the process of passaging the exfoliates as primary cell they observed that GLUT 2 isoform and SGLT 2 isoform protein expression were consistently increased in the proximal epithelial primary cells obtained from diabetic patients with increasing passage numbers. This was observed in cells identified as the S1 segment of the proximal tubules. It is important to note that this segment is involved with 90% of glucose re-absorption in the kidneys. Therefore, it suggests that diabetic patients retain more glucose than healthy individuals. Without considering genetic predisposition, this
implies that diabetic kidneys have the ability to increase glucose re-absorption from tubular lumen and also increase glucose delivery to the cortical areas of the kidney. Increased glucose in the cortical interstitium could lead to glycation and generation of ROS by glucoxidation. Glycation products in turn activate receptors that cause the transcription of inflammatory proteins and activate fibrotic pathways that cause the development and progression of DKD.

The function of SGLT expression is also hyperglycaemia dependent. Yesudas et al (Yesudas et al., 2012) observed that SGLT in high glucose conditions took a longer time to reach maximum glucose re-absorption. Also, protein kinases A and C are also known to modulate SGLTs. According to the results of Yesudas et al (Yesudas et al., 2012), PKA seemed to have more effect on SGLT activity; although PKC also increased SGLT activity significantly. Within this context, it can be proposed that hyperglycaemia increases the activity of SGLT and induces increase in glucose renal retention via increasing de novo synthesis of Diacylglycerol (DAG) which is an activator of PKC.

PKC activation is also associated with tubular hypertrophy, and extracellular matrix deposition which are markers for progression of DKD. Through this mechanism SGLT may contribute significantly to the progression of diabetic kidney complications. Moreover, SGLT activity can be influenced by angiotensin activity. For instance, increased renal SGLT 2 expression, elevated renal gluconeogenesis enzymes and some extent of insulin-resistance observed in diabetic rats were ameliorated by telmisartan (an angiotensin receptor blocker, ATR) after 24 hr starvlation (Tojo et al., 2015). This study also depicted an increase in activity of the renin-angiotensin-aldosterone system in diabetic conditions especially in relation to sodium and glucose retention. Considering that angiotensin II activity increases renal pressure and inflammation and fibrosis, more studies are required to understand the relationships between ATRs and
SGLT in the kidneys. Recently, synergism involving angiotensin antagonists and SGLT 2 inhibitors has been suggested (Gnudi and Karalliedde, 2016). This is in line with the opposing effects of SGLT inhibition on angiotensin II physiological effects in the kidneys. The authors propose that this synergy presents a new approach for the management and prevention of DKD (Gnudi and Karalliedde, 2016).

Furthermore, Na+ influences calcium ion concentration in heart cells and therefore modulates cardiac metabolism and contraction. Increased Na+ in the cytoplasm of heart cells has been shown to induce oxidative stress in cardiac cells. Also, increased Na+ is a component of diabetes induced heart failure. In myocytes from T2D patients with failing hearts, increased Na+ was related to increased expression of SGLT 1. According to the authors, increased expression of SGLT 1 may probably be an alternative pathway for glucose uptake in insulin resistant cardiac cells. This occurrence according to the authors could be specific to the underlying mechanism for heart failure in diabetes and obesity (Lambert et al., 2015).

SGLTs are also found in the intestines. This is the primary gateway for glucose entry into the blood stream (Schulze, 2014). This principal role of SGLTs in glucose absorption in the gut makes SGLTs useful targets for controlling post prandial blood glucose via insulin independent mechanisms. As a result of the broad prospects of inhibiting SGLTs in diabetes and recently in cancer treatment (Scafoglio et al., 2015), there is intensive search for novel compounds with SGLT inhibitory activity from natural products (Schulze, 2014).

In this study MPLE did not show any effect on glucose uptake inhibition in the first method. This could be because of the sensitivity of the cell lines during the application of the method. However, in the second method, MPLE showed significant inhibition of glucose uptake at 1mg/ml when compared to the control cells. This effect
observed was significantly different to 100μM PLDZ at p<0.05 and was comparable to 1.0mM PLDZ. A comparison of the activity of MPLE in both methods suggest that MPLE does interact with glucose transporters however, this interaction maybe weak compared to the natural standard SGLT inhibitor, Phloridzin.

In addition from the obtained data, it can be assumed that MPLE inhibits glucose uptake probably by direct interaction with the glucose transporters. This could be inferred as a potential mechanism through which MPLE may exert its antidiabetic effects. *Mucuna pruriens* leaves extracted in 95% ethanol has been observed to have hypoglycaemic activity in diabetic mice (Murugan and Reddy, 2009), yet the mechanisms remain unknown. The data demonstrated for the first time a potential anti-diabetic mechanism for the aqueous extract of *Mucuna pruriens* which is traditional method of consumption.

