Isolation and characterisation of anti-diabetic pharmacological activities of phytoestrogens and components of *Moringa peregrina* (Forssk) Fiori.

A thesis submitted for the Degree of Doctor of Philosophy

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Author's declaration

I attest that the work presented in this thesis entitled “Isolation and characterisation of anti-diabetic activities, of phytoestrogens and components of *Moringa Peregrina* (Forssk) Fiori” has not been submitted in support of any qualification in this or other educational institutions in the UK or elsewhere.

Davoud Roostaei

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Abstract

Diabetes is an endocrine disease characterised by a chronic increase in blood sugar levels caused by a deficiency of insulin production, which leads to type 1 diabetes, or by a loss of tissue response to insulin, which leads to type 2 diabetes. The disease leads to disruption of metabolism, vascular damage and damage to the nervous system, as well as damage to other organs and systems. Type 2 diabetes is becoming more common throughout the world, due to poor nutrition and lifestyle, and genetic background. Efforts have accordingly been increased towards developing and refining treatments as well as to addressing the underlying causes of the disease. Many parts of the world have a documented history of the use of plants to treat diabetes, and these can be an attractive, local, alternative to expensive pharmaceutical medicines. Accordingly there is an increasing interest in identifying new phytochemicals with proven pharmacological effects on diabetes.

The tree *Moringa peregrina* is commonly found throughout the Middle East and the oil from its seeds has been used for thousands of years. Other members of the *Moringa* family have recorded anti-diabetic family and *Moringa peregrina* was chosen for investigation in this work with the aim of characterising anti-diabetic activity from its leaves. Six extracts were prepared using solvents water, methanol, butanol, ethyl acetate, chloroform and hexane, based on standard extraction techniques. The study was designed to assess the effect of these six extracts on the uptake of glucose in a human hepatoma cell line (HepG2) using a well-studied fluorescent derivative of glucose, 2-2-[*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino glucose (2-NBDG). The cells can take up 2-NBDG instead of glucose via glucose transporters and its uptake is indicative of the capacity of cells to take up glucose,

i.e. of the number of active glucose transporters on the cell surface. The results revealed that the extracts prepared with ethyl acetate and chloroform increased glucose uptake significantly more than the other extracts. Moreover the effects of the extracts were rapid, with a one hour treatment producing a similar stimulation to a 24 hour treatment. These data were confirmed in a second method of investigation of the hypoglycaemic effect of the extracts, by measuring consumption of glucose from cell culture medium. A preliminary assessment of the effect of active extracts on expression of the main glucose transporter of HepG2 cells, GLUT1, by western blotting indicated no large changes in expression.

Dietary phytoestrogens have been shown to play a beneficial role in obesity and diabetes, so the second part of this study investigated the effect of phytoestrogens on glucose uptake. Three phytoestrogens (daidzein, formonontein and genestin, which are naturally occurring isoflavones) were chosen. HepG2 cells showed a significant increase in glucose uptake after treatment with phytoestrogens compared to the control.

In an attempt to identify the active phytochemicals that could account for the observed effects, extensive purification and characterisation of components from the ethyl acetate fraction was undertaken. Seven components were identified: (1) *O*-Ethyl 4-[(α-L-rhamnosyloxy)benzyl] thiocarbamate (*E*), (2) *O*-Butyl 4-[(α-L-rhamnosyloxy)benzyl] thiocarbamate (*E*), (3) 4-(α-L-Rhamnosyloxy)benzyl isothiocyanate, (4) β-Sitosterol, (5) Daucosterol, (6) 3'-methyl-quercetin-3-*O*-rutinoside (also known as isorhamnetin-3-*O*-rutinoside), (7) Rutin.

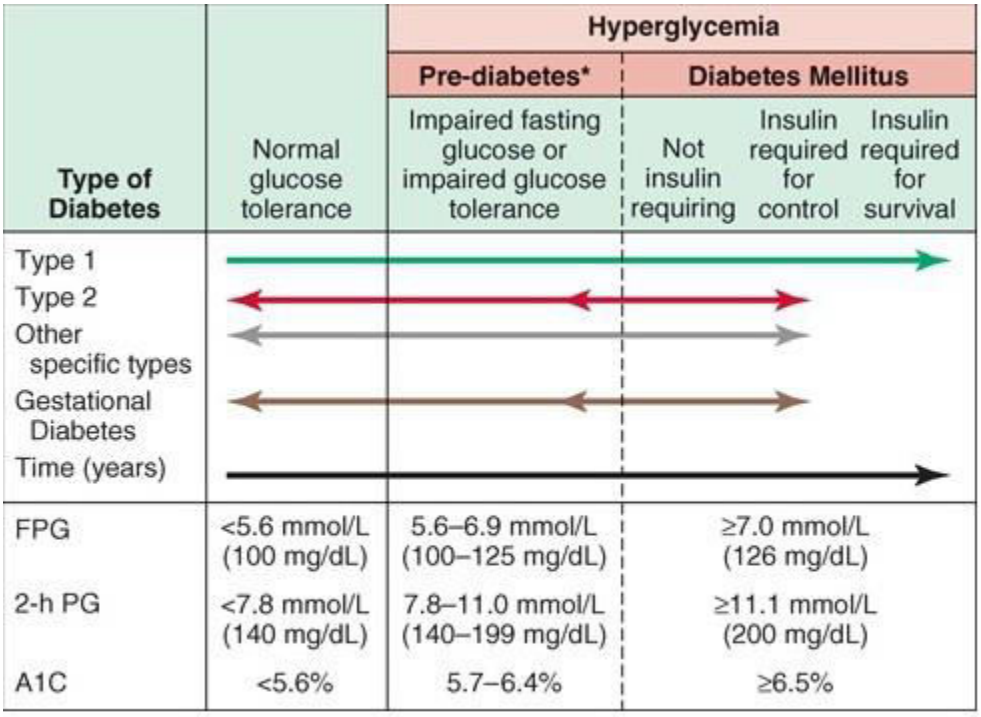
In a final analysis an attempt was made to assess the effect of a representative set of three out of the seven components on basic metabolic activity of HepG2 cells using a Seahorse XF-24 analyser. The three components chosen were *O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*), β-sitosterol from the phytosterols family and Rutin. Real-time monitoring of cell metabolism by a Seahorse XF-24 auto analyser after two hours incubation with the three chosen compounds revealed that maximal respiration, non-mitochondrial respiration and spare respiratory capacity have trended towards an increase with β-sitosterol and3-*O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*) treatments compared to control. As no similar trends were observed in ATP production, the increased maximal respiration could increase metabolic activity at higher concentrations of glucose and account, in part for the effects observed on glucose consumption.

Chapter I

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterised by hyperglycaemia and interruption of the metabolism of protein, carbohydrate and fat. It may be associated with distinctive symptoms, such as polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger).

DM is an important public health concern that affects more than 170 million individuals worldwide. It is expected that the number of people suffering from diabetes in the UK will reach approximately 5 million, or almost 10% of the population, by 2025 and it is one of the leading causes of death worldwide (Islam *et al*. 2009; UK Diabetes Association, 2014). Diabetes is a multi-organ disease independent of age, race and gender. A number of pathogenic actions are involved in the progress of diabetes, including destruction of the beta cells of the pancreas, which leads to insulin deficiency. Some of the processes can cause resistance to insulin, leading to increased plasma glucose levels and abnormalities of carbohydrate, fat and protein metabolism. Diabetes is classified into four groups: type 1, type 2, gestational and maturity onset diabetes of the young (MODY). Glucose profiles of these are shown in Figure 1.1.



Source: Longo DL, Fauci AS, Kasper DL, Hauser SL, Jameson JL, Loscalzo J: *Harrison's Principles of Internal Medicine, 18th Edition*: www.accessmedicine.com

Figure 1.1. Spectrum of glucose homeostasis and diabetes mellitus. The spectrum, from normal glucose tolerance to diabetes in type 1, type 2, other specific types of diabetes and gestational diabetes, is shown from left to right. In most types of diabetes, the individual traverses from normal glucose tolerance to impaired glucose tolerance to overt diabetes (these should be viewed not as abrupt categories but as a spectrum). Arrows indicate that changes in glucose tolerance may be bidirectional in some types of diabetes. *(Adapted from the American Diabetes Association, 2007.)*

1.1 Glucose metabolism

Glucose is a chemical substance that can be obtained from the diet and from endogenous pathways. From a chemistry point of view, it is a simple sugar that belongs to the group of monosaccharide carbohydrates and is one of the main energy sources for both plants and animal cells. Disaccharides such as lactose (a combination of glucose and galactose) and sucrose (a combination of glucose and fructose), and polysaccharides such as starch, a branched polymer of glucose are other types of carbohydrates and are also sources of glucose (Fox *et al*. 2003). The

main metabolic pathways of glucose in energy metabolism are outlined in Fig. 1.2. Glucose crosses cell membranes through glucose transporters, mainly members of the GLUT family of transporters, though which glucose moves by diffusion down a concentration gradient. Once taken up by cells, glucose enters the glycolytic pathway. The initial step is phosphorylation to glucose-6-phosphate by hexokinase or glucokinase, depending on the tissue. The conversion of glucose to glucose-6-phosphate prevents glucose leaving the cell, which it could do through the GLUT transporters if the diffusion gradient was reversed. Galactose also enters the glycolytic pathway after conversion to glucose-6-phosphate. Fructose can be converted to fructose-6-phosphate and then enter the glycolytic pathway.

Glycolysis produces pyruvate, which can enter the mitochondrial pathway of ATP generation after conversion to acetyl-CoA. Alternatively, under anaerobic conditions pyruvate is reduced to lactate which is then excreted by the cell.

Alternatively, and particularly in liver and muscle, glucose can be stored as glycogen, when cellular demands for ATP are low. The liver is also able to synthesise glucose in a reversal of glycolysis in the gluconeogenesis pathway (Fig. 1.2).

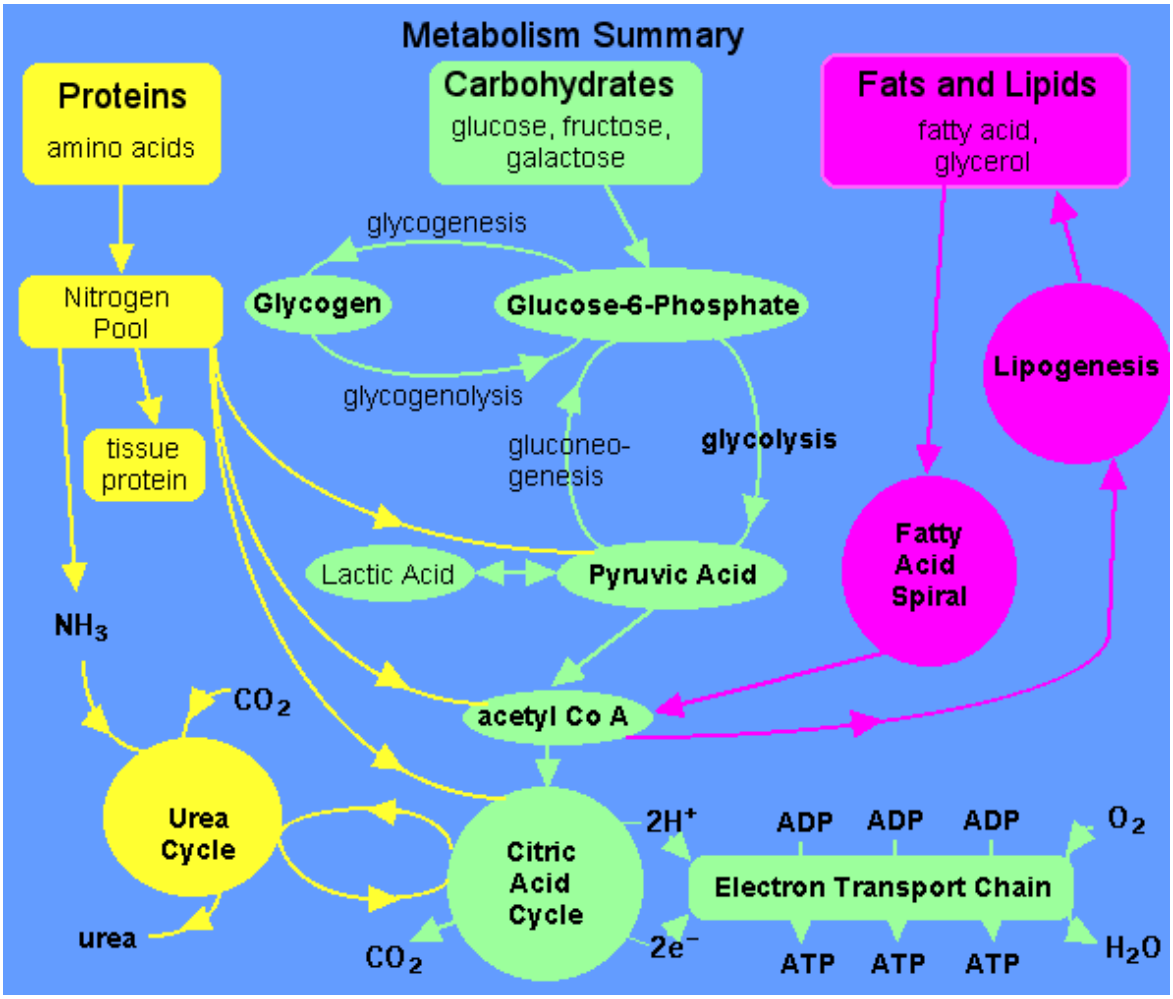


Figure 1.2 A summary of metabolism. Carbohydrate metabolism starts with digestion in the small intestine, where glucose (monosaccharide) is absorbed into the blood stream and the metabolism of glucose starts after uptake into cells.

The process of glucose metabolism is regulated by glucoregulatory hormones, which include insulin, glucagon, amylin, GLP-1, glucose-dependent insulinotropic peptide (GIP), epinephrine, cortisol and growth hormones. Of these, insulin and amylin are derived from the beta cells of the pancreas, glucagon from the alpha-cells of the pancreas, and GLP-1 and GIP from the L-cells of the intestine (Aronoff *et a*l. 2004).

1.2 Glucokinase

Glucokinase (GK), also known as human hexokinase IV, or hexokinase D, is one member of a group of ATP:D-hexose 6-phosphotransferases, and this form is expressed in the liver and pancreas. It has a high K_m for glucose and is active when glucose levels are high. It acts as a glucose sensor enzyme that controls carbohydrate metabolism in the liver, or as described above, insulin secretion from the pancreas. Glucokinase is not inhibited by its product glucose-6 phosphate, while other hexokinases are inhibited, and hence glucokinase is able to take part in a pathway of stimulating insulin release in response to increased glucose levels (Matschinsky 1996; Prokopenko *et al*.2009). It is possible that modulation of glucokinase activity could be one way in which anti-diabetic agents work. Insulin can stimulate liver glucokinase in a glucose-independent manner and perhaps some phytochemicals can mimic this action (Iynedjian *et al.* 1989).

1.3 Insulin

Structure

Insulin is produced in the beta cells of the Islets of Langerhans in the pancreas. It is a peptide hormone and the name comes from the Latin word *insula*, meaning island. Insulin synthesis starts with the production of preproinsulin from which the prohormone precursor, proinsulin is produced. Proinsulin consists of two chains A and B with 51 amino acid and the C-peptide with 31 amino acids. There are two disulphide connections between chains A and B in proinsulin. Insulin maturation happens after involvement of prohormone convertases 2 and 3 and carboxyl-peptidase H which facilitate the conversation of proinsulin to active insulin, as shown in Fig. 1.3 (Rung *et al*. 2009).

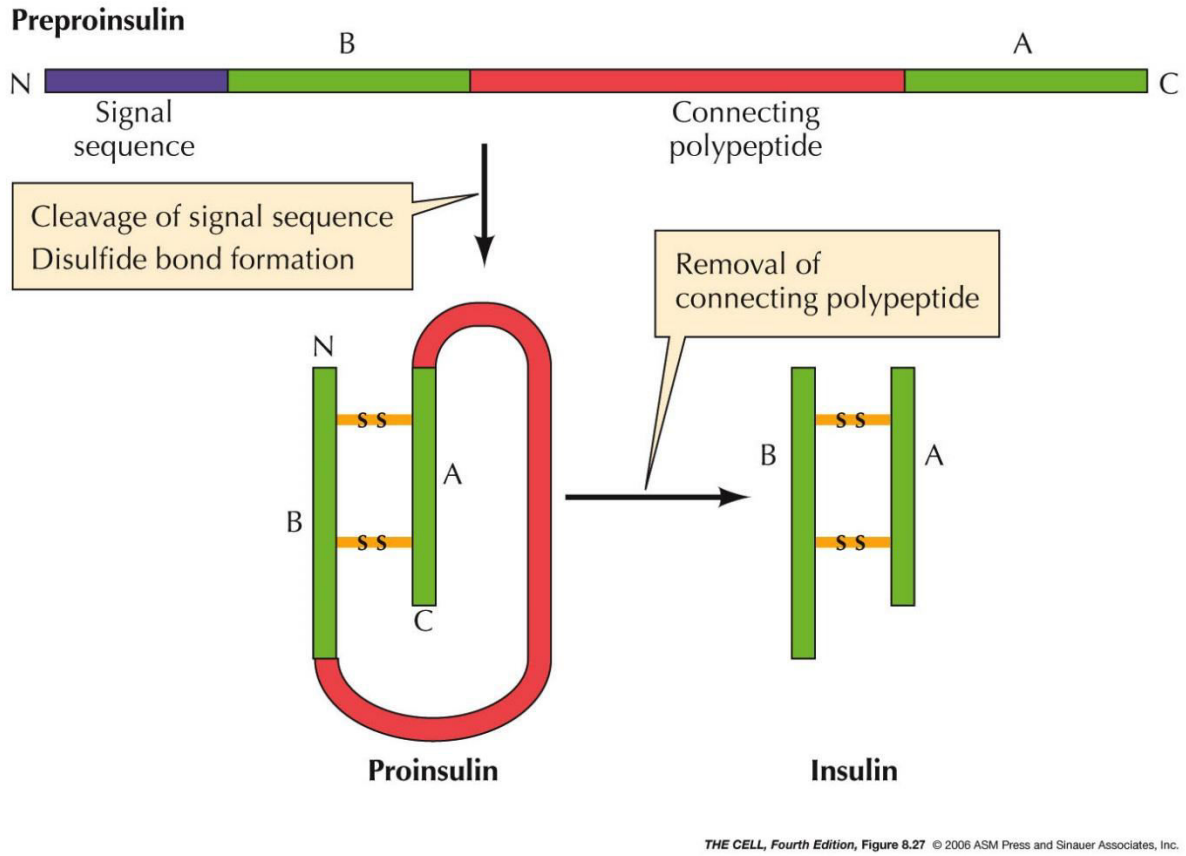


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Figure 1.3 Insulin biosynthesis. Insulin synthesis starts by cleavage of the signal peptide (SP) in preproinsulin that leads to formation of proinsulin. Removal of the connecting peptide assists the insulin maturation process.

Its effect in glucose haemostasis is based on its influence on glucose uptake from the general circulation while it has a preventive effect on the body against developing the metabolic disease diabetes mellitus, due to inhibition of hepatic gluconeogenesis. (Dupuis *et al*. 2010)

Secretion

Beta cells produce insulin in two phases; the first is a rapid, triggered phase and the second phase is slow release. The rapid triggered phase occurs in reaction to different types of factors such as glucose, glucagon-like peptide-1 (GLP-1), glucose-independent insulinotropic peptide (GIP), adrenaline via β2 receptors, and arginine, leucine, acetylcholine and cholecyctokinin (CCK) (Cawston *et al*. 2010: Layden *et al*. 2010).

Glucose, as the main triggering factor in releasing insulin, enters pancreatic beta cells through the glucose transporter GLUT2, and once inside the cells it is converted to glucose-6 phosphate (G6P) via a phosphorylation process by glucokinase (hexokinase IV). The process is rate-limiting and is responsible for glucose-related insulin secretion. The converted form of glucose, glucose-6-phosphate, enters glycolysis, in which it forms pyruvate, which then enters the pathway for ATP generation in mitochondria via the citric acid cycle. The increased influx of glucose into the beta-cells causes an increase in ATP production and hence the ratio of ATP/ADP consequently increases, and as a result closes the potassium channel which leads to depolarization of the cells. Cell depolarization opens voltage-

dependent calcium channels and an influx of calcium into the cell. An increase in calcium ion level induces the stimulation of phospholipase C which leads to insulin secretion. The pathway is illustrated in Figure1.4 (Gudmundsson *et al*. 2007; Brunton and Chabner 2011; Longo *et al.* 2011).

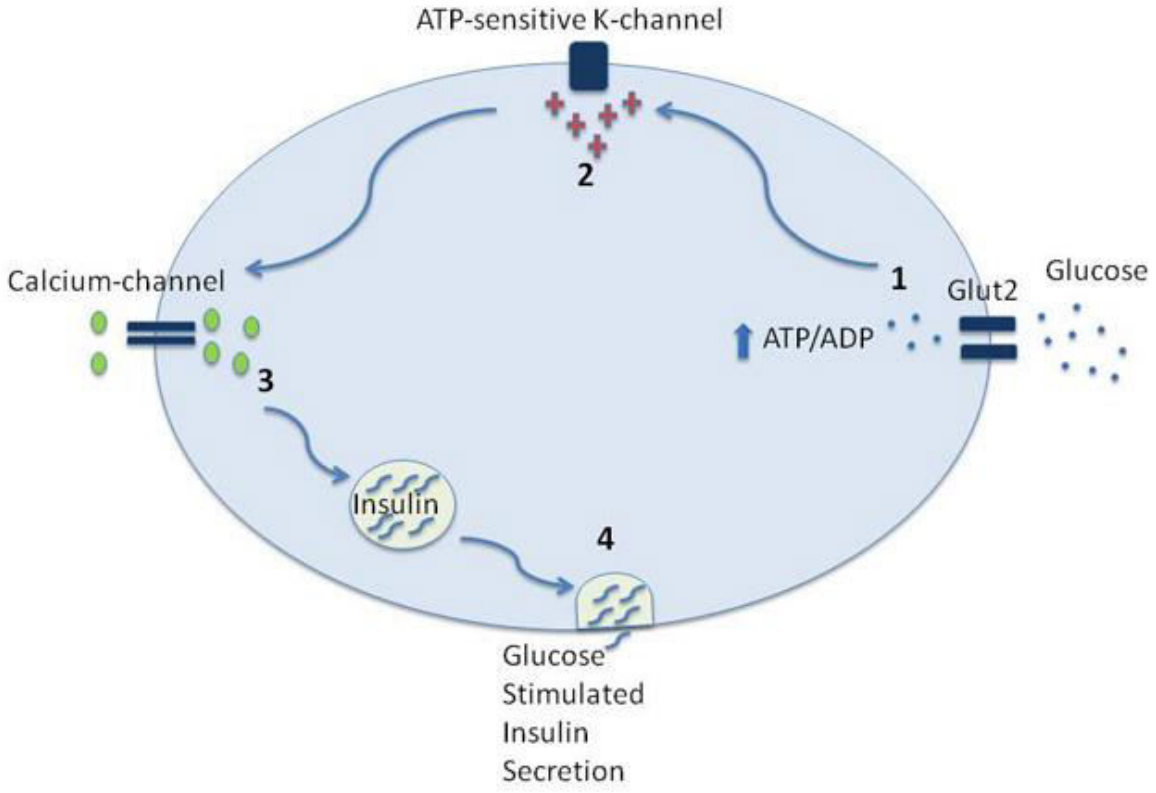


Figure 1.4 How pancreatic cells are induced to secrete insulin. When levels of glucose in the blood increase beyond normal, the flux of glucose into energy production in the pancreatic beta cell increases, resulting in an increase in the ratio of ATP/ADP (**1**). This is sensed by the ATP-sensitive K^+ channel which closes in response (**2**). K^+ can no longer flow into the cell and the cell becomes depolarised, an event which opens voltage-gated Ca^{2+} channels (**3**). The increase in intracellular Ca^{2+} triggers release of insulin from pre-formed granules in the early phase of insulin release (**4**).

1.4 Insulin mechanism of action

Insulin induces its effect by binding to the insulin receptors (IR) expressed on the cell plasma membrane. The receptors belong to the tyrosine kinase receptor group and in terms of functionality are similar to the insulin-like growth factor-1 (IGF-1) receptors (Lyssenko *et al.*2009).This transmembrane receptor is activated by insulin, IGF-I AND IGF-II (Bouatia-Naji *et al*.2009) and is composed of two subunits, alpha and beta, encoded by a single gene, INSR, on chromosome 19 (Miedema *et al*. 2005). Once the tyrosine kinase activity of the receptor is activated a series of biochemical pathways are triggered, mediated primarily by activation of insulin receptor signalling proteins (IRSs) (Fig. 1.5). Regulation of glucose uptake by peripheral tissues involves control of GLUT4 expression (see below), and phosphatidyl-inositol

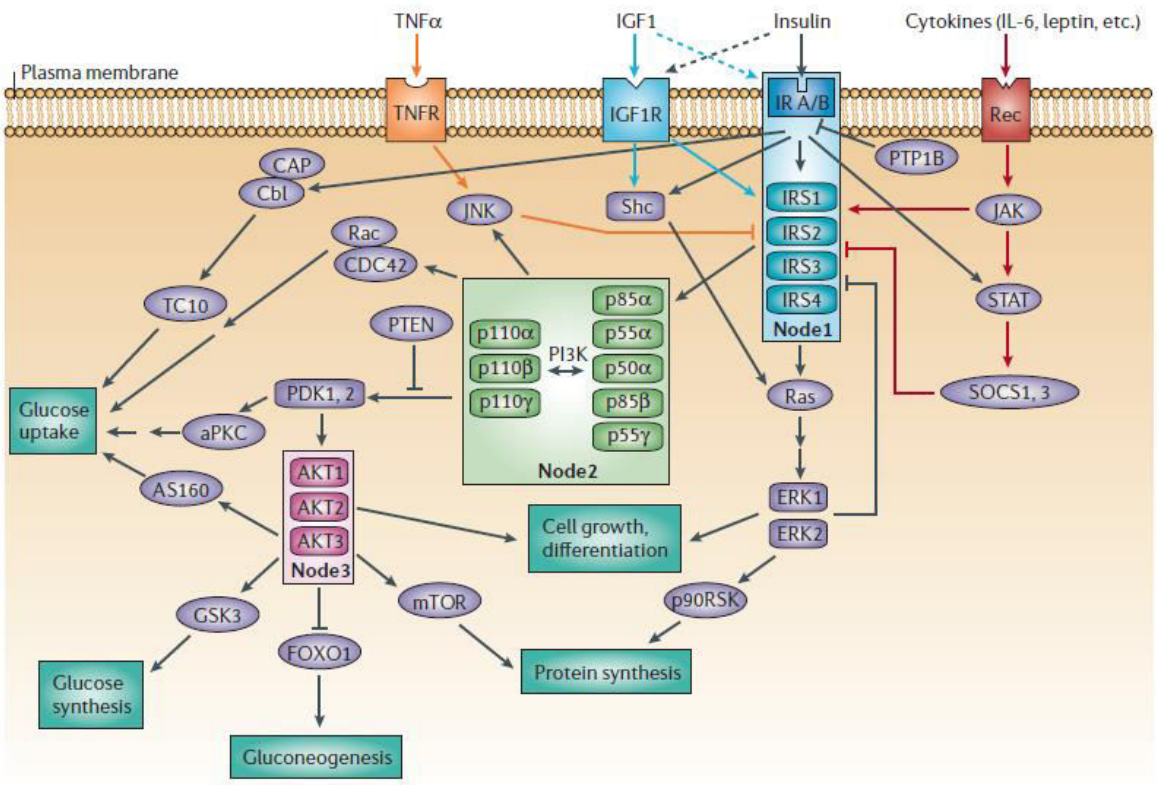


Figure 1.5 Insulin signalling pathways. The complexity of insulin signalling is illustrated by this scheme, taken from Taniguchi *et al.*(2007).

3-phosphate kinase (PI3K) plays a central role in mediating insulin signals that inhibit gluconeogenesis, stimulate glycogen synthesis and storage and stimulate protein synthesis and lipogenesis (Taniguchi *et al.* 2007).

1.5 Glucose Transport

Glucose is transported into cells through two types of carrier. One type is the Na^+-glucose linked transporter (SGLT) (Wright 2001) and the other are the Na^+-independent sugar transporters (GLUT group) (Mueckler *et al.*1994). The two groups differ in their functionality as well as their structures.

SGLT types comprise two sub-groups, based on their affinity for glucose. Most are high affinity, low capacity transporters and in the kidney the low-affinity, high capacity type is expressed as well. In these transporters Na+/K+ ATPase pumps promote glucose transport by maintaining the gradient of Na^+ needed. SGLTs are expressed mostly in small intestine and the proximal tubule of nephrons in the kidney (Wood *et al*. 2003).

The more widely expressed family are the facilitative glucose transporters (GLUT) are transmembrane proteins with 12 membrane –spanning helices that promote diffusion of glucose, and other sugars, down concentration gradients across the plasma membrane. When glucose attaches to the extracellular site of the transporter, a conformational change of the transporter is induced that allows the release of glucose from another, intra-membrane, site of the transporter (Hruz *et al.* 2001). The GLUT group is divided to three classes (Class I, II, and III) and has 13 members (Hruz *et al*. 2001; Joost *et al*. 2001). Class I GLUTs include GLUT 1 to GLUT4, the most important transporters in tissues affected by insulin signalling (Table 1.1).

Name	Distribution	Notes
GLUT1	Is widely distributed in fatal tissues. In the adult, it is expressed at highest levels in erythrocytes and also in the endothelial cells of barrier tissues such as the blood–brain barrier. However, it is responsible for the low level of basal glucose uptake required to sustain respiration in all cells.	Levels in cell membranes are increased by reduced glucose levels and decreased by increased glucose levels.
GLUT2	A bidirectional transporter, allowing glucose to flow in 2 directions. Is expressed by renal tubular cells, liver cells and pancreatic beta cells. It is also present in the basolateral membrane of the small intestine epithelium. Bidirectionality is required in liver cells to uptake glucose for glycolysis, and release of glucose during gluconeogenesis. In pancreatic beta cells, free flowing glucose is required so that the intracellular environment of these cells can accurately gauge the serum glucose levels. All three monosaccharides (glucose, galactose, and fructose) are transported from the intestinal mucosal cell into the portal circulation by GLUT2.	Is a high-frequency and low-affinity isoform. There is some evidence that GLUT 1 and 3 are actually the functional transporters in beta cells.
GLUT3	Expressed mostly in neurons (where it is believed to be the main glucose transporter isoform), and in the placenta	Is a high-affinity isoform, allowing it to transport even in times of low glucose concentrations
GLUT4	Found in adipose tissues and striated muscle (skeletal muscle and cardiac muscle).	Is the insulin-regulated glucose transporter. Responsible for insulin-regulated glucose storage

Table 1.1 Class I glucose transporters. (Bell *et al*. 1990 and Thornes *et al*. 2010)

GLUT3 is a very high affinity glucose carrier expressed in the brain, where glucose plays important role in central nerve system metabolism (Simpson *et al*. 2008, Vannucci *et al*. 1997,).

GLUT4 is the insulin sensitive glucose transporter present in skeletal muscle, heart and adipose tissue where it plays a crucial role in controlling levels of postprandial plasma glucose rises, where GLUT4 responds to insulin (Rayner *et al*. 1994). The uptake of glucose is rapidly enhanced by insulin via stimulation of GLUT4 translocation from intracellular vesicles to expression on the cell surface plasma membrane in skeletal muscle and adipose tissues (Shepherd *et al*. 1999). Translocation of GLUT 4 can also be stimulated through exercise in skeletal muscle tissues, known as insulin independent GLUT4 translocation stimulation (Ploug *et al*. 1998).

GLUT5 is the transporter for fructose and is widely present in intestinal tissue (Burant *et al*. 1992), as well as in kidney, brain, adipose tissue and skeletal muscle. This transporter has been a focus for research due to its selectivity for transporting fructose. The high level of fructose consumption in the diet suggests the involvement of fructose in metabolic disorders such as obesity, diabetes and insulin resistance. After uptake into cells, fructose will go through the pathways of ATP production. However it has a different metabolic path from glucose and does not increase insulin secretion from pancreatic beta cells in the way glucose does (Eliott *et al*.2002).

1.6 History of diabetes research

In the first century BC, the term diabetes and its diagnoses were given to patients with high levels of urine excretion by Greek physicians. Other doctors called diabetes “sweet urine disease”, whereby attraction of ants to a person’s urine was a key factor in diagnosing diabetes patients. In later stages, the sweet taste of diabetic patients’ urine was described by researchers and the word “mellitus” was added to the term “diabetes”.

In 1898, an important discovery after experimental pancreactomy revealed that the pancreas plays role in DM. (Minkowski *et al*.1890)

Following that, in 1922, Sir Frederick Grant Banting, a Canadian physician, announced insulin as a diabetic medication (Liabrary and Archives Canada 2000)

1.7 Type 1 Diabetes

Type 1 DM (T1DM) is an organ-specific autoimmune disease causing the selective destruction of beta cells of pancreatic islets of Langerhans, leading to progressive impairment of insulin production. Failure of insulin production means that control of glucose levels is largely lost. Consequently, after a meal, levels of glucose will become abnormally high. This in itself does not cause malaise but when microvasculature is chronically exposed to high levels of glucose, endothelial cells can become damaged and peripheral circulation impaired. Left untreated, the loss of micro-circulation can lead to tissue death at the peripheries, requiring amputation. Conversely if glucose levels are not regulated then in times of starvation, or more commonly, if food has not been eaten for several hours, then hypoglycaemia can

occur. The brain requires continual minimal levels, about 5 mM, of glucose for energy supply. If levels fall lower than this, even for a short period, brain function is impaired, loss of motor control and a state of coma can result. Prolonged hypoglycaemia induces utilisation of lipid to provide free fatty acids as a metabolic fuel. This can lead to complications, such as diabetic ketoacidosis (DKA) caused by the breakdown of adipose tissue, make T1DM a life-threatening condition if left untreated (Crawford 1991).

This condition is also as known as “insulin-dependent DM” (IDDM) or “juvenile diabetes” and can be activated by both genetic and environmental factors. This type of disease normally develops, and is diagnosed, in childhood and adolescence. It can however be diagnosed in adulthood.

IDDM normally starts at a young age, and presents with social disorders. Once diagnosed, patients need lifelong insulin treatment and can experience multiple disease-associated complications (Couch *et al.* 2008).

The HLA class II allele is the main genetic factor in autoimmune diabetes. The HLA locus encodes antigen presenting proteins that help immune cells attack cells infected by viruses or, less commonly, by bacteria, or to attack tumour cells. Inheriting particular types of HLA genes creates more opportunities for immune cells to attack the body’s healthy cells, which leads to autoimmune diseases such as DM type 1 (Dean *et al*. 2004).

There is currently no cure for T1DM. Promising cures could include transplant of beta cells, stem cell therapy, or gene therapy, but require resource intensive development to ensure long-term and reliable efficacy and safety. Research both in

rodent models of spontaneous diabetes and in humans has improved our understanding of the molecular basis of diabetes, and especially the role of the insulin receptor and associated signalling proteins (Wu *et al.* 2013).

1.8 Type 2 Diabetes

Type 2 diabetes is a group of progressive disorders characterised by high blood glucose levels caused by a lack of insulin activity which can arise from a combination of impaired insulin secretion and impaired response to insulin in key tissues and organs, known as insulin resistance. One consequence is that gluconeogenesis in the liver is no longer inhibited by insulin, and thus increases.

Insulin resistance means that the body is unable to use insulin efficiently, because target tissues become unresponsive to insulin. Type 2 diabetes develops most often in middle-aged and older adults, but increasingly is appearing in children, teenagers and young adults (Rasooly *et al*. 2015). It accounts for about 90% of cases of diabetes.

Patients with this type of diabetes may not require insulin to survive. Type 2 diabetes is often a result of excess body weight and physical inactivity in genetically predisposed individuals and is the most common type (Shojania *et al*. 2006).

Genetics and lifestyle are the two main causes of diabetes, and they can lead to insulin resistance, impaired insulin secretion, obesity and abnormal fat metabolism (Longo *et al.* 2011)

Therefore, in the early stage of type 2 diabetes, in response to impaired insulin action the level of serum insulin increases (Del Prato *et al*., 2002). At this time, the

cells responsible for production of insulin (pancreatic beta cells) lose their functionality due to constant stimulation to recover the insulin output. As a result, impaired insulin secretion occurs.

Numerous studies have proved the association of obesity and weight gain with an increased risk of diabetes (Ford *et al*. 1997; Resnick *et al*. 2000).

Research has shown that T2DM is a partially inherited disease. Family studies showed that individuals with a negative family history are three times less likely to develop the disease compared to those with a positive family history (Flores *et al*. 2003; Hansen 2003; Gloyn 2003).

Identification of disease susceptibility genes has been the focus of intensive research. Early studies (Barroso *et al.*, 2003; Stumvoll, 2004) identified the candidate susceptibility genes *PPARγ*, *ABCC8, KCNJ11* and *CALPN10*.

PPARγ (peroxisome proliferator-activated receptor-γ) is used as a target for some antidiabetic medications, such as thiazolidinediones, because this gene encodes a transcription factor important in adipocyte and lipid metabolism.

UABCC8 (ATP binding cassette, subfamily C, member 8) U and KCNJ11 are targets for sulfonylurea drugs and control the secretion of hormones, such as insulin and glucagon, by their involvement in the ATP-sensitive potassium channel. Therefore, any disturbance in their activities will affect their functionality, which can lead to T2D.

CAPN10 controls an intracellular calcium-dependent cysteine protease (Cox *et al.*, 2004).

Further studies showed an additional 20 confirmed loci associated with type 2 diabetes (Zeggini *et al.* 2008; Dupuis *et al*. 2010; Qi *et al*. 2010) and genome-wide association analyses that included patients from different ethnic groups have confirmed a total of almost 40 loci. These include one locus restricted to type 2 diabetes that was one of the first to be identified that has a role in mediating the action of insulin rather than its secretion, *IRS1* (encoding insulin-receptor substrate 1) (Rung *et al*. 2009). The hepatocyte nuclear factors *HNF1A* and *HNF1BI,* and variants in or near *WFS1* (Sandhu *et al*. 2007; Winckler *et al*. 2007; Gudmundsson *et al*. 2007; Franks *et al*.2008) the melatonin-receptor gene *MTNR1B* (Prokopenko *et al*.2009; Lyssenko *et al.*2009; Franks *et al.* 2008) are also implicated. The current state of GWAS findings has been recently reviewed and about 80 genetic loci are associated with development of T2D in European populations (Dorajoo *et al.* 2015). These findings have helped to understand the molecular basis of insulin secretion and action better, but also indicate genes that can affect treatment effectiveness and allow identification of potentially good or poor responders to a given treatment.

1.9 Other types of diabetes

In addition to types 1 and 2 diabetes, the two most common forms, other types of diabetes have been recognised, that have different aetiologies. These include congenital diabetes, caused by insulin secretion deficiency; cystic fibrosis-related diabetes arising as a complication of impaired *CFTR* function; steroid diabetes, which is influenced by high doses of glucocorticoids and gestational diabetes, which occurs during pregnancy (Lynn *et al.* 2012).

1.10 Diabetes complications

Complications related to any type of acute and chronic diabetes are classified into two types. Acute effects of short-term disruption in the balance of blood sugar are due to

Figure 1.6 Chronic complications arising from diabetes. DM is the main cause of a number of serious health conditions, such as cardiovascular disease**,** blindness**,** kidney failure and neuropathy that leads to lower limb amputation. (Reproduced from international diabetes federation).(http://www.idf.org/. international diabetes federation).

either too low or too high levels of glucose. When levels are low, a state of hypoglycaemia, diabetic ketoacidosis and nonketotic hypoglycaemic coma can occur. When levels of glucose are too high, diabetic hyperglycaemic hyperosmolar syndrome and hyperosmolar hyperglycaemic nonketotic syndrome (HHNS) can occur. The acute conditions include ketoacidosis, and hypoglycaemic reactions. The chronic complications include retinopathy, nephropathy, neuropathy (Henry *et al*.1997; Mard-Soltani *et al*. 2011).

Chronic complications of DM (Fig. 1.6) take 10 to 15 years to occur. One major type is caused by damage to microvasculature, which can lead to the common

complication of diabetic retinopathy, the major cause of blindness in diabetics (Font *et al.*, 2004). Another chronic complication is diabetic peripheral neuropathy (DPN) caused by damage to sensorimotor and autonomic nerves, and leads to erectile dysfunction (impotence) and foot ulcers (Andrew *et al*. 2005; Chu *et al.* 2001). Another microvascular complication is diabetic nephropathy, which is the most common reason for renal failure in countries such as the USA (Fowler, 2011).

Microvascular damage can also lead to macrovascular complications such as ischaemic heart disease, stroke and peripheral vascular disease. Macrovascular disease can also develop in the peripheral vasculature and can affect cerebrovascular flow (Chew 2006).

In the case of retinopathy, aldose reductase plays a key role in the pathogenesis. This enzyme is involved in the polyol pathway where glucose is converted to sorbitol. As glucose levels increase, the conversion of glucose to glucose alcohol (sorbitol) will increase as well. Sorbitol accumulation and consequently increased osmotic pressure are assumed to be the underlying mechanism in the pathogenesis of diabetic retinopathy (Gabbay 1975; Gabbay 2004; Fong *et al.* 2004).

