Trigonella foenum-graecum seed and 4-hydroxyisoleucine mediates glucose uptake via proximal insulin signaling activation and related downstream gene expression in liver cells

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ABSTRACT

Fenugreek (*Trigonella foenum-graecum*) is one of the oldest medicinal plants used worldwide to treat a variety of ailments, including hyperglycaemia. The seed and active compound – 4-hydroxyisoleucine (4-OH-lle) is thought to aid in the treatment of insulin resistance. This study investigated the effects of fenugreek aqueous seed extract and 4-OH-lle, on human liver cells (HepG2) compared to insulin (100ng/ml) and metformin (2mM) controls. Cells were treated with fenugreek seed extract (FSE) and 4-OH-lle: 10 and 100ng/ml under normogylcaemic (5mM glucose) and hyperglycaemic (30mM) conditions for 72h. Tyrosine phosphorylation of insulin receptor-β (IR-β), protein kinase B (Akt) and glycogen synthase kinase-3α/β (GSK-3α/β) protein extracts was determined by western blotting. Gene expression of sterol regulatory element binding protein 1c (SREBP1c), glucose transporter 2 (GLUT2), glycogen synthase (GS) and glucokinase (GK) was evaluated by qPCR. Under normogylcaemic and hyperglycaemic conditions, FSE, 4-OH-lle and insulin at 100ng/ml and metformin (2mM) caused tyrosine phosphorylation of IR-β ($p<0.0729$; $p<0.0121$), Akt ($p<0.0046$; $p<0.0005$) and GSK-3α/β ($p<0.0128$; $p<0.0048$). However, FSE showed the greatest ability in positively controlling GS ($*p<0.0262$; $*p<0.333$) and GK ($*p<0.333$; $*p<0.0213$), which regulates glycogen synthesis. Also, FSE increased SREBP1c ($*p<0.0157$; $*p<0.0012$) which positively regulates GLUT2 ($*p<0.0330$, $*p<0.0417$), allowing glucose into the cell. The data suggests that FSE and 4-OH-lle causes an up-regulation of insulin signaling proteins at a proximal level and related downstream gene expression. Taken together, the study suggests that FSE has potential application in the management of chronic hyperglycaemia.

DECLARATION

This dissertation represents the original work by the author and has not been submitted in any form to another University. The use of work by others has been duly acknowledged in the text.

The research described in this study was carried out in the Discipline of Medical Biochemistry, Faculty of Health Sciences, University of KwaZulu-Natal, Durban, under the supervision of Prof. A. A. Chuturgoon and Dr A Phulukdaree.

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PRESENTATIONS

The effect of *Trigonellla foenum-graecum* (fenugreek) aqueous leaf extract on insulin signaling and downstream gene expression in liver cells during hyperglycaemia.

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LIST OF ABBREVIATIONS

AIDS; Acquired immunodeficiency syndrome

AMPK; AMP-activated protein kinase

Akt; Protein kinase B

BCA; Bichonic assay

BSA; Bovine serum albumin

cAMP; Response element-binding protein

CCM; Complete culture media

cDNA; Copy deoxyribonucleic acid

Cm; Centimeter

Cu^{2+}; Cupric

Cu^{+}; Cuprous

dH2O; De-ionized water

dNTPs; deoxynucleotide triphosphates

ds; Double stranded

4-OH-lle; 4-hydroxyisoleucine

FSE; Fenugreek seed extract

GLUT2; Glucose transporter 2

GK; Glucokinase

g; Grams

-g; Gravitaional force

GSK-3α/β; Glycogen synthase kinase-3α/β

G-1-P; Glucose 1 phosphate

G-6-P; Glucose 6 phosphate

GS; Glycogen synthase

H2O; Water

Hepg2; Human liver cells

HIV; Human immunodeficiency virus

h; Hour

HRP; Horse radish peroxidase

HDL-C; high density lipoprotein cholesterol

IR; Insulin receptor

IR-β; Insulin receptor beta

IRS; Insulin receptor substrate

Kb; kilo base pairs

KCl; Potassium chloride

LDL-C; low-density lipoprotein – cholesterol

LDL; low-density lipoprotein

M; Molar

mg; Milligram

min; Minutes

ml; Milliliters

mM; Millimolar

mRNA; Messenger ribonucleic acid

NaCl; Sodium chloride

ng/ml; Nanogram/ml

NSPs; Non-starch polysaccharides

NSAIDS; non-steroidal anti-inflammatory drugs

pAkt; Phosphorylated protein kinase B

PBS; Phosphate saline buffer

pGSK-3α/β; Phosphorylated glycogen synthase kinase 3α/β

%; Perecentage

PI(3)K; Phosphoinositide 3-kinase

PTB; phosphotyrosine-binding

PDK-1; protein kinase 3-phosphoinositide-dependent protein kinase-1

PKCζ; Protein kinase C, zeta

PY20; Phosphotyrosine 20

RNA; Ribonucleic acid

RT; Room temperature

s; Seconds

SA; South Africa

SDS-PAGE; Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SREBP1c; Sterol regulatory element binding protein 1c