The results suggest that MPLE may be absorbed through active transport in the intestine since the transporters in the kidneys are similar to those in the intestine. Further tests using choline buffer instead of Na+ buffer (Fig. 4.4) shows lower glucose uptake by the NRK-52E cells (although this was not statistically significant). A similar observation was reported in experiments by Blodgett et al. (Blodgett et al., 2011), microscopic imaging revealed less glucose uptake in LLC-PK1 cells when experiments were performed in Na+ free buffer. As already mentioned, replacing Na+ with choline during glucose uptake experiments selectively inhibits the activity of SGLTs. In accordance with this, 0.1 and 1.0mM phloridzin which specifically inhibits SGLTs has no significant inhibitory effect on glucose uptake in NRK-52E cells (Fig. 4.5) while in the presence of Na+ buffer, 0.1mM phloridzin shows slight but insignificant inhibitory effect and 1mM phloridzin also shows slight but insignificant inhibitory effect on glucose uptake.
Due to formation of precipitates by phloretin, it was not included in these experiments; otherwise it could have been useful to further confirm selective activity of GLUT. However, in the previous experiments (Fig. 4.3) the effect 0.25mM phloretin (PHT) in experiments with Na\(^+\) buffer was very variable. This could depict a lack of potency in the presence of Na\(^+\).

In Fig. 4.4, the effects of the different doses of MPLE in the absence of Na\(^+\) are generally more variable compared to the data obtained in the presence Na\(^+\) even though the experiments were performed simultaneously. This could be because of the absence of SGLT activity. Furthermore, 2mg/ml MPLE shows slight inhibitory effect on glucose uptake in the cells in the presence Na\(^+\) and no effect in the absence of Na\(^+\). Considering that MPLE activity is similar to phlorizin, the data suggests that MPLE may have selective activity for SGLTs at high concentrations. In order to ensure that the concentrations of MPLE used were not toxic, further toxicity test was performed to ensure that the concentrations of MPLE used for glucose experiments were not toxic. Concentrations of MPLE below 3mg/ml (this concentration caused 50% necrosis after incubating the cells for 24 hr) were used for the study (Appendix 2).

The disparity between the data obtained could be due to the age of the extracts since the experiment in Fig. 4.4 was done 4 months after the data for Fig. 4.3 was obtained. Also the difference could be due to the use of cells with different passage numbers. Nevertheless, the data suggest that MPLE can interact with glucose transporters at high concentrations and limit intracellular glucose concentrations. In essence, MPLE could contain principles that could potentially prevent: glucose induced oxidative stress to cells and enhance glycaemic control by preventing spikes in blood glucose. Both effects in reality are useful for prevention of diabetic complications.
Although, the serum concentration of plant metabolites such as polyphenols is not usually in the range of the concentrations studied; however, in the gut they may exceed these concentrations. Therefore, the ability of MPLE to interact with glucose transporters at this concentration indicate that it could be useful for controlling post-prandial glycaemic index if consumed before proper meal or during meal. However, in relation to the kidneys, glucose inhibition depends on the concentrations that reach the kidneys after digestion. This in turn is dependent on the chemical characteristics of the active responsible for the ability of the extract to interact with glucose transporters. In the view of the potential interaction with glucose transporters, more studies are required to evaluate the safety of the plant extract when it is consumed with anti-diabetic drugs.

4.4 Conclusions

In summary, in this chapter the effect of MPLE on glucose uptake was evaluated using NRK-52E cell line as a model for glucose uptake studies. The study for the first time established a screening assay for screening for SGLT and GLUT inhibitory activity in NRK-52E cell line using 24 well plates under the specified conditions. In addition, the study has shown that MPLE can inhibit glucose uptake in vitro. Therefore, for the first time this study established a potential insulin independent mechanism for the anti-diabetic effect of the extract.
Chapter 5: Insulin Mimetic Effect of Aqueous Extracts of Mucuna pruriens Leaf Extracts
5.1 Introduction

Insulin dependent glucose uptake and storage in the adipocytes, liver and skeletal muscles is the principal element of glucose homeostasis in healthy individuals. Generally, majority of the glucose derived from diet is taken up by the skeletal muscles. Exercise is also known to stimulate glucose uptake in skeletal muscles. Different pathways are responsible for both insulin and exercise stimulated glucose uptake in the skeletal muscles. However, these different pathways converge to stimulate increase in glucose transporter (GLUT 4) activity (Pehmøller et al., 2009). Among the different glucose transporters, GLUT4 (GLUT isoform 4) in particular is exclusively found in mainly insulin-sensitive fat and muscle tissues, and is the main insulin-sensitive GLUT isoform (Cheng et al., 2010). Also, the proteins responsible for translocation of GLUT 4 from the cytoplasm to the cell membrane during glucose uptake in the muscles play similar role in stimulating GLUT 4 activity in adipocytes (Bryant et al., 2002).