Generally the continual, high levels of glucose experienced in untreated diabetes can cause generation of advanced glycation end-products in basement membranes of microvasculature. Glucose is a reducing sugar and can react, at a very slow rate, with proteins and other macromolecules. For example the blood marker for diabetes is Hb1Ac, a glycation product of haemoglobin. Glycation will occur more quickly when there is an oxidative environment and if protein turnover is slow (Goldin *et al.* 2006). The glycation causes cross-linking of proteins and eventually causes cell

dysfunction and local inflammation, deposition of scavenger cells, eventually leading to loss of flow and local tissue damage.

1.11 Diagnostic criteria

On basis of the information from Diabetes UK, who adopt that the WHO guidelines for diagnostic criteria, the following diagnostic categories for diabetes are recommended:

1 Diabetic symptoms such as polyuria, polydipsia and unexplained weight loss for Type 1 plus:
   - Random blood glucose level greater than 200 mg/dl (11.1 mM) in the presence of diabetes symptom including polydipsia, polyuria and polyphagia.
   - Fasting plasma glucose (FPG) level greater than 125 mg/dl (7.0 mM) from at least two independent measurements.
   - Two-hour plasma glucose greater than 200 mg/dl (11.1 mM) after 75 g of oral glucose load (Oral Glucose Tolerance Test).
   - Haemoglobin A1C (Glycated Haemoglobin) greater than 6.5%.
   - According to studies, a combination test of FPG and A1C is recommend for diagnosis of diabetes type (Sato *et al.*2009).

2 If diabetic symptoms are not present, then at least two tests need to be done to confirm the diagnosis (WHO 1999). There are, however, different diagnostic criteria for gestational diabetes, including:

- A fasting plasma glucose level of 5.6 mM or above or
- A 2-hour plasma glucose level of 7.8 mM or above (WHO 2006).

Glycated haemoglobin or glycosylated haemoglobin (haemoglobin A1c, HbA1c, A1c, or Hb1c) is a form of haemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods of time. It is formed in a non-enzymatic glycation pathway by haemoglobin's exposure to plasma glucose. Normal levels of glucose produce a normal amount of glycated haemoglobin. As the average amount of plasma glucose increases, the fraction of glycated haemoglobin increases in a predictable way. There is not particular level for HbA1c to identify whether patients are or not at risk of type 2 diabetes as this is continuous risk factor. According to WHO recommendation the cut-off point of $HbA_{1c}$for diagnosing type 2 diabetes in non-pregnant adults is a level of 48 mmol/mol (6.5%) and the range 42–47 mmol/mol (6.0–6.4%) is considered to be 'high risk' (www.nice.org.uk) .This serves as a marker for average blood glucose levels over the previous months prior to the measurement (Miedema 2005; Peterson 1998).

1.12 Treatment of Diabetes

DM can be managed by diet, exercise and drug therapy. In terms of diet, more than 50% of calorie intake should be from fibre-rich carbohydrates with minimal fat (especially saturated fat), refined carbohydrate and alcohol intake (Simon e*t al*. 2005). The total calorie intake for each individual should be adjusted according to their BMI to include a few portions of fresh fruit and vegetable in their daily diet; ready meals and alcohol should be avoided, as they are often sources of hidden sugar. In addition, regular exercise is encouraged as it can increase insulin sensitivity, decrease blood pressure and improve blood lipid control.

In terms of drug therapy, there are two main types used to treat DM.

1.13 Pharmaceutical (synthetic) drug therapy

The drug therapy for T1DM and for some patients with T2DM is insulin. Insulin therapy is a first-line treatment for T1DM whilst in T2DM it is considered only after diet and oral drug therapy have failed. Besides insulin, drugs currently used for T2DM treatment include sulfonylureas, biguanides and thiazides, followed by other medications like glitazon, ateglinide, repaglinide and acarbose (Royal Pharmaceutical Society 2015). The most widely prescribed medications for T2DM are sulfonylureas, which are derivatives of the antibacterial sulfonamides. Their mechanism of action is direct stimulation of the release of insulin from pancreatic beta cells, which leads to reduced blood glucose levels. This group of medications also decreases hepatic glucose production at higher doses. Second-generation sulfonylureas can have an additional pancreatic effects that result in increased insulin sensitivity (Briscoe *et al.* 2010; Basit *et al.* 2012).However, the mechanism of this effect is still unclear.

Second-line treatments for T2DM start with biguanides, for example, metformin. The mechanism of metformin likely involves a peripheral insulin sensitisation, which corrects most of the major symptoms characterising insulin resistance syndrome, and increases glucose uptake in muscle. Metformin is able to reduce gluconeogenesis in the liver as well as decrease intestinal absorption of glucose. All these effects result in lower insulin resistance and better glycaemic control. On the negative side, the main side effect that has made these medicines potentially dangerous is the possible induction of lactic acidosis, especially if over-prescribed.

Also, many patients, especially elderly patients may be contra-indicated for metformin due to the risk of lactic acidosis (Scheen and Paquot 2013).

Thiazolidinediones (glitazones or TZDs) are another class of drug for treating T2DM. The National Institute of Health and Clinical Excellence (NICE) guidelines recommend that TZDs could be used if diabetic patients cannot tolerate the combination therapy of metformin and sulfonylurea. The improvement of glucose and lipid metabolism is due to the agonist action of the PPAR gamma receptors, which increase transcription of certain insulin-sensitive genes, including those for lipoprotein lipase, the fatty acid transporter and the glucose transporter. Some examples of this class of drug are troglitazol, rosiglitazon and pioglitazon. On the negative side, this group of medications has several undesirable side effects, including inhibition of hepatic regeneration (Turmelle *et al.* 2006), induction of obesity (de Souza *et al.* 2001) and osteoporosis (Rzonca *et al.* 2004). Troglitazol was withdrawn from the market as a result of severe hepatotoxicity, unlike the aforementioned two drugs, which do not cause severe toxicity.

Another line of treatment of T2DM is meglitinides, which include nateglidine and repaglidine. Their mechanism of action is similar to anti-diabetic sulfonylureas, but clearly they are in different categories. These drugs should be used in combination with metformin and are not applicable for T1DM.

Glycoside inhibitors and glucagon like peptide-1 (GLP-1) are also used to treat T2DM.

Although these and similar drugs have been used widely and have shown good results in management of DM, they are either expensive or can have undesirable

side effects, such as weight gain, oedema, anaemia and hepatotoxicity (especially with TZD), and furthermore, all of them fail to restore full glycaemic control (Balakrishnan *et al.* 2009; Laville and Andreelli, 2000). Thus, such side effects discourage the correct and complete observation of the medication protocol by some patients. On the basis of these drawbacks, it is highly desirable to find new anti-diabetic compounds that increase the level of glucose uptake in different types of animal cells lines and do not have serious side effects like TZD (de Souza *et al*. 2001; Laville and Andreelli 2000).

1.14 Herbal and complementary therapies

Herbal medicine and plant derivatives have a long history of use as therapeutics for a variety of diseases and health conditions in many cultures around the world. There is an increasing trend in recent years towards the use of herbal-based therapeutics that originate from traditional medicines as an alternative or complementary treatment for some health conditions, such as hypertension, hyperglycaemia, hyperlipidaemia and cancer (Kim *et al.* 2011; Wazaify *et al.* 2013; Zhao *et al*. 2010; Ramkumar *et al.* 2008). There is also increasing interest in the field of herbal medicine research in investigating the efficacy and mechanisms of action of such derivatives.

In past two decades considerable numbers of complementary medicines have become available through the National Health Service (NHS), but the accessability of those services however depends on the level of support and local interest (Zollman and Vickers 1999). About 1200 plants are used globally for the treatment of T2DM. However, only around 350 of them are registered as anti-diabetic agents (José *et al*. 2005). Such traditional methods of medical therapy have been widely and successfully used in some eastern countries, such as China, Egypt and Iran.

It is well known that diabetes is one of the most disabling health conditions with increasing prevalence worldwide, which makes herbal derivatives with anti-diabetic properties increasingly attractive for further investigation. Studies on the effects of herbal derivatives on diabetes not only help us to identify new, active anti-diabetic molecules but may also provide us with better insight about the pathophysiology of diabetes.

1.15 Moringaceae family

A literature search for herbal derivatives with anti-diabetic effects highlighted *Moringa peregrina* as being relatively understudied. Its extracts have been suggested by some studies to have anti-diabetic effects (Ramkumar *et al.*, 2008; Elbatran *et al.*, 2005; Tsaknis 1998; Taha *et al*. 2011; Hanaa *et al*. 2013; Cederroth *et al.* 2009; Larijani *et al.* 2008), but active components had not been identified. The Moringaceae family is in the category of Brassicales or Capparales. There is only one genus recognised in this family, which is subdivided into 13 species, which can be found scattered around the tropical and semi tropical regions. All *Moringa* species originated mainly from India and from there they have found their way to many hot countries around the world.

Members of this group are quite differentiated from each other in terms of characteristics such as morphology, habitat requirements and pharmaceutical applications. Some species, such as *M. hildebrandtii*, have become extinct in nature. Other species, such as *M. arborea*, are in danger of extinction.

M. oleifera, the most common species of this group, is native to the Himalayan border regions and North West India, and can be found in many African countries, Arabian Peninsula, South East Asia, the Pacific Islands, the Caribbean and South America.

All known species of *Moringa* are listed below.

1. *M. arborea* Verdcourt
2. *M. borziana* Mattei
3. *M. concanensis* Nimmo
4. *M. drouhardii* Jumelle
5. *M. hildebrandtii* Engler
6. *M. longituba* Engler
7. *M. oleifera* Lamarck
8. *M. ovalifolia*
9. *M. peregrina* Forssk. ex Fiori 1
10. *M. pygmaea* Verdcourt
11. *M. rivae* Chiovenda
12. *M. ruspoliana* Engler
13. *M. stenopetala* (Baker f.) Cufodonti

In terms of structure evaluation, the genus *Moringa* is a class of dicotyledonous plants. The tree bears seed pods 20–40 cm long, each containing 8–15 unwigged seeds (Shojania *et al*., 2006). Young seeds of the plant are eaten in the way peas are eaten while mature seeds are fried or roasted like ground nuts.

Moringa peregrina (Forssk.) Fiori, Agricolt. [Syn.; *M. aptera* Gaertn., *M. arabica* (Lam)], is also known as Ben-nut tree, Ben-oil tree, wild drumstick tree, and is the second most important type of *Moringa* after *M. oleifera* Lam. It is important in the food, pharmaceutical and environmental industries. Its oil is used in the food industry due to the high content of mono-unsaturated fatty acids (like oleic acid),

which do not lose their quality when frying. In addition, the oil is used in pharmaceuticals, perfumes and even for streamlining parts in fine industries, such as watches or mobile laboratory devices that are only lubricated once in their lifetime to improve their resistance against heat and to avoid damage.

There have been far more studies on the species of *M. oleifera* in comparison to *M. peregrina*. *M. oleifera* is rich in nutrients and proteins and has multiple pharmacological effects, its use in diabetes and abortion was described by Gupta and his colleagues (Gupta *et al.* 2002),the crude ethanolic extract of its dry seed and hot water infusion of leaves, seed and roots of *M. oleifera* showed its anti-inflamatoy and diuretic effect (Caceres *et al*.1992).Purification of ethanolic extract of the leaves gave different components namely,niazinin A and B, niazimicin , niaziminin A and B. These isolated compounds showed *M.oleifera* cardiovascular effect by exerting hypotensive and bradycardiac effect (Gilani *et al.* 1994).Other bioactive components were found from extract of this plant including flavonoid group such as kaempferol and quercetin and exerted the antioxidant properties from phenolic constituents of *M.oleifera* (Siddhuraju *et al* 2003).

The leaves of this plant are a rich source of beta-carotene, protein, vitamin C, calcium and potassium. The plant is a natural source of antioxidant used to store high-fat foods. *M. oleifera* is a fast-growing species in tropical and subtropical areas, with extensive growth in Asian countries. This type of *Moringa* species is the only species that is cultivated intensively for commercial use in some countries. Because of its numerous uses of the plant, this species is also known as the miracle plant (Ashfaq *et al*. 2012).

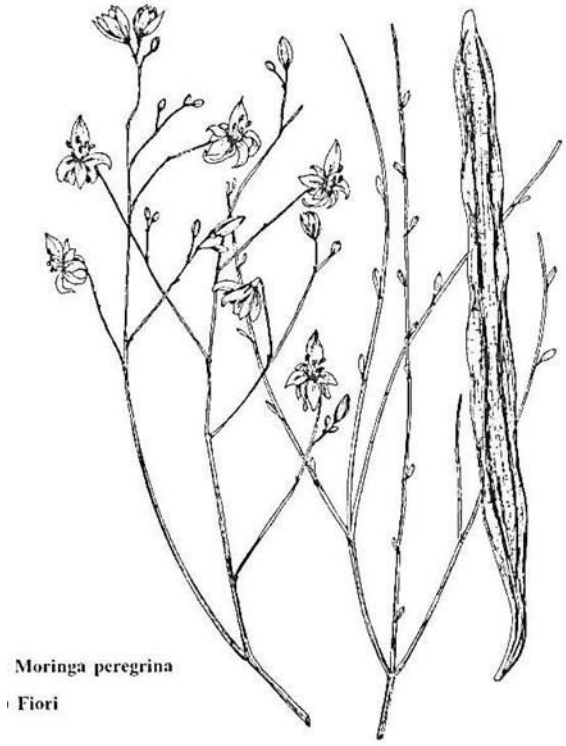


Figure 1.7 *Moringa peregrina* plant

1.16 Phytoestrogens

Phytoestrogens are a group of chemicals that naturally occur in plants with oestrogenic properties allow them to act as agonists and antagonists of oestrogen (Yildiz, 2005). The pharmacological effects are mediated through binding to oestrogen receptors. There are two types of oestrogen receptors in the human body, ER alpha and ER beta and phytoestrogens have higher affinity for ER beta than for ER-alpha (Turner, 2007). Phytoestrogens were first synthesised by Baker and Robinson in 1928 (Walter E. D. 1941), after which isoflavones, which is a group of phytoestrogens, were found in soybean meal (*Soja hispida*) by Waltz (Al-Othman *et al*., 1998). Another group of phytoestrogens called lignans were discovered by Haworth in 1941 (Marwah, 2006). Several studies were carry out to assess the reproductive effect of phytoestrogens particulary isoflavones (Bennetts *et al.*1946, Carter *et al.* 1955 and 1960 East J. 1955). Following these investigations, pharmacokinetic studies were carried out on animals as well as humans (Cederroth *et al.* 2009; Oergaard *et al*. 2008; Hintz *et al*. 2004; Bhathena *et al.* 2002; Stephenson *et al.* 2002; Jayagopal *et al*., 2002; Hepburn *et al*. 2002; Tourandokht *et al.* 2008; Sun *et al*. 2006; Hasani-Ranjbar *et al*. 2008; Shen *et al.* 2006; Santti *et al.* 1998; Curran *et al.* 2004; Harris *et al*. 2005; Zhou *et al*. 2009). Subsequently, the effects of phytoestrogens and their metabolites on estrogen receptors (ER) have been recognised and their estrogenic and anti-estrogenic activities confirmed, as well as their high affinity for uterine estrogenic receptors (Turner et al. 2007).

1.17 Classification of phytoestrogens

Phytoestrogens can be categorised into two main categories depending on their strutctures; the flavonoids and non-flavonoids as described in Figure 1.8. The isoflavones and coumestans from the flavonoids class have the highest estrogenic activity. Prenyl flavonoids are another member of this group that have been discovered recently and have not yet been fully investigated. In the non-flavonoids class of phytoestrogens, lignans have been well studied and have potent estrogenic activity, followed by stilbenes, which include the widely studied resveratrol.

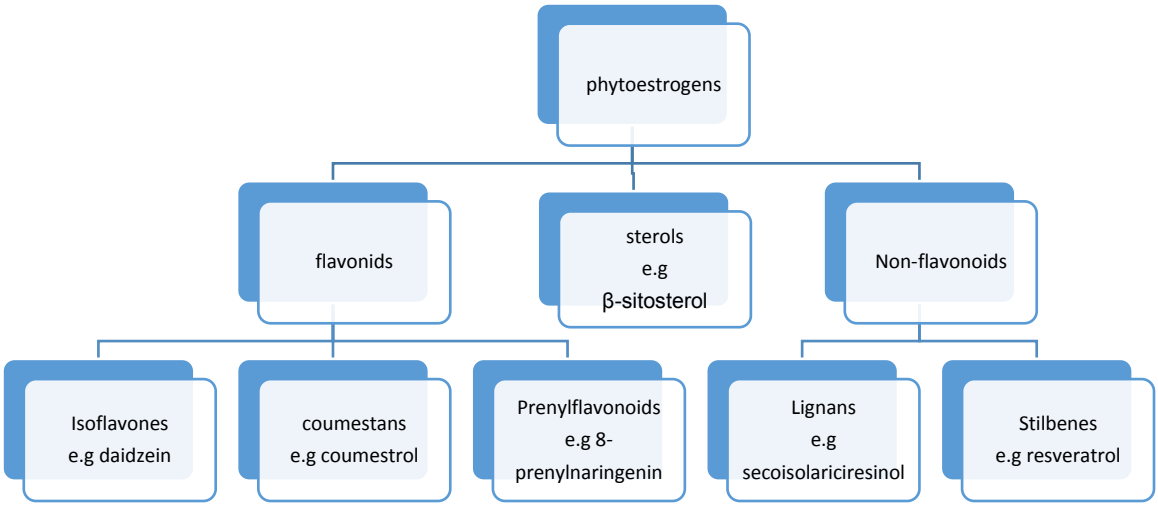
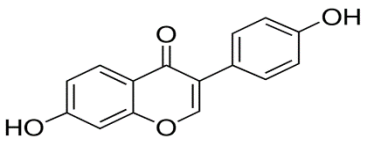
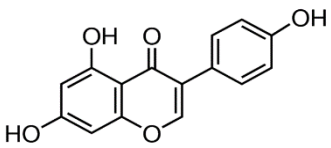


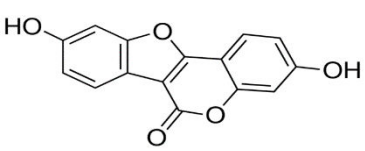
Fig.1.8. General classification and examples of phytoestrogens (adapted from Yildiz 2005, Sriraman et al. 2015, Brown et al. 2014, Touillaud et al. 2005 Nut)



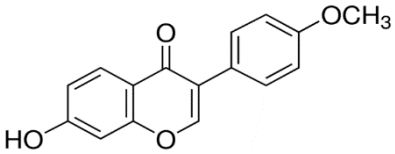
Daidzein



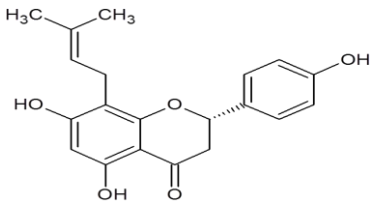
Genistein



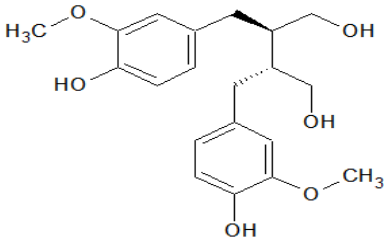
Coumestrol



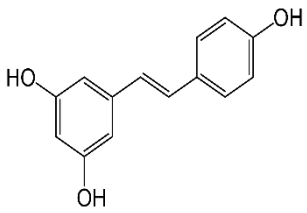
Formononetin



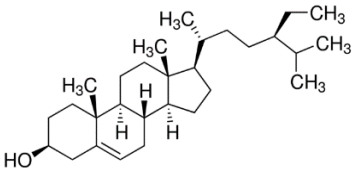
8-Prenylnaringenin (8-PN)



Secoisolariciresinol



Resveratrol



β-sitosterol

1.18 Aim of the work

This project aimed to investigate the anti-diabetic properties of *M.Peregrina* and phytoestrogens by *in-vitro* techniques, with a view to characterising new phyto-preparations with anti-diabetic properties. HepG2 cells were chosen for assaying glucose uptake, consumption and metabolism sterols.

The strategy was to prepare a series of extracts of *M.peregrina* leaves using solvents with a range of polarities. These would be screened for potential anti-diabetic activity to identify the most active fractions. The active fractions would undergo rigorous further extraction and analysis to identify individual phyto components, which could be assayed individually for anti-diabetic activity.

Chapter II
Material and Methods

Part I

Preparation and purification

Of

Moringa peregrina extracts

2.1 Preparation of Extracts of *M.peregrina*

In this part of the work there were two phases of preparation of extracts. One at the beginning of the work involving preparation of crude extracts for screening, and the second involving purification of isolated phytochemicals from the most active extract. The first phase was done at the Institute of Medicinal Plants in the city of Karaj, Iran, and the second phase was done at the University of Tarbiat Modares and Shahid Beheshti in Tehran, Iran.

2.1.1 Plant sources

Plant sources used in this project include aerial parts of *M.peregrina* that were collected from the province of Sistan-Baluchestan, city of Nikshahr in Iran, from a height of 300 meters above sea level in June 2010. This plant was previously registered in the Herbarium of Medicinal Plants in Karaj, Iran, with the registration number ACECR-44.

2.1.2 Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), beta-carotene and sodium carbonate were of analytical grade from FLUKA . Methanol, ethanol, dimethyl sulfoxide (DMSO) and other solvents, Folin reagent, Tween 40, linoleic acid, and butylated hydroxytoluene (BHT) were obtained from Merck .

2.1.3 Extraction and preparation of fractions

Dried aerial parts of *M. peregrina* (1kg) were extracted by successive percolation with solvents of increasing polarity at room temperature, starting with *n-hexane*. The extract then was concentrated using smooth finish paper and rotary evaporator and

dried in a Petri dish. Chloroform were added on the remaining amount of plant and extracted. After extraction with chloroform the remaining plant was dried in the dark and then extracted with ethanol 70% (each extract three times with 500 mL of solvent, for 24 hours). The ethanol extract was concentrated, suspended in water, and successively partitioned with EtOAc and *n*-butanol. The *n*-hexane extract (Hxe), chloroform extract, and ethyl acetate and *n*-butanol fractions (Btf) were obtained, along with the water fraction (Wtf). All extracts were filtered out, dried with a vacuum using a rotary evaporator, and brown viscous residues were obtained and stored in the dark at 4ºC prior to use, the extraction lasted 7 to 10 days.

Following the *in vitro* study in this project, positive biological activity was found in extracts of ethyl acetate and chloroform and further phytochemical investigation on these two active extracts were performed.

3. Purification of the active compounds of ethyl acetate and chloroform extracts

The extracts were purified by using silica gel chromatography (250g and 220g) with grade 60 silica gel (0.063-0.200mm, 70-230 mesh), column length 90 cm, internal diameter (i.d) 3.5 cm. Compounds were separated and monitored by thin layer chromatography (TLC) followed by preparative High Performance Liquid Chromatography. Proton nuclear magnetic resonance (^{1}H NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) were used for determination of compound structures.

NMR spectra were recorded at a target temperature of 18 °C on a Bruker Avance III 500 MHz spectrometer operating at 500.13 MHz for 1H and 125.77 MHz for 13C. A 1-mm TXI microprobe with a z-gradient was used for 1 H-detected experiments. 13C-NMR spectra were recorded with a 5-mm BBO probe head with z-gradient. Spectra were analyzed using Bruker TopSpin 2.1 software. Analytical HPLC separations were performed with a Knauer HPLC apparatus consisting of a 1000 Smartline pump, a 5000 Smartline manager solvent organizer, and a 2800 Smartline PDA detector. Injection was performed through a 3900 Smartline autosampler injector equipped with a 100 µL loop. Temperature control of the column was with a Jet Stream 2 Plus oven (Knauer, advanced scientific instrument). Data acquisition and integration was performed with EZchrome Elite software. The flow rate of the mobile phase was kept at 1.0 mL/min. Preparative HPLC was done on a Knauer Wellchrom series consisting of K-1800 pump, UV detector k-2501, and a Büchi fraction collector (Büchi Labortechnik AG). Knauer Eurospher 100-5 C18 (250 mm×4.6 mm, 5 µm), and Eurospher-100 (120×16 mm, 5 µm) HPLC columns with precolumn were used for analytical and preparative separations, respectively.

High resolution mass spectra(MS) was used , the samples was analysis with micrOTOF (bruker) , where Electrospray ionization and Time of flight (TOF) was used. .For Isolation of constituents from the EtOAc extract ,the column was eluted with a gradient of ethyl acetate/methanol (100:0 → 0:100). Fractions of 20 mL were collected and monitored by TLC (silica gel 60 F_{254}, Merck, ethyl acetate 100%; detection with H_2SO_4 3% reagent and heating) and eight fractions were obtained.

In the procees Isolation of constituents from the chloroform extract, the column was eluted with a gradient hexane/ethyl acetate (100:0 → 0:100) and followed by ethyl

acetate/methanol (100:0 → 80:20). Fractions of 20 mL were collected and monitored by TLC (silica gel 60 F_{254}, Merck, detection with H_2SO_4 3% reagent and heating). By adding the fractions with the same solvent and evaporating them at the end 15 fractions were obtained.

Part II

In vitro studies on glucose uptake

4.1 Introduction

Stimulation of glucose uptake is a key parameter of the hypoglycaemic effect of known anti-diabetic drugs (Kawamori *et al.*1995; Yu *et al*. 2015).

To establish the hypoglycaemic effect of the compounds in this part of the work two methods of study was designed for measurement of glucose metabolism in HepG2 cells, measurement of glucose uptake and measurement of glucose consumption. This part of the project was performed in the Research Laboratory of London Metropolitan University.

4.2 Materials

HepG2 cells were obtained from King's College Medical School Liver Unit. DMEM (Dulbecco's Modified Eagle Medium) glucose free and with glucose, methanol, dimethyl sulfoxide (DMSO), Costar 24 well plates, 2-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG) were obtained from Sigma Aldrich (Poole, Dorset, UK).

4.3 Cell culture

4.3.1. HepG2 cell line

The cell line HepG2 is a human liver carcinoma cell line. This is a perpetual cell line and have an epithelial morphology with stable chromosome number of 54 with variation between 54 to 56 in 90% of the cells (Simon *et al.*1982). HepG2 cells exhibit most cellular characters of normal human hepatocytes and produce a wide range of valuable biochemical molecules from proteins groups including albumin,

iron-binding blood plasma glycoprotein transferrin, glycoproteins such as fibrinogen, high molecular weight plasma proteins like α_2-macroglobulin, protease inhibitors (Alpha-1 antitrypsin) and plasmin which is degradation enzyme for blood plasma protein such as fibrin clots.

4.3.2. Cell Culture

The human hepatoma HepG2 cell line was used in this work, and was grown in DMEM with 11 mM glucose (Gibco) and 10% foetal calf serum. Glucose was added to 11 mM for most experiments unless otherwise stated. HepG2 cell cultures were started from frozen cells, using standard protocols (Hanaa *et al.*2011). A vial of cells was collected from liquid nitrogen storage and the lower half submerged in a water bath at 37 °C. After allowing to thaw until a small amount of ice remained in the vial (usually 1-2 minutes), the vial was transferred to a sterile safety cabinet. The vial was wiped with a tissue moistened with 70% alcohol, the cap removed and the cells were removed by slow pipetting to pre-warmed growth medium. The cells were centrifuged at 1000 rpm at room temperature for 5 minutes to remove the DMSO in the cryopreservation medium. The medium was carefully removed and replaced with new medium and the cells slowly pipetted to a flask. Cells were grown at 37 °C in 5% CO_2 / air.

The HepG2 cell line is anchorage dependent and must be cultured while attached to a solid or semi-solid substrate (adherent or monolayer culture). The fibroblastic morphology of the cells was examined by microscopy at each sub-culture. An example is shown in Figure 2.1

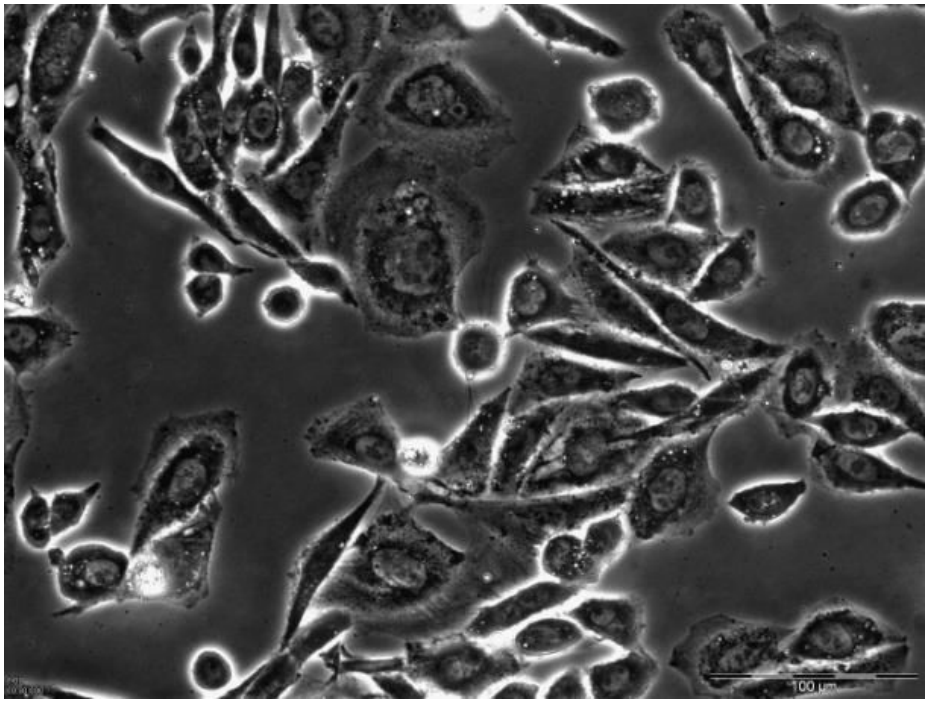


Figure 2.1. Fibroblastic morphology of HepG2 cells.
Fibroblastic (or fibroblast-like) cells are bipolar or multipolar, have elongated shapes, and grow attached to substrate. The image shows a reverse-phase microscopy picture of HepG2 cells taken during my PhD project. A scale bar of 100 μm is shown.

4.3.3. Subculture of HepG2 cells

When a flask of cells was at, or near confluency, the culture confluency as well as absence of bacterial and fungal contamination were checked by microscopy. Spent medium was removed, cells were washed with a half volume of culture medium. Trypsin-EDTA (1ml per 25 cm^2 of surface area) added to the flask, washed over the cells, then the flask was returned to the incubator and incubated until cells detached. The cells were examined by microscopy to ensure that they were fully detached and floating, then a small volume of the fresh serum-containing medium was added to inactivate the trypsin (Elbatran *et al.* 2005; Ramkumar *et al.* 2008). Cells were seeded into a fresh flask at a concentration of approximately $0.5x10^5$ in 5 mL medium per 25 cm^2 flask area. Viable cells were counted by trypan blue exclusion using a haemocytometer.

4.3.4 Glucose uptake in HepG2 cells

For *in vitro* glucose uptake assays using 2-NBDG, it was important to optimize the conditions of uptake of 2-NBDG by HepG2. 2-NBDG is a fluorescent derivative of glucose modified with a 2-[*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino group at the C-2 position (Molecular Weight = 342.26) (Yoshioka 1996). The probe shows intense fluorescence at 542 nm when excited at 467 nm. 5mg 2-NBDG was dissolved in 1.46 mL of methanol in order to make a 10mM as a stock solution. 5 mM, 1 mM, 0.5 mM and 0.1mM solutions were prepared in methanol, as shown in Table 2.1. Methanol only was used as a vehicle control.

.

Final concentration of 2-NBDG (mM)	Stock/Vol.	Vol MeOH
5	10mM/100 µL	100µL
1	5mM/40 µL	160 µL
0.5	1mM/100 µL	100 µL
0.1	0.5Mm/40 µL	160 µL

Table2.1 Dilutions of 2NBDG used for optimising the uptake assay.

HepG2 cells were sub-cultured using DMEM medium with glucose, 10mL of medium was added and cells were transferred into flasks, cells were seeded at a density 5.0 x10^4 cells per mL and incubated with 10 mL of medium. Cells were counted using a haemocytometer and diluted to 0.5x10^5 cells/mL. Then the cells were aliquotted at 0.5 mL per well in a 24-well plate and incubated for 24 hours. 5 µL of 0, 0.1, 0.5, 1, 5, or 10 mM 2-NBDG , was added to columns one to six respectively (from right to left), so that the 2-NBDG was diluted 100 times. Cells were incubated at 37 °C for 15 minutes then washed with PBS and fluorescent uptake was measured using an

Omega FluoStar (BMG LabTech, Ayelsbury, Bucks, UK) plate reader with λ_{ex} = 485nm, λ_{em} = 530nm, using the bottom optic and a gain of 1000.

Various modifications of the procedure were tried. One involved replacing normal medium with glucose-free medium after 24 hours. Another concern was the optimal period of incubation with 2-NBDG, and uptake was accordingly monitored from 15 minutes to 2 hours. The glucose uptake increase was checked by fluorescence microscopy.

4.3.5. Glucose uptake measurement after treatments

Preliminary experiments for testing the effect of extracts of *M. peregrina*, known anti-diabetic drugs and phytoestrogens on glucose uptake were carried out to test the influence of Incubation time, cell density and growth phase, concentration of 2-NBDG and plate-reader parameters for measuring fluorescence. These are described in the Results section.

According to previous studies the concentration of crude plant extracts used in cell culture experiments was in the range of 10 mg/mL to 20mg/mL, and was used as a reference for the current study (Mekonnen *et al.* 2005*;*Jung *et al. 2015 ;*Haasan *et al. 1995,* O'Neil *et al.* 2005).

10mg of dry extract was dissolved in 1.0 mL of DMSO to prepare a stock solution. HepG2 cells were cultured using various concentrations of cells in a 24-well plate and grown for 24 hours. After 24 hours the medium was replaced by 0.5 mL of DMEM glucose-free medium, supplemented with 5 µL of *M. peregrina* extracts prepared with hexane, chloroform, ethyl acetate, N-butanol, water and methanol and dissolved in DMSO at a concentration of 10 mg/mL. Three wells per plate were used

for each extract and three wells per plate received 5 μL DMSO only as vehicle control. Three wells were left without any treatment as a further negative control. Cells were treated for one hour (short-term treatment) or 24 hours (long-term treatment). 2-NBDG was added and cells incubated for 15 min, after which glucose uptake was measured as described above.

In one modification cells were cultured for 24 hours and treated with the extracts for 24 hours. After the incubation time medium replaced with glucose free medium. Then 2-NBDG added and incubated for 15 minutes after which the glucose uptake was measured by the fluorescent reader (Omega FluoStar plate reader).

4.3.6. Comparison of short term and long term treatment of three active extracts of *M.peregrina* (hexane, $CHCl_3$, EtOAc) on glucose uptake in confluent HepG2 cells.

The objective of this part of the project was to further study the effect of the three active extracts of *M.Peregrina* on regulating glucose uptake *in-vitro*.

HepG2 cells were seeded at $0.5x10^5$ cells/mL, and allowed to grow overnight. The next day cells were treated with 50 μg of hexane, $CHCl_3$ and EtOAc extracts and incubated for one hour as a short-term treatment, or 24 hours as a long-term treatment. At the end of the treatments normal DMEM medium was replaced by 0.5 mL glucose free medium, then 5 μL of 10 mM was 2-NBDG added into three wells in each row in order to have 3 wells as a control. After incubating for 15 minutes, the cells washed with PBS and fluorescent intensity measured.

4.3.7. Comparison of Glucose uptake effect of known anti-diabetic drugs with the three active extracts of *M.Peregrina*.

The aim of this part of the work is to compare the effect of the active extracts on glucose uptake with known anti-diabetic compounds such as metformin (Sahra *et al.* 2008), berberin (Yin *et al.* 2008; Yina *et al.* 2012) and ouabian (Reid *et al.*1996), as well as with a known inhibitor of glucose uptake, phloretin.

The cell culture, treatments and measurement methods were performed as described in section 2.4.

4.3.8. Glucose uptake measurement after treating confluent HepG2 cells with three phytoestrogens.

This part of the project was started by making serial dilutions of three phytoestrogens genestein, daidzein and formononetin (Sigma Aldrich, UK). Stock solutions of 10 mM, 1 mM, 100 μM, 10 μM, 1 μM, and 0.1μM were prepared in DMSO. HepG2 cells were cultured and treated as described in section 2.4.

5. Glucose consumption

A glucometer is a medical device for measurement of glucose levels in blood. However it can be used for research purposes in the laboratory as well, and was used in this study to measure changes in the levels of glucose in cell culture medium. The SD Codefree device used in this study is made by SD Biosensor Inc, Korea (Home Health UK, Watford, Herts) and uses a glucose oxidase biosensor with a measurement range of 0.6 ~ 33.3 mM. Each strip needs 0.9 μl of liquid sample for a single accurate measurement. The test strip was inserted into the device and the

surface of the cell culture medium was touched by the end of the strip, to receive a small volume of medium by capillary action.

The accuracy and reliability of glucose measurements using the SD Codefree glucometer was tested to establish the reading error rate. Cell culture medium with glucose concentrations of 1.6, 3.12, 6.25 and 12.5 mM were prepared by adding appropriate amounts from a 2.5 M stock solution (Sigma-Aldrich). Each medium was measured ten times with ten strips and the average readings are shown in Table 1.1. It was estimated that the glucometer shows the glucose concentration in cell culture medium with a value 7% to 10% above actual glucose concentration, over the range 1.6mM to 12.5 mM (Table 2.2).

	Actual Concentration (mM)	Glucometer reading (mM)	% of error	SD
Solution 1	12.5	13.5	+8	0.12
Solution 2	6.25	6.75	+8	0.03
Solution 3	3.12	3.33	+7	0.06
Solution 4	1.6	1.72	+7.5	0.04

Table 2.2 Comparison of actual glucose concentration with the reading from glucometer (average of ten readings). The average of the error is 7.6%. This percentage correction was deducted from values recorded experimentally.

5.1. Treatment of HepG2 cells with active *M. peregrina* extracts

The effects of the extracts of *M. peregrina* on HepG2 cells were assessed after two hours, 24 hours and 48 hours treatment, and were compared with a known anti-diabetic drug, metformin, that acts by promoting glucose uptake in peripheral tissues. 24 hours after seeding HepG2 cells into a 24-well plate, as described above, the medium of each well was removed and replaced with 0.5 mL fresh full DMEM

medium with a glucose concentration of 11mM. Cells were treated with the two most active extracts of *M. peregrina* found in previous work to enhance glucose uptake into HepG2 cells, described briefly in the Results section. 5μL of extract (10mg/mL) in DMSO were added to three replicate wells in each experiment. In addition one set of wells received 5μL of DMSO only as vehicle control. One set of wells received 10μL of 1mM metformin in DMSO (Sahra *2008*). The remaining wells of the plate were assayed as negative controls with no treatment.

5.2. Calculation of glucose consumption per cell

HepG2 cells were grown to more than 80% confluency and then the medium replaced by fresh medium containing 11 mM glucose and treatments at the same time. Glucose consumption by HepG2 cells was calculated by deducting the levels of glucose in the cell culture medium after incubation period (2, 24 and 48 hours in separate experiments) from 11mM as the amount of glucose in zero time. The glucose values were adjusted, based on 7.6% error rate. The medium was removed from the wells immediately after glucose measurements and replaced with 100μL trypsin solution, then kept in the incubator for 3-5 minutes. 400μL of medium was added to each well after cells were detached and cell counting was carried out using a haemocytometer. The glucose consumption in each group was normalised using cell count to eliminate the bias due to variation in cell number as much as possible.

6. Analysis of GLUT1 expression by western blotting

6.1 Preparation of cell lysates

Cell lysates were prepared for western blotting. Treated HepG2 cells were washed once with EDTA-PBS (phosphate buffered saline with concentration of137 mM NaCl, 10 mM phosphate, 2.7 mM KCl , pH 7.35, with EDTA, 2 mM), 5.0 mL trypsin solution added, and the flask incubated at 37 °C. Under these conditions HepG2 cells detached easily without the need to use a scraper. 5.0 mL full medium was added to the flask and cells were transferred to a 15 mL tube and spun 5 min. at 800 rpm, 4 °C; the cells collected as a pellet. The medium was removed carefully and the cells washed once with 5.0 mL ice-cold PBS, then collected by centrifugation as in the previous step. 1.0 mL of ice-cold PBS was added to the pellet and the re-suspended cells were transferred to a 1.5 mL microfuge tube; then the cells were collected by spinning for 10 seconds at 5000 rpm. The PBS was carefully removed and one volume (with respect to the cell pellet) of ice-cold RIPA buffer with protease inhibitor cocktail This mixture contains individual components, including AEBSF at 104 mM, Aprotinin at 80 µM, Bestatin at 4 mM, E-64 at 1.4 mM, Leupeptin at 2 mM and Pepstatin A at 1.5 mM (Sigma Aldrich, UK), was added then gently pipetted up and down to mix the cells thoroughly and left on ice for 5 min. The extract was spun at 12,000 rpm for 5 min at 4 °C; the supernatant, containing solubilised plasma membranes and cytosolic proteins, was stored at -20 °C.The soluble extracts (supernatants) were analysed by Western Blotting.

6.2. Western blotting

The procedure of this experiment followed a standard protocol; a shortened version is given here.