ss; Single stranded

SH2; Src homology 2

TB; Tuberculosis

TG; triglyceride

Tris-HCl; Tris hydrochloric acid

T3; triiodothyronine

T2DM; Type 2 diabetes mellitus

TTBS; Tris-buffered saline

µl; Microliter

FDA; United States Food and Drug Administration

V; Voltage

VLDL; Very-low-density lipoprotein

WHO; World health orgnaization

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INTRODUCTION

According to statistics South Africa (SA), diabetes is one of the main causes of death in SA following human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and tuberculosis (TB). The greatest increase in the incidence of diabetes is expected to be in Africa – it is predicted that the incidence of diabetes will have almost doubled by 2030 (WHO 2012). Latest reports on the prevalence of diabetes in SA read, "The diabetes tsunami is here. And we in SA are in trouble."

Type 2 diabetes mellitus (T2DM) is a progressive, multifactorial metabolic disease - a main cause of morbidity and mortality related to non-communicable diseases worldwide (Bhat et al. 2005; Laffel 1999; Hotamisligil 2010). However many of these cases go undiagnosed as there are very few symptoms initially (Alberti et al. 2006). As the symptoms can be very mild and develop gradually, many people fail to recognize them as warning signs of diabetes (Alberti et al. 2006). Researchers estimate an average of 7 years for a person to be diagnosed with diabetes for the first time (Alberti et al. 2006). However by the time they are diagnosed, about 30% of people with T2DM have already developed complications which include; blindness, heart disease, stroke, amputations and kidney failure (Alberti et al. 2006; Leng et al. 2004; Meyer et al. 2002). In most cases these complications could have been avoided entirely by early diagnosis and proper treatment (Alberti et al. 2006). The fact that many diabetic patients only acquire help when they already have complications, also poses a great burden on the health care system, whereas 80% of T2DM could have been avoided by a healthy eating plan and regular exercise (Alberti et al. 2006).

The main characteristic of T2DM is insulin resistance due to reduced expression and/or sensitivity of insulin receptors (IR) to insulin (Desvergne et al. 2006). This leads to a dysregulation of insulin mediated processes such as glucose uptake in muscle and fat, hepatic

glucose metabolism, glycogen synthesis and lipid metabolism (Gupta et al. 1995; Hazara et al., 1996). Insulin resistance is characterized by hyperglycaemia, where insulin is released from pancreatic beta cells, in response to elevated blood glucose levels (Bhat et al. 2005; Bhattacharya et al. 2007). Insulin binds IR, which is a tyrosine kinase that undergoes auto-phosphorylation and catalyzes the phosphorylation of cellular proteins. Upon phosphorylation, these proteins interact with signaling molecules, resulting in a diverse series of signaling pathways (Cheatham and Kahn 2005). The molecular mechanisms underlying insulin resistance is linked to a disruption in the insulin signaling cascade (Desvergne et al. 2006).

At present, the treatment of T2DM mainly involves a sustained reduction in hyperglycaemia by the use of drugs such as metformin (de Souza et al. 2014). However, due to common unwanted side effects the efficacies of these compounds are debatable, resulting in a demand for new compounds for the treatment of diabetes (Bhat et al. 2005; Greyber et al. 2010). Therefore, plants have been suggested as a rich, but unexplored source of potentially useful anti-diabetic drugs (Bhat et al. 2005; Causevic-Ramosevac 2013).

Trigonella foenum-graecum, commonly known as fenugreek, is a medicinal plant from the Papilionaceae, Leguminosae family and is native to Southern Europe and Asia (Belahcen et al. 2013). Fenugreek has been utilized as traditional medicine in countries such as North Africa, India and some parts of England (Belahcen et al. 2013). Documented medicinal benefits include reduced cholesterol levels and cardiovascular risk and controlled diabetes (Belahcen et al. 2013). The seeds of this plant are known to possess these medicinal properties (Tran 2003). Fenugreek seeds contain an unusual amino acid, 4-hydroxyisoleucine (4-OH-lle) - the biologically active compound exhibiting glucose–dependent insulin stimulating activity, hypoglycaemic and hypolipidaemic properties (Tran 2003; Broca et al. 1999; Chempakam et al. 2008). However no detailed study to elucidate the mechanism of

action of these extracts at the cellular and molecular level has been previously performed. In this study we investigated the effect of fenugreek seed extract (FSE) and 4-OH-lle on insulin signaling and subsequent gene expression in liver cells (HepG2) under hyperglycaemic conditions.