Adipocytes also express GLUT isoform 1 (GLUT1). GLUT1 can be stimulated via pathways different from GLUT4. Thiazolidinediones (TZDs) which are peroxisome proliferative activator receptor gamma (PPARγ) agonists were observed to stimulate GLUT1 basal activity and potentiate insulin effect on GLUT1 mediated glucose uptake but they did not have effect on GLUT4 (Nugent et al., 2001). This further suggests the selectively of the GLUT4 transporter to insulin stimulation.

Furthermore, GLUT4 is the most extensively studied transporter in metabolic diseases compared to GLUT1. A clinical study in T2D patients observed that GLUT4 expression in adipocytes of T2D patients is 43% lower than GLUT4 expression in healthy subjects (Hussey et al., 2011). Reduction in glucose transporter expression may underline insulin sensitivity of adipocytes in hyperglycaemic conditions. In fact, insulin
insensitivity in adipocytes is a major contributor to development of T2D in obesity because in this condition, adipocytes become unable to absorb plasma glucose and continually release fatty acid into the blood stream. The result is; important organs responsible for glucose homeostasis especially the β-islet cells are damaged due to exposure to high plasma lipids and glucose levels. Indeed, overexpressing GLUT4 in the adipose tissue of mice with selective negative expression of GLUT4 in skeletal muscles improved insulin sensitivity and diabetes in these mice (Carvalho et al., 2005). In line with this view, improving glucose transporters activity enhances insulin mechanisms for glycaemic control. This approach currently contributes to majority of the current treatment for T2D.

Besides the use of drugs, exercise and modification of diet (calorie restriction) has been shown to improve glucose transporter protein expression and overall insulin sensitivity. Wheatley et al (Wheatley et al., 2011) compared the effect of exercise and calorie restriction on insulin sensitivity in obese mice and they found that calorie restriction had more effect on GLUT 4 transcription and protein expression than exercise.

In the light of this, dietary intervention with plants could be useful for enhancing insulin sensitivity. Coffee consumption has been associated with improved insulin sensitivity in overweight study subjects (Sarriá et al., 2016). The study also observed an inverse relationship between coffee consumption with reduction in fasting blood glucose (Sarriá et al., 2016). Although recent clinical studies on the effect of green tea for insulin resistance report no effect on glycaemic markers or a positive relation with consumption of green tea (Rebello et al., 2011), molecular studies on the effect of major green tea chemical constituents reported that green tea catechins caused translocation
of GLUT 4 from the cytoplasm to the cell membrane of skeletal muscle cell lines (Hamlin, 2015). Moreover, clinical studies proved that consumption of Chinese green tea by diabetic patients may be beneficial for prevention of diabetic retinopathy (Ma et al., 2015). Taken together, regular consumption of edible plants as beverages could have benefits for prevention of diabetes and diabetic complications.

The phytochemical constituents of tea and coffee have been associated with their hypoglycaemic effect. For instance ferulic and chlorogenic acids are found in coffee are also (Farah et al., 2008) and Ferulic acid increased GLUT 4 expression in diabetic rats after short term treatment (Liu et al., 2000). In in vitro experiments chlorogenic acid increased translocation of GLUT 4 in skeletal muscle cell lines (Ong et al., 2012).

Other plant phytochemicals in addition to polyphenols have been associated with improving glucose transport activity. For example, Ginsenoside Re a saponin isolated from Panax ginseng, was observed to improve insulin sensitivity and translocation of GLUT 4 to membrane of adipocytes (Gao et al., 2013). Panax ginseng is a popular Korean herbal plant used for treatment of various diseases and is also consumed as tea.