6.3. SDS Polyacrylamide Gel Electrophoresis

1-1 Preparation of the Running and Stacking Gels

A very clean and dry glass plate, an aluminium backing plate and two spacers (0.75 mm) are assembled into a Vertical Slab Gel and placed into a gel caster carefully to prevent leaks and water added to test for leaks.

The running gel solution (RGS) containing 10% acrylamide was prepared in a plastic tube, TEMED (*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine

colorless liquid and soluble in water, methanol and aceton) and APS (Ammonium persulfate soluble in water (100 mg/ml), yielding a clear, colorless solution)added last (APS and TEMED are catalysts for polymerization of acrylamide) A volume of RGS was pipetted into the vertical gel caster to a level that left 1 cm to the teeth of the comb, to give enough space for the stacking gel.

A volume of water saturated with n-butanol solution was added on the top of the running gel to prevent the possible reaction between the gel solution and the atmospheric oxygen and the RGS was left for 20-30 min at room temperature to polymerise. The n-butanol was removed from the gel. The stacking gel solution (SGS) was prepared and TEMED and APS added last. Then a comb inserted and the SGS is pipetted into the space at the top of the aluminium and was left for 20 min to polymerise.

1.2 Sample preparation:

One volume of sample was added to one volume sample buffer and heated at

95 °C for 5 min to denature the proteins; the sample was allowed to cool before loading. Denatured samples were stored at –20 °C for long term storage. Loadings were typically 10 or 20 μL per well.

1.3 Loading Samples onto the Gel Apparatus

The gel unit (glass and aluminium plates and spacers in the cassette) was placed into the electrophoresis tank containing running buffer. Buffer poured into the cassette chamber and the comb was slowly removed from the stacking gel.

The same volume, 20 μL, of chloroform and ethyl acetate extract treated samples and controls were loaded into the wells; 5 μL of BioRad markers were used.

1.4 Running the Gel

The stacking gel was run at 10mA and the power increased to 20mA after the samples moved into the running gel, constant current (power supply, POWER PAC 300, BioRad).

6.4. Blotting

The aluminium plate and the gel (still united) was submerged in Towbin Transfer Buffer (TTB) with composition of 3.03g Tris-base, 14.41g Glycine, 10 mL 10% SDS,up to 800 mL with H_2O, 200 mL methanol , for 5 – 10 min. Then the stacking gel removed and the running gel separated from the plastic plate. Four pieces of blotter paper (Whatman, 3MM), the same size as the sponges for the blot, and one piece of nitrocellulose membrane were cut (e.g. Hybond – C extra, Amersham). All the papers, the sponges and the membrane were wetted with TTB.

The order for the contents of the transfer cassette (“sandwich”):

1- grey side of the cassette
2- sponge
3- two pieces of blotter paper
4- membrane
5- running gel
6- two pieces of blotter paper
7- sponge
8- black side of the cassette

The cassette was closed and inserted into the transfer apparatus (TE22, Hoefer Scientific Instruments), the grey side with the positive pole (anode). The proteins go from the gel (- side) to the membrane (+ side). Proteins transferred overnight at room temperature with a constant voltage of 10V.

6.5. Immunodetection of a GLUT1

After transfer of proteins the membrane was washed with TBS for 5 – 10 min and loose acrylamide removed. Then the TBS is removed and molecular weight markers were marked on the membrane with an indelible ink pen. Blocking Buffer (3 % w/v “Marvel” milk powder in TTBS) was added and the membrane incubated on a shaker (4RT Rocking Table, LuckHam) at room temperature for at least 30 min. The blocking buffer was discarded and the membrane washed three times in wash buffer. The wash buffer was poured off and the membrane incubated with the primary antibody solution, GLUT1- antibody (C-terminal) is produced in rabbit using as immunogen and categorically recognise rat GLUT1. The antibody can be used in numbers of immunochemical techniques such as immunoblotting (55-70 kDa).
The membrane was incubated with constant shaking at room temperature for at least 2 hours. Then the primary antibody solution is discarded and the membrane is washed three times for 5 min in wash buffer. The wash buffer is poured off and the

membrane incubated with the secondary antibody solution (usually a 1:1000 dilution) – for example an anti-rabbit IgG labelled with horse-radish peroxidase (HRP). The membrane was incubated on a shaker at room temperature for at least one hour.

6.6. Incubation with the substrate (for horse-radish peroxidase)

The membrane was washed five times in wash buffer. After this the membrane was placed into a plastic sleeve sealed at the bottom with the top open. 2 to 4 mL luminescent substrate solution was freshly prepared according to the manufacturer's instructions, and pipetted into the sleeve over the membrane, the volume depending on the size of membrane. It was kept in the dark for two minutes, the solution poured off / squeezed out, and fresh solution added. The membrane was incubated for two minutes in the dark, the excess liquid poured off, leaving the membrane moist. The moist membrane was placed into a plastic sleeve for image analysis using a UVitec (Cambridge, UK) documentation system. The intensity of each band in the blot is measured using image J software and the intensity of bands compared for analysis.

Part III

Studies on cell metabolism

7.1 Cell metabolism and mitochondrial respiration analysis

The Seahorse Bioscience (Massachusetts, US) XF24 machine was designed to test mitochondrial function of cells in real time, with high throughput and as an alternative to using a much more labour intensive and low throughput, but well-established methodology using the classical oxygen electrode.

The XF cell Mito stress Kit was developed to assess mitochondrial function on the basis of the rate of change of dissolved oxygen and pH in the media immediately surrounding living attached cells cultured in a microplate in real time mode. This provides an accurate measurement of the rate of cellular metabolism in detail. Exclusive factors in the technology of the XF include calculation of oxygen and proton exchange rate based on short periods (5 minutes) of measurement, achieved with a small volume of medium.

The apparatus uses 24 optical fluorescent heads fixed in sterile disposable cartridges which locate into a 24 well tissue culture microplate. Each head becomes immersed in the medium of each well and can transmit optical signals independently, and simultaneously, with the other heads. The test runs every 2-5 minutes approximately and generates the data for oxygen consumption rate (OCR) in pmol/min which shows mitochondrial respiration, and extracellular acidification rate (ECAR) in mpH/min that is due to glycolysis. The experiment was performed at the University of Westminster.

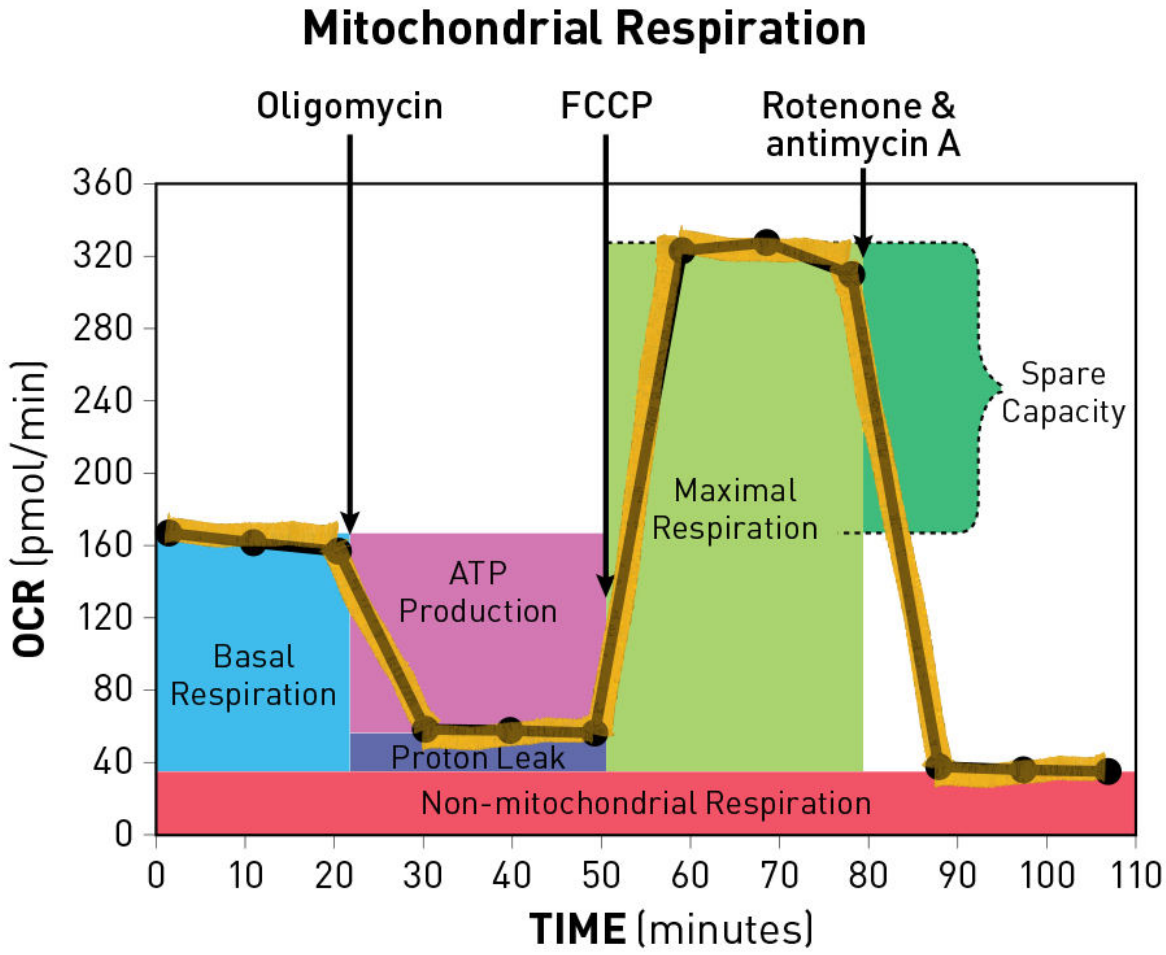


Figure 3.1 | XF Cell Mito Stress Test. Profile of the key parameters of mitochondrial respiration. Sequential compound injections measure basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration.(*Adopted from www.Seahorsebio.com*)

Three respiration modulators are used in the XF Cell mito stress test. Each modulator works on a specific target complex of the electron transport chain (ETC). Oligomycin is used first, and inhibits ATP synthase (complex V) and thereby decreases the oxygen consumption rate (OCR) by mitochondrial respiration, which is directly linked to cellular ATP production. The uncoupling modulator, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) uncouples the mitochondrial

membrane potential and destroys the proton gradient across the membrane. This means respiration is nolonger linked to production of ATP and electron discharge via the ETC is only limited by oxygen availability and complex IV oxygen consumption is at a maximal level. The third agent is a combination of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor. This mix completely inhibits mitochondrial respiration allows measurement of non-mitochondrial respiration induced by processes outside the mitochondria.

The cell's ability to react to increased energy demand is called spare respiratory capacity, and can be calculated from the difference between maximal respiration and basal respiration. This can be calculated by using the FCCP-stimulated OCR (Figure 3.2, Table 2.3).

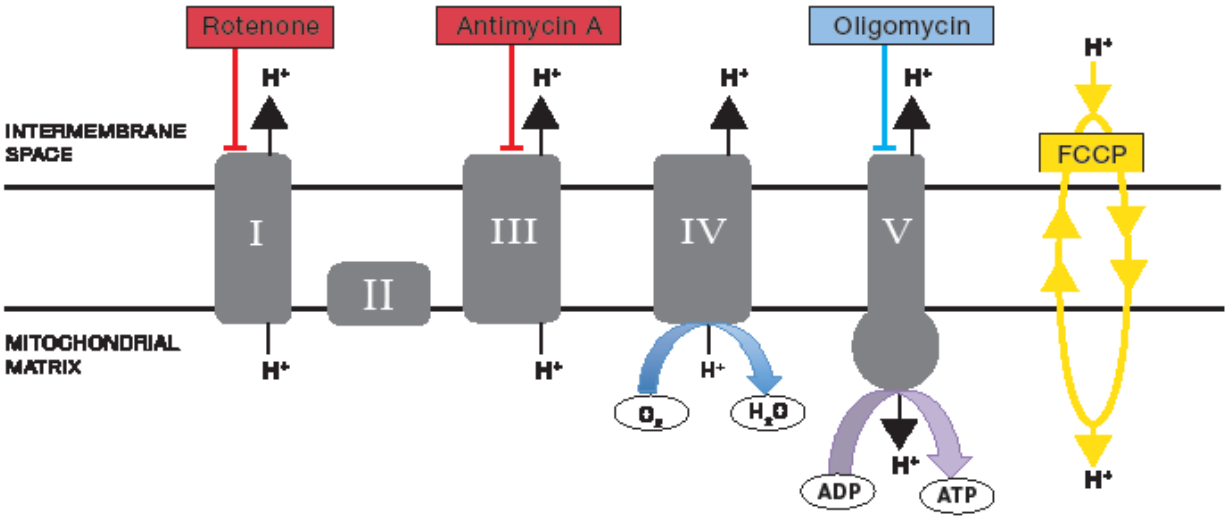


Figure 3.2| XF Cell Mito Stress Test Modulators of the ETC. This diagram illustrates the complexes of the ETC and the target of action of all of the compounds in the XF Cell Mito Stress Test Kit. Oligomycin inhibits ATP synthase (complex V), FCCP uncouples oxygen consumption from ATP production, and rotenone/ antimycin A inhibit complexes I and III, respectively.(*Adopted from www.Seahorsebio.com*)

7.1.1 Materials

Uridine, penicillin/streptomycin, DMEM, glucose, glutamine, sodium pyruvate, buffering agent and HEPES were from Sigma-Aldrich; trypsin was from Invitrogen and fetal bovine serum from Hyclone. Other consumables were from Seahorse Bioscience (XF24-3 FluxPak cat. No. 102070-001; XF Cell Mito Stress Test Kit cat. No. 101706-100.

7.1.2 Cell culture and Treatments: Cells were maintained in DMEM, 10% FBS + 22 mM glucose, in 75-cm^2 T-flasks in a controlled incubator at 37°C, 95% humidity, and 10% CO_2. Every two to three days, HepG2 cultures were detached from the flasks using a 0.25% solution of trypsin and subcultured. All cultures were maintained at less than 80% confluence at the time of subculture. For XF24 assays, cells were seeded at 30,000 cells/well in an XF24 cell culture microplate.

Cells were incubated with treatments including control, 8μM (*O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*)) (MA4P2), 30 μM β- sitosterol, and 10 μM rutin (M8P2) for two hours before the start of the experiment (Figure 3.3, Figure 3.4).

Basal respiration in the presence of the treatments, proton leak and ATP turnover by adding oligomycin (10μM) to inhibit ATP synthesis, maximal mitochondrial respiratory capacity by adding FCCP (carbonyl cyanide 4-trifluoromethoxy phenylhydrazone) (10μM) as uncoupler, and non-mitochondrial respiration by adding rotenone and antimycine (5μM) to stop mitochondrial respiration were measured as OCR. Protein extraction was carried out for each well at the end of the experiment. OCR readings were normalized to total protein per well which was measured using a

Pierce BCA Protein Assay Kit (ThermoFisher Scientific, UK), with triplicate samples assayed per well.

Background	Control	Control	Control	Control	Control
O-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*)	*O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*)	*O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*)	Background	*O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*)	*O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*)
beta-sit	beta-sit	Background	beta-sit	beta-sit	beta-sit
rutin	rutin	rutin	rutin	rutin	Background

Figure 3.3 Seahorse assay plate layout. The layout of the three chosen compounds in the seahorse 24-well microplate

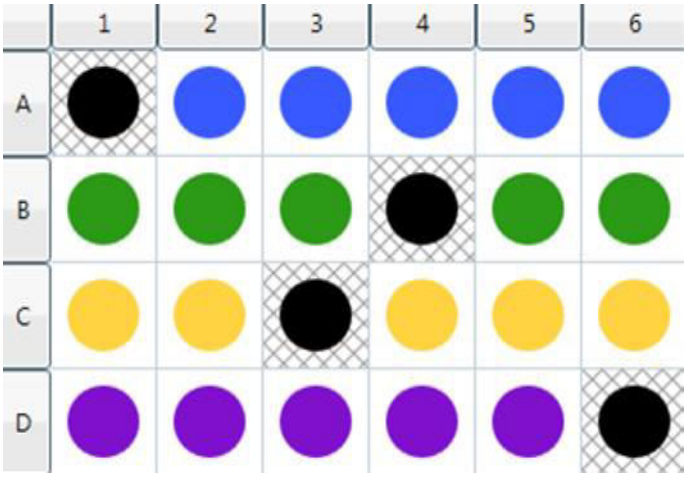


Figure 3.4 Plan of the plate used in the Seahorse experiment according to the layout in Figure 3.3,the colours are indicate the treatment in each well; blue is for control, black shows backgrounds , green is for *O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*),yellow is for β-sitosterol and purple is for rutin

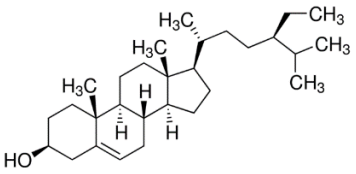
Chapter III

Characterisation of Phytochemicals from Extracts of *M.peregrina*

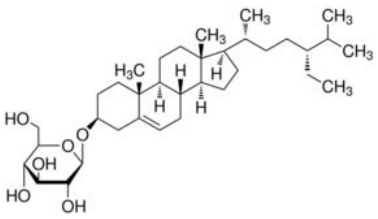
8.1 Introduction

The *in vitro* study of *M.peregrina* extracts in this work revealed that the ethyl acetate and chloroform extracts have the most hypoglycemic effect on HepG2 cells among the six extracts. A phytochemical analysis process was designed for the purification and characterization of components from these two active extracts.

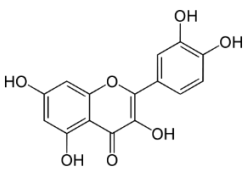
Essential oil purification for *M.peregrina* from its seed and leaves for first time showed that the majority of components are isobutyl isothiocyanate and isopropyl isothiocyanate (Afsharypuor *et al*. 2010), and further studies on the volatile constituents of the seed coat and stem of *M.peregrina* also reported isobutyl isothiocyanate as a main component (Dehshahri *et al.* 2012). Another study on *M.peregrina* assessed levels of glucosinolates in leaves (Bellostas *et al*. 2010). Characterisation of *M.peregrina* extracts described in the latest studies identified a number of components including, β-sitosterol , β-sitosterol-3-O-glucoside, quercetin, quercetin-3-O-rutinoside, β-amyrin, α-amyrin, lupeol acetate, thiocarbamates and isothiocyanate (Taha *et al*. 2011; Ayyari *et al*. 2013).



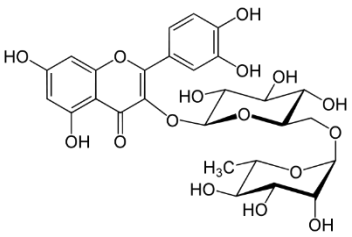
β-sitosterol,



β-sitosterol-3-O-glucoside

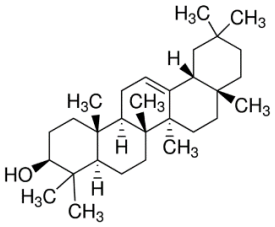


quercetin

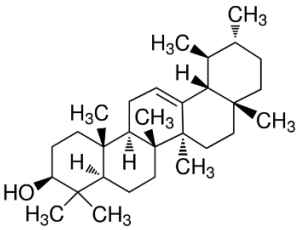


quercetin-3-O-rutinoside

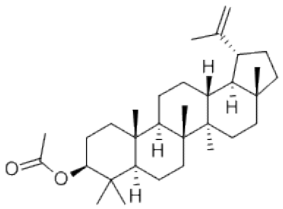
(rutin)



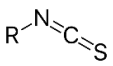
β-amyrin



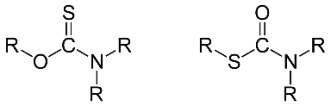
α-amyrin



lupeol acetate



Isothiocyanate



1 **2**

O-organyl (**1**) and *S*-organyl (**2**) Thiocarbamates

In this work column chromatography was chosen as the main technique for the purification process of the ethyl acetate and chloroform extracts while other studies used vacuum liquid chromatography (VLC) for isolation of *M.peregrina* extracts.

Other separation techniques and identification methods includied preparative HPLC, TLC and NMR were also used in this project.

8.2. Isolation of constituents from the EtOAc extract

11 g of ethyl acetate fraction was prepared as described section 2.1.3 and 2 of of this study and separated by column chromatography on silica gel (250g). Two fractions were chosen from the eight on the basis of their purity, quantity and numbers of components - they had fewer constituents in comparison to other fractions according to their chromatograms.

8.3. Fraction 4

From 11 g of Ethyl acetate the total of 20 mL fractions including F1 to F8 , were collected, after evaporating the solvents from the fractions , 1 g of fraction 4 (F-4, 1 g) in semisolid form was obtained. F4 showed three UV active spots on the RP-TLC (MeOH/H_2O 13:7) as shown in Fig. 3.1 .

The purification of a portion of F-4 (400 mg) was achieved by preparative RP-HPLC using isocratic elution with ACN-H_2O 40:60 (flow rate 6.0 mL/min, detection at 247 nm) as mobile phase. Three main constituents were resolved (Figure 3.2).

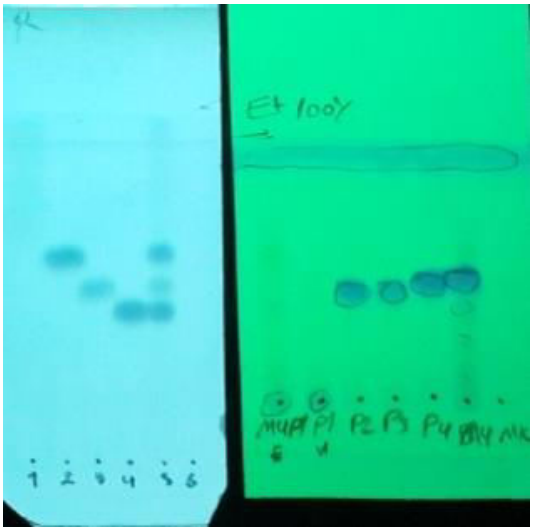


Figure 3.1 Analysis of F-4. Fraction F-4 of Ethyl acetate fraction , before and after preparative HPLC. The right TLC in normal phase could not separate resolve components of F-4 , shows as F4 in first lane from right hand side, lane P4 presence *O*-butyl (2) 4-[(α-L-rhamnosyloxy benzyl] thiocarbamate (*E*) , P3 shows 4(α-L-rhamnosyloxy) benzyl isothiocyanate and P2 shows *O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*) in Fig.31, the right TLC shows the three isolated compounds in normal phase, where all bands are almost have the same mobility while the left TLC in Fig.3.1. is in reverse phase ,number 5 shows F4 seperation , number 2 shows *O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*)),number 3 presence (4(α-L-rhamnosyloxy) benzyl isothiocyanate) and number 4 is for (*O*-butyl (2) 4-[(α-L-rhamnosyloxy benzyl] thiocarbamate (*E*)). The left TLC, presents the three components found in F-4 (shows in number 5 in left TLC) , the bands in lanes 2,3.and 4 are in the same level with their relevent bands in lane 5 , meaning the separation method using preparative HPLC occurred successfully . The TLC plates were visualized at 254 nm.

Further to TLC, Preparative HPLC was performed and revealed three separate peaks, named as 1, 2 and 3 (Fig. 3.2). Following analysis by NMR the structures was identified as described below. (Fig 3.3)

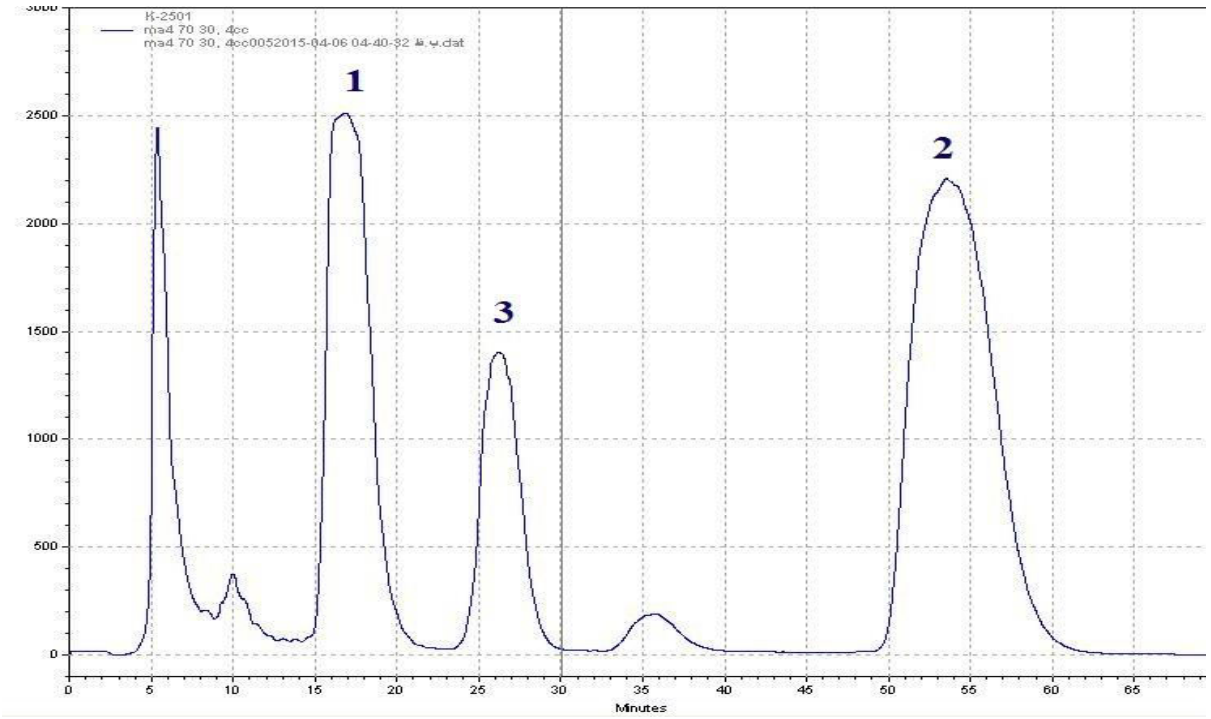


Figure 3.2. Preparative HPLC chromatogram of F-4. , using isocratic elution with ACN-H_2O 40:60 (flow rate 6.0 mL/min, detection at 247 nm) as mobil phase . NMR in DMSO-d6 solvent ,was used to identify the compounds from the peaks. Peak 1 and 2 are thiocarbamate and 3 was the isothiocyanate the separation was monitored at 247 nm.

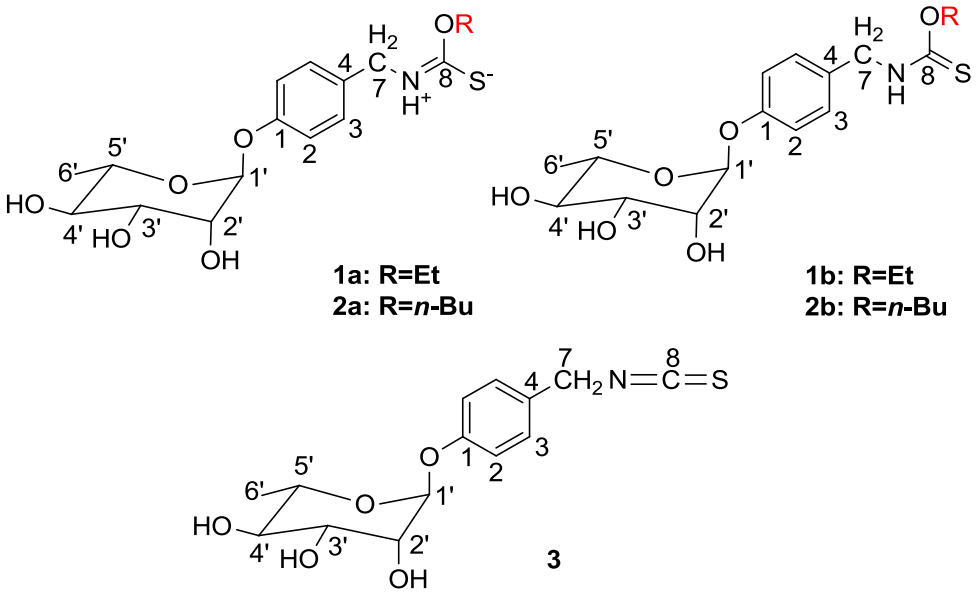


Figure 3.3 the structure of the *O*-ethyl (**1**) and *O*-butyl (**2**) 4-[(α-L-rhamnosyloxy benzyl] thiocarbamate (*E*) in two resonance hybrids, a and b, and 4-(α-L-rhamnosyloxy) benzylisothiocyanate (**3**)

The structures of the three components fractionated by RP-HPLC are shown in Figure 3.3 and their identification is described below.

8.3.1.The first compound, recovered from peak 1 of fraction 4 (compound 1)

According to previous studies, when thiocarbamate phytochemicals were isolated for the first time from *M. Oleifera* two resonance hybrid forms, A and B in Fig. 3.4, were suggested (Faizi*,* 1992*)*. This compound, *O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*) is also called niazimicin.The data for ^{1}HNMR and ^{13}CNMR were compared with previous NMR data from previous study (Ayyari *et al*. 2014) and the following data interpretation is suggested;

^{1}HNMR data are shown in section 1 of Table 3.1 . The compound has two triple gaps in the aliphatic region of 1.22,1.26 ppm and two mixed quadruples in the area of 4.41and 4.42 ppm, that indicate an ethoxy group in the molecule. The chemical shift of the NH group in the region of δ 9.45, also confirms the structure of s-*trans* two forms of resonance. The benzyl hydrogens chemical shift of NH and benzyl molecules in this structure were comparable with those of methoxy derivative forms. ^{13}CNMR spectrum in the area of 192.2 ppm indicates for ethyl thiocarbamate in Fig.3.6.

The structure of these two hybrids resonance is shown in Fig. 3.4., Heating to 40 °C could not alter the structure of these two hybrids or produce only one form. Fig. 3.5 shows the ^{1}HNMR spectrum at 30 °C.

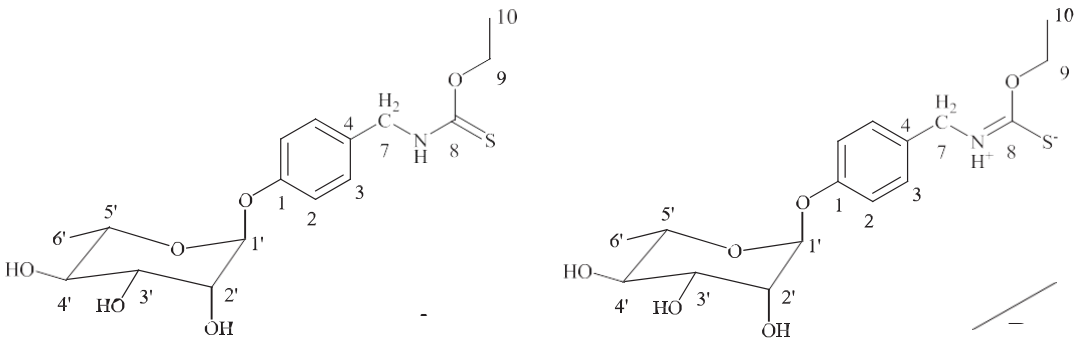


Figure 3.4 Structures of two forms of *O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*) (niazimicin), isolated from the ethyl acetate extract of *M.peregrina*. The difference between these two molecules is the location of the double bond between **C** 8, **N** and **S.** In molecule A the double bond is between carbon number 8 and the nitrogen atom while in molecule B the double bond is between carbon number 8 and the sulphur atom.

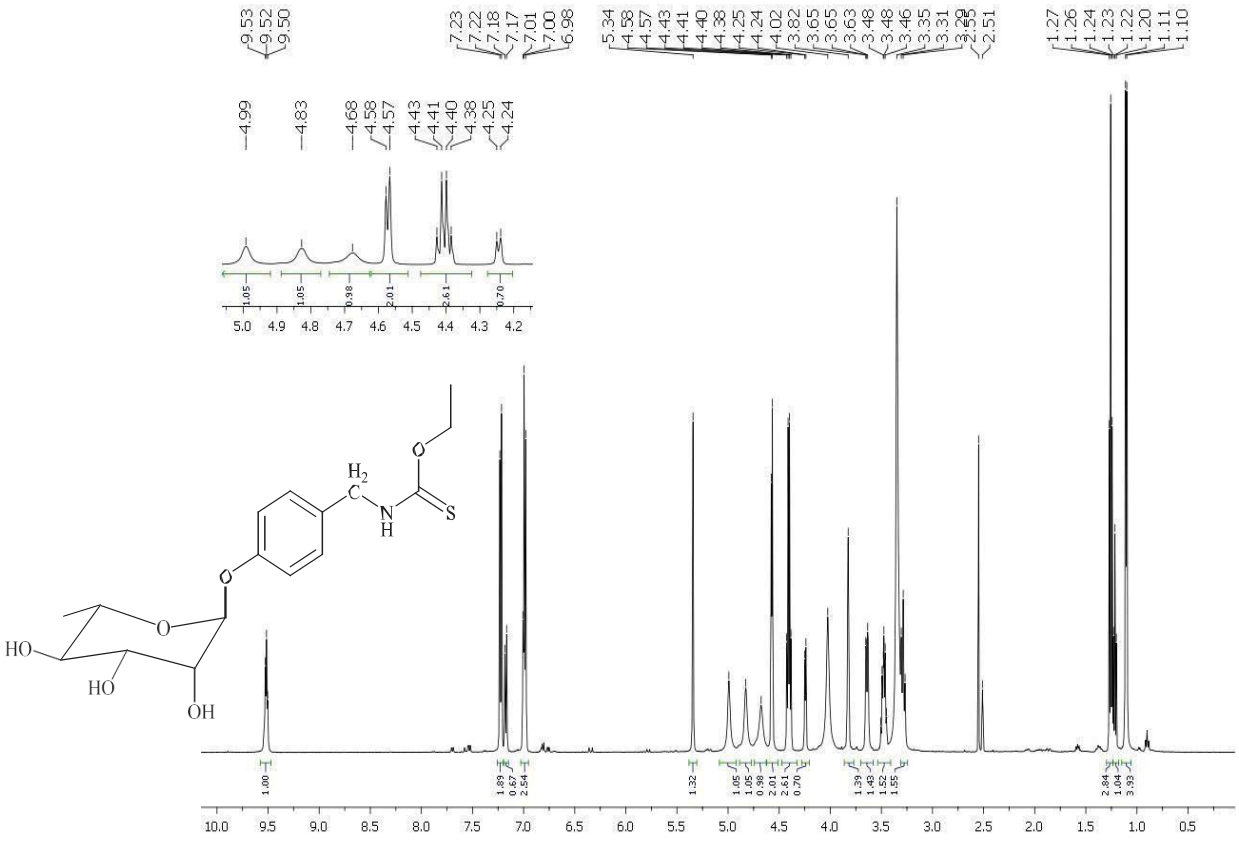


Figure 3.5 ^{1}H-NMR spectrum for compound 1 in DMSO-d$_6$ solvent at 30 °C and 500 MHz. The analysis of the spectrum is discussed in the text.

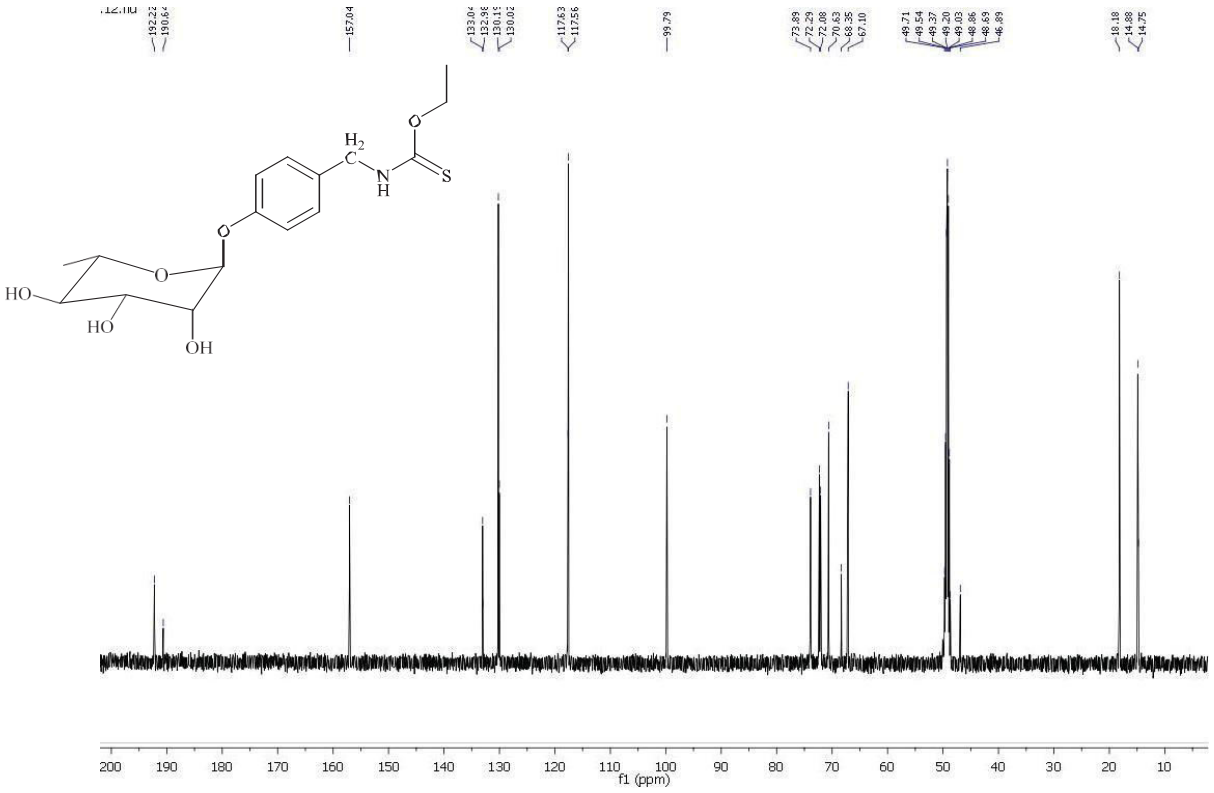


Figure 3.6 ^{13}C-NMR spectrum for compound 1 in DMSO-d6 at 125 MHz

Table 3.1 ^{1}H and ^{13}C NMR spectroscopic data for thiocarbamates glycosidic **1** and **2** and isothiocyanate **3** (δ in ppm and *J* in Hz).

No	**1**		**2**		**3**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1		157.0		157.1		155.9
2	6.99 (d, 8.5) [a]	117.6	6.99 (d, 8.6) [a]	117.5	7.07 (d, 8.6)	116.7
	7.00 (d, 8.5) [b]	117.6	7.00 (d, 8.6) [b]	117.6		
3	7.24 (d, 8.5) [a]	130.2	7.23 (d, 8.6) [a]	130.2	7.31 (d, 8.6)	128.8
	7.18 (d, 8.5) [b]	130.0	7.17 (d, 8.6) [b]	129.8		
4		133.1		133.1		127.9
		133.0		133.1		
5	7.24 (d, 8.5) [a]	130.2	7.23 (d, 8.6) [a]	130.2	7.31 (d, 8.6)	128.8
	7.18 (d, 8.5) [b]	130.0	7.17 (d, 8.6) [b]	129.8		
6	6.99 (d, 8.5) [a]	117.6	6.99 (d, 8.6) [a]	117.5	7.07 (d, 8.6)	116.7
	7.00 (d, 8.5) [b]	117.6	7.00 (d, 8.6) [b]	117.6		
7	4.58 (d, 6.0) [a]	49.0	4.58 (d, 6.0) [a]	49.1	4.84 s	47.5
	4.25 (d, 5.9) [b]	46.9	4.25 (d, 6.1) [b]	46.9		
8		192.2		192.4		128.9
		190.6		190.8		
1'	5.35 (d, 1.5)	99.8	5.35 (d, 1.6)	99.8	5.39 (d, 1.6)	98.3
2'	3.85 (dd, 3.6, 1.5)	72.1	3.84 (dd, 3.6, 1.6)	72.1	3.84 (dd, 3.6, 1.6)	70.1
3'	3.67 (dd, 9.4, 3.6)	72.3	3.65 (dd, 9.4, 3.6)	72.3	3.65 (dd, 9.5, 3.6)	70.4
4'	3.31 (t, 9.4)	73.9	3.30 (t, 9.4)	73.9	3.30 (t, 9.5)	71.7
5'	3.51 (qd, 9.4, 6.1)	70.6	3.49 (qd, 9.4, 6.2)	70.6	3.46 (qd, 9.5,6.1)	69.5
6'	1.12 (d, 6.1)	18.2	1.12 (d, 6.2) [a]	18.2	1.10 (d, 6.1)	17.9
			1.11 (d, 6.2) [b]			
1"	4.42 (q, 7.1) [a]	67.1	4.37 (t, 6.7) [a]	71.1		
	4.41 (q, 7.1) [b]	69.35	4.36 (t, 6.3) [b]	72.2		
2"	1.26 (t, 7.1) [a]	14.88	1.64 (quin, 7.2) [a]	32.1		
	1.22 (t, 7.1) [b]	14.75	1.58 (quin, 7.2) [b]	31.9		
3"			1.37 (sex, 7.5) [a]	20.2		
			1.25 (sex, 7.5) [b]	20.2		
4"			0.91 (t, 7.5) [a]	14.3		
			0.85 (t, 7.4) [b]	14.2		
NH	9.45 (t, 6.0) [a]		9.45 (t, 6.1) [a]			
	9.46 (t, 5.9) [b]		9.49 (t, 6.1) [b]			
2-OH	4.99 br		4.92 br		5.07 br	
3-OH	4.68 br		4.60 br		4.76 br	
4-OH	4.83 br		4.76 br		4.89 br	

Table 3.1 ^{1}H and ^{13}C NMR spectroscopic data for thiocarbamates glycosidic **1** and **2** and isothiocyanate **3** (δ in ppm and *J* in Hz).