AIMS AND OBJECTIVES

Aim:

To determine the effects of FSE and 4-OH-lle - in comparison to insulin and metformin, on the insulin signaling pathway of HepG2 hepatoma cells.

Objectives:

1. To analyze the effects of FSE and 4-OH-lle on the insulin signalling pathway of human hepatoma cells. The study will focus on the proximal step in the cascade, which includes the tyrosine phosphorylation of IR-β as well as some of the more distal steps – tyrosine phosphorylation of Akt and GSK-3α/β.

2. To evaluate the effect of FSE and 4-OH-lle on SREBP1c and GLUT2 expression relevant to glucose uptake of liver cells.

3. To analyze the effects of FSE and 4-OH-lle on the expression of key genes – GS and GK involved in glycogen synthesis.

CHAPTER ONE

1. LITERATURE REVIEW

1.1 Introduction

Plants are natural producers of medicinal compounds – which, when classified, has led to the discovery of new, inexpensive drugs with high therapeutic potential (Abubakar et al. 2013; Huie 2002). The exploitation of such medicinal compounds is very old, for example, in the Middle Ages in Europe, *Achillea millefolium (*yarrow tea) was taken to stop internal bleeding and to treat upper respiratory infections (Causevic-Ramosevac 2013). Hippocrates prescribed *Salix* leaves to reduce fever, and garlic and onions were thought to have antibiotic properties and substances that lower blood sugar, serum cholesterol and blood pressure (Causevic-Ramosevac 2013). Interestingly, salicin (an extract from the white willow tree), aided in anti-inflammatory and pain-relieving properties and is now synthetically produced and used as a staple over-the-counter drug (Houghton and Raman 1998; Huie 2002). The leaves and seeds of *Trigonella foenum-graecum* (fenugreek) are not only used as a nutritional source but are also commonly prescribed in traditional medicine. Fenugreek is indigenous to Western Asia and Southern Europe but cultivated worldwide. In ancient times fenugreek was used by the Egyptians together with honey - for the treatment of rickets, diabetes, dyspepsia, rheumatism, anaemia and constipation. This plant has also been described in early Greek and Latin pharmacopoeias for hyperglycaemia and T2DM by Yemenite Jews, Indians and Chinese (Kavishik et al. 2010).

In rural areas, plants possessing medicinal properties are important in traditional medicine. Apart from the traditional healers practicing herbal medicine, there has been an increase in its demand and use with limited knowledge of side effects and therapeutic efficacy. The World Health Organization (WHO) estimated that 80% of the world's population is dependent on medicinal plants for their primary health care (WHO 2008; Abdo et al. 2008; Mwaniki et al. 2011; Gupta et al. 2010; Prakash and Sandhu 2012). Between 1983 and 1994 the United States Food and Drug Administration (FDA) approved 78% of the new drugs derived from unmodified natural products or semi-synthetic drugs obtained from natural sources (Mwaniki et al. 2011; Nepomuceno et al. 2006). A survey was carried out on the usage of medicinal plants and revealed an increase from 3% (1993) to 37% (1998) (Briskin 2000). The low cost of herbal drugs has facilitated this shift in its increased usage accompanied by the developing world; the 'green' movement in the developed world that campaigns on the essential safety of natural products and the individualistic philosophy of western society that encourages self-medication, with countless people choosing to treat themselves with herbal remedies (Sharma and Raghuram 1990; Houghton and Raman 1998).

Trigonella foenum-graecum commonly known as fenugreek is a promising medicinal plant from the Papilionaceae, Leguminosae family (Belahcen et al. 2013). The seed of the plant is known to possess documented medicinal properties (Tran 2003). Fenugreek seeds contain an unusual amino acid, 4-hydroxyisoleucine (4-OH-lle) – which is a biologically active compound (Tran 2003; Broca et al. 1999; Chempakam et al. 2008). Medical uses of fenugreek in Indian and Chinese medicine, include labour induction, aiding digestion, and as a general tonic to improve metabolism and health. Preliminary studies have suggested possible hypoglycaemic and anti-hyperlipidaemic properties of fenugreek seed powder (Ara

et al. 2006). However, at this time, the evidence is insufficient to recommend fenugreek for or against the treatment of hyperglycaemia or hyperlipidaemia.

1.2 The *Trigonella foenum-graecum* (fenugreek) plant

Fenugreek is a self-pollinating annual leguminous bean. The seeds are sown in well-prepared soil which sprouts in three days. The seedling grows erect, semi-erect or branched based on its variety and attains a height of approximately 30 to 60cm (Chempakam et al. 2008). It has compound pinnate, trifoliate leaves, axillary white to yellow flowers, and 3-15cm long thin pointed hoop-like beaked pods (Causevic-Ramosevac 2013; Chempakam et al. 2008). Every pod contains 10-20 oblong greenish-brown seeds with unique hooplike groves (Chempakam et al. 2008; Basu and Sricjamroen 2010). Pods, number of seeds in a pod, seed shape-size and plant height varies from one fenugreek variety to another.