Similarly, Mucuna pruriens leaves are consumed as a tea after hand washing the leaves and warming the aqueous extract from the leaves for a few minutes. In such occasions it is used to relieve anaemia (E.U. Madukwe, 2014), and for general wellbeing in the eastern parts of Nigeria. In some parts of Asia, the leaves are used for management of diabetes. In rat diabetic models, the organic extracts of Mucuna pruriens leaves were reported to have hypoglycaemic and hypolipidaemic activity (Eze et al., 2012, Murugan and Reddy, 2009). In this study, the anti-diabetic effects were observed with the chloroform fraction of an ethanol extract. Ethanol is miscible with water and
may extract similar compounds as would water, albeit with varying yields depending on
the chemical properties of the actives. Consequently, evaluating the effect of the
aqueous extract on glucose uptake in a cell system that expresses GLUT 4 insulin
responsive transporter could provide preliminary data that could explain the anti-
diabetic mechanisms of *Mucuna pruriens* leaves. The study could also suggest its use in
diet for prevention or management of diabetes and related diseases. Therefore, the aim
of this chapter was to investigate the effect of MPLE on glucose uptake in adipocyte cell
line in order to propose potential insulin mimetic effects as a mechanism of its anti-
diabetic action.
5.2 Results

The effect of MPLE on glucose uptake was studied in differentiated adipocytes. Florescent glucose (6NBDG) was used as a marker for glucose for glucose transport. The details of the methods used for the study is detailed in section 2.8.4

Acid hydrolysed fractions of the crude extract were prepared according to the method described in section 2.8.1. These extracts were also evaluated for their effect on glucose uptake in adipocyte cells using the method described in section 2.8.4

5.2.1 Effect of MPLE and acid hydrolysed fractions on glucose uptake in adipocytes

Pre-treatment of cells with insulin significantly increased glucose uptake by 138% compared to negative control at p<0.05. When adipocytes cells were pre-treated with CE 50 and 100µg/ml for 3hr, CE caused a significant stimulation of glucose uptake in the adipocytes at p<0.05 (Fig. 5.1 and 5.2) when compared to the negative control. The increase in glucose uptake was 57.06 and 86.24 % respectively.

The diethyl ether fractions obtained at 85°C heating temperature for 1hr retained stimulatory effect on glucose uptake. Although, 3hr pre-treatment of adipocytes with DE 50µg/ml did not have any significant effect on glucose uptake (Fig. 5.1), pre-treatment of adipocytes with DE 100, NDE 50 and NDE 100µg/ml significant glucose uptake was observed at p<0.05 compared to the negative control (Fig. 5.1 and 5.2). The increase in glucose uptake was 49.1, 68.22 and 76.30% respectively.

On the contrary, no significant effect was observed for chloroform fractions (CL and NCL) obtained after acid hydrolysis at heating temperature of 100°C for 2hr.
Fig. 5.1: Comparison of the effect of 50µg/ml MPLE and 50µg/ml MPLE acid hydrolysed fractions on glucose uptake in 3T3-L1 adipocytes. * Shows significant difference in glucose uptake stimulatory effect compared to the negative control at p<0.05 (one way ANOVA followed by a Bonferroni’s post test). Data represents mean value ± S.D 8 replicates (n=8).
Fig. 5.2: Comparison of the effect of 100µg/ml MPLE and 100µg/ml MPLE acid hydrolysed fractions on glucose uptake in 3T3-L1 adipocytes. * Shows significant difference in glucose uptake stimulatory effect compared to the negative control at p<0.05 # shows significant difference in glucose uptake stimulatory effect between DE 100µg/ml and CE 100µg/ml at p<0.05 (one way ANOVA followed by a Bonferroni’s post test). Data represents mean value ± S.D 8 replicates (n=8).
5.3 Discussion

In the previous chapter, it was observed that the aqueous leaf extract of *Mucuna pruriens* inhibited glucose uptake in renal NRK-52E cell lines and this effect was proposed as an insulin independent mechanism of its anti-diabetic effect. In this chapter, the aim was to evaluate the effect of the same extract on glucose uptake in 3T3-L1 cell lines. The data suggests for the first time that MPLE can stimulate glucose uptake in 3T3-L1 cell lines and therefore may also mimic insulin as one of its mechanisms for its anti-diabetic effect.