8.3.2.A second compound, recovered from peak 2 of fraction 4 (compound 2)

Compound number 2 was another compound isolated from the extract that belongs to the thiocarbamate family. There are similarities between the analytical data of this compound with data from the previously isolated thiocarbamate compound in this project, (Table 3.1) including spectral characteristics, such as carbon, hydrogen, benzylic carbon, sugar group of α-L-rhamnose without acetyl, para-substitution of the benzyl ring with NH and carbon region of 194.4 ppm (Table 3.1).The only difference is that compound 2 has a busy aliphatic area that shows a chain with four carbons, n-butyl, in the molecule. A triplet in the area 4.37 ppm indicated that a CH_2 of this group was bound to oxygen, which has a connection with carbon 71.07 ppm in the HSQC spectra. Similarity five in 1.64 , six in 1.37 and triple in 0.91 ppm related to other hydrogens of butyl group that are bonded to carbons 14.30, 20.21 and 32/05 ppm respectively. Hybrid resonance in these compounds can be seen clearly. This combination, as with the two other compounds, is s-trans type and the ratio of hybrids forms are 2.5 and 1. Form A constitutes a major form, and the compound was identified and named as *O*-Butyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*). ^{13}C-NMR and ^{1}H-NMR data are provided in table3.1.

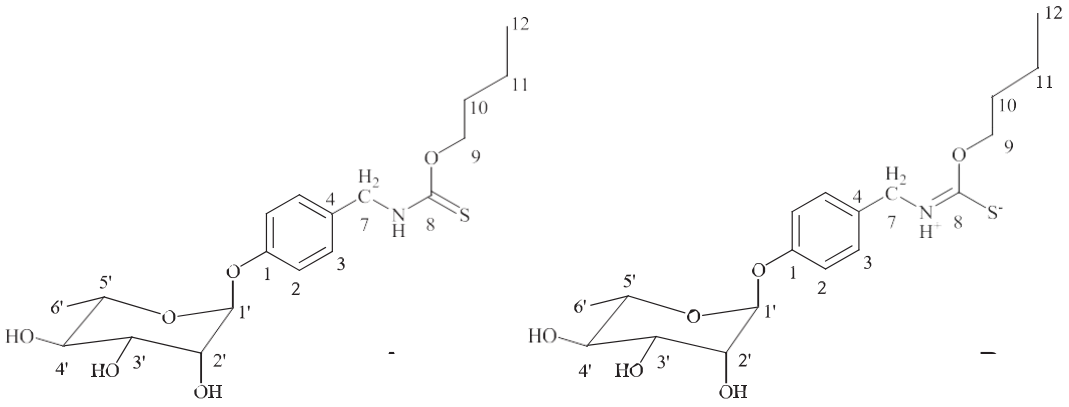


Figure 3.7 Structures of two forms of *O*-Butyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*) isolated from the ethyl acetate extract of *M.peregrina*.

Comparing the analytical data obtained for compound number 1 and 2 with data from previous studies (Ayyari *et al*. 2013) has confirmed the structures. Other details can be extracted from the data.

For *O*-ethyl (**1**) and *O*-butyl (**2**) 4-[(α-L-rhamnosyloxy benzyl] thiocarbamate (*E*) (compound number 1), the resonance hybrid proportion is 2 to 1 and resonance hybrid A is the main structure with molecular weight of 357.1246, calculated according to to its molecular formula,$C_{16}H_{23}NO_6S$.

For compound 2, *O*-Butyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*), the resonance hybrid proportion is 2.5 to 1 and resonance hybrid A is the main structure with molecular weight of 385.1559 calculated on the basis of its molecular formula $C_{18}H_{27}NO_6S$.

8.3.3. A third compound, recovered from peak 3 of fraction 4 (compound 3)

This is peak number 3 from the preparative HPLC of fraction 4 (Fig. 3.2) with a peak absorption of 1400. The structure of this compound is different from two others.

The spectrum in both the ^{1}H-NMR and ^{13}C-NMR in the area of sugar molecules are same. There was no evidence of carbonyl thiocarbamate in the area of 193 ppm, in the glycon part. In the ^{1}H-NMR spectrum the hydrogen benzyl spectrum appeared slightly to the lower side in the area of 4.86 ppm. This molecules does not have hybrid resonance; it was as a single, pure component. The aromatic part of this molecule has a benzyl structure with para substitute same as the other two molecules. According to previous studies on the isolation of *Moringa* extracts (Dehshahri *at el.*2012),the spectral data are very similar. From that follows that the structure must be similar to the structure of isothiocyanate, also confirmed by carbon chemical shifts. Carbon number 8 in the structure of the isothiocyanate in the area of 128.9 ppm appeared to be very weak. The spectrum for this carbon sometimes did not appear in the ^{13}C-NMR spectrum therefore other scans were required to show the different spectra for this molecule such as HSQC, APT,^{13}C-NMR, ^{1}H-NMR (Fig 3.9 to Fig.3.12). Spectrum data are shown in Table 3.1.

The calculated molecular weight for this compound is 311.0827, and the weight of 2M+H and 2M-H can be observed on the positive and negative mode of ITMS (Fig. 3.13, Fig.3.14).

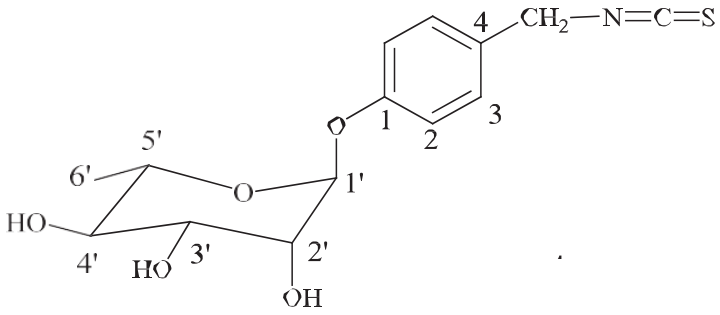


Figure 3.8 Structure of 4(α-L-rhamnosyloxy) benzyl isothiocyanate isolated from the ethyl acetate extract of *M.peregrina*.

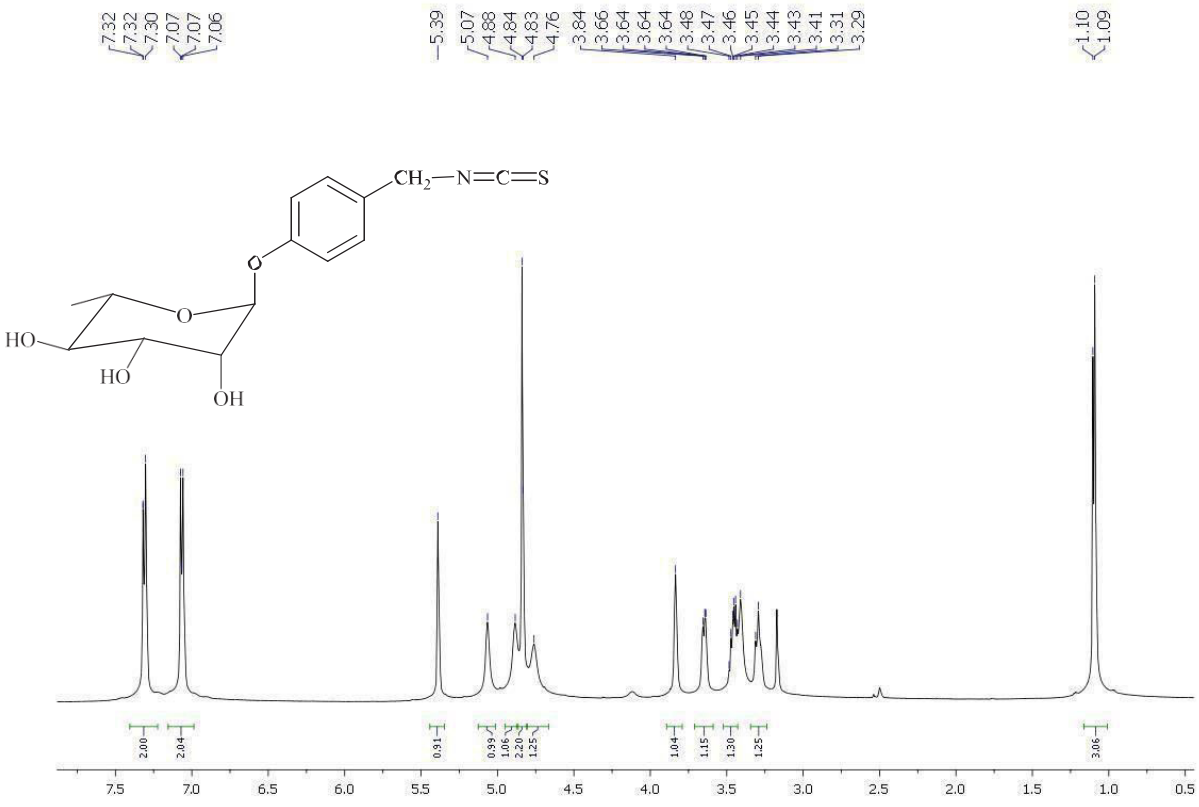


Figure 3.9 ^{1}H-NMR spectrum for compound 3 in DMSO-d6 at 500 MHz.

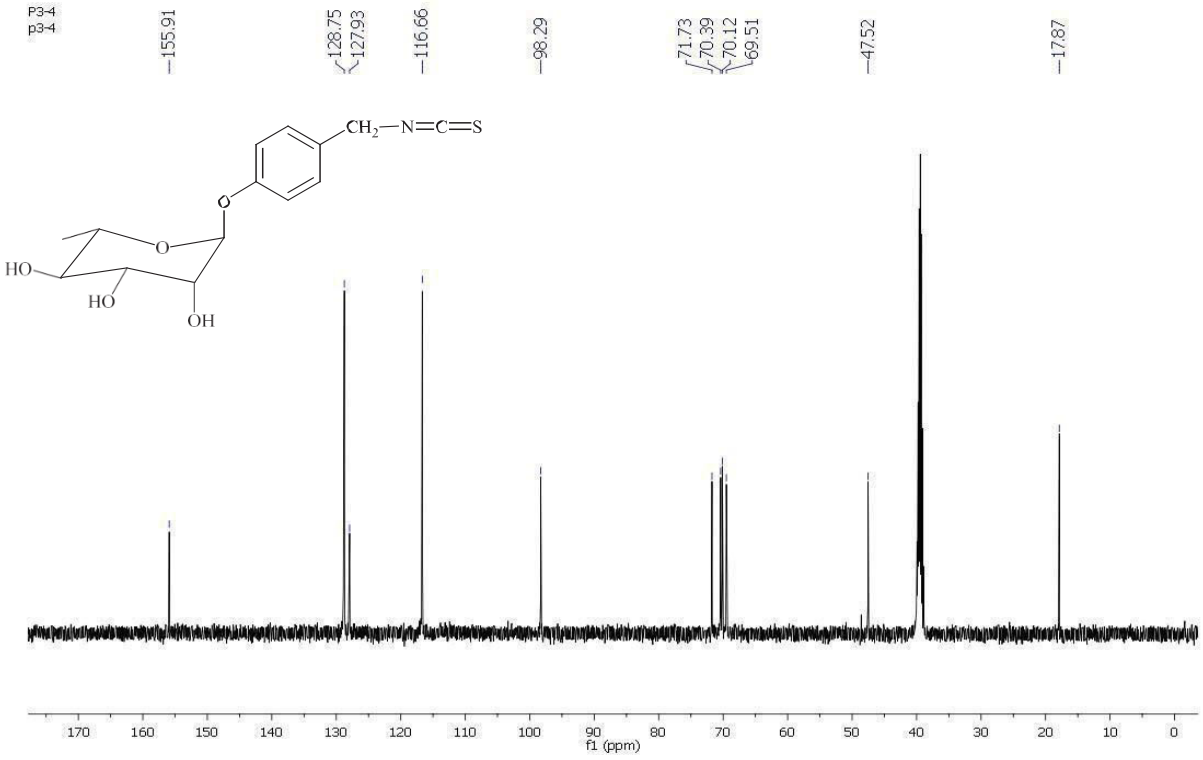


Figure 3.10 ^{13}C-NMR spectrum for compound 3 in DMSO-d_6 at 125 MHz.

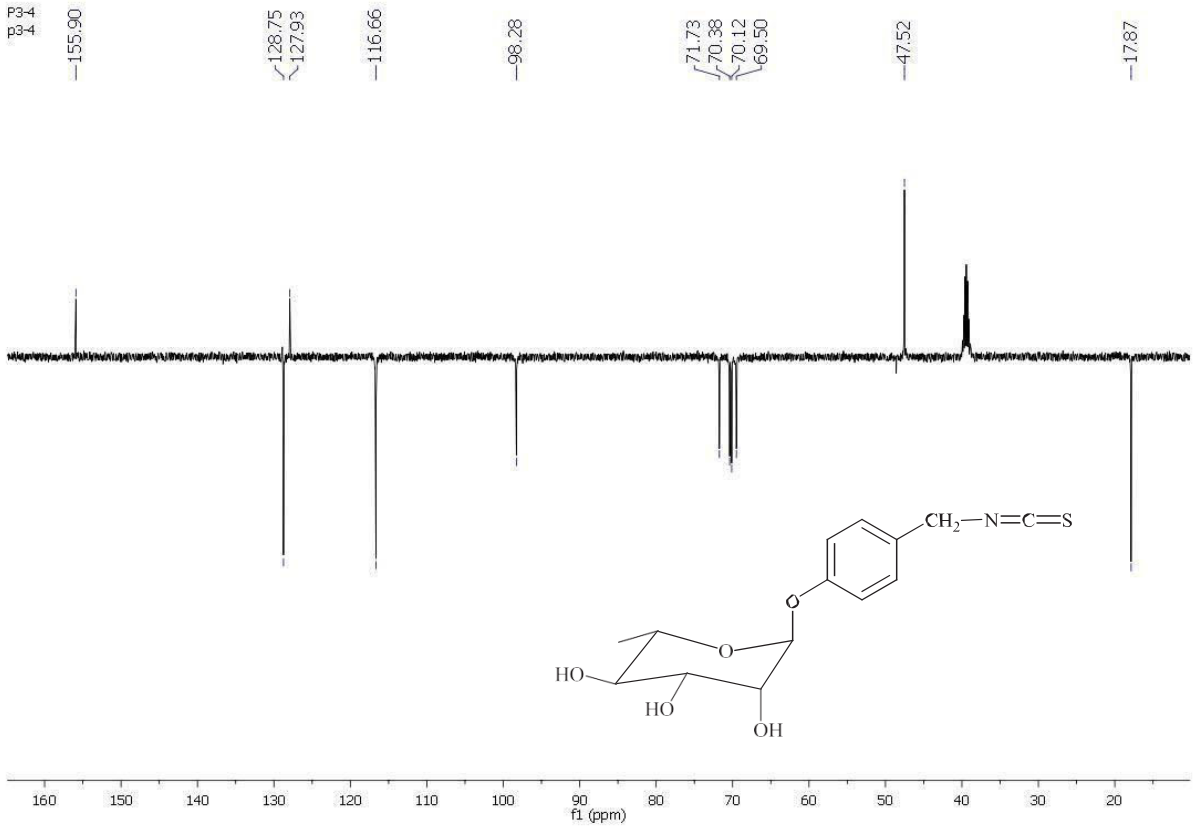


Figure 3.11 APT spectrum for compound 3 in DMSO-d6 at 125 MHz.

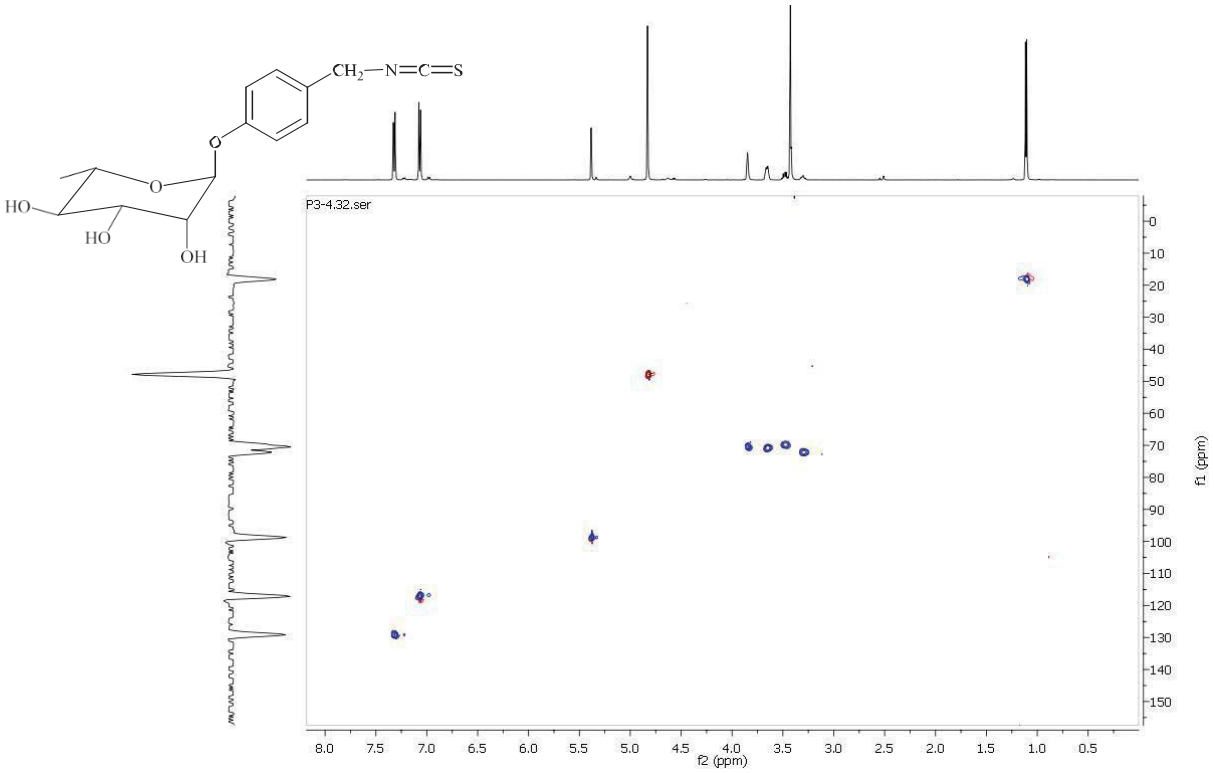


Figure 3.12 Two dimensions HSQC spectrum, hydrogen carbon bond for compound 3 in DMSO-d6 .

Compound 3 is identified as 4-(α-L-rhamnosyloxy) benzyl isothiocyanate, and has known strong anti-bacterial effects, having been previously isolated form *M. stenopetala*, *M. oleifera* and *M. peregrina* (Eilert, 2007).

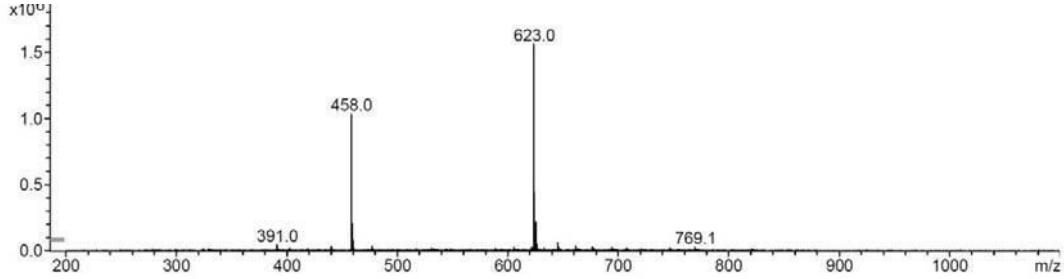


Figure 3.13 The mass spectrum in plus mode of ITMS for compound 3 isolated from the ethyl acetate fraction of *M.peregrina*.

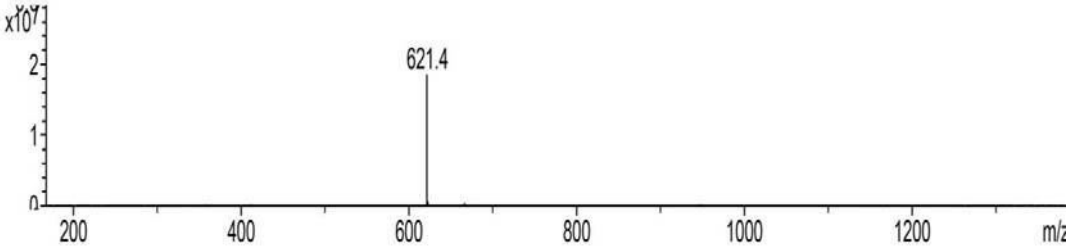


Figure 3.14 The mass spectrum in minus mode of ITMS for compound 3 isolated from the ethyl acetate fraction of *M.peregrina*.

8.4. Fraction 8

150 mg of fraction 8 (F-8) were separated by preparative RP-HPLC using isocratic elution with MeOH-H_2O 45:55 (flow rate 6.0 mL/min, detection at 360 nm) as mobile phase and after completion of analysis by [^{1}H-NMR and ^{13}CNMR] two compounds were identified.

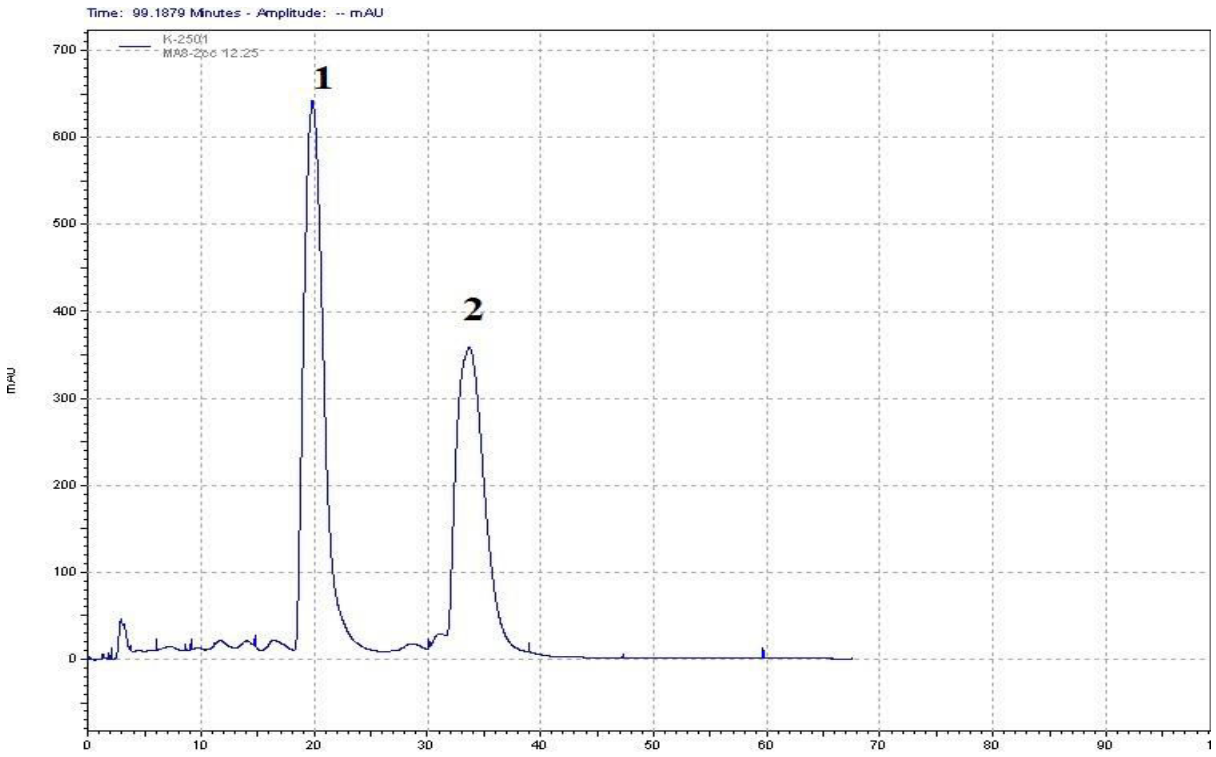


Figure 3.15 Separation of two flavonoids from fraction 8 of the ethylacetate extract of *M. peregrina*, detected at 360 nm, by preparative HPLC.

5.4.1. First compound isolated from fraction 8 (compound 4)

A fourth compound was isolated from fraction 8 using preparative HPLC (peak 1) and its flavonoid glycoside structure was identified on the basis of ^{13}C-NMR in Fig.3.18, ^{1}H-NMR shows in Fig.3.17 , ATP spectrum shows in Fig. 3.19 and Mass Spectrometry (MS) in Fig. 3.20 and Fig. 3.21 data .The UV analysis of this compound also confirmed the flavonoid structure. Hydrogens 6 and 8 with 2.0 Hz

shows the *meta* gap and as a result the substitution of carbon 5 and 7 was confirmed. If the sugar molecules were connected to carbon 7 then the chemical shift of carbon 2 would be transferred to 147 ppm; therefore the sugar molecules are connected to carbon 3 (in this case the chemical shift of carbon number 2 was 156/8 ppm). The ^{1}H-NMR spectrum shows another three hydrogens in the aromatic region, a doublet for hydrogen 5 with a gap of 8.4 Hz has only one *ortho* neighbour, and hydrogen 6, which appears in the form of two doublets with gaps of 8.4 and 2.0 Hz, shows two neighbours, *ortho* and *meta*. Hydrogen 2 with only one 2 Hz *meta* gap shows its connection with hydrogen 6. In the sugar part, glucose with a beta bond also attached to the flavonoid is confirmed with 5.4 shift in 7.4 Hertz gap. The rhamnose connection is in the alpha configuration as indicated by the 4.41 shift and the hydrogen anomeric gap of 1.1 Hertz. The carbonyl carbon and the methoxy group, appeared at 177.6 and 55.9 ppm, repectively. Carbon data and types of the carbons are listed in Table 3.2. Fig. 3.17 to Fig. 3.19 , show the spectra obtained by ^{13}C-NMR, ^{1}H-NMR and APT for this compound. The chemical shifts of this compound are consistent with the name 3′-methyl-quercetin-3-*O*-rutenoside. In flavonoids with two sugars attached to the glycon, rhamnose is always in the second position (Agrawal 1989).

Figure 3.20 shows the molecule mass of 624.7 in the positive mode of ITMS related to M+H. In the negative mode of ITMS related mass to M-H was 623/8 (Figure 3.21). The structure of the molecule is shown in Figure 3.16 , Rutinoside is the connection between 1 from α-L-rhamnopyranosyl and 6-β-D-glucopyranoside

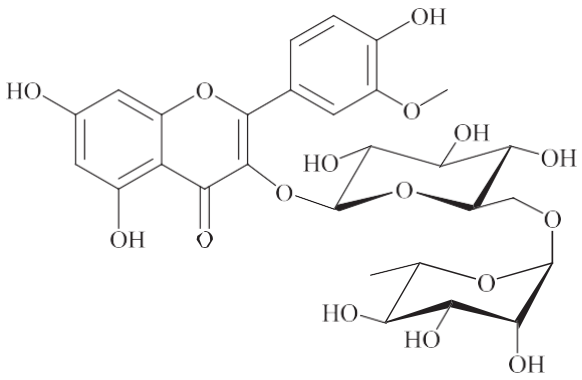


Figure 3.16 Chemical structure of 3′-methyl-quercetin-3-*O*-rutinoside.

^{1}H-NMR and ^{13}C-NMR data for compound 4, 3′-methyl-quercetin-3-*O*-rutinoside (Fig.3.16, Fig.3.17).

^{1}HNMR (500 MHz, DMSO-d_6) δ 7.83 (d, *J* = 2.0 Hz, 1H), 7.50 (dd, *J* = 8.4, 2.0 Hz, 1H), 6.90 (d, *J* = 8.4 Hz, 1H), 6.43 (d, *J* = 2.0 Hz, 1H), 6.20 (d, *J* = 2.0 Hz, 1H), 5.41 (d, *J* = 7.4 Hz, 1H), 4.40 (d, *J* = 1.1 Hz, 1H), 3.82 (s, 3H), 0.95 (d, *J* = 6.2 Hz, 3H).

^{13}CNMR (125 MHz, DMSO-d_6) δ 177.6, 164.5, 161.4, 156.9, 156.8, 149.6, 147.2, 133.3, 122.6, 121.3, 115.5, 113.5, 104.3, 101.4, 101.2, 99.1, 94.1, 76.6, 76.1, 74.5, 72.0, 70.8, 70.6, 70.4, 68.6, 67.2, 55.9, 17.9.

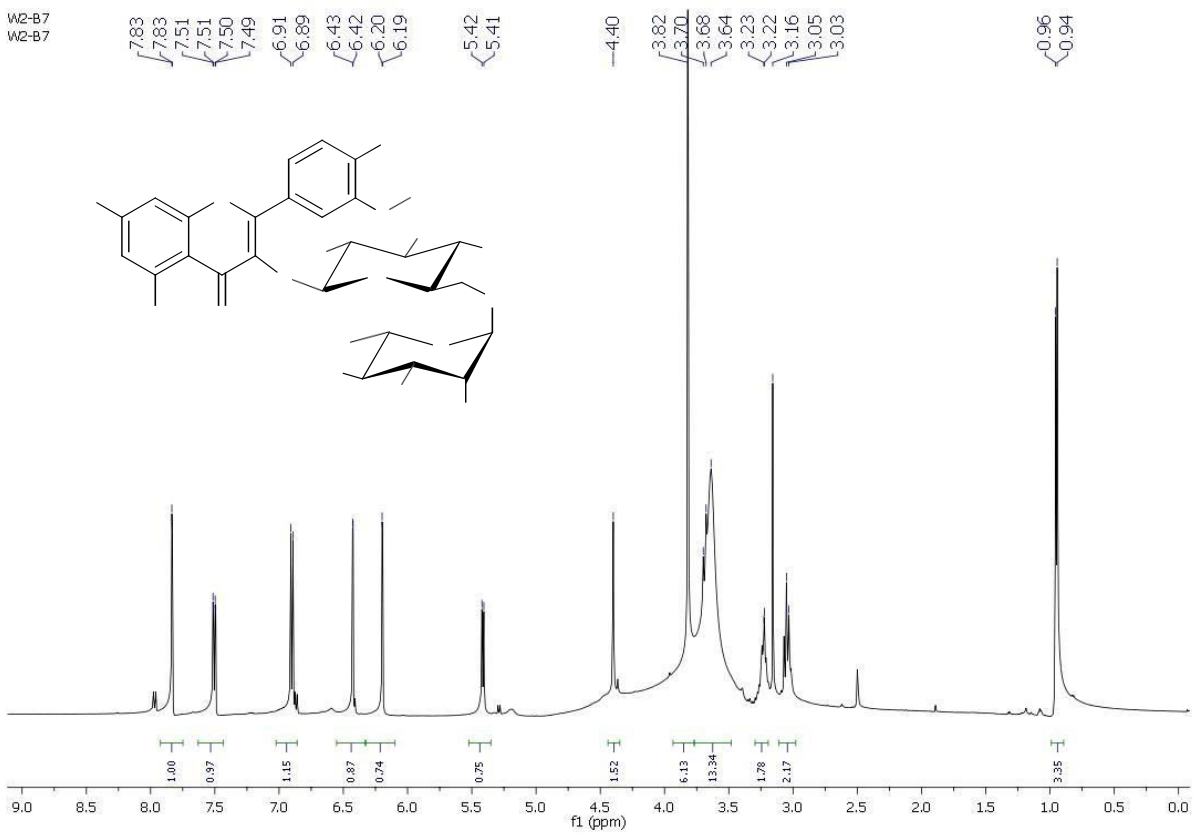


Figure 3.17 ^{1}H-NMR spectrum of compound 4 in DMSO-d$_6$ at 500 MHz

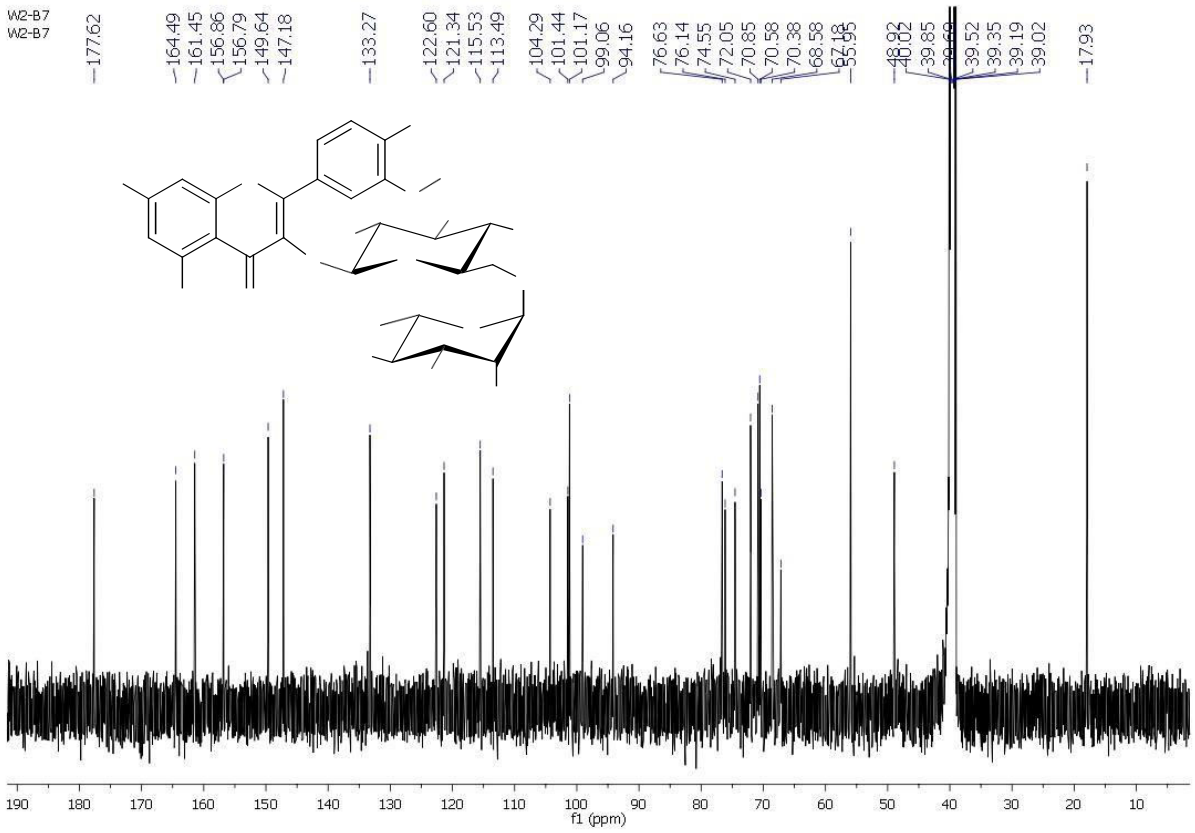


Figure 3.18 ^{13}C-NMR spectrum in DMSO-d6 at 125 MHz

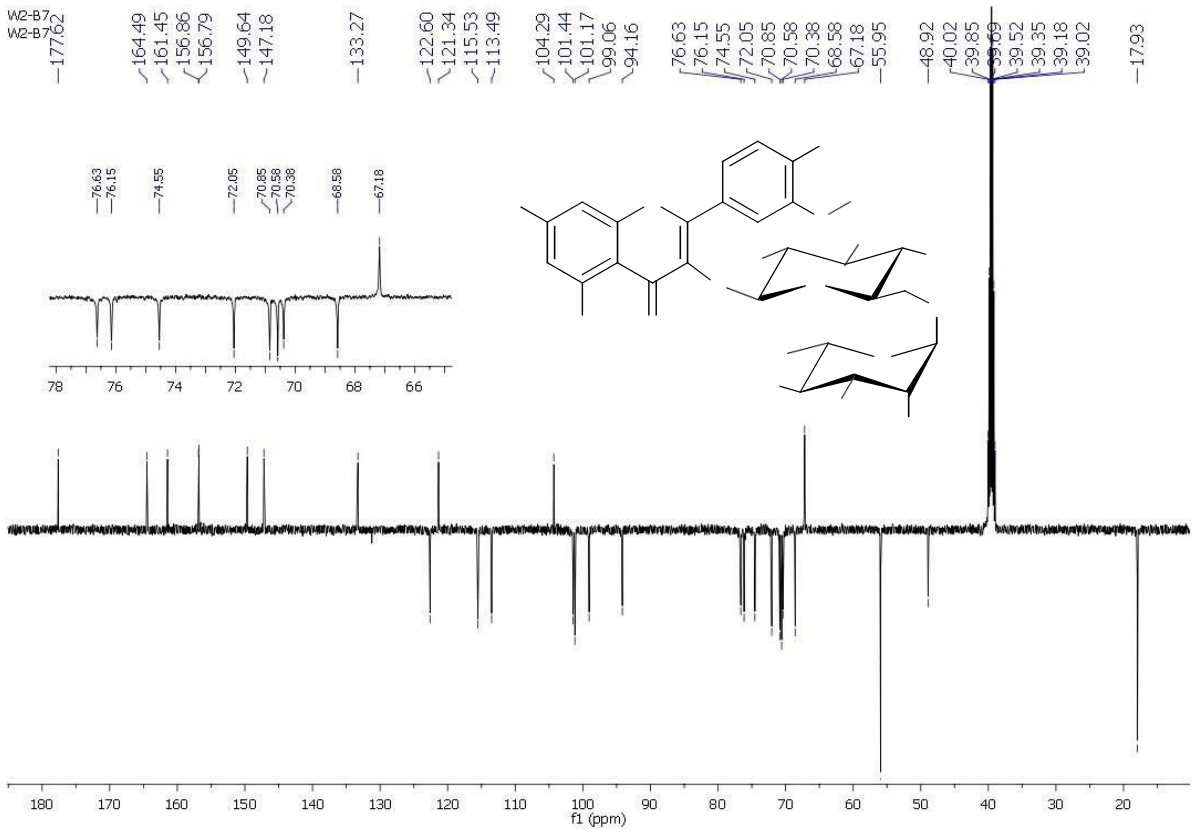


Figure 3.19 APT spectrum of compound 4 in DMSO-d$_6$ at 125 MHz.

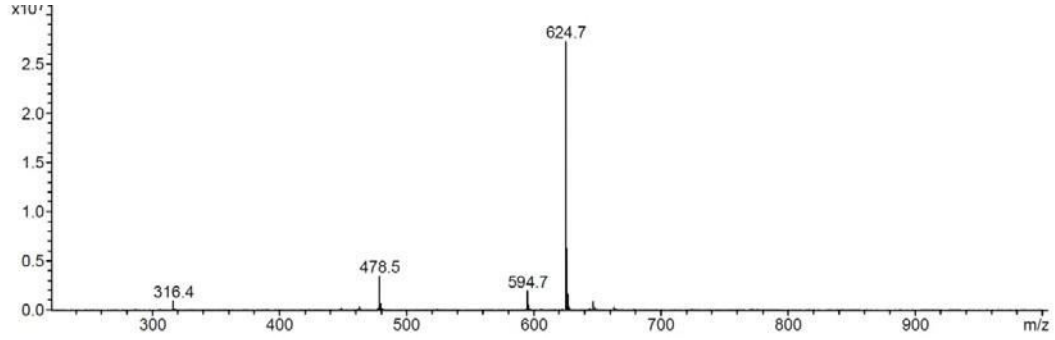


Figure 3.20 Mass spectrum of compound 4 in positive mode of ITMS isolated from the ethyl acetate fraction of *M.peregrina.*

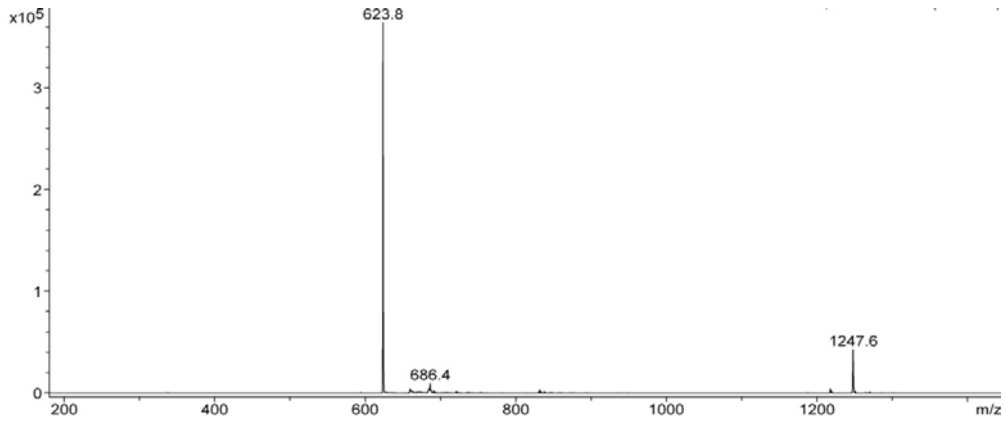


Figure 3.21 Mass spectrum of compound 4 in minus mode of ITMS from the ethyl acetate fraction of *M.peregrina.*

8.4.2. Second compound isolated from fraction 8 (compound 5)

The next constituent was recovered from the second peak shown in Fig.3.15 . After removal of solvent a yellow solid was redissolved for HPLC analysis, the compound is substantially pure more than 90% purity , as indicated by a separate major peak. The observed UV pattern was the flavonoid pattern that usually appears, with two spaced peaks in the area of 265 and 366 nm (Fig. 3.22 upper right panel and lower right panel).