Figure 1 *Trigonella-foenum graecum* seed, leaves and pods (http://www.shutterstock.com/pic-80569651/stock-photo--fenugreek-leaves-pods-and-seeds-on-white-background.html; ediblyasian.info/cultivation.php)

1.3 The *Trigonella foenum-graecum* (fenugreek) seed

Strongly aromatic and pungent flavoured fenugreek seeds are popular spices widely employed for their culinary as well as for medicinal properties. Fenugreek seeds are the most important and useful part of the fenugreek plant. These seeds are golden-yellow in colour, small in size, hard and have a four-faced stone like structure (Causevic-Ramosevac 2013; Basu and Sricjamroen 2010). The seed is 3-6mm long, 2-5mm wide and 2mm thick. They are a rich source of minerals, vitamins, and phytonutrients - a 100g of seeds provide 323 calories (Chempakam et al. 2008). They are also a good source of soluble dietary fiber – a 100g of seeds provide 24.6g or over 65% of dietary fiber (Chempakam et al. 2008). Non-starch polysaccharides (NSPs) which constitute major fiber content in the plant include saponins, hemicellulose, mucilage, tannin, and pectin (Chempakam et al. 2008). These compounds help lower blood low-density lipoprotein – cholesterol (LDL-C) levels by inhibiting bile salts re-absorption in the colon. They also bind to toxins in food and help to protect the colon mucus membrane from cancers (Causevic-Ramosevac 2013; Chempakam et al. 2008). Non-starch polysaccharides increase the bulk of the food and augment bowel movements. Altogether, NSPs assist in smooth digestion and help relieve constipation ailments. It has been established that amino-acid – 4-OH-lle present in the fenugreek seeds has facilitator action on insulin secretion. The seeds contain fiber which aid in lowering the rate of glucose absorption in the intestines thus controls blood sugar levels. The seeds contain many phytochemical compounds such as choline, trigonelline, diosgenin, yamogenin, gitogenin, tigogenin and neotigogens (Causevic-Ramosevac 2013; Chempakam et al. 2008). Together, these compounds attribute for the medicinal properties of fenugreek. This spice is an excellent source of minerals like copper, potassium, calcium, iron, selenium, zinc, manganese, and magnesium (Causevic-Ramosevac 2013; Chempakam et al. 2008). Potassium is an important

component of cell and body fluids that help to control heart rate and blood pressure by countering action on sodium. Iron is essential for red blood cell production and as a co-factor for cytochrome-oxidases enzymes. It is also rich in many vital vitamins that are essential nutrients for optimum health, including thiamin, pyridoxine (vitamin B6), folic acid, riboflavin, niacin, vitamin A, and vitamin C (Causevic-Ramosevac 2013; Chempakam et al. 2008).

Figure 2 Illustration of fenugreek seed (http://www.saraogiandco.com/fenugreek-seeds.htm)

1.4 Biologically active components of *Trigonella foenum-graecum*

The composition of fenugreek includes a large number of chemical components. They include proteins and amino acids, flavonoids, saponins and steroidal saponins, coumarin, lipids, vitamins, minerals, galactomannan, fiber and alkaloids, such as trigonelline. Active compounds of fenugreek include soluble fiber, saponins, trigonelle, diosgenin and 4-OH-Ile

(Basch et al. 2003; Baquer et al. 2011). Hypoglycaemic activities have mainly been attributed to dietary fiber, saponin and 4-OH-Ile.

The natural non-proteinogenic amino acid 4-OH-Ile possesses insulinotropic biological activity (Baissac et al. 1998; Broca et al. 1999). It is extracted from fenugreek seeds (Fowden et al. 1973) and its absolute stereo configuration was determined as (2S, 3R, 4S) (Alcock et al. 1989). 4-Hydroxyisoleucine increases glucose-induced release of insulin. In contrast to several types of pharmacological drugs that have been used for the treatment of type II diabetes (e.g. sulfonylureas), the insulin response mediated by 4-OH-lle is strictly dependent on the glucose concentration. This unique property of 4-OH-lle allows us to avoid undesirable side-effects such as hypoglycaemia in the therapy of type II diabetes (Bessler and Jackson 1981; Jennings et al. 1989). 4-Hydroxyisoleucine increases glucose-induced insulin release, in the concentration range of 100µmol/1 to 1mmol/1, through a direct effect on isolated islets of Langerhans from both rats and humans. The stimulating effect of 4-OH-lle is strictly glucose dependent; indeed, ineffective at low (3mmol/1) or basal (5mmol/1) glucose concentrations, the amino acid potentiates insulin secretion induced by supranormal (6.6 - 16.7mmol/1) concentrations of glucose. In addition, in the isolated perfused rat pancreas, it was shown i) that the pattern of insulin secretion induced by 4-OH-lle was biphasic, ii) that this effect occurred in the absence of any change in pancreatic cell activity, and iii) that the more glucose concentration was increased, the more insulin response was amplified (Bhat et al. 2005; Li et al. 2005). Moreover, 4-OH-lle did not interact with other agonists of insulin secretion (leucine, arginine, tolbutamide, and glyceraldehyde) (Bhat et al. 2005).