The method used in this study involved the use of the 6-NBDG a non-metabolizable analogue of the 2-NBDG. 6-NBDG was the first fluorescent glucose derivative developed to probe the behaviour of glucose transport systems (Kim et al., 2012). It has been employed for glucose transport in cells that are sensitive to insulin stimulated glucose uptake and express GLUT 3 and 4 isoforms (Dimitriadis et al., 2005). Some theoretical studies suggest that 6-NBDG may not be suitable for studying glucose uptake in GLUT 1 isoform especially in cerebral tissues (DiNuzzo et al., 2013). According to the authors, 6-NBDG transport into the cells may be more of an exchange of glucose for 6-NBDG and its uptake into the cells is a reflection of the glucose levels in the cells that express GLUT 1 isoform and not a response to stimulation of these cells (Mangia et al., 2011). However, 6-NDBG was discovered to have 300 times higher affinity for GLUT 1 isoform than glucose, even though the rate of cell permeation of 6-NBDG is slower compared to glucose in unstimulated state (Barros et al., 2009). In relation to the slow rate of permeability in cells, intracellular uptake of 6-NBDG into cells expressing GLUT 1, has been suggested to be due to diffusion (Mangia et al., 2011). Nonetheless, a comparison between 2-NBDG and 6-NBDG revealed that NBDG produced better
flourescent read outs when stimulated with insulin and insulin mimetic zinc sulphate in differentiated adipocytes (Jung et al., 2011). Therefore within this context, 6-NBDG is a suitable glucose tracer for GLUT 4 insulin dependent glucose transport. Also, considering that glucose uptake by 6-NDBG it is not easily displaced by glucose (due its high affinity for the similar GLUT 1 isoform) it would not be affected by glucose in experimental medium. Moreover, it would give a better signal since it does not get into the glycolytic chain of reaction unlike 2-NBDG analogue. Furthermore, since it is not metabolized within the cells the 6-NBDG will indicate the extent of glucose uptake by the cell. This could depict the extent of influence of the test compound/insulin on the activity of GLUTs in the cell during the duration of the assay.

GLUT 1 isoform may not be sensitive to insulin stimulation in some cell models but GLUT 4 isoform is sensitive to insulin stimuli and GLUT 4 isoform is an insulin-regulated glucose transporter in adipose tissue (Dimitriadis et al., 2005, Huang and Czech, 2007). Thus, using the adipocytes for this study is a suitable model for evaluating potential insulin mimetic activity. Analysis of the data obtained showed that there was no significant difference between the activity of CE50μg/ml and 100μg/ml. Similarly, the activity of NDE did not increase with increasing concentrations. The effect of DE50μg/ml was not significantly different to DE 100μg/ml. In essence, increasing concentrations of both MPL and diethyl ether acid hydrolysis fractions did not cause a corresponding increase in glucose uptake in adipocytes.

Statistical comparison of DE and CE concentrations revealed that the effects of DE50μg/ml was not significantly different to CE50μg/ml while the activity of DE100μg/ml was significantly lower when compared to CE100μg/ml at p<0.05. In contrast, there was no significant difference in activity when CE100μg/ml and NDE50
and NDE100µg/ml were compared. Considering that CE50µg/ml did not have any significant effect on stimulation of glucose uptake, it can be inferred from the data that DE fractions generally had lower potency compared to CE and NDE fractions.

Bearing in mind that the DE fraction is a non-polar fraction of the extract after acid hydrolysis, the DE fraction, represents hydrophobic constituents after acid hydrolysis. The method applied during acid hydrolysis in this study is a method developed for isolating and identifying aglycones of polyphenolic glucosides. Typically, the aglycones in the non-polar fractions obtained after acid hydrolysis are identified using various chromatographic techniques (Paterson, 1999). Aglycones of common polyphenols have been shown to have different effects on glucose uptake. In adipocyte cell lines, they have been shown to inhibit glucose uptake while in liver cell lines, they have been shown to enhance glucose metabolism and expression of GLUT transporters after 12 hour pre-treatment (Claussnitzer et al., 2011, Kerimi et al., 2015). The disparity in the data may be cell specific. On the other hand, it may suggest that absorption of this group of compounds occurs mainly via interaction with GLUT and they can modulate intracellular signalling pathways in pre-treated cells to cause an increase in glucose uptake. For instance, gallic acid was observed to increase translocation of GLUT 4 in differentiated adipocytes after 30 minute pretreatment (Vishnu Prasad et al., 2010). Currently, most of the studies of aglycones from polyphenols are carried out using concentrations that may not be reached in vivo. Therefore, further studies are required to understand the effects of polyphenols at physiological concentrations.

Polyphenols are ubiquitous compounds in most plant families. Dietary polyphenols are currently a topic of intensive research for their benefits for prevention and therapy of several diseases including diabetes and diabetic vascular complications.
For example, a recent clinical trial demonstrated that chronic intake of flavan-3-ols and isoflavones improved biomarkers of risk of cardiovascular diseases (CVD) (Curtis et al., 2012). This study underscores the additional benefit of flavonoids to standard drug therapy in managing CVD risk in type 2 diabetic patients.