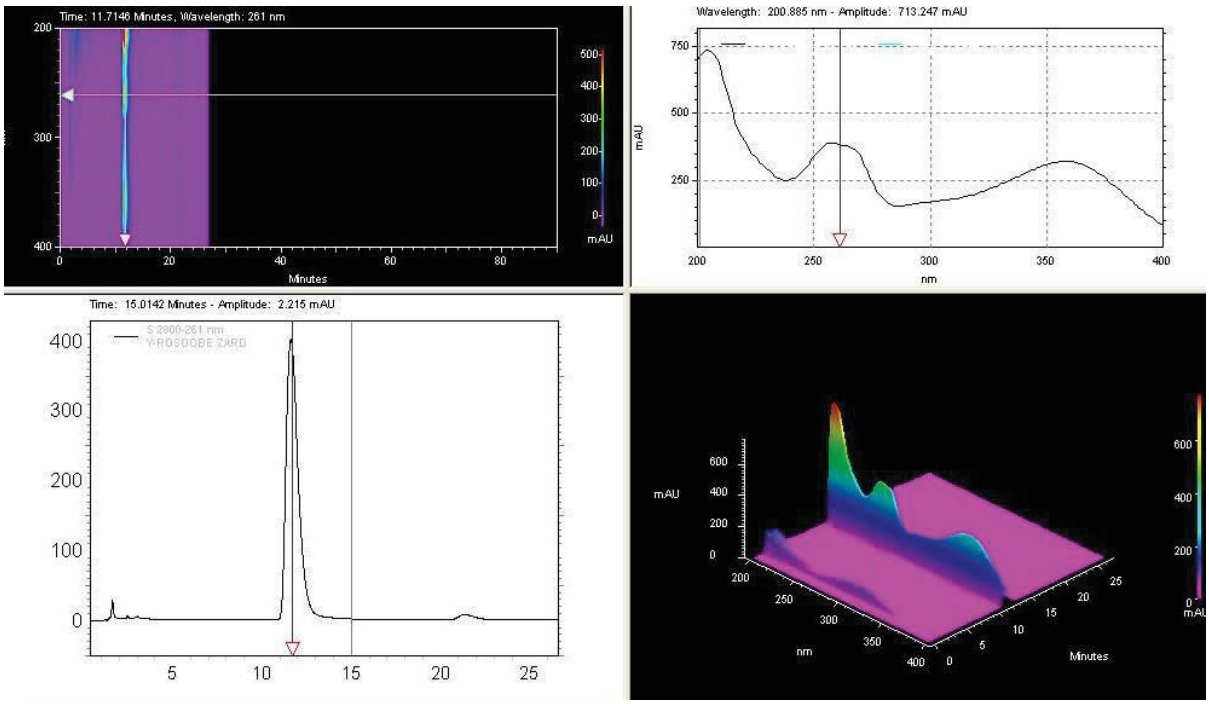


Figure 3.22 UV pattern of flavonoid structure for the second compound isolated from F8 (Compound 5). Right side of the panel shows flavonoid pattern ,including top right for two dimentions chromatogram and lower right for three dimentions UV pttern. Left side top is one dimention view of lower right of the panel and lower left is a two dimentions chromatograms of top left.

In the ^{1}H-NMR spectrum of compound 5, splits are not clearly identified due to peaks from sugers regions of the molecules, from 3 to 4 ppm shows in Fig. 3.24 ,but on the basis of hydrogen chemical shifts and also ^{13}C-NMR in Fig. 3.25 and ATP spectrum shows in Fig 3.26, identification of this compound could be completed. The absence of a methoxy group in the area of 55 ppm shows the difference between this molecule and 3ˈ-methyl-quercetin-3-*O*-rutnoside. Substitution of hydroxy instead of methoxy caused a chemical shift of some carbons in ring B. This compound was identified as rutin which is a combination of glucose ruteinoside and glycon quercetin. The chemical structure of the compound is shown in 3.23. The list of carbons and their chemical shifts for compounds 5 and 6 are shown in Table 3.2. These two compounds have been previously isolated from *M.peregrina* (Elbatran *et al*. 2005; El-Alfy *et al*. 2011).

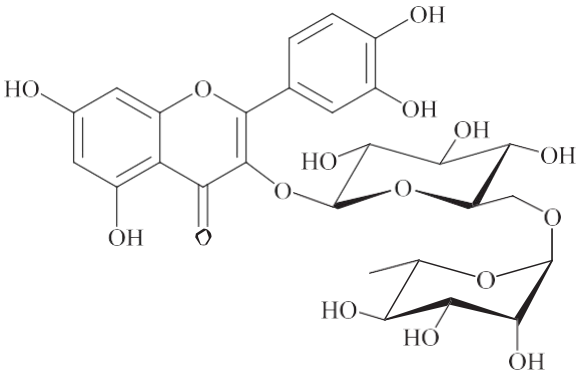


Figure 3.23 Chemical structure of compound 6 (rutin).

The complete name of rutin is quercetin-3-*O*-α-L-rhamnopyranosyl-1-(1→6)-β-D-glucopyranoside.

^{1}H-NMR and ^{13}C-NMR data for compound five (Rutin)

^{1}HNMR (500 MHz, DMSO-d_6) δ 7.55 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.54 (d, *J* = 2.0 Hz, 1H), 6.86 (dd, *J* = 8.3, 2.0 Hz, 1H), 6.41 (d, *J* = 2.0 Hz, 1H), 6.21 (*J* = 2.0 Hz, 1H), 5.34 (d, *J* = 7.0 Hz, 1H), 4.39 (d, *J* = 1.1 Hz, 1H), 0.99 (d, *J* = 6.1 Hz, 3H).

^{13}CNMR (125 MHz, DMSO-d_6) δ 177.3, 163.9, 161.1, 156.6, 156.4, 148.2, 144.6, 133.2, 121.6, 121.1, 116.2, 115.1, 103.9, 101.1, 100.7, 98.6, 93.5, 76.3, 75.8, 74.0, 71.7, 70.4, 70.3, 69.9, 68.1, 66.9, 17.6

		δ ^{13}CNMR (ppm)	
	Carbon number	**Compound 4**	**Compound 5**
C	2	156/8	156/4
C	3	133/3	133/2
C	4	177/6	177/3
C	5	161/5	161/1
CH	6	99/1	98/6
C	7	164/5	164/0
CH	8	94/2	93/6
C	9	156/9	156/6
C	10	104/3	103/9
C	1'	121/3	121/1
CH	2'	113/5	116/2
C	3'	149/6	144/6
C	4'	147/2	148/3
CH	5'	115/5	115/1
CH	6'	122/6	121/6
CH	1''	101/4	101/1
CH	2''	74/6	74/0
CH	3''	76/6	76/3
CH	4''	70/6	70/3
CH	5''	76/1	75/8
CH_2	6''	67/2	66/9
CH	1'''	101/2	100/7
CH	2'''	70/4	69/9
CH	3'''	70/9	70/4
CH	4'''	72/1	71/7
CH	5'''	68/6	68/2
CH_3	6'''	17/9	17/6
OCH_3	3'	55/9	-

Table 3.2. Carbon data and types of the carbons for the first and second purified compounds from F8.

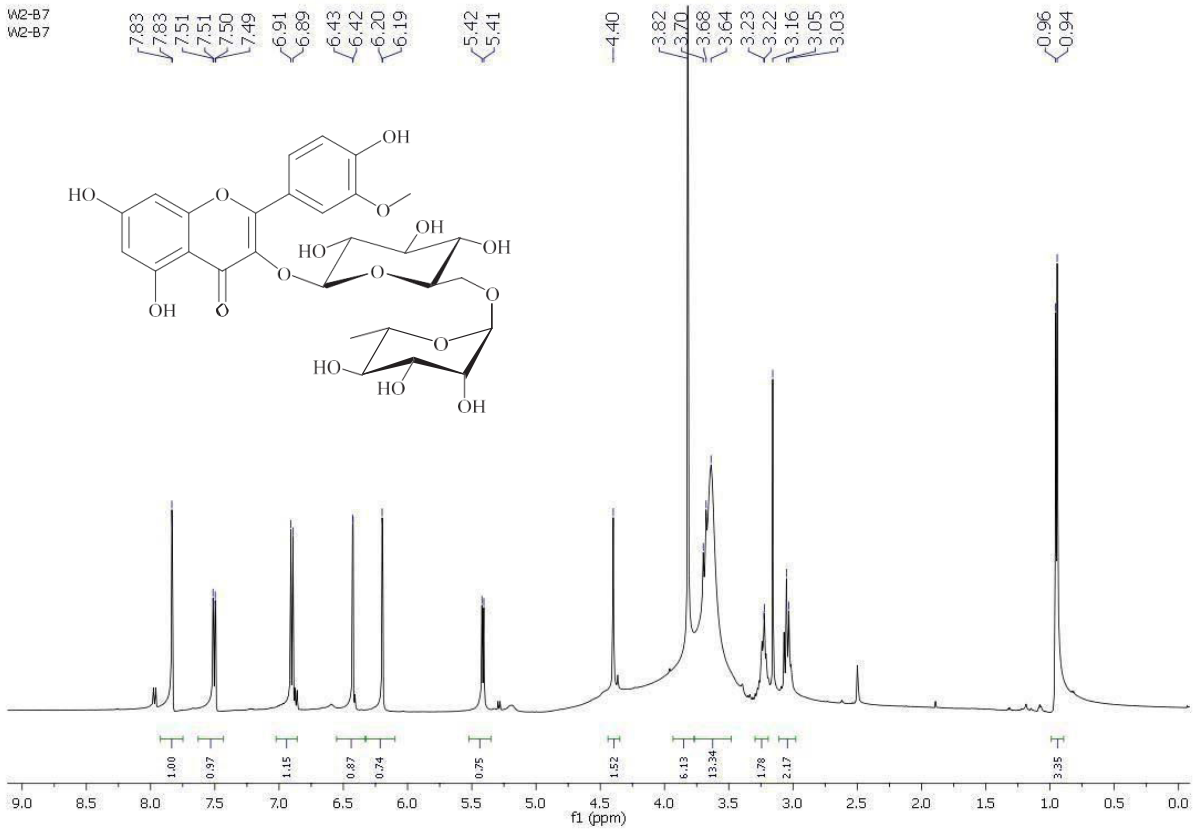


Figure 3.24 ^{1}H-NMR spectrum for rutin in at DMSO-d$_6$ 500 MHz

The splits between 3 to 4 ppm are not cleare due to suger molecules.

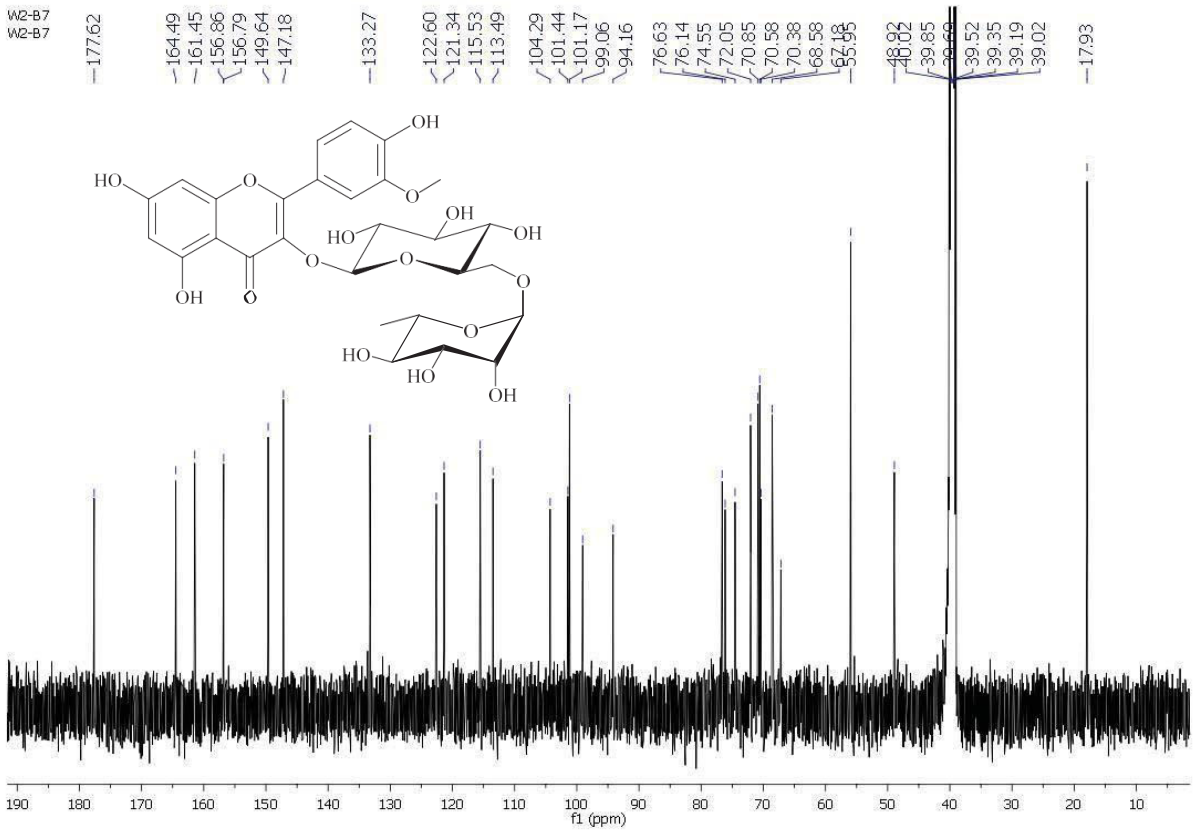


Figure 3.25 ^{13}C-NMR spectrum for rutin in DMSO-d$_6$ at 125 MHz

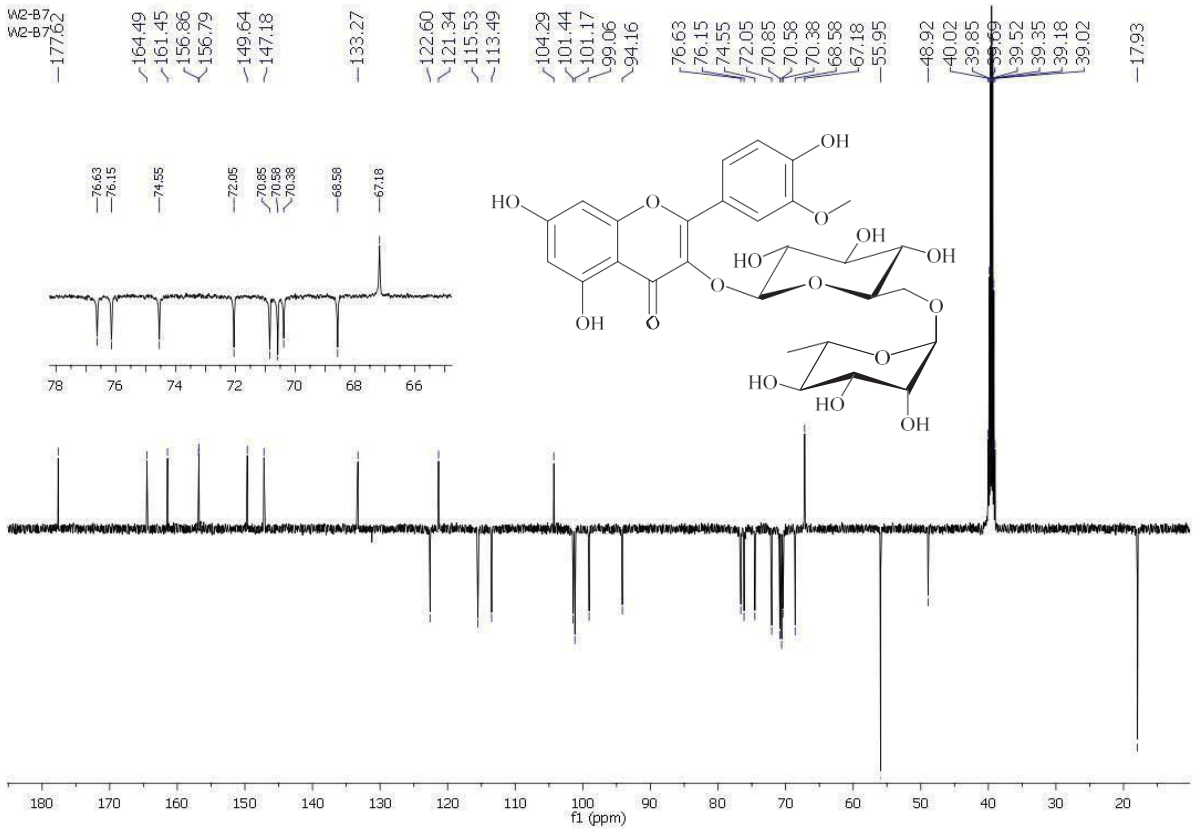


Figure 3.26 ATP spectrum for rutin in DMSO-d6 at 125 MHz

8.5. Isolation of constituents from the chloroform extract

10 g of Chloroform fraction was prepared as described in section 1.3 and fractionated by column chromatography using silica gel (220 g).. Two fractions, **C-4** and **C-13** were chosen for further study and identification of constituent molecules.

8.5.1. First compound isolated from the chloroform extract (compound 6)

The fraction **C-4** from the hexane/ethyl acetate gradient at (85:15) gave a white precipitate (30 mg) after removal of solvent. It was confirmed by comparison with the standard of β–sitosterol in our laboratory by TLC.

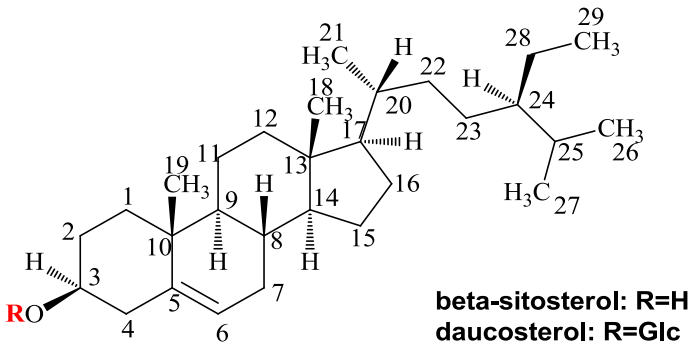


Figure 3.27 Structure of β-Sitosterol and daucosterol isolated from chloroform extract of *M. peregrina*.

8.5.2.Second compound isolated from the chloroform extract (compound 7)

The fraction **C-13** from the 95:5 part of the ethyl acetate/methanol gradient gave a white precipitate by adding methanol to the material concentrated from **C-13**. The TLC spot of the compound and the ^{1}H-NMR and ^{13}C-NMR analysis established the

structure as that of daucosterol, which is a glycoside of β-sitosterol (3.7 mg, >97%, ^{1}H-NMR).

Different NMR spectra of compound C-13 showed the structure of a steroid glycoside and the presence of a double signal in the region of 5/02 ppm with coupling index of 7.7 hertz are sign of beta anomeric proton connection in the region of 3.9 to 4.5.

Proton of sugar glucose with other protons attached to an oxygen atom, was observed. One olefin proton in the area of 5.34 ppm has appeared. ^{13}C-NMR in pyridine solvent was therefore carried out and revealed a triple peak in the area of 124,136,150 ppm, and two olefin carbons also at 141.4 and 122.4 ppm. One signal, related to the anomeric carbon, was seen at 103/0 ppm. Sugar carbons and one other carbon were seen in the region of 63-78 ppm (Fig. 3.26 and Fig. 3.27).

According to the TLC spot, NMRs (Fig. 3.26 and Fig. 3.27) and ATP (Fig.3.28) data and reference standards available in the laboratory this compound was identified as β-sitosterol-3-O-β-D-glucoside or daucosterol, with the structure shown in Fig. 3.24 ,Daucosterol also had been isolated and identified from other plants in the past, including *Phyllenthus emblica*, *Arctotis arctotoides* and *Cryptolepis obtuse* (Paulo *et al*. 2000; Sultana, *et al.* 2007).

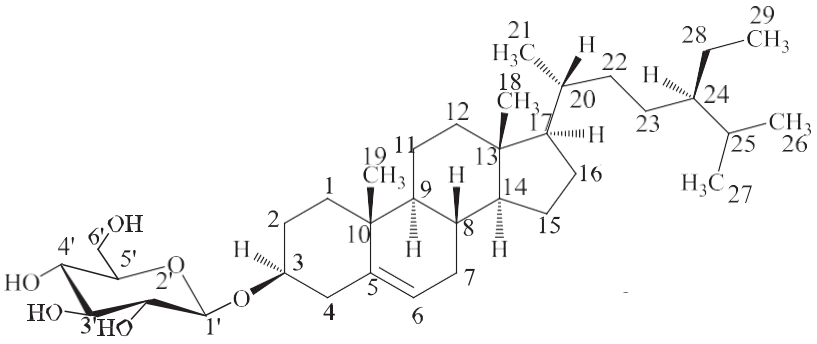


Figure 3.28 Structure of daucosterol isolated from chloroform extract of *M. peregrina*.

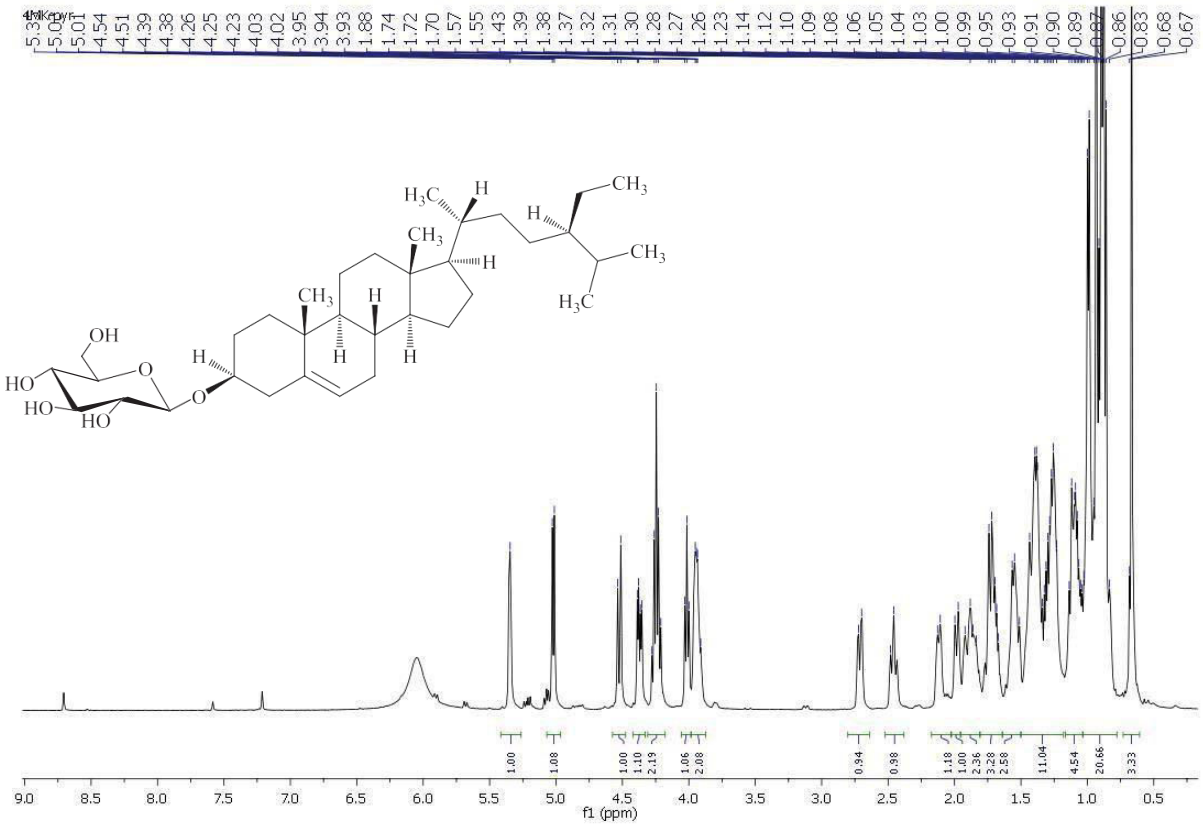


Figure 3.29 ^{1}H-NMR spectrum for daucosterol in pyridine-d5 and 5% of CD_3OD at 500 MHz.

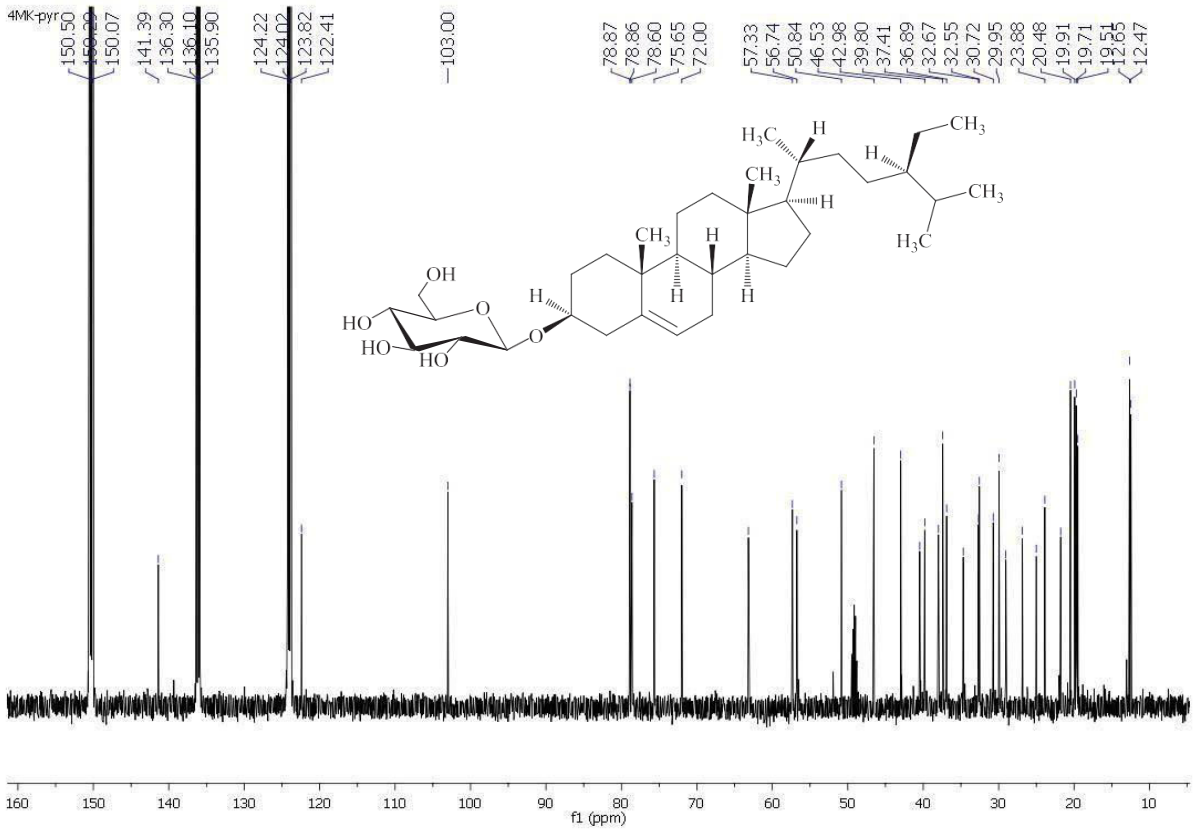


Figure 3.30 ^{13}C-NMR for daucosterol, in pyridine-d5 with 5% of CD_3OD at 125 MHz.

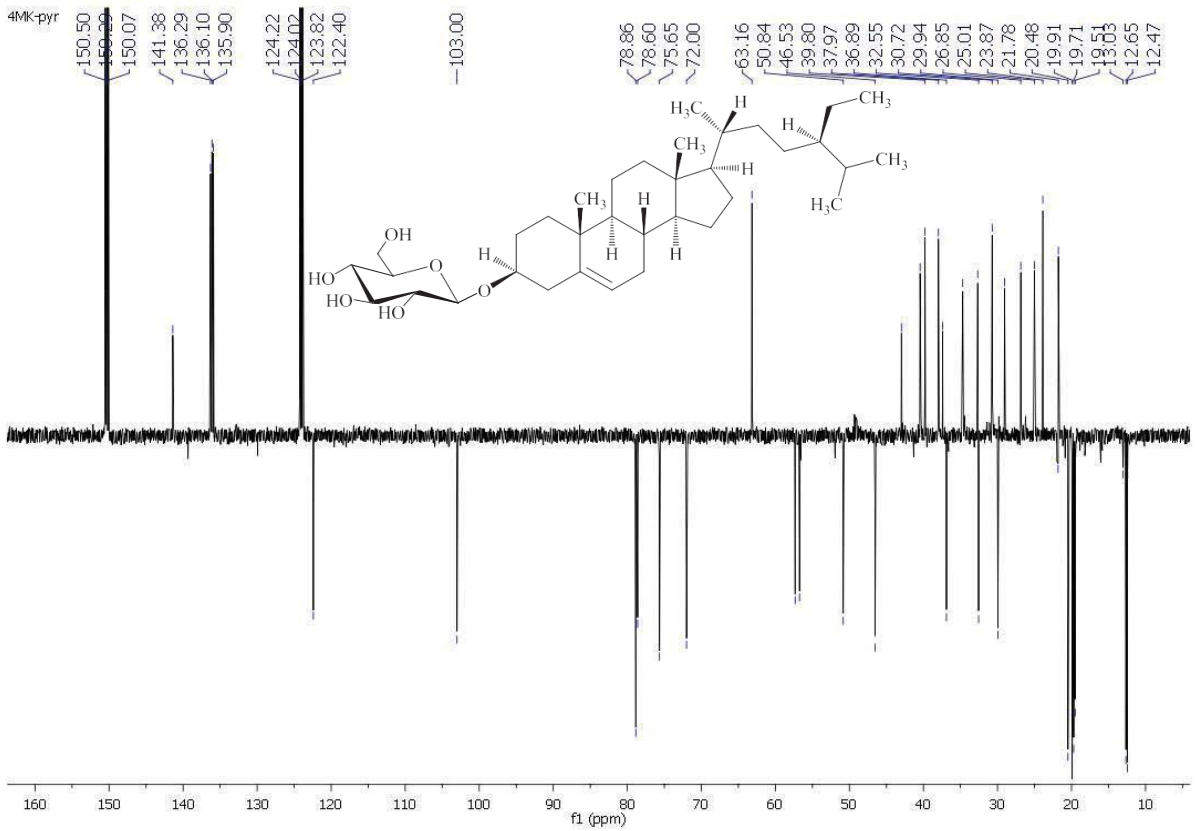


Figure 3.31 ATP spectrum for daucosterol in pyridine-d5 with 5% of CD_3OD at 125 MHz.

Chapter IV
In-Vitro Study

Part I
Glucose uptake

9.1.1. Introduction

Generally, *in vitro* and animal studies that assess herbal and chemical compounds for potential anti-hyperglycaemic activity focus on measuring stimulation of glucose uptake (Hui *et al.*2009; Bösenberg *et al*.2008). The anti-diabetic compounds, including herbals and western medicines, may have different modes of action, but they all work to achieve the same goal, that is, restoring normal glucose levels in blood (Fig 4.1).

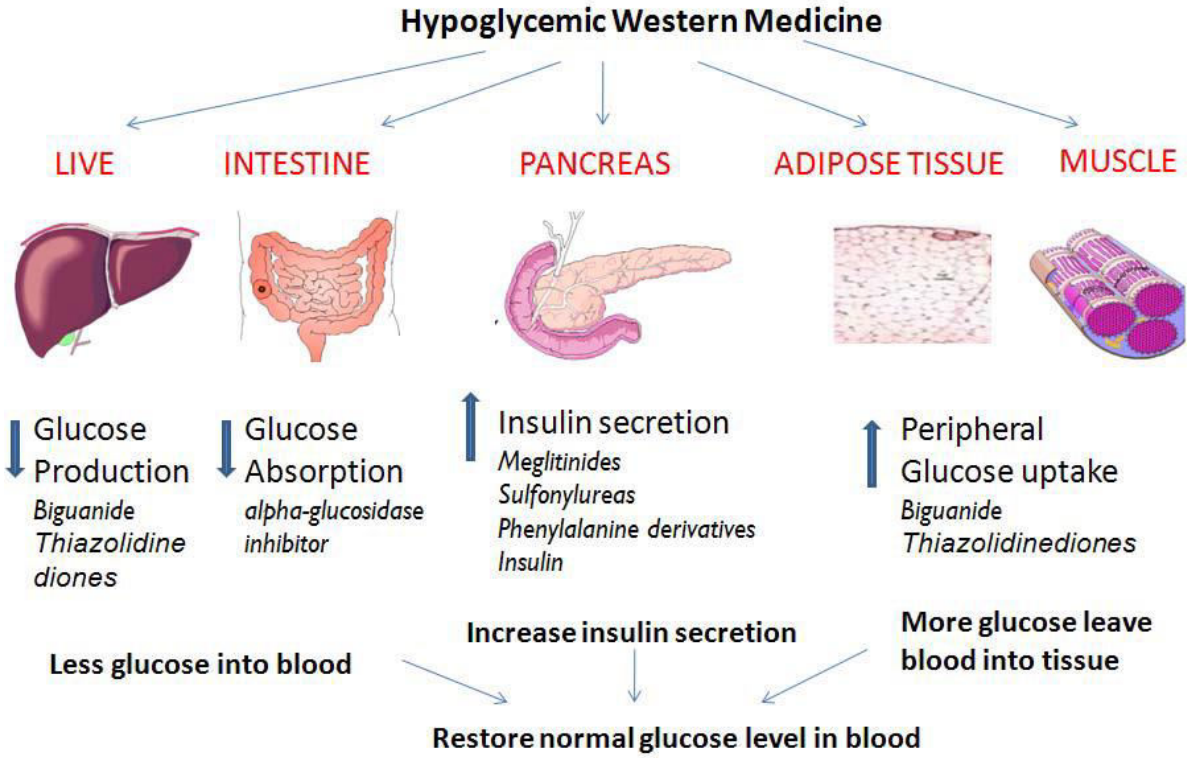


Figure 4.1 Action sites of western medicine in diabetes treatment. Hypoglycaemic medicines restore euglycemia via several modes of action, including insulin secretagogues (sulfonylureas, meglitinides), insulin sensitizers (biguanides, metformin, thiazolidinedione), and alpha-glucosidase inhibitors (miglitol, acarbose). (Adopted from Bösenberg *et al*. 2008)

Cells' capability of glucose uptake, and its evaluation, plays an elemental part in diabetes research. There are different ways of assaying glucose uptake, for example using a labelled form of glucose to track uptake, as with 2-deoxy-[1,2-^{3}H]-D-glucose

(Asano *et al*.1989; Mazibuko *et al*. 2011). Another approach is to measure glucose content inside cells, or changes in glucose in cell media. The former requires a sensitive assay capable of measuring small changes in glucose, for example using glucose-oxidase (Wu *et al*. 2009). In our study we assessed the glucose uptake in HepG2 cells using 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG), a fluorescent D-glucose analogue that produces detectable fluorescence when taken up by cells (Zou *et al.* 2005). This is a non-radioactive and non-invasive test for fast and direct evaluation of glucose uptake.

9.1.2. Development of Glucose uptake Assay

A previous study showed that when cells are treated with 2-NBDG concentrations higher than 0.25 mM, a high degree of self-quenching may occur, which would give a falsely low signal (Ball *et al*. 2002). Since it was the first time that 2-NBDG had been used in our laboratory , some preliminary experiments were carried out to optimise parameters for measuring 2-NBDG uptake, including 2-NBDG concentration, presence of glucose in the medium, incubation time and plate reader settings.

9.1.3. Effect of 2-NBDG concentration and the presence of glucose

HepG2 cells were seeded 2 x 10^5 cells in 0.5 mL full DMEM medium per well of a 24 well plate. Cells were allowed to grow overnight. 2-NBDG was added in methanol to final concentrations 1, 5, 10, 50 and 100 µM, with methanol at 1% (v/v) in all wells. Vehicle control cells received methanol only. Cells were incubated for one hour at 37 °C and fluorescence measured immediately, using the bottom optic of the plate reader with λ_{ex} 485 nm and λ_{em} 530 nm, and without changing the medium. There

was no difference in the fluorescence between samples. This means that even though the reader was assessing fluorescence at the bottom of each well where cells will be attached, the differences in fluorescent signal between samples could not be differentiated. The 2-NBDG was still present in the medium, but even though cells were confluent, the fluorescent signal from the 2-NBDG in the medium was stronger than the cell signal and effectively masked detectable cellular uptake.

Consequently the medium containing 2-NBDG was removed, cells washed once with PBS, then 0.5 mL PBS per well was added. The fluorescence signal in cells was detected through the bottom optic of the plate reader and was about 30% higher in cells treated with 100 µM 2-NBDG compared with vehicle only. The assay of glucose uptake in this conventional format was insensitive therefore as a next step the assay was modified by using medium with no glucose.

In the next experiment HepG2 cells were seeded and grown as described above. The next day the full DMEM medium, which contains 5 mM glucose was replaced with glucose-free DMEM. 2-NBDG in methanol was added to final concentrations 1, 5, 10, 50 and 100 µM, with methanol at 1% (v/v) in all wells and 1% (v/v) methanol

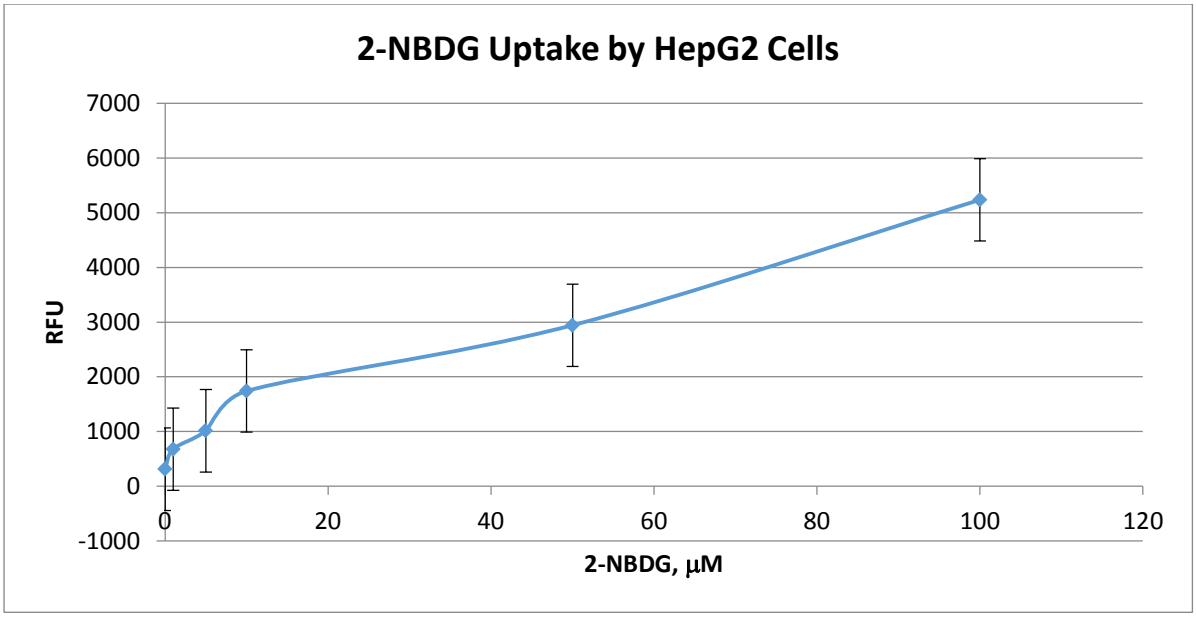


Figure 4.2 Concentration dependence of 2-NBDG uptake by HepG2 cells
Cells were incubated with indicated concentrations of 2-NBDG for four hours. Data are the mean ±SD of triplicate measurements, the uptake enhanced considerably when no glucose was present in the medium during treatment with 2-NBDG.

only in control wells and cells were incubated for a further two hours. The medium was removed, cells washed with PBS then 0.5 mL PBS added and fluorescence measured in the cells. The data (Fig. 4.2) show a clear trend of monotonic increase in RFU with concentration of 2-NBDG when no glucose is present. Moreover the signal at the highest concentration of 2-NBDG is about 15 times higher than negative controls, compared with a 30% when glucose was present.