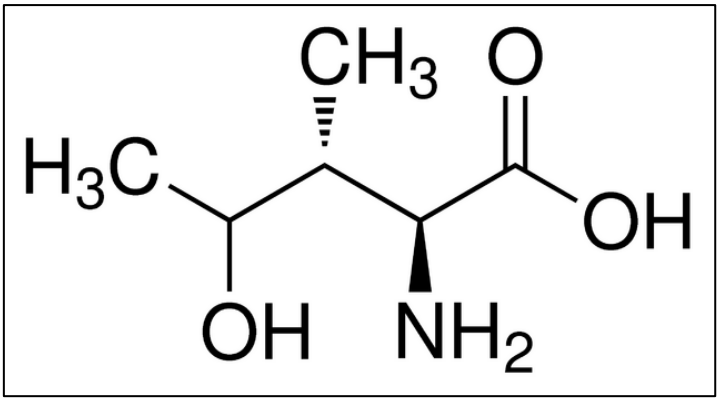

Figure 3 Chemical structure of 4-hydroxyisoleucine

(http://www.sigmaaldrich.com/catalog/product/sigma/50118?lang=en&region=ZA)

1.5 Adverse effects and safety

Although fenugreek has traditionally been considered safe and well-tolerated, some side effects have been associated with its use (Basch et al. 2003; Chiba et al. 2011). Patients using fenugreek, who are allergic to the plant or chickpeas, should take necessary caution due to possible cross reactivity (Bapat et al. 1997). The use of fenugreek during pregnancy and lactation or those with severe liver or kidney impairment should be avoided due to inadequate data suggesting use. Other reported side effects include transient diarrhoea, flatulence and dizziness, maple syrup urine, hypoglycaemia with large doses and decrease in blood urea to low normal after 12 weeks of ingestion of fenugreek seed powder (Abdel-Barry et al. 2000; Sharma and Raghuram 1990). Fenugreek can cause allergic reactions including nasal congestion, wheezing, persistent coughing, hoarseness, facial angioedema, and shock. Repeated topical application, chronic use and excessive ingestion of fenugreek should be avoided to prevent undesirable reactions or toxicities and due to long-term data being unavailable. Hypoglycaemia is an expected effect, and therefore, upon fenugreek supplementation care should be taken to monitor blood glucose levels (Abel et al. 1988;

Sharma and Raghuram 1990). Decrease in body weight has also been reported which has been attributed to a decrease in triiodothyronine (T3) (Kar et al. 1999). The data generated to date on the above in regard to fenugreek use in patients are sparse however future studies will lead to the development of well-designed, randomized, adequately powered clinical trials to evaluate the effect of fenugreek seed powder on measures of insulin resistance, insulin secretion and cholesterol metabolism.

1.6 Pharmacological action of *Trigonella foenum-graecum* (fenugreek)

Fenugreek has anti-diabetic and anti-lipidaemic effects. However, the exact mechanism of action is still unclear, as more work is needed. The anti-diabetic effect of fenugreek was thought to be due to formation of a colloidal-type suspension in the stomach and intestines when the mucilagenous fiber of the seeds is hydrated, therefore affecting gastrointestinal transit, slowing glucose absorption (Bhat et al. 2010). The anti-lipidaemic effects of fenugreek was thought to be due to inhibition of intestinal cholesterol absorption due to saponin-cholesterol complex formation, increased loss of bile through faecal excretion due to saponin-bile complexes, thus increasing conversion of cholesterol to bile by the liver, and effects of amino acid pattern of fenugreek on serum cholesterol (Enjoji et al. 2008; Au et al. 2003). Fenugreek contains coumarins and other constituents that might affect platelet aggregation, but this might not be clinically significant (Au et al. 2003). Fenugreek constituents also show evidence of cardiotonic, diuretic, anti-inflammatory, anti-hypertensive, and anti-viral properties.