*Mucuna pruriens* belongs to the family of Fabaceae or Leguminosae in which isoflavonoids are widely distributed (Wang et al., 2014). Therefore, it is logical to predict that the leaf extract used for this study may contain polyphenols. Although, the identification and quantification of the polyphenols present in the extract was not studied during the time frame of the project, the data obtained with DE in this current study, suggests that DE may contain aglycones of polyphenols that retain insulin mimetic activity after acid hydrolysis. However, the potency of the DE extract is reduced when compared to the effect of the crude extract after acid hydrolysis. This may point to the fact that anti-diabetic activity of polyphenols may be influenced by glycosidic bonds. Further research is required to evaluate this relationship as it may inform future designs for synthetic supplements that can be used in preventive treatment of diabetes.

The significant difference observed between the non polar fraction DE and the polar fraction NDE on glucose uptake, could be explained as follows: If it is taken into account that this NDE fraction contained impurities such as ascorbic acid, and salt from the neutralized acid after acid hydrolysis, it is possible that the activity of this fraction could have been influenced by these impurities. On the other hand, considering that there was no significant difference observed between the activity of NDE fraction and CE, the presence of impurities may not have had any effect. This comparable activity could also mean that other actives besides polyphenols may be responsible for the activity of
extract. Further studies are therefore required to determine the active principles in the extract.

The chloroform fractions did not show any effect on glucose uptake. This could be due to the acid hydrolysis conditions. Complete oxidation of the extract constituents may have occurred at the temperature and duration of the acid hydrolysis.

Each of the extracts that showed significant glucose uptake activity and their respective concentrations were compared to insulin. In terms of percentages, DE 100µg/ml was 62.65% of the insulin activity observed, CE 50µg/ml was 65.97%, NDE 50µg/ml was 70.68%, NDE 100µg/ml was 74.08% and CE 100µg/ml had the highest activity at 78.25%.

Finally, the entrance of glucose into cells is generally via glucose transporters. Therefore, based on the cell model used and on the data obtained, an increase in glucose uptake suggests that MPLE crude extracts and the diethyl ether acid hydrolysis fractions of the crude extract, may trigger insulin signalling pathways that probably increase GLUT4 or GLUT1 dependent glucose uptake. Nevertheless, further studies are required to elucidate the molecular pathways responsible for the observed effects.

5.4 Conclusion

The aqueous leaf extract of *Mucuna pruriens* and the diethyl ether acid hydrolysis fractions may possess insulin mimetic activity by stimulating glucose uptake in differentiated adipocyte cell lines. Therefore, it may also exert some anti-diabetic effect via insulin dependent mechanisms.
Chapter 6: General Discussion and Conclusion
6.1: General Discussion and Conclusion

The concept of consuming functional foods is on the increase because functional foods are a class of foods that have additional medicinal benefits beyond nutritional values (Liu, 2003). The biologically active constituents of functional foods, offer health-enhancing effects beyond nutrition (Ajiboye et al., 2014). Functional foods play a useful role in the prevention of diseases of metabolic imbalances such as obesity, type 2 diabetes, hypertension, inflammatory disorders as well as cancer (Ajiboye et al., 2014). Therefore, they can be consumed with normal diet, serving a dual role of food and medicine.

Considering that most functional foods are rich in plant secondary metabolites, certain herbal plants can be considered as candidates for functional foods. For example, *Ficus racemosa* Linn. (Moraceae) is a popular medicinal plant in India, which has long been used in Ayurveda for diabetes (Ahmed and Urooj, 2010). Extracts from the fruits of this plant have been reported to possess both hypoglycaemic and antioxidant activity (Jahan et al., 2009). Date fruits, *Phoenix dactylifera*, which are consumed commercially, are known to be rich in polyphenols and minerals and fibre (Vayalil, 2012). In some parts of Morocco dates are used for treating diabetes and hypertension (Vayalil, 2012). Diosmetin Glycosides isolated from Date fruits have been observed reduce hyperlipidaemia and to improve levels of antioxidant enzymes in the liver of diabetic rats (Michael et al., 2013). Long pepper, *Pipper longum*, is a plant used as an Indian spice. Piperine isolated from the extracts from the plant have been observed to have both antioxidant and anti-diabetic effect (Kumar et al., 2013).