In the next experiment the concentration dependence was re-assessed, this time using cells seeded at 1.2 x 10^6 / mL, 0.5 mL per well in 24-well plate and grown as described in pervious experiment overnight. This is a much higher concentration and cells were tightly confluent after overnight incubation. The full DMEM medium was replaced with glucose-free DMEM and then the cells were incubated with 2-NBDG at 1, 5, 10 and 100 μM for 4 hours at 37 °C. The changes in assay condition,

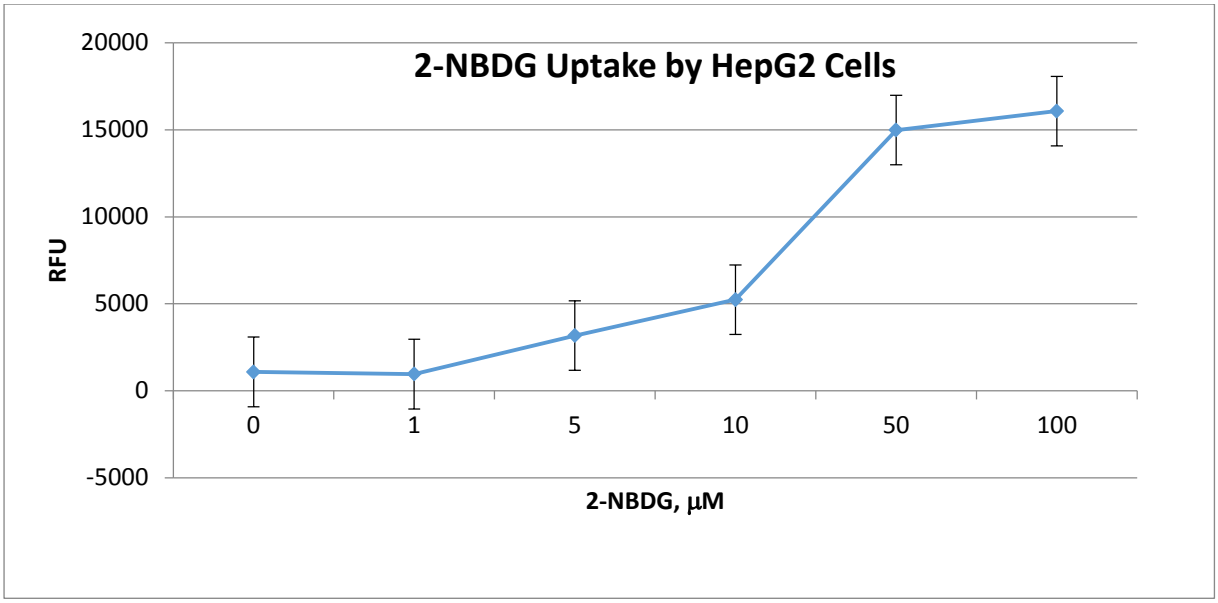


Figure 4.3 Concentration dependence of 2-NBDG uptake in confluent HepG2 cells.
Cells were incubated with indicated concentrations of 2-NBDG for four hours. Data are the mean ±SD of triplicate measurements.

including incubation time with 2-NBDG and increased cell density led to a change in the trend (Fig. 4.3) compared with the previous experiment (Fig. 4.2), but at the highest concentration of 2-NBDG used, 100 μM, the fluorescence increased about 15 times higher than that of negative control.

9.1.4.Time course measurement of 2-NBDG uptake

In this assay HepG2 cells were cultured in a 24-well plate and incubated overnight. The medium was replaced by glucose free medium and 100 μM of 2-NBDG in duplicate wells at time points: 0, 30, 60, 90 minutes. At 90 minutes medium was removed from all wells and washed once with PBS, then fluorescence was

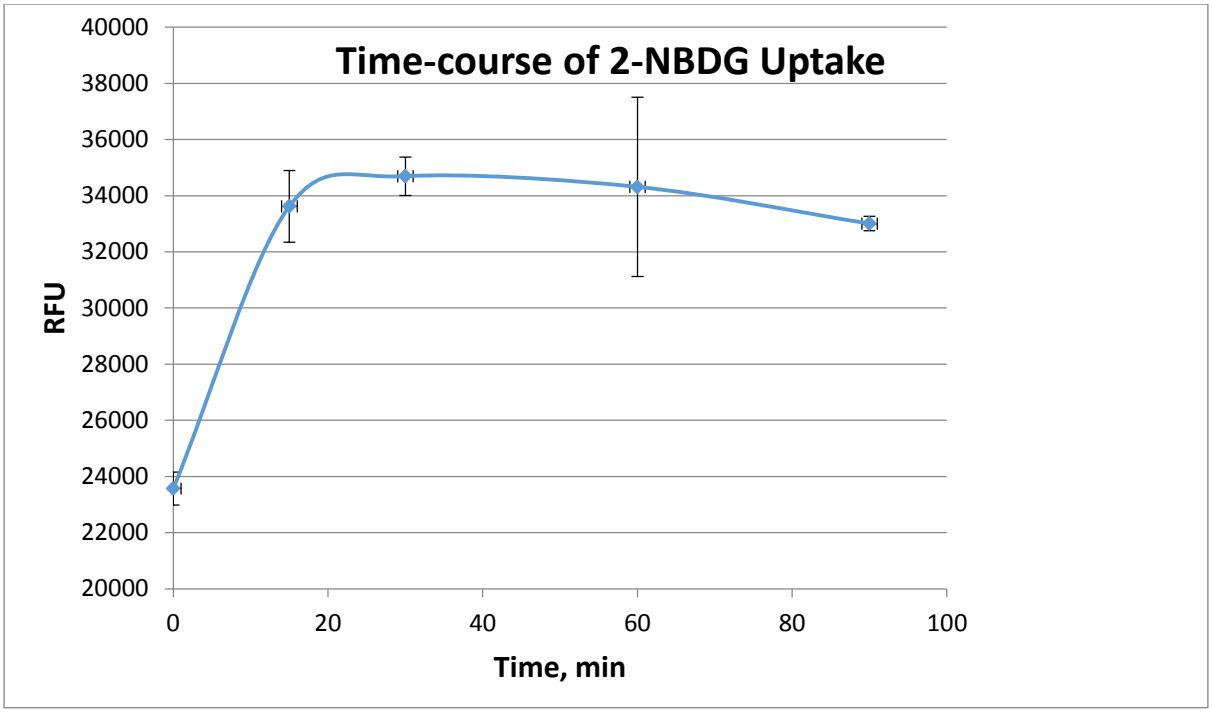


Figure 4.4 Time-course of 2-NBDG uptake by HepG2 cells.
Uptake of 2-NBDG was monitored in cells incubated with DMEM containing 2mM pyruvate and 100 μM 2-NBDG. Data are mean ±SD of duplicate measurements. The data indicate maximal uptake of 2-NBDG within 15 min.

measured (Fig. 4.3). 2-NBDG uptake was maximal by 15 min.

In view of these preliminary findings, the assay of glucose uptake was carried out routinely with glucose-free medium, 2-NBDG at 100 μM and with an incubation with 2-NBDG for 15 min. Although it is possible that higher concentrations of 2-NBDG could have been used to improve sensitivity, it was too costly to do so routinely.

9.1.5. Effect of extracts of *M.peregrina* on glucose uptake by HepG2 Cells

2- NBDG uptake by HepG2 cells was used to evaluate the potential anti-diabetic effects of *Moringa peregrina* extracts. HepG2 cells were treated with six different types of *M. peregrina* extracts for one hour then incubated with 2-NBDG for 15 minutes. After incubation, free 2-NBDG was cleared from cultures and fluorescence associated with the cells was measured in a fluorescent reader. The results are presented as the mean+S.D. of six independent experiments in triplicate (Fig. 4.4).

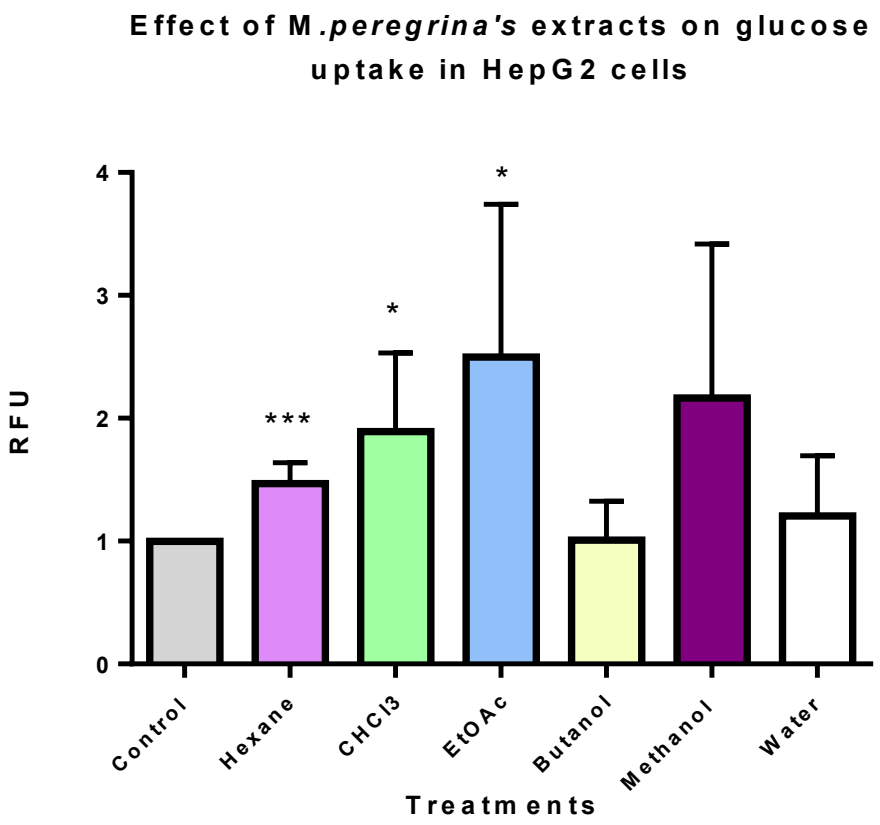


Figure 4.5 Effect of six extracts of *M.peregrina* on glucose uptake by HepG2 cells. Glucose uptake was measured in HepG2 cells after treatment with 5µL of 10mg/ml of each *M.peregrina* extracts for one hour. The results are mean ±S.D of five independent experiments in triplicates. Asterisks indicate $p<0.05$ (*) or $p<0.005$ (***) compared with control, determined by t-test or by one-way ANOVA.

The data demonstrate the effect of six extracts of *M.peregrina* on glucose uptake in HepG2 cells, of which EtOAc is the most effective extract, stimulating a 2.5 times increase on glucose uptake with respect to control. Methanol, hexane and chloroform extracts increased glucose uptake 2.1, 1.9 and 1.5 times, respectively, compared with control. The water extract induced only a 20% change in glucose consumption with respect to control and the effect of the butanol extract was negligible with respect to control.

9.1.6.Comparison of short term and long term treatment of three active extracts of *M.peregrina* (Hexane, $CHCl_3$, EtOAc) on glucose uptake in confluent HepG2 cells

To understand the mechanism of stimulation of glucose uptake in HepG2 cells by *M. peregrina* extracts, we designed two experiments. A short-term treatment of HepG2 with the extracts and a long-term treatment. HepG2 cells were grown over three days to ensure confluency and the medium was replaced with fresh medium every day. The three active extracts of *M. peregrina* (Hexane, $CHCl_3$ and EtOAc) were added for one hour then 2-NBDG uptake was assessed. A second batch of HepG2 cells were treated with the three extracts for 24 hours after which 2-NBDG uptake was measured. The data, shown in Fig. 4.5, revealed that there is a significant difference between duration of the treatments of HepG2 cells with active extracts of *M.peregrina* with short term treatments being more effective than long term treaments. The one hour treatment with the chloroform extract increased the uptake around three times more than control (300%) and the ethyl acetate extract increased uptake by approximately 70%, whereas the hexane fraction showed about 10% improvement in the uptake in respect with control. After 24 hours treatment

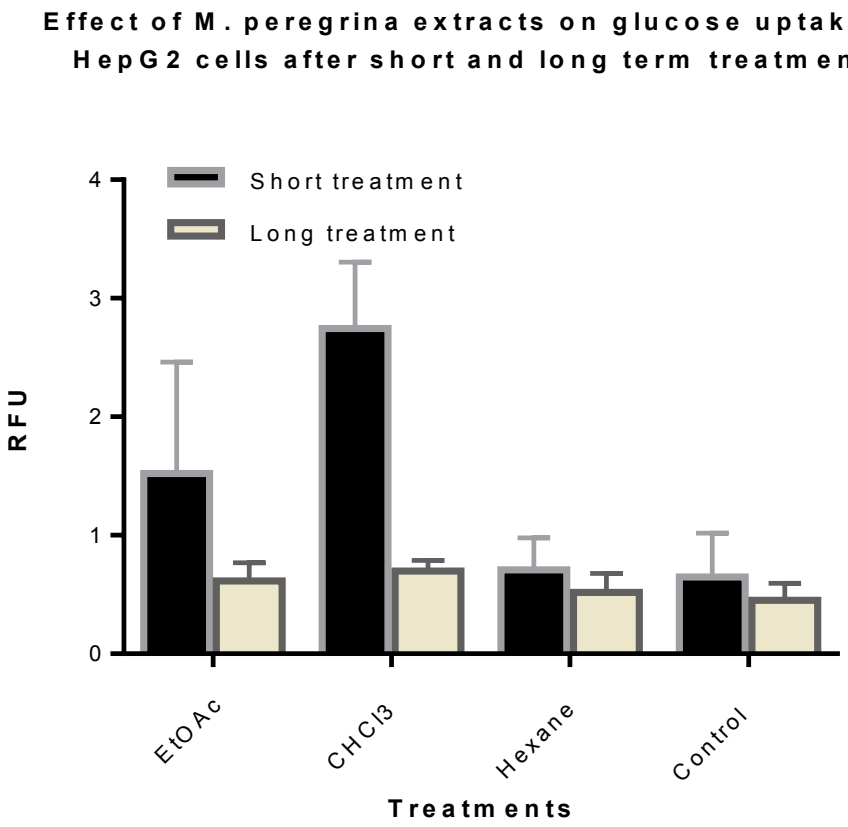


Figure 4.6. Effect of *M. peregrina* extracts on glucose uptake by HepG2 cells after short and long term treatments. Glucose uptake was monitored in confluent HepG2 cells incubated with ethyl acetate, chloroform or hexane extracts for short term (1 hr) or long term (24 hr) treatments. The results are mean+S.D. of four independent experiments in triplicate. $p^{****} < 0.0001$ for differences in duration of treatment as determined by two-way ANOVA.

the chloroform extract increased the uptake by about 30%, the ethyl acetate by less than 15% and there was almost no effect from hexane extract on glucose uptake. Analysis of the data by two-way ANOVA confirmed that there was a significant difference ($p<0.0001$) in glucose uptake between the two periods of treatment. The quick acting effect of the extracts suggests that the mechanism of action does not involve modulation of gene expression at the level of transcription. Instead there could be a post-transcriptional effect involving modulation of glucose transporter expression at the protein level. HepG2 cells, in common with many cancer cells, have a high capacity for glucose metabolism due to a high requirement of generating

energy by aerobic glycolysis, often called the Warburg effect. The possibility of regulation of gene expression as a major mechanism of action of the extracts has been eliminated. Instead the data raise the possibility that levels of glucose transporter, or transporter activity have been modulated. This could involve translocation, but the main glucose transporter of HepG2 cells, GLUT1, does not undergo an insulin-dependent translocation regulation. such a mechanism previously was shown for other anti-diabetic drug like insulin in osteosarcoma cells (Cifuentes *et al.* 2011).

9.1.7.Evaluation of effect of known anti-diabetic drugs against active extracts of *M.peregrina*

To test the effectiveness of *M.peregrina* on glucose uptake in HepG2 cells a comparison was carried out between the extracts and known ant-diabetic medications, both synthetic and phytochemical, including metformin, berberin and ouabain, and using phloretin as a negative control. HepG2 cells were seeded at a concentration of $0.5x10^5$ cells/mL, incubated overnight then treated with ethyl acetate, chloroform and hexane extracts of *M.peregrina* and anti- diabetic compounds as described in chapter 2 and incubated for one hour. The required concentrations for the known anti-diabetics and phloretin and was found from previous studies, including 10μM for berberine (Yeb *et al.*2012), 0.1 mM for metformin (Jacobs *et al*.1986), Ouabian (Reid 1996), 1 mM of ouabain (Kajikawa *et al.* 2002) and 200μM of phloretin (Wu C.H.,*et al.* 2009). In one hour treatment, normal DMEM medium replaced after one hour incubation by glucose free medium, then 5 μL of 2-NBDG added into three wells in each row in order to have

3 wells as a control. After incubation for 15 minutes, the cells were washed with PBS and fluorescent intensity measured using an Omega FLUOStar plate reader.

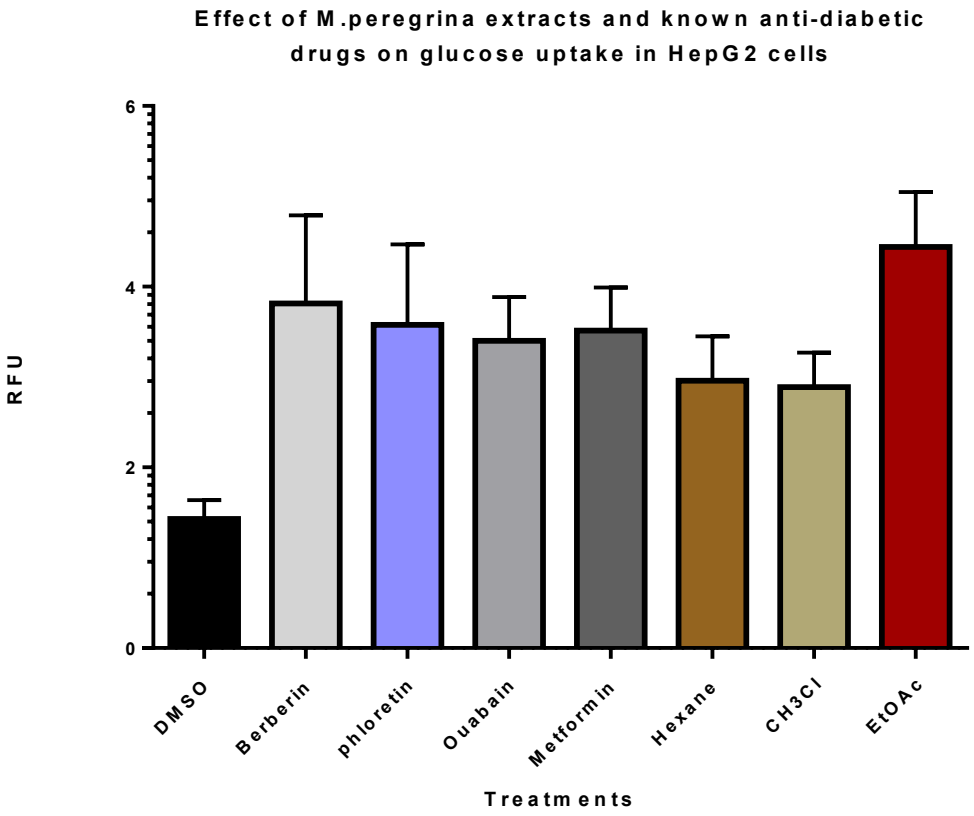


Figure 4.7. Effect of *M.peregrina* extracts and known anti-diabetic drugs on glucose uptake in HepG2 cells. Uptake of 2-NBDG was measured in HepG2 cells incubated with metformin, ouabain, berberin, and ethyl acetate and chloroform extracts of *M.peregrina*, and phloretin. The data are mean +S.D. of four independent experiments in triplicate. $p^{****} < 0.0001$, difference between the means of treatments compared with control are statistically significant as determined by one-way ANOVA.

According to the data presented in Fig. 4.6 all treatments stimulated glucose uptake and the most effective anti-diabetic agent that stimulates glucose uptake after one hour is berberin with a 167% increase with respect to control ,followed by metformin and ouabain with 145% and 138% respectively. The EtOAc extract of *M.peregrina* proved to have the highest effect among all the treatments in this experiment, with 210% increased on glucose uptake compare to control while chloroform and hexane appeared to have the least effect on glucose uptake in compare to rest of the treatment, with 102% and 106% respectively in respect to control. An inhibitory effect of phloretin was not observed in this assay. Instead, it too stimulated glucose uptake quite markedly, by about 150%.

9.1.8.Evaluation of phytoestrogens effect on glucose uptake in HepG2 cells.

In previous studies consumption of phytoestrogens in the human diet and their effect on human health have been investigated (Adlercreutz *et al* .1997; Anderson *et al.* 1998; Setchell *et al*.1998; Anthony *et al*.1998; Tham et at.1998; Lissin *et al*. 2000; Velasquez *et al.*2001). Due to structural similarity between this biologically active group of phytochemiclas and the structure of estradiol, phytoestrogens bind to estrogens receptors and apply estrogenic and antiestrogenic effects (Martin et al.1978; Wang et al. 1996; Kuiper et al. 1998; Miksicek et al.1994). These plant derived compounds can be divided in three major types including, isoflavones, lignans, and coumestans. The two most common phytoestrogens in the human daily diet are isoflavones and lignans, and one of the rich sources of isoflavones are soy beans with approximately 0.2% isoflavones content (Reinli *et al.* 1996). A number of trials have been done in type 2 diabetic patients to evaluate the effect of

phytoestrogens, mainly isoflavones, on diabetes management (Jayagopal *et al*. 2002; Azadbakht *et al*. 2008; Hermansen *et al.* 2001; Liu *et al.* 2009; Clerici et al. 2011; Anderson *et al.* 1998; Teixeira *et al.* 2004; Gobert *et al.* 2010).

The most bioactive isoflavones include genistein and daidzein, that are derived from biochanin A and formononetin. We used our current optimised method of monitoring glucose uptake to measure the hypoglycaemic effect of genistein , daidzein and formononetin in HepG2 cells.

9.1.9.Glucose uptake measurement in HepG2 cells treated with daidzein

HepG2 cells were cultured and seeded in a 24 well plate at a concentration of $0.5x10^5$ cells/mL, and the next day the cells were treated with 5µL diadzein with six different concentrations, started from 10^{-4} M to 10^{-9}M. For short-term treatments the cells were incubated only for one hour after treatment, while for long term treatment the cell were treated for 24 hours. After the treatment period the medium was replaced with glucose free medium and 2-NBDG added. The measurement was performed after 15 minutes of incubation with the fluorescent analogue.

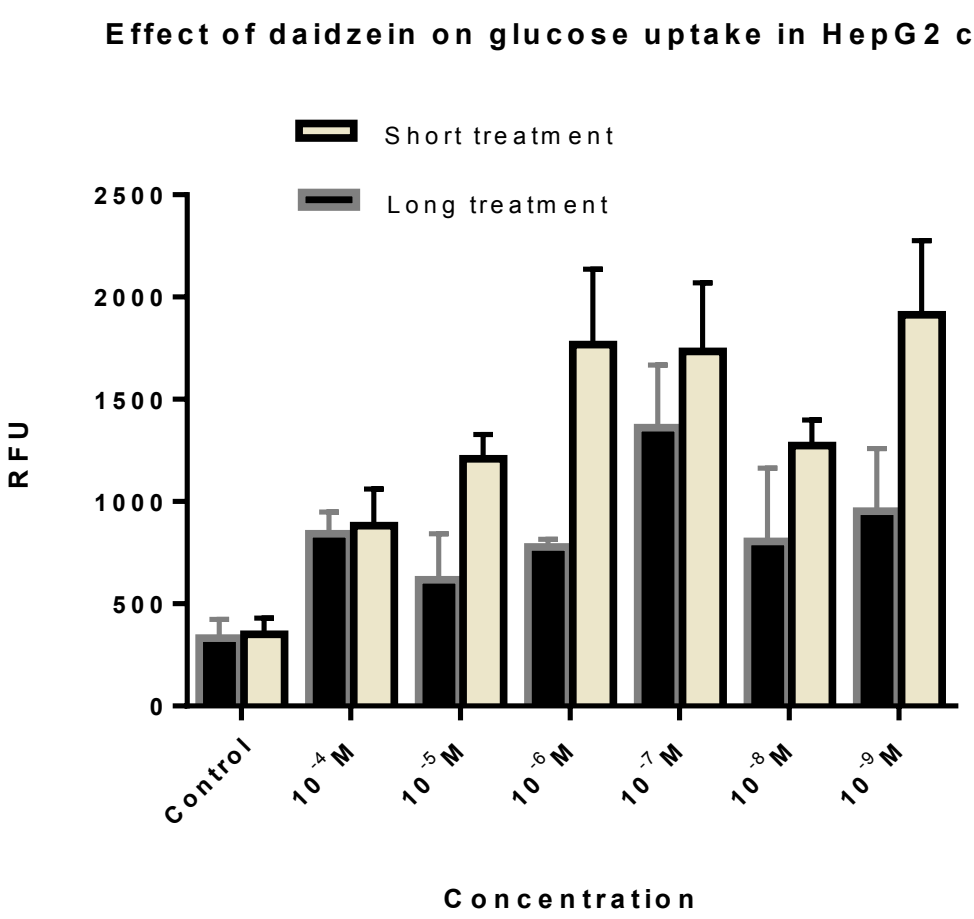


Figure 4.8. Effect of daidzein on glucose consumption in HepG2 cells. Uptake of the fluorescent glucose analogue 2-NBDG was measured in HepG2 cells treated with daidzein forr short and long term periods at the indicated concentrations and compared to control untreated cells. The data are mean+S.D. of three independent experiments in triplicate. *p***** < 0.0001 comparing duration of treatment, *p*****<0.0001 comparing daidzen with control, as determined by two-way ANOVA.

The data (Fig. 4.7) show that daidzein stimulates uptake of glucose, with a greater effect after a short treatment of one hour compared with tretament 24 hours. The effect was broadly higher at the lower concentrations tried, 1 μM to 1nM, at which glucose uptake was increased four to five fold after a one hour treatment. Interestingly the higher concentrations tried, 10 and 100 μM, showed less effect. Longer treatments resulted in a lesser stimulation of glucose uptake, and a peak effect was found at 0.1 μM.

9.1.10.Glucose uptake measurement in HepG2 cells treated with genistein

Another isoflavone phytoestrogen that was used in this study was genistein. HepG2 cells were cultured at $0.5x10^5$ cells/mL, incubated overnight, and then treated with 5µL genistein with range of concentrations starting from 10^{-4} M to 10^{-9}M, for one hour or 24 hours.

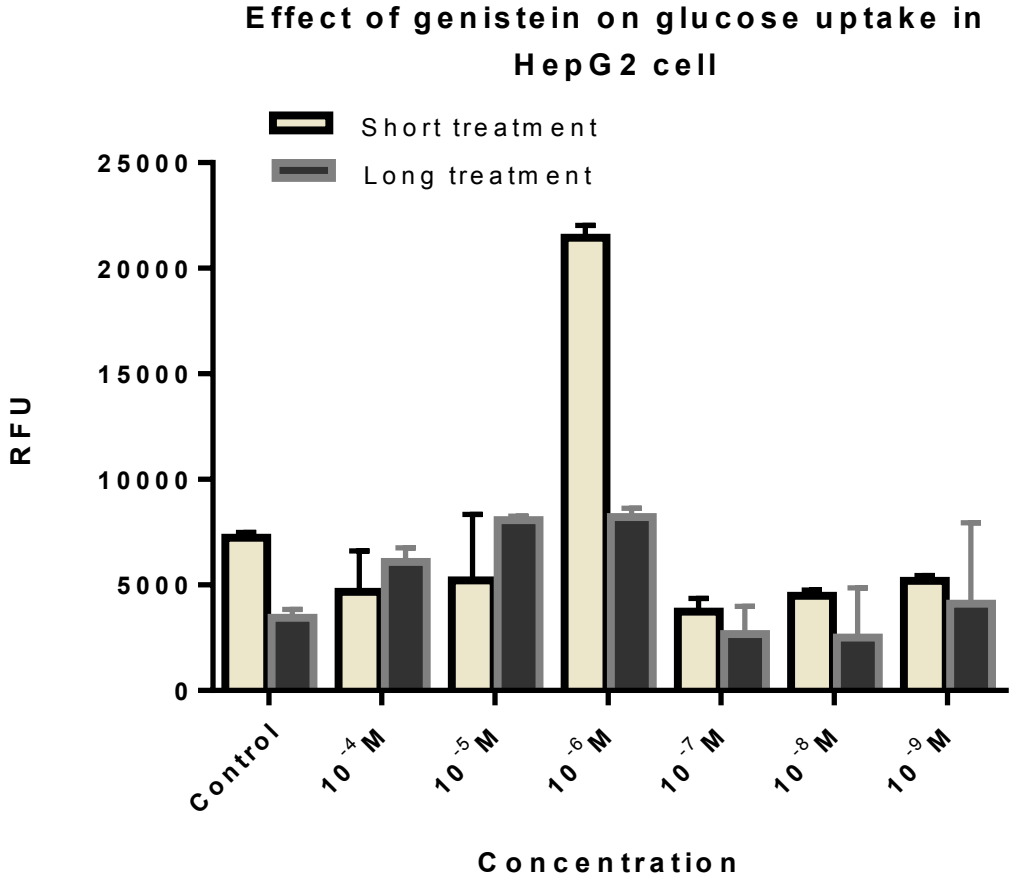


Figure 4.9. Evaluation of genistein on glucose uptake in HepG2 cells. Glucose consumption test was performed in HepG2 cells treated with range of genistein concentrations from 10^{-4} M to 10^{-9}M, for one hour and 24 hour compared with untreated control . The data are mean+S.D. of three independent experiments in triplicate. $p^{****} < 0.0001$ comparing duration of treatment, $p^{****}<0.0001$ comparing genistein with controls determined by two-way ANOVA.

The data (Fig. 4.8) indicated much less effect of genistein compared with diadzein. Generally one hour treatments had a slight inihibitory effect compared with control, with the exception of treatment at 1 µM, which stimulated uptake about htree-fold

compared with control. Longer treatments with 10^{-4}M to 10^{-6} M genistein resulted in gradual increase in glucose uptake to 2.4 times more than control, and thereafter a decline. Overall there was significant difference in glucose uptake between short and long term treatements,as indicated by two-way ANOVA, but the differences depend on the concentration of genistein, with higher concentrations of 10 and 100 μM inducing greater uptake after 24 hours treatment but at lower concentrations, uptake was stimulated more after a one hour treatment.

9.1.11. **Glucose uptake measurement in HepG2 cells treated with formononetin**

Formononetin is the last isoflavone phytoestrogen that was studied in this project. The hypoglycaemic effect of this compound was assessed in the same way as the two others.

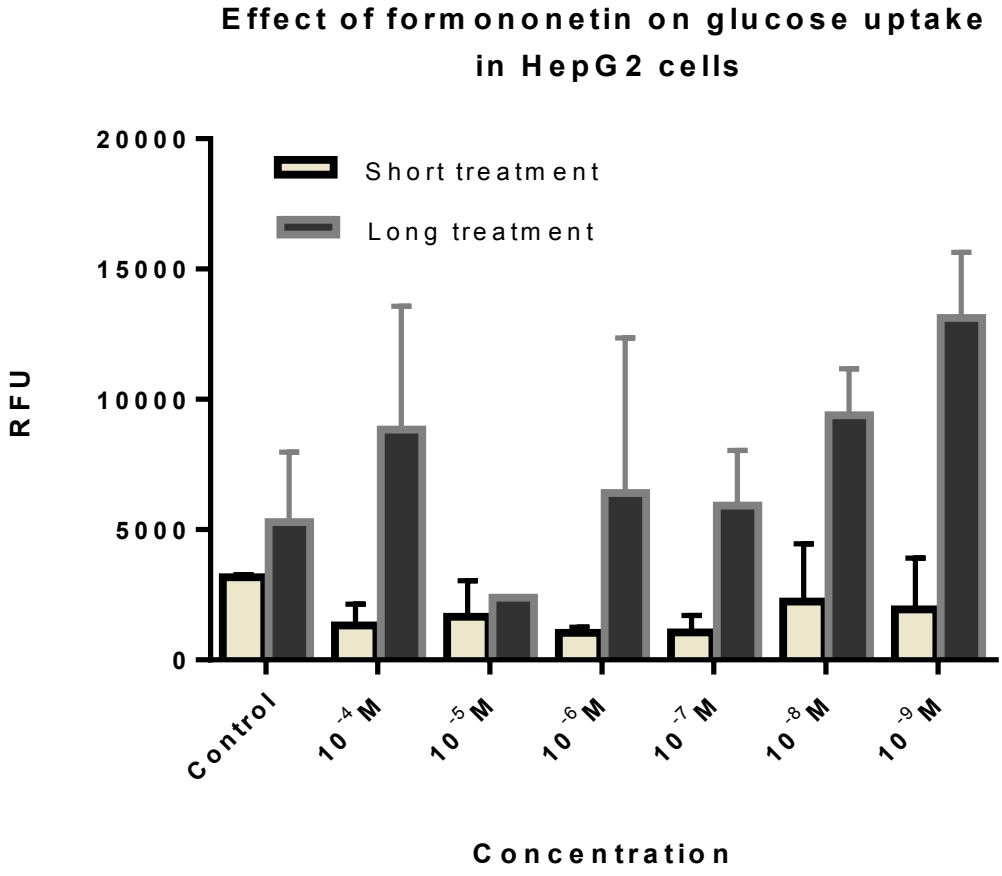


Figure 4.10. Effect of formononetin effect on glucose uptake in HepG2 cells. Glucose uptake measured in HepG2 cells after short and long treatments with six different concentrations of formononetin compared with untreated control. The data are mean+S.D. of three independent experiments in triplicate. *p***** 0.0001 comapring duration of treatment, *p****<0.0001 as determined by two-way ANOVA.

A short treatment with formononetin inhibited glucose uptake (Fig. 4.9), while longer treatments had varaible effects, with an aprroximately two fold stimulation across a range of concentrations.

Part II

Glucose consumption

9.2.1.Introduction

The glucose uptake studies described in part one showed the effect of *M.peregrina* extracts on glucose uptake in HepG2 cells as assayed using a short kinetic method that gives a relative measure of the uptake capacity of the cells at the time of measurement. It was of interest to see whether treatments with extracts of *M.peregrina* would affect glucose consumption to a measurable degree, by assessing changes of glucose concentration in the cell culture medium. A glucometer was used, based on a glucooxygenase strip, which had a measurement range of 0.5 to 22 mM.

In the series of experiments described in this section, changes in glucose levels were monitored in the cell culture medium of HepG2 cells after 2 to 48 hours of treatment with two *M. peregrina* extracts, prepared with ethyl acetate or chloroform. The extracts are compared with metformin, a drug used to treat diabetes, which is known to act by stimulating glucose uptake in the liver and peripheral tissues. In the work described here it is assumed that there is no involvement of insulin in the mechanism of action – no exogenous insulin is added.

To model diabetic levels of glucose, HepG2 cells were grown in full medium containing 11mM glucose. To take into account variations in cell numbers in the wells of the 24-well plate, cells were counted in each well, by trypsinisation and counting with a haemocytometer, after measurement of glucose. The changes in glucose in the medium are used to calculate glucose uptake and are normalised to 500,000 cells. There was not much variation in cell numbers between samples, since for most measurements cells were allowed to grow to near confluency.

9.2.2.Two hour treatments

In these experiments HepG2 cells were seeded as previously described and incubated overnight, and then the medium was replaced with 0.5ml of fresh full DMEM medium with glucose concentration of 11mM and treated with 5µl of each *M.peregrina's* extract (10mg/ml) in DMSO, 10µl of 5mM metformin in DMSO, and one set of wells received DMSO only as a vehicle control. The rest of the wells received no treatment. The cells were then incubated for two hours and the glucose level in the medium was measured using the glucometer.

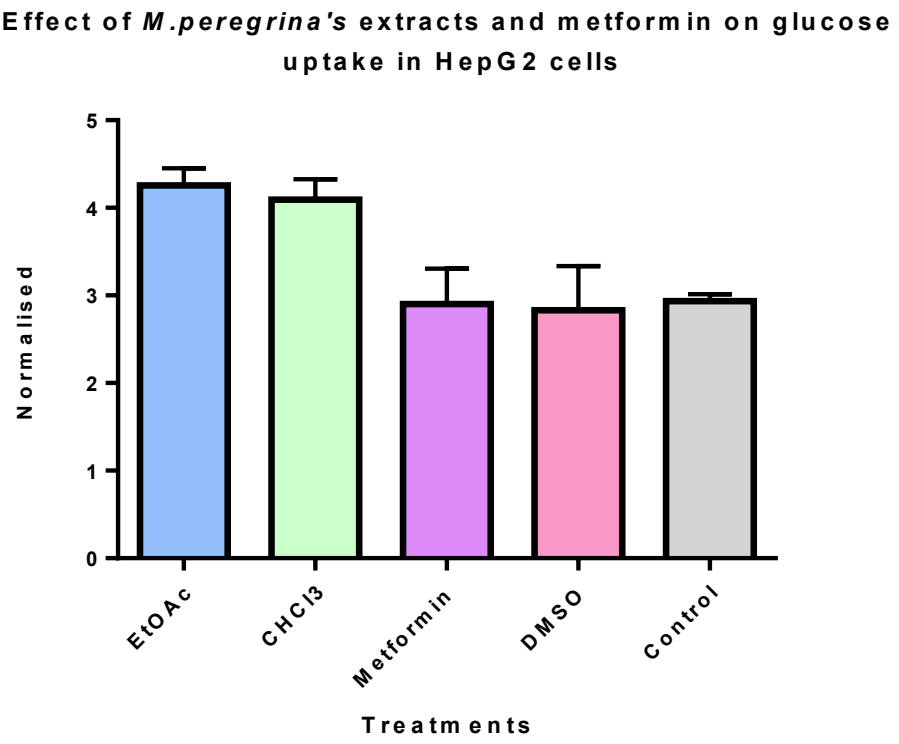


Figure 4.11 Glucose consumption measurement in HepG2 cells treated with ethyl acetate chloroform and metformin after two hours treatment. Effect of two active extracts of M.*peregrina,* metformin and DMSO only was evaluated in HepG2 cells for two hours treatment compared with untreated control. The data are mean+S.D. of two independent experiments in triplicate. *p*****< 0.0001 comparing treatments with control, as determined by one-way ANOVA. Data are expressed as decrease in glucose concentration in cell culture medium, normalised for cell number. The glucose uptake data shows in the graph as normalised per cell count.

Data from two independent experiments are shown in Figure 4.10.
Cells incubated for two hours with extracts of *M.peregrina* showed a marked increase, with an average of 42%, in glucose consumption compared with unstimulated controls. At this time point metformin had slight increase with around 8% in glucose uptake and differences among means are statistically significant *(p* < 0.0001).The effect from metformin explains its mechanism of action that is expected to start usually after 24 hours (Miyoshi *et al*. 2014, Mitsuhashi *et al.*2014, Luo *et al.*2012

9.2.3. 24 hour treatments

In this part of the work HepG2 cells were cultured and treated with the two extracts as described in the previous section. After 24 hours incubation of HepG2 with the treatments there is a trend towards increased glucose uptake (Fig. 4.11), and this time metformin induced a similar response to the *Moringa* extracts, a 52% increase in glucose consumption with respect to control. The ethyl acetate and chloroform *moringa* extracts produced about the same level of stimulation.

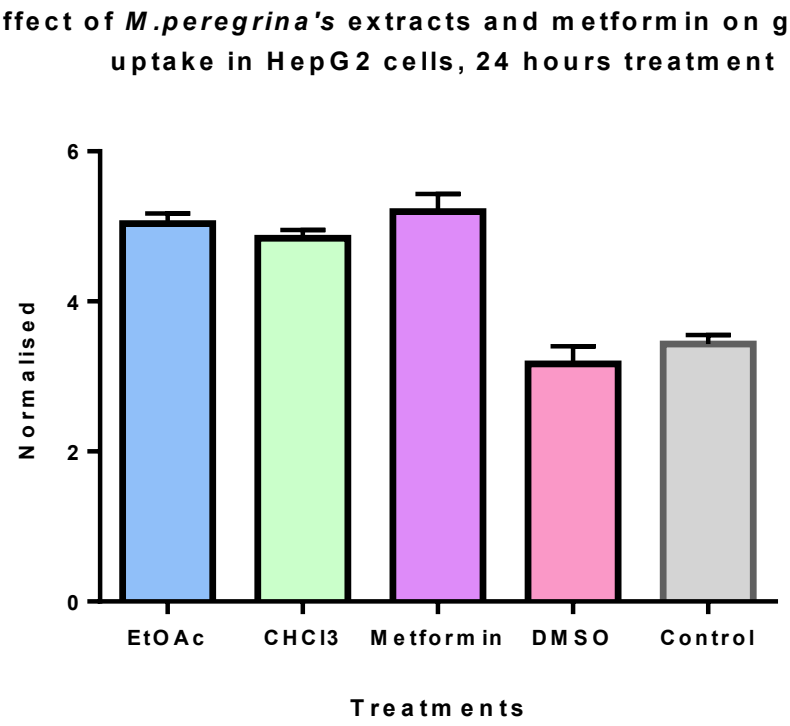


Figure 4.12 Glucose uptake measurement in HepG2 cells treated with ethyl acetate and chloroform extracst from *Mroinga*, and metformin, for 24 hours treatment. Evaluation of hypoglycaemic effect of two active extracts of M.*peregrina,* metformin and DMSO only was tested in HepG2 cells for 24 hours treatment compared with untreated control. The data are mean+S.D. of two independent experiments in triplicate. $p < 0.0001$ as determined by one-way ANOVA comparing means of treatment with DMSO or control. Data are expressed as decrease in glucose concentration in cell culture medium, normalised for cell number.