1.6.1 Evidence based studies on the anti-diabetic properties of fenugreek

The action of fenugreek on lowering blood glucose levels was said to be almost comparable to that of insulin, mimicking its effects (Baqueer et al. 2011). Baqueer et al. (2011) and Ardekani et al. (2009) reported that the unusual amino acid, 4-OH-lle displays *in vitro* insulinotrophic activity and anti-diabetic properties in animal models. According to Ardekani et al. (2009), the amino acid is a useful and well-tolerated treatment for insulin resistance. It acts as a hypoglycaemic agent and a protective agent for the liver. A meta-analysis of the effect of herbs on glucose balance in type II diabetes patients, by Bar Dayan et al. (2011), and colleagues, stated that glycated hemoglobin (HbA1c) was significantly reduced in the group that used fenugreek as a food supplement (Bar Dayan et al. 2011). Further-more, it was reported by Prabhakar and Doble that ocular histopathological and biochemical abnormalities that are relevant to diabetic retinopathy were controlled when using fenugreek and sodium orthovandate alone or in low dose combination (Doble and Prabhakar 2011). A review article by Assad and Morse (2013) included three separate studies that assessed the effect of fenugreek in patients with diabetes. The first study assessed the effect of fenugreek use in patients with type 1 diabetes. This study showed a significant reduction of fasting blood glucose level, triglycerides; and total cholesterol, very-low-density lipoprotein (VLDL), and low-density lipoprotein (LDL) levels. Also, it showed an improvement of glucose tolerance test, and a reduction in 24h urinary glucose excretion (Assad and Morse 2013). The second study assessed the effect of fenugreek use (25g of fenugreek powder daily) in patients with T2DM. This study showed that fasting blood glucose level was reduced, and glucose tolerance was improved. Furthermore, there was a significant reduction in 24h urinary glucose excretion, and HbA1c after eight weeks of fenugreek intake (Assad and Morse 2013).

The third study showed that fenugreek improved glycaemic control, insulin sensitivity, and hypertriglyceridaemia in newly diagnosed patients with T2DM (Assad and Morse 2013).

1.6.2 Evidence based studies on the anti – lipidaemic properties of fenugreek

In the Hazra et al. (1996) trial on type 1 diabetes cited above, small but statistically significant reductions were noted in triglyceride (TG) (approximately 1.3 mMol/L; $p<0.001$) and low-density lipoprotein – cholesterol (LDL-C) levels (approximately 1.0 mMol/L; $p<0.01$), but the level of high density lipoprotein cholesterol (HDL-C) remained unchanged. Without an adequate description of blinding and randomization, the results of this study can only be considered preliminary. Several case series have also found hypocholesterolemic effects associated with oral fenugreek. Hazra et al. (1996) investigated 15 non-obese, asymptomatic, hyperlipidemic adults. After the subjects had ingested 100g defatted fenugreek powder per day for three weeks, their TG and low-density lipoprotein – cholesterol (LDL-C) levels were lower than baseline values. Slight decreases in HDL levels were also noted. In a later study, normalization of lipid profiles was observed in 60 patients with T2DM whose diets were supplemented with 25g powdered fenugreek seeds per day for 24 weeks. While mean LDL-C, and TG levels decreased by 14-16 percent during the study period, mean HDL-C levels increased by 10 percent. Similarly, Sowmya and Rajyalakshmi (1999) observed significant reductions in TG and LDL-C levels in 20 adults with hypercholesterolemia who received 12.5-18.0g powdered, germinated fenugreek seeds for one month, although no changes in HDL-C, very-low-density lipoprotein (VLDL), or TG levels were observed. In another study, Sharma et al. (1990) also reported a decrease in total cholesterol levels in 5 diabetic patients treated with fenugreek seed powder (25g orally per day) for 21 days. Bordia et al., 1997 studied the effects of fenugreek seed powder (2.5g administered twice daily for

three months) in a subgroup of 40 subjects. In the subjects who had coronary artery disease and T2DM, significant decreases in the TG levels were observed, with no change in HDL-C level. The methodology for this study was not clearly documented. Most available studies are case series lacking proper controls, randomization, or blinding.

1.7 Drug interactions

Since fenugreek powder is rich in fiber, it can interfere with the absorption of oral medication. Prescription medicines should be taken separately from fenugreek-containing products. Simultaneous use of fenugreek with other hypoglycaemic agents might lower serum glucose level more than expected (Basch *et al.* 2003). Toxicological evaluation of diabetic patients taking fenugreek seed powder at a dose of 25g per day for 24 weeks showed no clinical hepatic or renal toxicity and no haematological abnormalities (Hazara et al. 1996). In an animal study, fenugreek powder failed to induce any signs of toxicity or mortality in mice and rats that received acute and sub-chronic regimes (Muralidhara *et al.* 1999). There were no significant haematological, hepatic or histopathological changes in weanling rats that were fed fenugreek seeds for 90 days (Rao et al. 1996). Simultaneous use of antiplatelet or anticoagulant drugs might increase the risk of bruising and bleeding because fenugreek constituents contain coumarins that have antiplatelet effects. Some drugs with anticoagulant or antiplatelet effects include aspirin, clopidrogel, non-steroidal anti-inflammatory drugs (NSAIDS) such as ibuprofen, diclofenac, naproxen, and others. Also, heparin, dalteparin and enoxaparin increase might increase the risk of bleeding. Simultaneous use of fenugreek and anti-diabetic agents including insulin, metformin, acarbose, glipizide, glyburide, pioglitazone, rosiglitazone, and others may cause hypoglycaemic episodes. Fenugreek can also interfere with corticosteroid drug activity, warfarin, and insulin, hormone therapy, monoamine oxidase

inhibitors, which may be due to high content of mucilagenous fiber in fenugreek and high viscosity in the gut (de Souza et al. 2014).