Similarly, *Mucuna pruriens* L. (Fabaceae) as mentioned earlier, are used in the eastern part of Nigeria West Africa for a variety of diseases such diabetes and anaemia
and they are traditionally consumed as aqueous or alcoholic beverages. The project was designed to evaluate the potential benefits of consuming the tea from the leaves (MPLE) within the context of diabetes. Initially, MPLE showed free radical scavenging activity for superoxide anion which is implicated in development of oxidative stress induced damage in diabetes (Mohora et al., 2007). However as discussed earlier, it caused a paradoxical pro-oxidant activity in vitro. The pro-oxidant effect of MPLE observed can be explained as an indication that the MPLE altered redox state of the cells. Although, in vivo experiments with the aqueous extract of *Mucuna pruriens* leaves was reported to have antioxidant effect by causing an increase in concentration of endogenous free radical scavenging enzymes. Specifically, superoxide dismutase and Catalase where increased and lipid peroxidation was reduced in liver of rats pretreated with the *Mucuna pruriens* leaves aqueous extract after induction of oxidative stress with carbon tetrachloride (Agbafor and Nwachukwu, 2011).

This contrasting results could be explained by the recent theory of Forman et al (Forman et al., 2014). They propose that antioxidants in fruits and vegetables act like toxins at low concentrations, stimulating the activity of endogenous antioxidants via pro-oxidant mechanisms. Therefore, the increase in antioxidant enzymes observed in the liver of rats pretreated with *Mucuna pruriens* leaves aqueous extract could have been due to its pro-oxidant activity which was observed. Bearing in mind that the extract after consumption would be more concentrated in the liver during absorption. In addition, Agbafor at al (Agbafor and Nwachukwu, 2011) reported that the water and methanol extracts of *Mucuna pruriens* leaves aqueous extract contained flavonoids, saponins, tannins, cardiac glycosides, and anthraquinones. Also a recent study reported high total phenolic content in the methanolic extract of the leaves (Cortelazzo et al.,

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2014). The aforementioned compounds are known natural antioxidants. Therefore the results in this study support the Forman theory that nutritional antioxidants are most likely to be pro-oxidants in reality. Taken together these reports and the pro-oxidant activity observed suggest that the MPLE (the extract tested) contains bioactive substances that could have benefits for metabolic diseases such as diabetes.

On the other hand, the pro-oxidant activity observed could be related to the glucose inhibitory effect of the extract. Considering that, glucose uptake inhibition of the extract would lower intracellular glucose levels in the kidney cells during the period of pre-incubation. Low glucose levels in theory would reduce the krebs cycle substrates and therefore electron carriers to the mitochondria. Also there would be a reduction in electron carriers used to regenerate endogenous antioxidants such as Gluthathione.

Consequently, the cells would be in a pro-oxidant state and more predisposed to oxidant injury. This could be reason why the cells pre-treated with the extract had increase oxidative stress when incubated with paraquat. Furthermore, glucose inhibitory is indicative of the mechanism by which the cell internalize the extract. This could directly affect the intracellular concentrations of the extract, especially at the mitochondria (the main point of oxidant injury for paraquat). In the light of this, it can be assumed that unlike tempol which is membrane permeable, the extract is dependent on the activity of the cells and in the event of depleted energy stores as explained above, the cells may not be able to accumulate enough of the extract intracellularly, to stall oxidative stress that is induced by paraquat.

Conversely, in relation to diabetic complications such as diabetic kidney disease; reduction of glucose entry into cells by inhibiting glucose transport can be related to antioxidant activity. In the first instance, oxidative stress is increased in the kidneys of
both diabetic animals and humans. According to a study by Thuraisingham et al. (Thuraisingham et al., 2000) increased levels of peroxinitrate radical was observed in biopsies of proximal tubules and thin loop of Henles from patients with diabetic kidney disease. Recently, treatment of diabetic rats with phlorizin (an SGLT inhibitor) reduced levels of peroxinitrite in the cortex and medulla of the diabetic rats (Osorio et al., 2012). These are the positions were the proximal tubules and the loop of Henles can be found. This implies that the effect of inhibiting sodium dependent glucose transport in the proximal tubules has antioxidant effect. Similarly, the ability of MPLE to interfere with glucose transport in proximal tubules as observed in this study, could be interpreted as an antioxidant property.

Therefore within this context, biological antioxidant can be redefined as compounds that either activate endogenous antioxidant systems or alter intracellular metabolic pathways in order to maintain optimum intracellular redox state. This definition accommodates the pro-oxidant properties of phytochemicals. Nevertheless, the pro-oxidant activity of MPLE does carry potential health benefits as well.