9.2.4. 48 hours treatment

The glucose uptake measurements were repated on cells treated for 48 hours with either *Moringa* extracts or metformin. The *Moringa* extracts were still active, producing an overall stimulation of glucose uptake, after 48 hours, whereas the

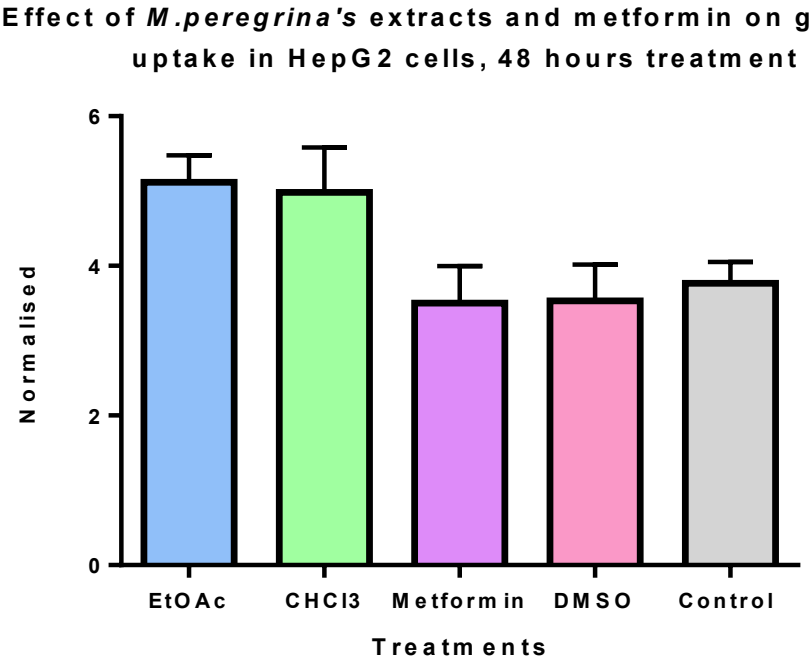


Figure 4.13. Effect of ethyl acetate and chloroform extracts from Moringa,and metformin, on glucose uptake in HepG2 cells compared with control after 48 hours treatment. Glucose consumption was measured with a glucometer. The data are mean+S.D. of two independent experiments in triplicate. *p*****< 0.0001 as determined by one-way ANOVA Data are expressed as decrease in glucose concentration in cell culture medium, normalised for cell number.

effect of metformin was no longer apparent, when compared with control.

9.2.5. Time course effect of extracts on glucose uptake in HepG2 cells

The data from the glucose consumption measurements was combined (Fig. 4.13) Shows the effect of the extracts of *M.peregrina* started immediately and continues after 24 hours and 48 hours and the effect of metformin started within 24 hours of treatments with continues decrease to 48 hours. Statistical analysis, two-way ANOVA, shows a significant difference between the effect of the treatments at different time points *(p*****< 0.0001) and between the treatments also is significant (*p*****<0.0001). t-test , comparing ethyl acetate with contro (p**<0.01) and chloroform with control (p**<0.01) ,showed significant difference between the treatments with active extracts compare to control .

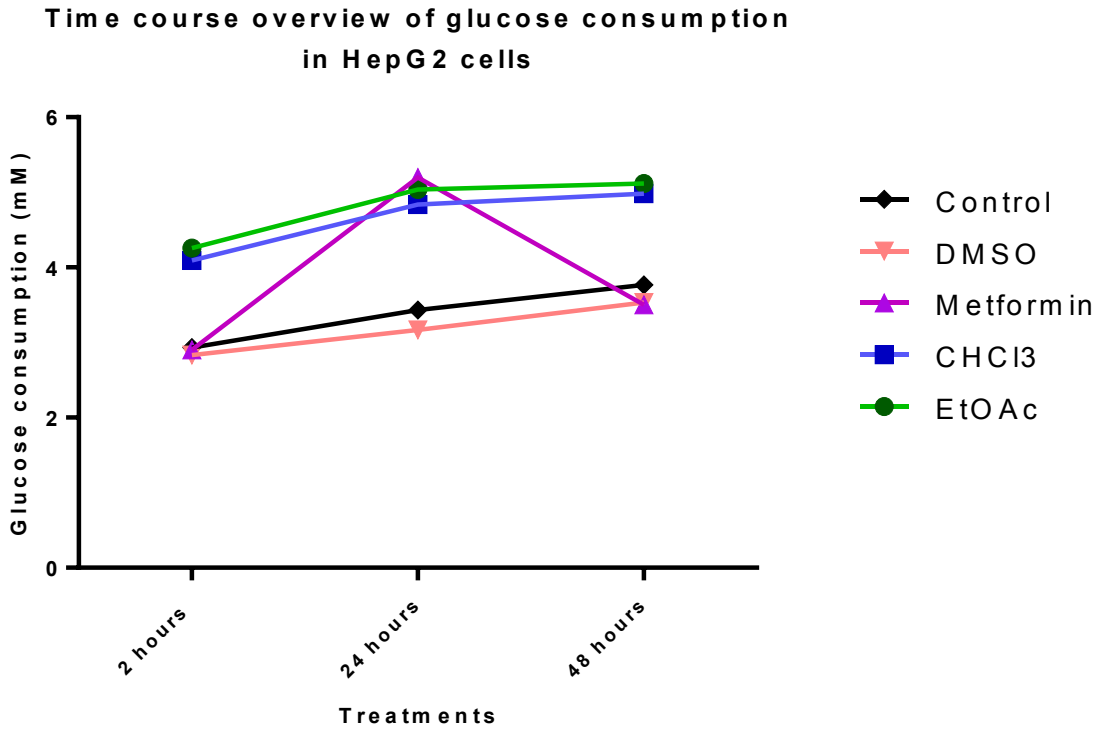


Figure 4.14. Time course overview for effect of two active extracts of *M.peregrina* (prepared using ethyl acetate or chloroform) compared with metformin and untreated control on glucose uptake in HepG2 cells for 2, 24, 48 hours of incubation with treatment. The effect of active *M.peregrina* extracts prepared using ethyl acetate or chloroform, metformin or DMSO on consumption of glucose from the cell culture medium over two, 24 and 48 hours. Data are expressed as decrease in glucose concentration in cell culture medium, normalised for cell number. The data are mean ±S.D of three experiments with six replicates. P**** 0.0001 for duration of treatment, P****<0.0001 for concentration as determined by two-way ANOVA.

9.2.6. Western Blotting

The western blot is a widely used analytical technique to detect specific proteins in samples such as cell extracts. HepG2 cells after incubation with treatment extracts for 24 hours were lysed using lysis buffer. The protein content of each lysate was measured using the BCA assay and 20 µg of protein from each lysate was analysed. Proteins were blotted to nitrocellulose membrane after completion of electrophoresis according to standard protocol as described in part II. GLUT1 protein was detected using primary anti-glut1 antibody and secondary anti-body solution of anti-rabbit IgG labelled with horse-radish peroxidase (HRP).

The bands were detected in all loaded lanes as a single band in the region of 50kDa as expected molecular weight for GLUT1 (Fig 4.14), although the quality of the blot precludes a clear analysis but the impression is that the EtAc fraction increases GLUT1 expression.

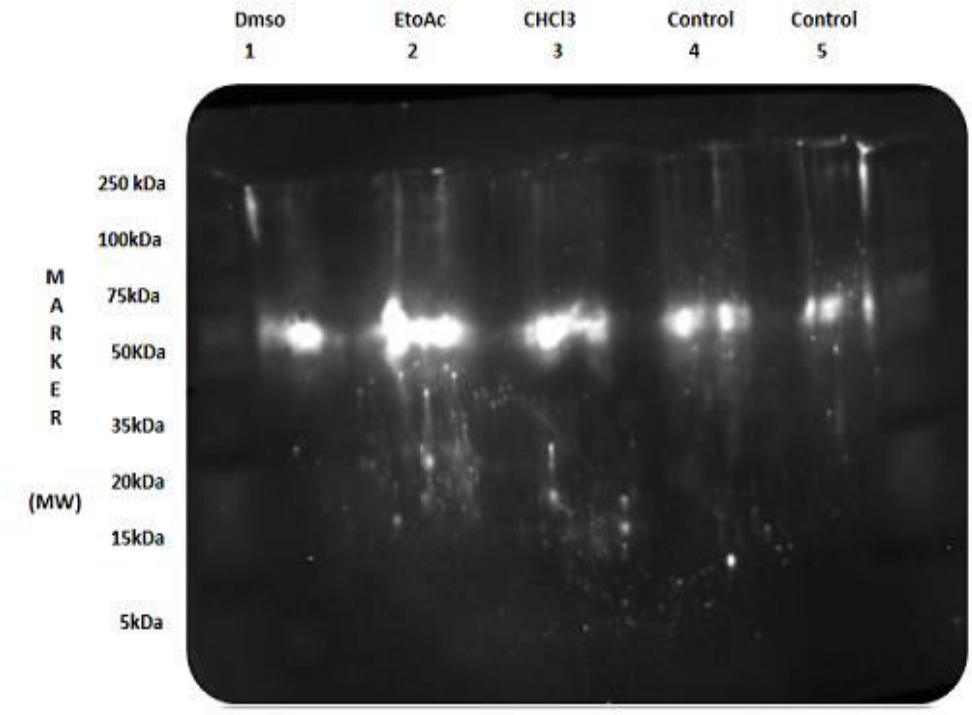


Figure 4.15. GLUT1 expression in HepG2 cells. GLUT1 is detected in the region of 50kDa in HepG2 cells. Extracts from cells treated with DMSO (lane 1), two active extracts of *M.peregrina* (prepared using ethyl acetate or chloroform) (lanes 2 and 3 respectively), DMSO (lane 4) and unstimulated controls (lanes 5 and 6).

The gel was analysed using ImageJ software to compare the expression of GLUT1 in each sample, by selecting a rectangular region around each band and quantifying the intensity of each sample (Fig. 4.15). These data are a preliminary analysis and

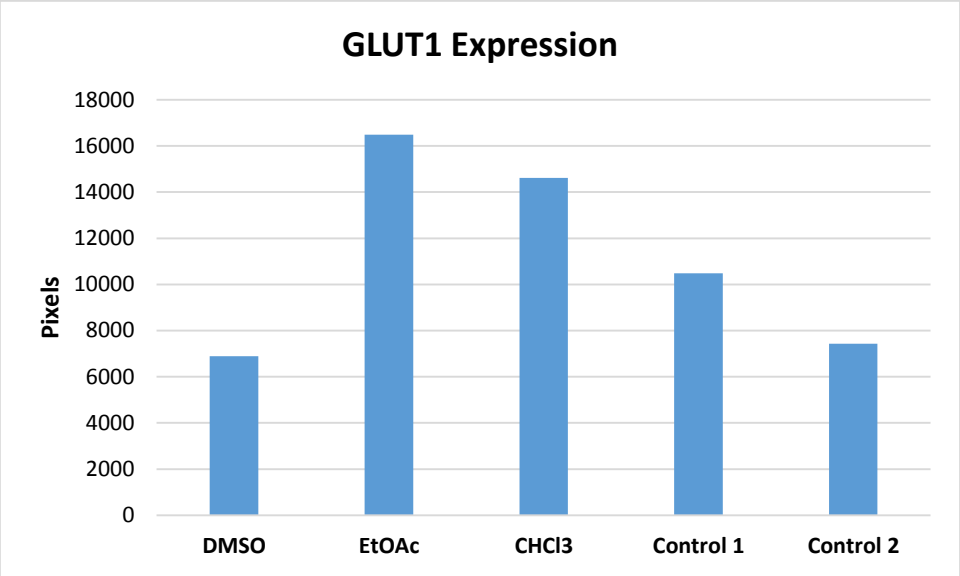


Figure 4.16. Quantitation of expression of GLUT1 in HepG2 cells. A plot of the intensity of the bands of GLUT1 from the image of the blot shown in Fig. 4.14, quantified using ImageJ.

suggest that both extracts from *M.peregrina* could stimulate an increased expression of GLUT1. However these are data from a single experiment and although care was taken to load equal amounts of protein from each extract, the data do not take into account a control for the blotting and detection process, such as measurement of β-actin.

Part III

Metabolic Activity Measurements using a Seahorse XF^{e}24 Analyser

9.3.1. Analysis of the effect of three *Moringa* compounds on HepG2 metabolism

A Seahorse Bioscience XF^{e}24 Extracellular Flux analyser was used in this part of the project to measure the oxygen consumption rate (OCR) and glycolysis within the HepG2 cells as indicators of primary metabolic activity though glycoysis and oxidative phosphorylation. Three compounds purified from the two active extracts of *Moringa peregrina* were selected, chosen as representative of the three classes of phytochemical identified in the extracts, namely a thiocarbamate (*O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*)) and a flavonoid (rutin) from the EtOAc extract and a sterol (β-sitosterol) from the $CHCl_3$ extract.

Cells were incubated with the three compounds or vehicle control for two hours before the start of the experiment.

The Seahorse XF analyser is a platform for metabolic assays which, simultaneously and in real time, measures the two major energy producing pathways of the cell, mitochondrial respiration and glycolysis, in a microplate. The analyser measures oxygen consumption rate (OCR) as the indicator of mitochondrial respiration, and extracellular acidification rate (ECAR) which is predominately the result of glycolysis, at intervals of approximately 2-5 minutes. We evaluated the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in HepG2 cells after two hours incubation with control, 8μM [*O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*)], 30 μM β-sit and 10 μM (rutin) over a period of 30 minutes in three replicates per group, to investigate functional transformation in glycolysis and mitochondrial respiration.

The Thermo Scientific™ Pierce™ BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method had been used to measure the protein level of the cells for normalising the data from the Seahorse experiment.

The standard curve of the assay is shown in Fig. 5.3.1. and the mean protein content.

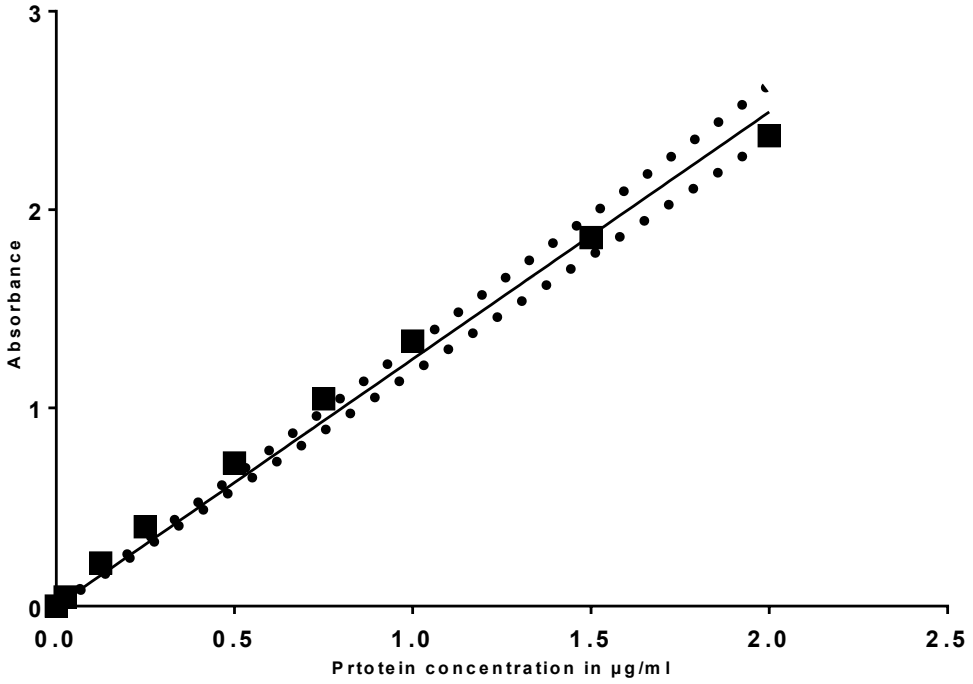


Figure 5.3.1. **Standard curve of BCA protein assay.** The line represents the best linear fit.. The dotted lines either side represent linear regression for the entire set of standard points.

of cells grouped according to treatment are shown in Fig. 5.3.2. The treatments did not induce any significant changes in proteins content, as indicated by comparison of means by ANOVA or by student's t-test comparing treatments with control (p>0.05).

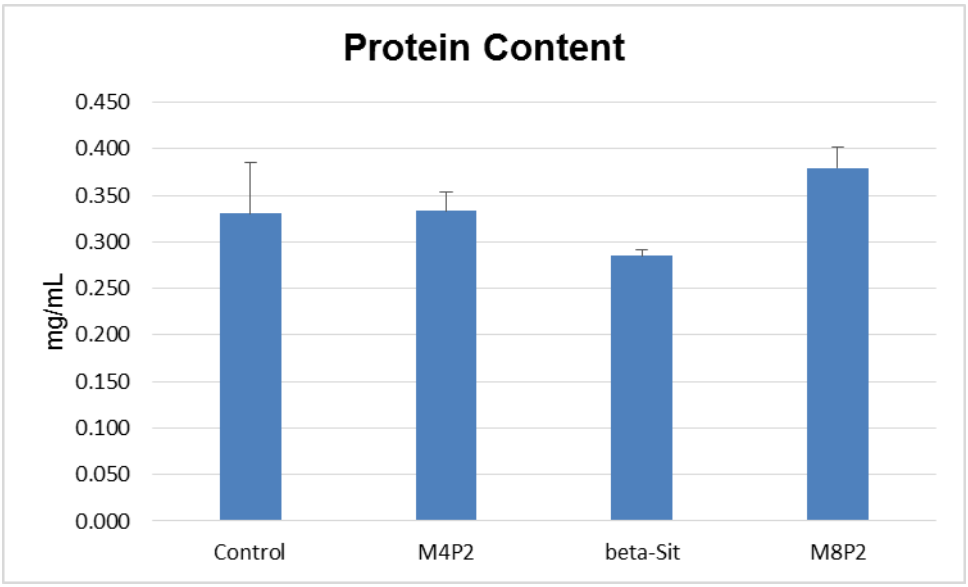


Figure 5.3.2. Protein content of HepG2 cells groups in the Seahorse analysis after treatment of HepG2 cells with β-sitosterol, MA4P2 (*O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*))and M8P2 (rutin). Data from the BCA assay using the standard curve in Fig. 5.3.1, and the mean+SD of five cell replicates, each sampled for the assay in triplicate. There are no significant differences between the means as assessed by one-way ANOVA and student's t-test comparing treatments with control.

In order to assess the mitochondrial metabolic capacity of the cells maximal respiration can be measured. This in turn is linked to spare respiratory capacity and mitochondrial ATP production. These three sets of measurements, derived from measuring oxygen consumption in the presence of appropriate inhibitors (described in chapter 2), are presented in Fig. 5.3.3. There are no significant differences between treatments and control (one-way ANOVA and student's t-test give $p>0.05$).

One mechanism that could account for increased glucose consumption could be increased metabolism through the TCA cycle which is uncoupled from ATP generation. This is a well known mechanism by which energy metabolism can be increased, resulting in an increase in heat rather then ATP. Known uncouplers include 2,4-ditrophenol and FCCP, but these compounds are either potentially or

actually toxic. If a herbal or other treatment acts by this mechanism it must do so in a mild way otherwise it would be toxic.

When the measurements of basal respiration and proton leak are examined it appears that some significant effects of the *Moringa* compounds have occurred.

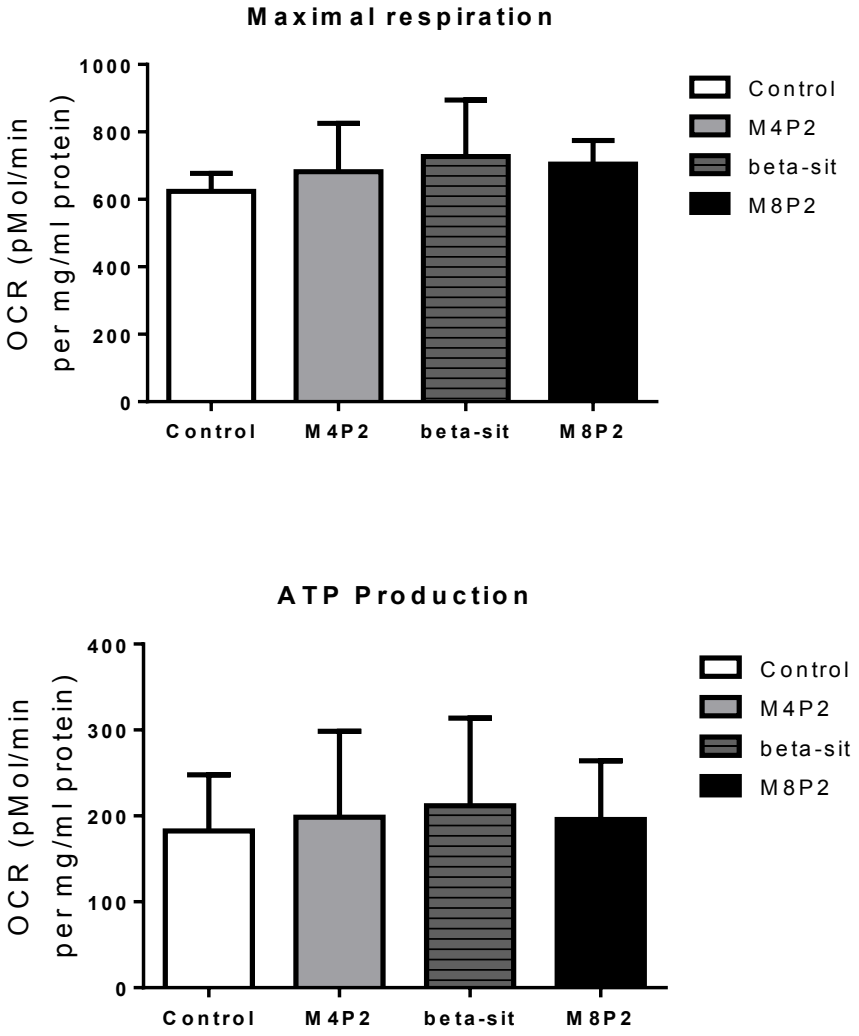


Figure 5.3.3. Effect of selected *Moringa* phytochemicals on mitochondrial energy metabolism in HepG2 cells. Oxygen consumption rate measurement of maximal respiration (upper chart), ATP production (middle chart) and spare respiratory capacity (lower chart) after treatment of HepG2 cells with β-sitosterol, MA4P2 (*O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*))and M8P2 (rutin).

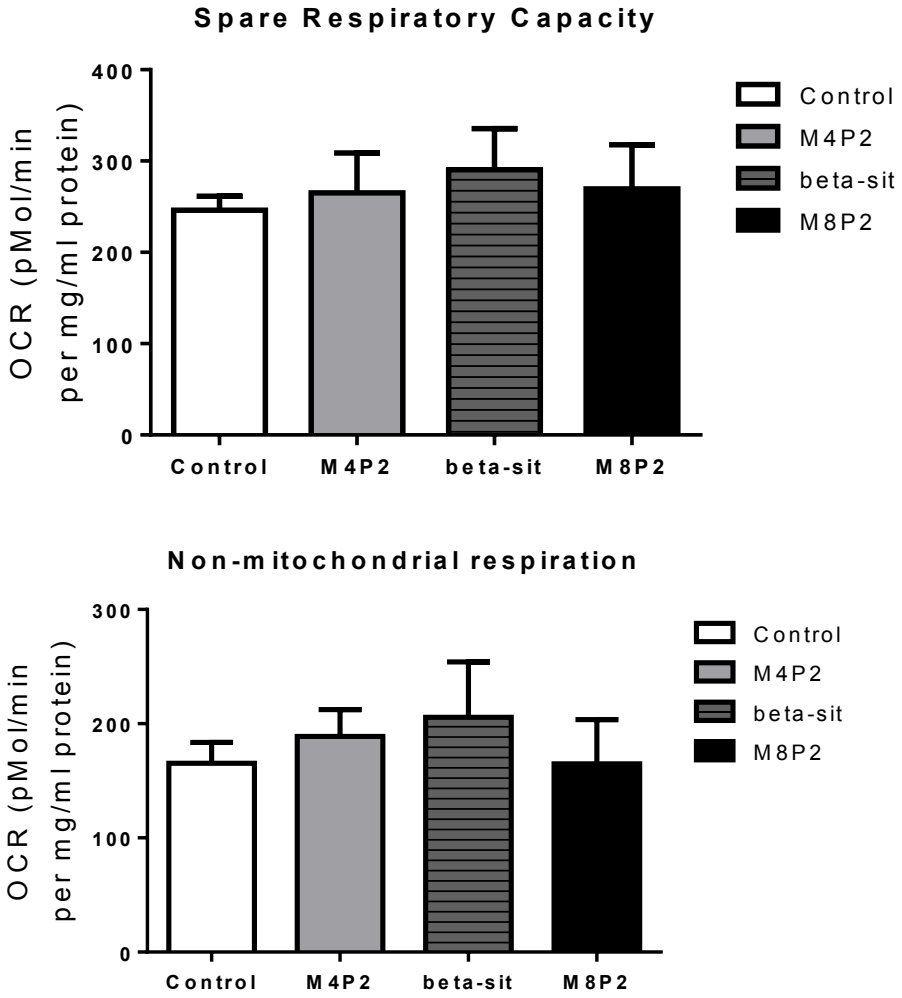


Figure 5.3.4. Effect of selected *Moringa* phytochemicals on non-mitochondrial resipiration in HepG2 cells. Oxygen consumption rate measurement of non-mitochondrial respiration after treatment of HepG2 cells with β-sitosterol, MA4P2 (*O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*)) and M8P2 (rutin). β-sitosterol induces a significant increase in oxygen consumption compared with control (student's t-test $p<0.01$).

The effect of the *Moringa* phytochemicals on non-mitochondrial respiration is shown in Fig. 5.3.4. β-Sitosterol significantly increases the rate of oxygen consumption, by about 15%, compared with control (student's t-test $p<0.01$), and *O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*) ,does this too, but not quite significantly (student's t-test $p=0.053$). These effects are not likely linked to energy metabolism and may reflect an increase in P450 metabolic activity that may be induced by the phytochemicals.

Basal respiration is defined as the normal rate of mitochondrial oxygen consumption, made up of two components, ATP production (oxidative phosphorylation) and leakage of protons across the mitochondrial membrane - the latter causes consumption of oxygen because the protons are generated by the TCA cycle and electrons also donated to the electron transport chain will combine with oxygen and protons to form water.

The effect of the *Moringa* phytochemicals on basal respiration and leakage of protons is shown in Fig. 5.3.5. β-Sitosterol causes a significant increase, by 38%, in basal respiration compared with control (student's t-test $p<0.05$). Since there are no significant differences in ATP production amongst the groups, the increased basal respiration could reflect an increase in proton leakage induced by β-sitosterol. This turns out to be the case. Both β-sitosterol and rutin cause a significant (student's t-test $p<0.05$) increase in proton leakage, by 56% and 49% respectively. This effect is the largest found in any of the measurements made in this experiment.

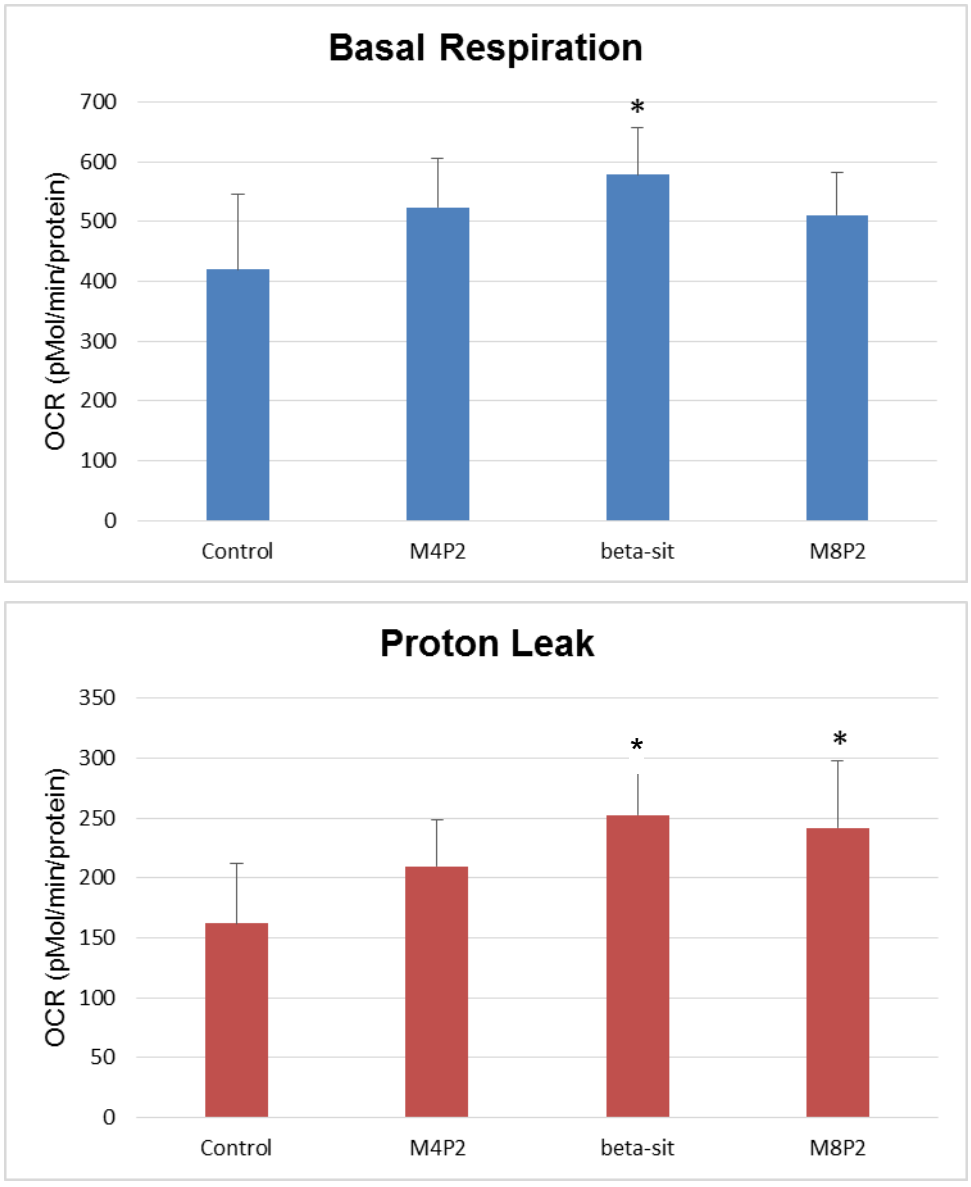


Figure 5.3.5. The effect of *Moringa* phytochemicals on mitochondrial basal respiration and proton leak. Oxygen consumption rate measurement of mitochondrial basal respiration and proton leak after treatment of HepG2 cells with β-sitosterol, MA4P2 (*O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*)) and M8P2 (rutin). Significant increases in oxygen consumption compared with control (student's t-test $p<0.01$) are indicated by asterisks.

Chapter v
Discussion and conclusion

10.1. Discussion and conclusion

The data from this part of the project have guided us about hypoglycaemic effect of *M.peregrina* extracts and phytoestrogens. Data from optimisation of the method of use for 2-NBDG as fluorescent glucose analogue in glucose uptake assay was the first data generated in this project, and it helped us to determine the most suitable concentration for 2-NBDG in terms of its effect and cost which is 100 μM. Also cell concentration and types of medium with/ without glucose (Fig.4.1, 4.2 and 4.3) and incubation time were established. After deploying the optimised method to the first experiments for evaluation of *M.peregrina* extracts on glucose uptake in HepG2 cells (Fig.4.4) the most effective extracts in stimulating glucose uptake were identified, namely those made with ethyl acetate, chloroform and hexane. These extracts induced an increase in glucose uptake that was significant with respect to control *(p*<0.05 for ethyl acetate, chloroform and hexane extracts) while the effects of the three other extracts were not significantly different compared to control. Although the methanol extract appeared to make a significant difference on uptake with respect to control, the standard deviation was high (*p*= 0.710, methanol vs control). We then focused on the three active extracts and have tested their effect on glucose uptake as assyed using 2-NBDG, after long term and short term treatments. We chose to use confluent cells for this part of the assay to make the model similar to the quiescent phase of hepatocytes in liver. The data in Fig 4.5 shows that the treatments exerted more effect on glucose uptake after one hour compared with 24 hours treatment. According to a two-way ANOVA test the difference between the

duration treatment in this test is significant *(p*< 0.0001) and the difference between the treatments also is significant *(p*<0.001).

As the effect of *M.peregrina* extracts on stimulation of glucose uptake had been shown using the 2-NBDG assay it was of interest to compare the effect with well-known anti-diabetic drugs metformin, berberin and ouabain, and with, phloretin, a known inhibitor of glucose uptake. The ethyl acetate extract expressed the highest effect on stimulating of glucose consumption among all the treatments while chloroform and hexane extracts were less effective, they however increased the uptake, on the basis of a one-way ANOVA, differences between the means are statistically significant (*p*<0.001).

Measurement of glucose consumption was the second method to analyse the efficacy of the extracts. The finding from these experiments Fig. 4.10-Fig. 4.14 also showed the effectiveness of the ethyl acetate and chloroform extracts as the most potent in modulating glucose consumption. The data also confirmed that the best effect was achieved with a short term treatment and there was not much change on stimulation of glucose consumption after 24 and 48 hours treatment with ethyl acetate or chloroform extracts. In Fig 4.10, metformin data revealed that there is no effect from metformin on glucose uptake after a short treatment, while after 24 hours treatment Fig 4.11, shows the efficacy of metformin which is in line with its known pharmacokinetics, which was not observed in 48 hours treatment Fig.4.12.

On the basis of our findings in this study, the hypoglycaemic effects of *M.peregrina* ethyl acetate and chloroform extracts was confirmed with two different methods (Fig.4.4 and Fig.4.10). Further *in vitro* investigation with these methods indicate that

the two active extracts of *M.peregrina* are fast acting with no increase in their efficacy with longer incubation time (Fig.4.5 and Fig 4.13). Therefore an increase in gene expression could not be a mechanism by which an increase in glucose uptake is induced. It suggests a fast-acting mechanism is operating for example the translocation or stabilisation of glucose transporters.

Since the active extracts from *M. peregrina* had been identified, it became important to isolate and characterise the components in them. Following purification of the ethyl acetate and chloroform extract as fully described in chapter 3, seven components were identifed, three of which, *O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*), β-sitosterol and rutin were chosen to be investigated for further investigation for more clarification of their mechanism of effect. As the extract assessed for their anti-diabetic effects, a study was designed to assay the components' effect on HepG2 cell metabolism.

Previous studies showed anti- diabetic, hypotension and antioxidant effect of Moringa species specially M. *peregrina* (Ullah et al. 2015, Ullah et al. 2016, Dehshahri *et al.* 2012) and M. *oleifera* (Gupta *et al*. 2012),Bennett *et al.* 2003), different parts of this plant use as vegetables in many countries which is relevant to the effects of this plant on many diseases.

Ullah and his team investigated hypoglycaemic effect of hydro-alcoholic extract of *M.peregrina* by using *in-vitro* enzyme model to assess inhibitory effect of three important enzymes in diabetic treatment including α-amylase, α-glucosidase and dipeptyl peptidase 4.the results of inhibitory effect on pancreatic α-amylase showed persistent intervention of the extract with action of the enzyme in response to increasing the concentration of the extract with IC_{50} value of 1,335.89 µg/mL. Values

reported are ± SEM of three independent experiments*P ≤ 0.05, * *P ≤ 0.01 significant when compared with the control (in the absence of tested concentration of the extract), this inhibitory effect leading to decrease in glucose absorption and lowering the postprandial serum glucose levels.

The data from analysis of *M.peregrina* inhibitory effect on Dipeptidyl peptidase-4 (DPP4) revealed that the extract regularly inhibited the enzyme function in a dose-dependent manner with IC_{50} value of 1,218.12 µg/mL. Values reported are ± SEM of three independent experiments*P ≤ 0.05, * *P ≤ 0.01 significant when compared with the control (in the absence of tested concentration of the extract). The inhibitory effect improves glucose homeostasis by achieving longer half-life and biological activity for Glucagon-like peptide-1 (GLP-1) and Glucose-dependent insulinotropic polypeptide (GIP).

Oxidative stress is an etiological factor for diabetes mellitus hence it is trusted antidiabetic treatment may contain anti- peroxidative/cytoprotective potential. Antioxidant effect of *Moringa* as a part of human dietary in many countries was assessed using $ABTS^+$ radical scavenging assay. The data showed *M. peregrine* extract led to continuous scavenging of $ABTS^+$ radical in a dose dependent manner with IC_{50} value of 20.56 µg/mL and the positivity of the assay were due to presence of components that were isolated from the extract such as; tannins , phenolics and saponins.Values reported are ± SEM of three independent experiments*P ≤ 0.05, * *P ≤ 0.01 significant when compared with the control (Ullah *et al.* 2015).They also evaluated hypotension effect of *M.peregrina* by examining its inhibitory effect against

angiotensin converting enzyme (ACE) that is one of the critical enzyme in management of hypertension and cardiovascular disease, in this study dose-response of *M*. peregrine extract with R. *stricta, M. Peregrin*a and *A. fragrantissima* was tested and the results showed the increase of ACE inhibitory activity with increase in the extract concentration. Captopril as ACE inhibitor drug was used as a standard in this experiment, IC_{50} values presented the ACE inhibitory effect of the extracts in the following order*, R. stricta > M. peregrina > A. fragrantissima*. Values reported are ± SEM of three independent experiments. *P ≤ 0.05: significant when compared with the control (Ullah *et a*l. 2016).

Dehshahri and her colleagues studied antioxidant effect of phenolic group as one of the major constituents using he DPPH radical scavenging assay. The data revealed that *M. peregrina* leaf extract reduced DPPH radicals significantly as compared to the control (*P*<0.05). The extract showed IC_{50} value of 8.06 ± 0.29 µg/ml compared with polyphenol, EGCG from green tea, which was used as the positive control with IC_{50} value of 1.54 ± 0.19 µg/ml. The extract was significantly (*P*<0.05) less active than EGCG. There were significant differences (*P*<0.05) between DPPH scavenging activity of different concentrations of extract except 30 and 40 µg/ml (Dehshahri *et al* 2012)

Adisakwattana and his team studied other *Moringa* species including *M. oleifera.* Anti- diabetic and hypolipidemia effect of the extract was evaluated by testing its inhibitory effect on of alpha-glucosidase and pancreatic alpha-amylase . *M.oleifera* extract contained a specific inhibitor of intestinal sucrose than intestinal maltase with IC_{50} value of 0.78 +/- 0.21 mg/ml, values reported are ± SEM of three independent experiments*P ≤ 0.05, significant when compared with the control. Considering it

slightly inhibited pancreatic alpha-amylase and pancreatic cholesterol esterase, the extract did not show inhibitory effect on pancreatic lipase. Results indicated that the leaf extract of *M. oleifera* may be used for the control of blood glucose and lipid concentration and prevention of hyperglycaemia and hyperlipidaemia (Adisakwattana and Chanathong 2011).

Sangkitikomol and his colleagues determined total antioxidants and inhibitory effect of *Moringa oleifera* extract (MOE) on AGE formation using the ORAC assay, the FCP assay, and the total flavonoids assay. HepG2 cells treated with varying concentrations of MOE (0 - 10 mg/mL; 0 = control) was evaluated using AGE formation assay and amino guanidine was used as an inhibitor. Values are reported as means with their standard error (SE). All experiments were performed in triplicate (N = 3). *P < 0.05 for significant change in AGE formation as compared to control (untreated) (Sangkitikomol *et al.* 2014).

In this current work we also have focused on a phenolic (isoflavone) group as well as thiocarbamates and phytosterols (β-sitosterol).

β-sitosterol was previously isolated from the chloroform extract as a major component of the aerial parts of *Aristolochia indica* (CEAI). Excellent anti- diabetic effect of CEAI was shown through *in vitro* as well as animal study. The efficacy of the extract was compared with standard drug, Glibenclamide (10 mg/kg p.o.) in diabetic mice. Antioxidant effect of the extract in the level of 1, 1- diphenyl-2-picrylhydrazyl (DPPH) and superoxide radical with IC_{50} value being 7.325 and 8.498 µg/ml, respectively. The data showed that *A. indica* has anti-diabetic property as well as high level of natural antioxidants (Sanjay *et al.* 2012).

Another traditional herbal medicine that has been studied intensively for treatment of diabetes is *Momordica charantia*. Some researchers investigated effect of *M. Charantia* aqueous leaf extract on fasting blood glucose (FBG), where administration of the *M. Charantia* aqueous leaf extract in the diabetic rats significantly ($p<0.05$) reduced FBG level after two and four weeks treatment in compared to untreated diabetic control in dose-response manner (Sani *et al.*2015). Numbers of studies have suggested its mechanism of anti-diabetic effect through stimulation of peripheral glucose utilization , inhibition of gluconeogenesis, stimulation of key enzyme of HMP pathway and conservation of islet β cells and their activity (Hasan *et al*.2012, Joseph *et al*. 2013).Due to multitude medical conditions that can *M.charantia* treat , scientists were interested to study its bioactive compounds ,charantin, polypeptide-p and vicine were isolated as main compounds with hypoglycemic effect from *M.charantia* (Joseph *et al*. 2013).

Charantin hypoglycemic effect in fasting rabbits showed gradual decrease in blood sugar level within one to four hours and recovered slowly to initial level. Blood sugar level was dropped by 42% at the 4th hour with oral dose of 50 mg/kg .The average blood sugar decline during 5 hours was 28%. Charantin was found to be more potent than tolbutamide nonetheless both compounds have changed blood sugar level in the same way (Desai *et al.* 2015). Charantin is steroidal glycoside and exist as equal mixture of stigmasterol glucoside and β- sitosterol glucoside (Pitiphanpong *et al.* 2007).These information are supporting the usage of isolated phytoestrol (β-sitosterol) from our extracts for testing its effect on HepG2 cell metabolism.