1.8 *Trigonella foenum-graecum* (fenugreek) capsules

Fenugreek seed capsules are readily available at pharmacies and health stores as over the counter complementary medicine. The directions for use include one to two capsules daily, or as prescribed. Each capsule contains 500mg of 100% pure *Trigonella foenum-graecum* seed. The indications include assistance in the stimulation of lactation, used for the treatment of dyspepsia, gastritis and diabetes and aids in the digestive process, treatment of high cholesterol and should only be used as directed by a health care practitioner.

Figure 4 Fenugreek capsules available at local pharmacies and health stores (https://inspiredlivingnig.wordpress.com/2013/01/14/fenugreek-red-clover-natural-breast-enlargement-do-work/)

1.9 The liver – an organ involved in the metabolism of glucose

1.9.1 Function of the liver

Glucose is an essential nutrient for the human body. It is the main energy source for various cells, which depend on the bloodstream for a steady supply. Blood glucose levels, therefore, are carefully maintained. The liver plays an essential role in this process by balancing the uptake and storage of glucose via glycogenesis and the release of glucose via gluconeogenesis and glycogenolysis. The numerous substrate cycles in the major metabolic pathways of the liver play crucial roles in the regulation of glucose production (Jungermann and Keitzmann 1996). This includes the short- and long-term regulation of glucose-6-phosphatase and its substrate cycle counter-part, GK (Francini et al. 2011). The substrate cycle enzyme glucose-6-phosphatase catalyzes the terminal step in both the gluconeogenic and glycogenolytic pathways and is opposed by the glycolytic enzyme GK (Francini et al. 2011). In addition, the regulation of GLUT2, this facilitates the final step in the transport of glucose out of the liver and into the bloodstream (Ahn et al. 2005).

1.9.2 Functional unit of the liver: the hepatocyte

Hepatocytes are metabolic super-achievers in the body. They play critical roles in synthesizing molecules that are utilized elsewhere to support homeostasis, in converting molecules of one type to another, and in regulating energy balances (Berthiaume et al. 2003). To facilitate the exchange of a wide variety of substances between the blood and hepatocytes, the hepatocytes are directly exposed to the blood passing though the organ, by being in close contact with the liver blood sinusoids (Decker 1990). The sinusoids are lined by two types of cells - Kupffer cells and sinusoid lining cells (Decker, 1990). The blood enters through portal

tracts at the outer edge of the liver lobule, and filters through the sinusoids which are in close connection with the liver hepatocytes, until it reaches the central hepatic vein, where it drains away (Decker 1990). Thus the flow of blood is from the outside to the inside of the lobule. Under hormonal control, hepatocytes can respond to either feeding or fasting conditions by storing or producing glucose as necessary. In the fasting state, the effects of glucagon avoid hypoglycaemia by stimulating glucogenesis and glycogenolysis and initiating hepatic glucose release. In the post prandial state, insulin prevents hyperglycaemia, in part, by suppressing hepatic gluconeogenesis and glycogenolysis and facilitating hepatic glycogen synthesis. Both transcriptional regulation of rate limiting enzymes and modulation of enzyme activity through phosphorylation and allosteric regulation are involved (Guillou et al., 2008).

1.9.3 Biochemical activity of liver cells

1.9.3.1 Metabolism

The hepatocytes of the liver are tasked with many of the important metabolic jobs that support the cells of the body. Because all of the blood leaving the digestive system passes through the hepatic portal vein, the liver is responsible for metabolizing carbohydrate, lipids, and proteins into biologically useful materials (Jungermann and Keitzmann 1996; Felig 1975).

The digestive system breaks down carbohydrates into the monosaccharide glucose, a primary energy source. Blood entering the liver through the hepatic portal vein is extremely rich in glucose from digested food (Jungermann and Keitzmann 1996). Hepatocytes absorb much of this glucose and store it as the macromolecule glycogen, a branched polysaccharide that

allows the hepatocytes to pack away large amounts of glucose and quickly release glucose between meals (Jungermann and Keitzmann 1996). The absorption and release of glucose by the hepatocytes helps to maintain homeostasis and protects the rest of the body from dangerous changes in the blood glucose level.