Glucose transport is implicated in tumour cell growth. Increase in expression of GLUT is associated with metastasis in certain cancers (Shen et al., 2010, Kawamura et al., 2001). Inhibiting glucose transport in such cancer cells could induce cytotoxicity and reduce growth of such cancer cells. In order words, MPLE may also posses chemopreventive properties due to its ability to inhibit glucose transporters into cells. However, further studies are required to confirm this.
In addition to glucose inhibition, MPLE also increased glucose uptake in adipocytes. This is not uncommon for certain natural products which interact with glucose transporters. For example, the GLUT inhibitor, Phloretin has been shown to increase differentiation in adipocytes and glucose homeostasis in mice (Shu et al., 2014)

Overall, the data reported in this study suggest that: MPLE may contain active compounds that belong to the class of phytochemicals generally known as natural antioxidants which have nutritional value and for the first time the data suggest that the chemical constituents of MPLE via multiple mechanisms of lowering plasma glucose levels, could be beneficial for management of metabolic related diseases such as T2D. In view of this, the consumption of MPLE could be of great value in prevention of diseases related to metabolic imbalance such diabeties, diabetic vascular complications and by extension cancer.

Finally, *Mucuna pruriens* L. (Fabaceae) is a common weed that grows in different parts of Africa, Asia and America. This makes it a cheap and accessible plant. In the light of the evidence presented in this project, consumption of its leaves as tea, in a similar way as the green tea or rooibus tea is consumed could present another functional food that could be useful for the management of T2D and diabetic complications as well as help promote health and longevity.
6.2 Future Work

The above study is a preliminary study and therefore further studies are required to harness the benefits of MPLE as herbal remedy and functional food. To further validate anti-diabetic effect future studies on the work could include:

- **Bioassay Guided Fractionation of MPLE**

  The ideal step after the preceding study should be bioassay guided fractionation of MPLE. In view of the data in chapter 5, the assay described in chapter 5 can be used as guide to elucidate the chemical constituents of the tea and the active principle(s) responsible for stimulating glucose uptake. Extraction of the tea in other solvents to optimise activity accompanied by use of chromatographic and nuclear magnetic resonance, is one way to identify the actives in the compound (Cazarolli et al., 2012). Alternatively, a complete metabolic profiling of the crude extract using hyphenated techniques such Liquid Chromatography (LC)/Mass spectrophotometry to avoid isolation of known metabolites (Queiroz et al., 2009), could be done and then the assay described in chapter 5 could be used to determine the biological activity of any new compounds observed.

- **Evaluating the Insulin Mimetic Activity of MPLE**

  Insulin mimetic activity can be confirmed by evaluating the molecular pathways for increasing glucose uptake in adipocytes. Protein expression studies such as western blotting and or polymerase chain reaction for gene expression studies are useful techniques for molecular studies. Insulin receptor substrate (IRS-1) is directly involved with insulin signalling pathway (Rondinone et al., 1997) and
GLUT 4 facilitates glucose uptake at the adipocyte membrane (Pessin et al., 1999). Accordingly, molecular studies can focus on the effect of the extract on translocation of GLUT4 to the plasma membrane of adipocytes and activation of insulin receptor substrate IRS-1 (Choi et al., 2004).

- **Evaluating the anti-diabetic effect of MPLE using *in Vivo* studies**

  Determination of the effect of the tea on post-prandial glucose levels in animals would give insight to the effect hypoglycaemic effect of MPLE (Meddah et al., 2009). Furthermore, evaluating the effect of the extract of glucose excretion in diabetic and non-diabetic subjects would further confirm effect of the extract on SGLTs in the kidneys. Animal studies can also include effect of MPLE on lipid profile, weight gain in diabetic animal models (Kumar et al., 2012).

- **Investigating the effect of MPLE on intracellular ROS generation**

  Finally, as extension of the experiments in chapter 3, future studies on the effect of MPLE on mitochondrial ROS can be performed. This would give a general insight on the effect of MPLE on intracellular metabolism. Techniques such as fluorescent microscopy or flow cytometry in combination with fluorescent dyes (e.g. rhodamine dye) can be used to measure the effect of the extract on mitochondrial membrane potential (Petit, 1992). Intracellular superoxide anion can also be measured by using fluorescent ethidium bromide (Carter et al., 1994) in combination with flow cytometry or spectrofluorometric techniques.
Appendix 1: Effect of MPLE on cell viability. 5mg/ml MPLE significantly reduces cell viability below 80%. *Shows significance at p<0.05. Data represents mean±S.D of at least three replicates (n=3).
Appendix 2: Effect of MPLE on cell necrosis measured as Lactate dehydrogenase (LDH) release. 3 and 5mg/ml MPLE cause significant cell lysis above 50%. *Shows significance at p<0.05. Data represents mean±S.D of at least three replicates (n=3).
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