An isoflavone was studied as one of the isolated compounds from *M.peregrina* extracts and as a member of the phytoestrogen family in this project.
Seahorse bioanalysis was chosen to measure effect of the constituents on oxygen consumption rate in HepG2 cells. Beta-sitosterol and *O*-ethyl 4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (*E*) have shown an increase trend in maximal respiration, non-mitochondrial respiration and spare respiratory capacity while there was no change for oxygen consumption rate in ATP production .
As we use cancer cells for our experiment (HepG2 cells) and after taking into consideration Otto Warburg's finding in cancer cell metabolism (Koppenol et al. 2011), that cancer cells have increased aerobic glycolysis (the so-called "Warburg effect") (Michelle *et al.*2013), the outcome of the Seahorse assay can be supported in the sense that there should not occur any changes in mitochondrial respiration as in tumour cell aerobic glycolysis is the main pathway of energy production.

Analysing the hypoglycaemic effect of phytoestrogens was next part of this work where we applied the optimised method for usage of 2-NBDG to assess glucose uptake effect of the phytoestrogens. HepG2 cells were treated with Isoflavones phytoestrogens including daidzein, genistein and formononetin and each treatment was separately performed and analysed. The daidzein data, Fig.4.7 revealed that in both modes of study the uptake were stimulated and all the concentrations exerted increased effect on the uptake. However a better effect came from short term treatment, and concentration of 10^{-9} M is the most potent treatment. With genistein (Fig. 4.8) the situation is different, as in both modes of study, the change in glucose uptake fluctuated. In the one hour test the only concentration of genistein that

showed an increase effect on the uptake was 10^{-6} M and it was about 2 times more than control. The rest of the concentrations did not have a positive effect on glucose consumption in HepG2 cells. In long treatments only two of the genistein concentrations appeared to have a positive effective on modulating the uptake, 10^{-6} M and 10^{-5} M.

The information from formononetin assay and its effect on glucose uptake (Fig 4.9), shows that only the long term method of treatment can stimulate the consumption of glucose, where the lowest concentration 10^{-9} M is the most potent compared with the rest of concentrations, and increased the uptake around 2.5 times more than control. The effect of phytoestrogens, mainly in type 2 diabetes, have been extensively studied previously (Jayagopal *et al*. 2002; Azadbakht *et al*. 2008; Hermansen *et al.* 2001; Liu *et al.* 2009; Clerici *et al*. 2011; Anderson *et al.* 1998; Teixeira *et al*. 2004; Gobert *et al*. 2010). In this work we assessed the effect of daidzein, genistein and formononetin on glucose uptake in an *in vitro* study. The finding in this section in terms of the isoflavones' hypoglycaemic effect is supported by previous studies.

The aim of the western blotting part of this study was to analyse the effect of our extracts on expression of *GLUT1* in our model hepatocellular carcinoma cell line. Previous studies on the expression of *GLUT1* in human and rat HCC tissue had different outcomes. In most studies a strong and increased expression of *GLUT1* had been found in HCC (Yamamoto *et al.* 1990; Grobholz *et al.*1993). While other studies failed to detect GLUT1 protein expression by immunohistochemical staining in HCC tissue (Su *et al.* 1990; Younes et al. 1996; Zimmerman et al. 2002; Burke et.al.2002; Roh *et al*.2004). We designed an experiment to establish Western

blotting technique for our targeted protein GLUT1. On basis of the results from the experiment we could see that the bands was detected in all loaded lanes as a single band in the region of 50kDa as were expected. These data show the GLUT1 was detected and the GLUT1 transporters are abundantly expressed in HepG2 cells. The effect of the active extracts was assessed once and very preliminary data suggest they may increase expression.

References

Abeeleh M.A., Ismail Z.B., Alzaben K.R., Abu-Halaweh S.A., Al-Essa M.K.,Abuabeeleh J., Alsmady M.M. Induction of diabetes mellitus in rats using intraperitoneal streptozotocin: a comparison between two strains of rats. *Eur. J. Sci. Res*. 32(2009) 398–402.

Anderson J.J.B., Garner S.C., Phytoestrogens and bone. *Balliere's Clin Endocrinol Metab.,* 12(1998)543–557*.*

Adisakwattana S., Chanathong B. Alpha-glucosidase inhibitory activity and lipid-lowering mechanisms of Moringa oleifera leaf extract. *Eur Rev Med Pharmacol Sci.,*15(2011) 803-808.

Adlercreutz H., Mazur W., Phyto-oestrogens and western diseases. *Ann Med* 2(1997) 95–120.

Ae Park S., Choi M.S., Cho S.Y., Seo J.S., Jung U.J., Kim M.J., Sung M.K., Park Y.B., Lee M.K. Genistein and daidzein modulate hepatic glucose and lipid regulating enzyme activities in C57BL/KsJ-db/db mice. *J. Life Sciences* 79(12) (2006) 1207–1213.

Afsharypuor, S., Asghari, G., Mohagheghzadeh, A., Dehshahri, S., Determination of volatile glucosinolate degradation products in seed coat, stem and *in vitro* cultures of *Moringa peregrina* (Forssk.) Fiori. *Iran. J. Pharma.Sci*., 6(2010) 141-144.

Agrawal, P. K., *Carbon-13 NMR of flavonoids*. Central Institute of Medicinal and Aromatic Plants Lucknow, India, 1989

Akanaga H, Chaudhuri B, Frommer WB.T GLUT1 and GLUT9 as major contributors to glucose influx in HepG2 cells identified by a high sensitivity intramolecular FRET glucose sensor. *Biochim Biophys Acta .,* 4(2008) 1091-1099.

Al-Othman A.A., Al-Kahtani H.A., Hewedy F.M., Al-Khalifa A.S. Plasma lipid profiles of rats fed seed oils of Moringa peregrine and Salicornia. *J. Applied Nutrition* 1 & 2(1998) 3–11

Anderson J.W., Blake J.E., Turner J., Smith B.M., Effects of soy protein on renal function and proteinuria in patients with type 2 diabetes. *Am J Clin Nutr*, 68(1998) 1347-1353.

Anthony M.S., Clarkson T.B., Williams J.K., Effects of soy isoflavones on atherosclerosis: potential mechanisms. *Am J Clin Nutr*., 68(1998) 1390–1393.

Asano T., Shibasaki Y., Ohno S., Taira H., Lin J.L. Kasuga M., Kanazawa Y., Akanuma Y., Takaku F., Oka Y. Rabbit brain glucose transporter responds to

insulin when expressed in insulin-sensitive Chinese hamster ovary cells. *J Biol. Chem.*, 64(2005) 207-215

Ashfaq M., Shahzad M.A., Ashfaq B.,Ashfagh U., Moringa: A Miracle Plant for Agro-forestry, *Journal of agriculture & social sciences,*13(2012) 115–122.

Ayyari M., Salehi P., Ebrahimi S.N., Zimmermann s.,Portmann L.,KrauthSiegel L., Kaiser M.,Brun R., Rezadoost H., Rezazadeh S., Hamburger M., Antitrypanosomal Isothiocyanate and Thiocarbamate Glycosides from Moringa peregrine, *J.Plant Med*. 80 (2014) 86-89

Azadbakht L., Atabak S., Esmaillzadeh A., Soy protein intake, cardiorenal indices, and C-reactive protein in type 2 diabetes with nephropathy: a longitudinal randomized clinical trial. *Diabetes Care,*31(2008) 648-654.

Balakrishnan V., Prema P., Ravindran K.C., Robinson P.J. Ethnobotanical studies among villagers from Dharapuram taluk. Tamil Nadu. *Indian Global J. Pharmacol*. 3(2009) 8–14.

Ball S.W., Bailey J.R., Stewart J.M., Vogels C.M., Westcott S.A., A fluorescent compound for glucose uptake measurements in isolated rat cardiomyocytes , *Can J Physiol Pharmacol*.,80(2002) 205-209

Baluchnejadmojarad Tourandokht; Roghani Mehrdad. Chronic administration of genistein improves aortic reactivity of streptozotocin-diabetic rats: mode of action.

J. Vascular Pharmacology 49(1) (2008) 1–5.

Barroso I., Luan J., Middelberg R.P.S. *et al.* Candidate gene association study in type 2 diabetes indicates a role for genes involved in B-Cell function as well as insulin action. *PLoS Biol*,. 1(2003) 41–55.

Bell G., Kayano T., Buse J., Burant C., Takeda J., Lin D., Fukumoto H.,
Seino S., Molecular biology of mammalian glucose transporters, *Diabetes Care*.
13(1990) 198–208.

Bellostas, N., Sørensen, J. C. ,Nikiema, A., Sørensen, H., Pasternak, D., Kumar, S., Glucosinolates in leaves of Moringa species grown and disseminated in Niger.
Afr. J. Agr. Res. 5 (2010) 1338-1340.

Bennetts H.W., Underwood E.J., Shier F.L., A specific breeding problem of sheep on subterranean clover pastures in Western Australia. *Australian Veterinary Journal* 22 (1946) 2–12

Bethesda , *National Center for Biotechnology Information* (US) (2004).

Bhathena S.J., Velasquez M.T. Beneficial role of dietary phytoestrogens in obesity and diabetes. *American Journal of Clinical Nutrition* 6 (2002) 1191–1201.

Boland M.L., Chourasia A.H., Macleod K.F., Mitochondrial Dysfunction in Cancer. *Front. Oncol.,* 3(2013) 292.

Bösenberg L.H., van D. G., The mechanism of action of oral antidiabetic drugs: A review of recent literature. *Journal of Endocrinology, Metabolism and Diabetes of South Africa*, 13(2008) 80-88, Bouatia-Naji N., Bonnefond A., Cavalcanti-Proenca C., et al. A variant near MTNR1B is associated with increased fasting plasma glucose levels and type 2 diabetes risk. Nat Genet 41(2009) 89-94

Brown A.C.,StevensonL.M., Leonard H.M, Nieves-Puigdoller K. and Clotfelter D.E Phytoestrogens β-Sitosterol and Genistein Have Limited Effects on Reproductive Endpoints in a Female Fish, Betta splendens. *Biomed Res.Int. ; Article ID681396* (2014) .

Burant C.F., Takeda J., Brot-Laroche E., Bell G.I., Davidson N.O.,Fructose transporter in human spermatozoa and small intestine is GLUT5,*J Biol Chem.* 25;267(1992) 14523-14526.

.

Caceres A., Saravia A., Rizzo S., Zabala L., De L.E., Nave F.,. Pharmacologic properties of Moringa oleifera. 2: Screening for antispasmodic, antiinflammatory and diuretic activity,J *Ethnopharmacol.* 3(1992) 233-237

Carter M., Matrone G., Smart W.W.G.J. Effect of Genistein on Reproduction of the Mouse*, J. Nutr.* 55(1955) 639–645

Carter M., Matrone G., Smart W.W.G.J. The Effect of Genistin and Its Aglycone on Weight Gain in the Mouse, *J. Nutr.*,14(1960) 301–304

Cederroth C.R., Nef S., Soy, phytoestrogens and metabolism: A review. *Molecular and cellular endocrinology* 304 (2009) 30–42.

Chuang P.H., Lee C. W., Chou J.Y., Murugan M., Shie h.B.J., Chen H.M., *Bioresour. Technol.,* 98(2007) 232-236

Cifuentes M., García M.A., Arraba P.M., Martínez F., Yañez M.J., Jada N., Weil B., Domínguez D.,Medina R.,Nualart F., insulin regulates GLUT1-mediated glucose transport in MG-63 human osteosarcoma cells. *journal of Cellular Physiology*,226(2011)1425-1432

Clerici C., Nardi E., Battezzati P.M., Asciutti S., Castellani D., Corazzi N., Giuliano V., Gizzi S., Perriello G., Di Matteo G., Galli F., Setchell K.D. ,Novel soy germ pasta improves endothelial function, blood pressure, and oxidative stress in patients with type 2 diabetes. *Diabetes Care,34(* 2011) 1946-1948.

Couch R., Jetha M., Dryden D.M., Diabetes education for children with type 1 diabetes mellitus and their families. Rockville (MD, USA): Agency for Healthcare Research and Quality (US) (2008).

Crawford J.M. Liver, biliary tract, and pancreas. In: Robbins S.L., Cotran R.S., Kumar V., *Pocket companion to Robbins pathologic basis of disease*. Philadelphia, (PA, USA): WB Saunders (1991) 313–317.

Curran E.M., Judy B.M., Newton L.G., Lubahn D.B., Rottinghaus G.E., Macdonald R.S., Franklin C., Estes D.M. Dietary soy phytoestrogens and ERalpha signalling modulate interferon gamma production in response to bacterial infection. *J. Clin. Exp. Immunol.* 2(2004) 219–225.

Dean L., McEntyre J. The genetic landscape of diabetes Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications (PDF). World Health Organisation. (1999).

Defeng W., Arthur I., Cederbaum, Ethanol Cytotoxicity to a Transfected HepG2 Cell Line Expressing Human Cytochrome P4502E1*, J. Biological Chemistry* , 271(1996) 23914 –23919

Dehshahri S., Afsharypuor S., Asghari G., Mohagheghzadeh A.,
Antioxidant activity of methanolic leaf extract of *Moringa peregrina* (Forssk.) Fiori.
*Res. Pharm. Sci.,7(*2012) 51.

De Souza C.J., Eckhardt M., Gagen K., Dong M. Effects of pioglitazone on adipose tissue remodeling within the setting of obesity and insulin resistance. Diabetes, Am Diabetes Assoc. (2001).

Desai S.,Tatke P., Charantin: An important lead compound from Momordica charantia for the treatment of diabetes*, Journal of Pharmacognosy and Phytochemistry* 3 (2015) 163-166.

Dorajoo R., Liu J., Boehm, B.O. Genetics of Type 2 Diabetes and Clinical Utility. *Genes* 6 (2015), 372-384.

Dupuis J, Langenberg C, Prokopenko I, et al. New genetic loci implicated in fasting glucose homeostasis and their impact on type 2 diabetes risk. Nat Genet 42(2010) 105-116

East, J. The Effect of Genistein on the Fertility of Mice*, J. Endocrinol*. 13(1955) 94–100

Efrat S., Making sense of glucose sensing, *Nat. Genet* 17(1997) 249–250.

Eilert U., Wolters B., Nahrstedt A., The antibiotic principle of seeds of Moringa oleifera and Moringa stenopetala**.** *Planta Med.,* 42 (1981) 55-61.

El-Alfy T.S., Ezzat S.M., Hegazy A.K., Amer A.M., Kamel G.M., Isolation of biologically active constituents from *Moringa peregrina* (Forssk.) Fiori. (Family: Moringaceae) growing in Egypt*. Pharmacogn. Mag.* 7(2011) 109–115*.*

Elbatran S.A., Abdel-Salam O.M., Abdelshfeek K.A., Nazif N.M., Ismail S.I., Hammouda F.M. Phytochemical and pharmacological investigation on *Moringa peregrina* (Forssk)Fiori. *Nat. Prod. Sci.* 11(2005) 199–206.

Elliott S., Keim N., Stern J., Teff K., Havel P., Fructose, weight gain , and the insulin resistance syndrome, *American Journal of Clinical Nutrition*, 76(2002) 911-922.

Faizi S., Siddiqui B. S., Saleem R., Siddiqui S., Aftab K., Isolation and structure elucidation of novel hypotensive agents, Niazinin A, Niazinin B, Niazimicin and Niaziminin A + B from Moringa oleifera: the first naturally occurring thiocarbamates. *J. Chem. Soc. Perkin Trans 1*, (1992) 3237-3241

Flores-Saenz J.L., Alarcon-Aguilar F.J., Studies on hypoglycemic activity of Mexican medicinal plants. *J. Unbound MEDLINE* (2002)

Flores J.C., Hirschhorn J., Altshuler D.,The inherited basis of diabetes mellitus: implications for the genetic analysis of complex traits. *Annual Review of Genomics and Human Genetics* 4(2003) 257–291.

Fowler M.J. Microvascular and Macrovascular Complications of Diabetes,*Clinical Diabetes* ,26(2008) . 2 77-2782.

Fong D.S., Aiello L.P., Ferris F.L. 3rd, Klein R., Diabetic retinopathy. *Diabetes Care* 27(2004) 2540–2553.

Ford E.S., Williamson D.F., Liu S.,Weight change and diabetes incidence: findings from a national cohort of US adults. *Am. J. Epidemiol.* 146(1997) 214–222

Food and Agriculture Organization, FAO Food and Nutrition, In *Traditional Food Plants*, 42(1988) 369-373.

Franks P.W., Rolandsson O., Debenham S.L., Replication of the association between variants in WFS1 and risk of type 2 diabetes in European populations. *Diabetologia,51(* 2008)458-463

Gabbay K.H. Hyperglycemia, polyol metabolism, and complications of diabetes mellitus. *Annu. Rev. Med.* 26(1975) 521–536.

Gabbay K.H. Aldose reductase inhibition in the treatment of diabetic neuropathy: where are we in 2004?, *Curr. Diab. Rep.* 4(2004) 405–408.

Gloyn A.L., The search for T2D genes. *Ageing Research Reviews* 2(2003) 111–127.

Gilani A.H., Aftab K., Suria A., Siddiqui S., Salem R., Pharmacological studies on hypotensive and spasmolytic activities of pure compounds from *Moringa oleifera, Phytotherapy Research* 9(1994) 87-91 .

Goldin A., Beckman J.A., Schmidt A.M., Creager M.A., Advanced glycation end products. Sparking the development of diabetic vascular injury. *Circulation* 114 (2006), 597-605.

Gobert C.P., Pipe E.A., Capes S.E., Darlington G.A., Lampe J.W., Duncan A.M.,Soya protein does not affect glycaemic control in adults with type 2 diabetes. *Br J Nutr*., 103(2010)412-421.

Gudmundsson J., Sulem P., Steinthorsdottir V.,Two variants on chromosome 17 confer prostate cancer risk, and the one in TCF2 protects against type 2 diabetes.*Nat Genet* 39(2007) 977-983

Gupta R.,Mathur M.,Bajaj V.K.,Katariya P.,Yadav S.,Kamal R.,Gupta R.S.,Evaluation of antidiabetic and antioxidant activity of Moringa oleifera in experimental diabetes. *J. Diabetes*.,4(2012) 164-171

Hanaa H.A., El B., Gamal S., El B., Biological activity of the Egyptian *Moringa peregrina* seed oil. *In.J. of Management Sciences and Business Research* 2(2013), 2226-8235.

Hansen L., Candidate genes and late-onset T2D mellitus. Susceptibility genes or common polymorphisms?. *Danish Medical Bulletin* 50(2003) 320–346.

Harris D.M., Besselink E., Henning S.M., Go V.L.W., Heber D.,Phytoestrogens induce differential estrogen receptor alpha- or beta-mediated responses in transfected breast cancer cells. *J. Biol. Med*. 230(2005) 558–568

Hasani-Ranjbar S., Larijani B., Abdollahi M., A systematic review of Iranian medicinal plants useful in diabetes mellitus. *J.* Arc*. Med. Sci.* 4(3) (2008) 285–292.

Hasan I., Khatoon S., Effect of *Momordica charantia* (bitter gourd) tablets in diabetes mellitus: Type 1 and Type 2. *Prime Res Med (PROM)* 2 (2012) 72–74.

Henry W., The Complications of Diabetes Mellitus. *J Natl Med Assoc*. 79(6)(1987) 677–680.

Hermansen K., Søndergaard M., Høie L., Carstensen M., Brock B., Beneficial effects of a soy-based dietary supplement on lipid levels and cardiovascular risk markers in type 2 diabetic subjects. *Diabetes Care, 24(2001*) 228-233 .

Hintz K.K., Ren J. Phytoesrtogenic isoflavones daidzein and genistein reduce glucose-toxicity-induced cardiac contractile dysfunction in ventricular myocytes. *J. Endocrine Research.* 30(2) (2004) 215–223.

Hongxiang H., George T., Vay L., Go W., Hypoglycaemic herbs and their action mechanisms *Chinese Medicine 4(*2009).

Hruz P., Mueckler M., Structural analysis of the GLUT1 facilitative Glucose transporter, *Mol. Membr. Biol*. 18(2001) 183–93.

Iynedijan P.B., Jotterand D., Nouspikel T., Asfari M., Pilot P.R. (1989) Transcriptional induction of glucokinase gene by insulin in cultured liver cells and its repression by the glucagon-cAMP system. *J. Biol. Chem*. 264, 21824–21829.

Jaiswal D., Rai P., Kumar A., Mehta S.,, Watal G., Effect of Moringa oleifera Lam. leaves aqueous extract therapy on hyperglycaemic, *Journal of Ethnopharmacology* 123 (2009) 392–396

Jayagopal V., Albertazzi P., Kilpatrick E.S., Howarth E.M., Jennings P.E., Hepburn D.A., Atkin S.L. Beneficial effects of soy phytoestrogens intake in postmenopausal women with type 2 diabetes. *J. Diabetes Care* 25(10) (2002) 1709-1714.

Jacobs D.B., Hayes G.R., Truglia J.A., Lockwood D.H., Effects of metformin on insulin receptor tyrosine kinase activity in rat adipocytes.*J. Diabetologia* , 29(1986) 798-801.

Joost HG & Thorens B The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members (review). Molecular Membrane Biology 18 (2001) 247–256.

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

Jun Y., Jianping Y., Weiping J., Effects and mechanisms of berberine in diabetes treatment *Acta Pharmaceutica Sinica*, 2(2012) 327–334

Kajikawa M., Fujimoto S.,Tsuura Y., Mukai E., Takeda T.,Hamamoto Y. Takehiro M., Fujita J.,Yamada Y., Seino Y., Ouabain Suppresses Glucose-Induced Mitochondrial ATP Production and Insulin Release by Generating Reactive Oxygen Species in Pancreatic Islets. *J.DIABETES*, 51(2002)

Kawamori R., Morishima T., Kubota M., Matsuhisa M., Ikeda M., Kamada T.,Influence of oral sulfonylurea agents on hepatic glucose uptake. *Diabetes Res Clin Pract.* 28 (1995) 109-113.

Kim Y.S., Bae J.H., Nah D.Y., Kim Y.K., Lee M.M., Kim H.Y., Effect of Korean red ginseng on arterial stiffness in subjects with hypertension. *J. Altern. Complement. Med.* 17(2011) 45–49.

Koppenol W.H., Bounds P.L., Dang C.V., Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer,*5(2011) 325-337

Kuiper G., Lemmen J.G., Carlsson B., et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor . *Endocrinology*, 139(1998) 4252–4263.

Larijani B., Abdollahi M., A systematic review of the impact of n-3 fatty acids in bone health and osteoporosis. *Medical* (2008).

Laville M., Andreelli F. Mechanisms for weight gain during blood glucose normalization. *Diabetes & metabolism* (2000) .

Lebovitz H.E., Feinglos M.N., Mechanism of action of the second-generation sulfonylurea glipizide. *Am J Med.* 30(1983) 46-54.

Libraray and archive Canada,*www.collectionscanada.gc.ca/physicians/030002-2000-e.html*2000.

.Lissin L.W., Cooke J.P., Phytoestrogens and cardiovascular health. *J Am Coll Cardiol.,35(* 2000)1403–141

Liu Z.M., Chen Y.M., Ho S.C., Ho Y.P., Woo J. Effects of soy protein and isoflavones on glycemic control and insulin sensitivity: a 6-mo double-blind, randomized, placebo-controlled trial in postmenopausal Chinese women with prediabetes or untreated early diabetes. *Am J Clin Nutr*., 91(2010) 1394-140

Longo D.L., Fauci A.S., Kasper D.L., Hauser S.L., Jameson J.L., Loscalzo J. *Harrison's Principles of Internal Medicine, 18th Edition* 2011 McGraw Hill

.

Luo W., Zhao M., Yang B., Shen G., Rao G., Identification of bioactive compounds in *Phyllenthus emblica* L. fruit and their free radical scavenging activities. *Food Chem., 114(*2009**)** 499-504

Luo Q., Hu D., Hu S., Yan M., Sun Z., Chen F., *In vitro* and *in vivo* anti-tumor effect of metformin as a novel therapeutic agent in human oral squamous cell carcinoma*, BMC Cancer*, (2012) 12: 517.

Lye H.S., Kuan C.Y., Ewe J.-A., Fung W.-Y., Liong M.-T. The improvement of hypertension by probiotics: effects on cholesterol,diabetes,renin and phytoestrogens. *International Journal of Molecular Sciences* 10(9) (2009) 3755–3775.

Lyssenko V., Nagorny C.L., Erdos M., Common variant in MTNR1B associated with increased risk of type 2 diabetes and impaired early insulin secretion. *Nat Genet.* 41(2009)82-88

Maori L., Ezekiel D., Bilal J., Prevalence of Diabetes in Zambuk General Hospital. *Rep Opinion,* 4(10)(2012) 54-57.

Martin P.M., Horwitz K.B., Ryan D.S., McGuire W.L.. Phytoestrogen interaction with estrogen receptors in human breast cancer cells. *Endocrinology,* 103(1978) 1860–1867.

Marwah R.G., Fatope M.O., Al Mahrooqi R., Varma G.B.,Abadi A., Al-Burtamani H., Suad Khamis S. Antioxidant capacity of some edible and wound healing plants in Oman. *J. Food Chemistry* 101(2) (2006) 465–470.

Mazibuko, Sithandiwe E., An in vitro study of the effects of free fatty acids on insulin mediated glucose uptake and metabolism by myocytes and fibroblast derived adipocytes, URI: http://hdl.handle.net/10530/618 ,2011.

Mekonnen N., Houghton P., Timbrell ., The toxicity of extracts of plant parts of Moringa stenopetala in HEPG2 cells in vitro*, Phytother Res.*,19(2005):870-875.

Miksicek R.J. Interaction of naturally occurring non-steroidal estrogens with expressed with recombinant human estrogen receptor. *J Steroid Biochem Mol Biol.,49(*1994)153–160

Miedema K. , "Standardization of HbA1c and Optimal Range of Monitoring". *Scandivavian Journal of Clinical and Laboratory investigation*, 240(2005) 61–72.

Morton J.F.,The horseradish tree, Moringa pterigosperma (Moringaceae). A boon to arid lands. *Econ Bot* 45(1991) 318–333.

Mueckler M (1994) Facilitative glucose transporters. European Journal of Biochemistry 219, 713–725

Nelson D., Cox M., *Lehninger Principles of Biochemistry,* 978(2008)-7167-7108-1.

Oergaard A., Jensen L., The effects of soy isoflavones on obesity. *Experimental Biology and Medicine* 233(9) (2008) 1066–1080.

Peterson K.P., Pavlovich J.G., Goldstein D., Little R*.,* What is hemoglobin A1c? An analysis of glycated hemoglobins by electrospray ionization mass spectrometry". *J.Clinical Chemistry* 44 (9)(1998)1951–1958.

Paulo A., Jimeno M. L., Gomes E. T., Houghton P. J., Steroidal alkaloids from cryptolepis obtuse. *Phytochemistry*, 53(2000) 417-422

Pitiphanpong J., Chitprasert S., Goto M., Jiratchariyakul W., Sasaki M., Shotipruk A. New approach for extraction of charantin from Momordica charantia with pressurized liquid extraction. *Sep Purif Technol* 52 (2007) 416-422.

Ploug T., van Deurs B. A. H., Cushman S.W., Ralston E., Analysis of GLUT4 Distribution in Whole Skeletal Muscle Fibers: Identification of Distince Storage Compartments That Are Recruited by Insulin and Muscle Contractions, *The Journal of Cell Biology*, 142(1998) 1429-1446.

Prokopenko I., Langenberg C., Florez J.C.,Variants in MTNR1B influence fasting glucose levels. *Nat Genet* 41(2009) 77-81.

Qi L, Cornelis M.C., Kraft P., Genetic variants at 2q24 are associated with susceptibility to type 2 diabetes. *Hum Mol Genet 19(*2010) 2706-2715.

Ramkumar K.M., Vijayakumar R.S., Ponmanickam P., Velayuthaprabhu S., Archunan G., Rajaguru P., Anti-hyperlipidaemia effect of Gymnema montanum: a study on lipid profile and fatty acid composition in experimental diabetes. *Basic Clin. Pharmacol. Toxicol.* 103(2008) 538–545.

Rasooly R.S., Akolkar B., Spain L.M., Guill M.H., Del Vecchio CT, Carroll LE.,The National Institute of Diabetes and Digestive and Kidney Diseases Central Repositories: a valuable resource for nephrology research. *Clin J Am Soc Nephrol*. 10(2015) 710-715.

Rayner D.V., Thomas M.E., Trayhurn P., Glucose transporters (GLUTs 1–4) and their mRNAs in regions of the rat brain: insulin-sensitive transporter expression in the cerebellum. *Can J Physiol Pharmacol*. 72 (1994) 476–479.

Reid J. M., Paterson D. J., Role of K+ in regulating hypoxic cerebral blood flow in the rat: effect of glibenclamide and ouabain, American *Journal of Physiology - Heart and Circulatory Physiology* 270 (1996) 45-52.

Reinli K., Block G., Phytoestrogens content of food ,*Nutr Cancer* 26(1996) 123-148.

Resnick H..E., Valsania P., Halter J.B., Lin X., Relation of weight gain and weight loss on subsequent diabetes risk in overweight adults. *J. Epidemiol. Community Health* 54(2000) 596–602.

Royal Pharmaceutical Society and BMJ, *British National Formulary*, 70(2015).

Rung J., Cauchi S., Albrechtsen A., Genetic variant near IRS1 is associated with type 2 diabetes, insulin resistance and hyperinsulinemia. *Nat Genet.41(* 2009) 1110-1115.

Rzonca S.O., Suva L.J., Gaddy D., Montague D.C., Bone is a target for the antidiabetic compound rosiglitazone*. Endocrinology 145(2004) . 401-406*

Sahra B., Laurent K , Loubat A , Giorgetti-Peraldi S. , Colosetti P., Auberger P. , Tanti J.F. , Marchand-Brustel Y. L. ,Bost F., The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level I . *Oncogene,* 27(2008) 3576–3586.

Sandhu M.S., Weedon M.N., Fawcett K.A., Common variants in WFS1 confer risk of type 2 diabetes. *Nat Genet*. 39(2007) 951-953.

Sangkitikomol W. , Rocejanasaroj A., Tencomnao T., Effect of Moringa oleifera on advanced glycation end-product formation and lipid metabolism gene expression in HepG2 cells, *J.Gen. Mol.Res.*13 (2014) 723-735.

Sani F. A., Atiku M. K., IMAM, A. A.,Effect of administration of aqueous leaf extract *of Momordica Charantia* (Bitter melon) on serum glucose and lipid profile in alloxan – induced diabetic rats, *Bayero Journal of Pure and Applied Sciences*, 8 (2015) 170 – 174.

Sanjay K.K.,, Sagar K.M., , Dilipkumar P., Arijit M., Isolation of β-sitosterol and evaluation of antidiabetic activity of Aristolochia indica in alloxan-induced diabetic mice with a reference to in-vitro antioxidant activity, *Journal of Medicinal Plants Research* 6 (2012) 1219-1223

Santti R., Mäkelä S., Strauss L., Korkman J., Kostian M.L. Phytoestrogens: potential endocrine disruptors in males. *J. Toxicol. Ind. Health* 14 (1998) 223–237.

Sato K.K., Hayashi T., Harita N., Yoneda T., Nakamura Y., Endo G., Kambe H., Liver enzymes compared with alcohol consumption in predicting the risk of type 2 diabetes: the Kansai Healthcare Study. *Diabetes Care*. 31(2008) 1230-1236.

Scheen A.J., Paquot N., Metformin revisited: a critical review of the benefit-risk balance in at-risk patients with type 2 diabetes. *Diabetes Metabolism* 39(3) (2013) 179-190.

Setchell K., Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *Am J Clin.Nutr*. 68(1998)1333–1348

Shen P., Liu M.H., Ng T.Y., Chan Y.H., Yong E.L. Differential effects of isoflavones, from Astragalus membranaceus and Pueraria thomsonii, on the activation of PPARalpha, PPARgamma, and adipocyte differentiationin. *J. Nutration* 136 (2006) 899–905

Shepherd P.R., Gould G.W., Colville C.A., McCoid S.C. Gibbs E.M.,Kahn B.B.,Distributioan of GLUT 3 gluocse transporter protein in human tissues,*Biochemical and Biophysical Research Communications*, 188 (1999) 149-154

Shojania K.G., Ranji S.R., McDonald K.M. Effects of quality improvement strategies for type 2 diabetes on glycemic control: a meta-regression analysis. *JAMA* 296(2006) 427–440.

Siddhuraju P., Becker K., Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (Moringa oleifera Lam.) leaves, *J Agric Food Chem.*8 (2003) 2144-2155.

Simon C., Everitt H., Kendrik T.,Oxford handbook of General practice, 2nd edn , 2005.

Simon D., Aden D.P., Knowles B.B.,Chromosomes of human hepatoma cell lines. *Int J Cancer*.30(1982) 27-33.

Simpson I.A., Dwyer D., Malide D., Moley K.H., Travis A., Vannucci SJ. The facilitative glucose transporter GLUT3: 20 years of distinction. *American Journal of Physiology. Endocrinology and Metabolism*.295 (2008) E242–E253

Sriraman S, Ramanujam G.M, Ramasamy M, Dubey G.P. Identification of beta-sitosterol and stigmasterol in Bambusa bambos (L.) Voss leaf extract using HPLC and its estrogenic effect in vitro*, J Pharm Biomed Anal.* 115 (2015) 55-61.

Stephenson T.J., Anderson J.W. Phytoestrogens: diabetic nephropathy. *J. Phytoestrogens and Health* (2002) 488–496*.*

Stumvoll M. Control of glycaemia: from molecules to men. Minkowski Lecture 2003. *Diabetologia* 47(2004) 770–781

Sultana, N., Afolayan, A. J., *Nat. Prod. Res.,*21(2007) 889-896.

Sultana N., Afolayan A., A review of the phytochemistry, botany , pharmacology and toxicology of ARCTOTIS ARCTOTOIDES (L.F.) O. HOFFM. *J. Nat. Prod. Res.,* 21(2007) 889-896*.*

Taniguchi C.M., Emanuelli B., Kahn C.R., Critical nodes in signalling pathways: critical insights into insulin action. *Nature Reviews: Molecular Cell Biology*, 7 (2006) 85-96.

Teixeira S.R., Tappenden K.A., Carson L., Jones R., Prabhudesai M., Marshall W.P., Erdman J.W., Isolated soy protein consumption reduces urinary albumin excretion and improves the serum lipid profile in men with type 2 diabetes mellitus and nephropathy. *J Nutr*., 134(2004) 1874-1880.

Tende J.A.,Ezekiel I., Dikko A.A.U., Goji A.D.T., Effect of Ethanolic Leaves Extract of Moringa oleifera on Blood Glucose Levels of Streptozocin-Induced Diabetics and Normoglycemic Wistar Rats, *British Journal of Pharmacology and Toxicology* 2(2011) 1-4.

Tham D.M., Gardner C.D., Haskell W.L., Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *J Clin Endocrinol Metab*.86 (1998) 2223–2235.

Thorens B., Mueckler M., Glucose transporters in the 21st Century, *American Journal of Physiology - Endocrinology and Metabolism* 298(2010) 141-145.

Touillaud M.S., Pillow P. C., Jakovljevic J., Bondy M.L.,Singletary S.E., Li D., and Shine Chang. Effect of Dietary Intake of Phytoestrogens on Estrogen Receptor Status in Premenopausal Women With Breast Cancer, *Nutr. Cancer Int.J.* 51(2005) 162-169.

Turner J.V., Agatonovic-Kustrin S., Glass B.D. Molecular aspects of phytoestrogen selective binding at estrogen receptors *J. Pharm. Sci.* 96 (2007) 1879–1885.

Tsaknis J., Characterization of Moringa peregrine Arabian seed oil. *Grasas y Aceites (Sevilla)* 49(1998) 170–176.

Turmelle, Y.P., Shikapwashya O., Tu S., Hruz P.W., Yan Q., Rudnik D.A., Rosiglitazone inhibits mouse liver regeneration. *Federation of American Societies for Experimental Biology Journal,* 20(2006) 2609–2611

Turner J.V., Agatonovic-Kustrin S., Glass B.D., Molecular aspects of phytoestrogen selective binding at estrogen receptors. *J Pharm Sci.* 96(8)(2007)1879-1885.

Ullah M.F. , Abuduhier F.M., Inhibition of angiotensin converting enzyme by *Rhazya stricta*, *Moringa peregrina* and *Achillea fragrantissima*, used in traditional system of

medicine in Arabian Peninsula: Implication in the management of hypertension,*J. of Medicinal Plants Research,*10 (2016) 93-99

Ullah M.F., . Bhat S.H.,. Abuduhier F.M., Antidiabetic Potential of Hydro-Alcoholic Extract of *Moringa peregrina*Leaves: Implication as Functional Food for Prophylactic Intervention in Prediabetic Stage, J.Food Chem. 39 (2015) 360-367

Vannucci SJ, Maher F, Simpson IA. Glucose transporter proteins in brain: delivery of glucose to neurons and glia, 21 (1997) 2–21.

Velasquez M.T., Bhathena S.J.,Dietary phytoestrogens: a possible role in renal disease protection. *Am J Kidney Dis.,37(* 2001) 1056–1068.

Voight B.F., Scott L.J, Steinthorsdottir V, et al. Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis.*Nat Genet* 42(2010) 579-589.

Von Mering J., Minkowski O., Diabetes mellitus nach pankreasexstirpation. *Arch. f. exp. Path. u. Pharm*. 26(1890) 371–387.

Walter E. D. , Genistin (an Isoflavone Glucoside) and its Aglucone, Genistein, from Soybeans. *Journal of the American Chemical Society* 63 (1941) 3273–3276.

Wang T.T.Y., Sathyamoorthy N., Phang J.M., Molecular effects of genistein on estrogen receptor mediated pathways. *Carcinogenesis* , 17(1998) 271–275.

Wazaify M., Alawwa I., Yasein N., Al-Saleh A., Afifi F.U., Complementary and alternative medicine (CAM) use among Jordanian patients with chronic diseases. *An. Int. J. I.,* 19(2013) 153–157.

Winckler W., Weedon M.N., Graham R.R., et al. Evaluation of common variants in the six known maturity-onset diabetes of the young (MODY) genes for association with type 2 diabetes.*Diabetes*, 56(2007) 685-693.

Wood S., Trayhurn P., Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. *British Journal of Nutrition*. 89(2003) 3–9.

World Health Organisation."Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications" (PDF). 1999.

World Health Organisation,"Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia" ,2006

Wu Y.L., Ding Y.P., Gao J., Tanaka Y., Zhang W.,Risk factors and primary prevention trials for type 1 diabetes *Int J Biol Sci*. 9(7) 2013 666-679.

Wu H.S., Zhu D.F., Zhou C.X., Insulin sensitizing activity of ethyl acetate fraction of Acorus calamus L. in vitro and in vivo, *Journal of Ethnopharmacology*, 123(2009) 288–292.

Wu C., Ho Y.,Tsai C., Wang Y., Tseng H, Wei P., Lee C., Liu R., Lin S.,In vitro and in vivo study of phloretin-induced apoptosis in human liver cancer cells involving inhibition of type II glucose transporter. *Int J Cancer*.124(2009) 2210-2219

Yildiz F.,Phytoestrogens in functional food.*Taylor&Francis* (2005) 3-5.

Yin J., Gao Z., Liu D., Liu Z., Ye J., Berberine improves glucose metabolism through induction of glycolysis. J *Physiol Endocrinol Metab.,* 294 (2008) 148–156

Yoshioka K. , Saito M., Oh K.B., Nemoto Y., Matsuoka H., Natsume M., Abe H., Intracellular fate of 2-NBDG, a fluorescent probe for glucose uptake activity, in Escherichia coli cells. *Bioship Biotechnol Biochem*., 60(1996); 1899-1901

Yu H., Fujii N.L., Toyoda T., An D., Farese R.V., Leitges M., Hirshman M.F., Mul J.D., Goodyear L.J. Contraction stimulates muscle glucose uptake independent of atypical PKC, *J.Physiol Rep*, 11(2015) 12565,

Zeggini E., Scott L.J., Saxena R. et al. Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes. *Nat Genet 40(*2008) 638-645.

Zhao A.G., Cao W., Xu Y., Zhao G., Liu B.Y., Cai Y., Yang J.Z., Gu Y., Yuan W., Zhu Y.J., Han Y.Y., Yang J.Y. Survival benefit of an herbal formula for invigorating spleen for elderly patients with gastric cancer. *Zhong Xi Yi Jie He Xue Bao. Obstruct.* 8(2010).

Zollman Catherine and Vickers ,Complementary medicine in conventional practice, *BMJ*., 319(1999) 901–904.

Zhou l., Tang Y.,Gao L., Fan X., Liu C., Wu D., Separation, characterization and dose-effect relationship of the PPARs-activating bio-active constituents in the Chinese herb formulation ‘San-Ao Decoction’. *Molecules* 14(2009) 3942–3951.

Zou C., Wang Y., Shen Z., 2-NBDG as a fluorescent indicator for direct glucose uptake measurement, *Biochem Biophys Meth*., 64(2005)207-215.

http://www.nature.com/scitable/content/the-key-steps-leading-to-glucose-stimulated-14457827.

https://www.nice.org.uk/guidance/ph38/chapter/8-glossary#glycated-haemoglobin-hba1c

http://www.seahorsebio.com/resources/pdfs/manual-xf24-cell-mito-stress-test-kit.pdf