Fatty acids in the blood passing through the liver are absorbed by hepatocytes and metabolized to produce energy in the form of ATP (Beylot et al. 2004). Glycerol (another lipid component) is converted into glucose by hepatocytes through the process of gluconeogenesis. Hepatocytes can also produce lipids like cholesterol, lipoproteins and phospholipids that are used by other cells throughout the body (Beylot et al. 2004). Much of the cholesterol produced by hepatocytes is excreted from the body as a component of bile (Beylot et al. 2004).

Dietary proteins are broken down into their component amino acids by the digestive system before being passed on to the hepatic portal vein (Jungermann and Keitzmann 1996). Amino acids entering the liver require metabolic processing before they can be used as an energy source. Hepatocytes first remove the amine groups of the amino acids and convert them into ammonia and eventually urea (Jungermann and Keitzmann 1996). Urea is less toxic than ammonia and can be excreted in urine as a waste product of digestion. The remaining parts of the amino acids can be broken down into ATP or converted into new glucose molecules through the process of gluconeogenesis (Jungermann and Keitzmann 1996).

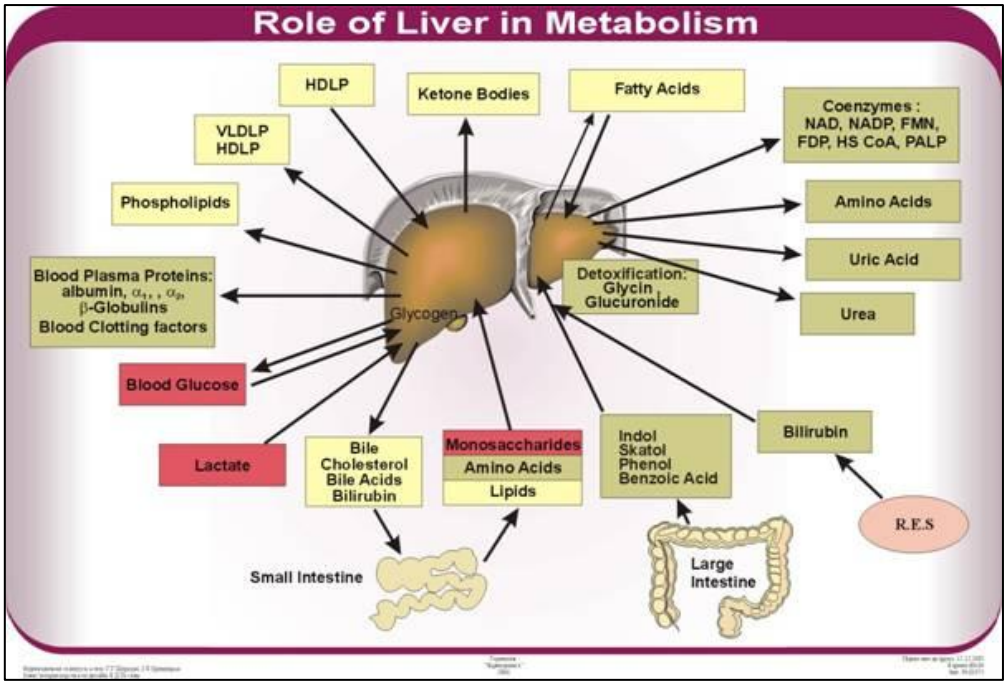

Figure 5 Role of the liver in metabolism

(http://intranet.tdmu.edu.ua/data/kafedra/internal/chemistry/classes_stud/en/pharm/prov_pharm/ptn/3/11.%20BIOCHEMICAL%20FUNCTION%20OF%20LIVER.htm)

1.9.3.1.1 Carbohydrate metabolism

Carbohydrate metabolism begins with digestion in the small intestine where monosaccharides are absorbed into the blood stream (Danielsson et al. 2005; DeFronzo et al. 1979). Blood sugar concentrations are controlled by insulin, glucagon and epinephrine. If the concentration of glucose in the blood is too high, insulin is secreted by the pancreas. Insulin stimulates the transfer of glucose into the cell, especially in the liver and muscle, although other organs are also able to metabolize glucose (Pandey and Srivastava 1998).

In the liver and muscle, majority of the glucose is converted to glycogen by the process of glycogenesis (Jungermann and Keitzmann 1996). Glycogen is stored in the liver and muscle

until needed when glucose levels are low. When blood glucose levels are low, the hormones epinephrine and glucagon are secreted to stimulate the conversion of glycogen to glucose. This process is called glycogenolysis (Jungermann and Keitzmann 1996). If glucose is required immediately upon entering the cells to supply energy, it begins the metabolic process called glycolysis. The end products of glycolysis are pyruvic acid and ATP (Jungermann and Keitzmann 1996). Since glycolysis releases relatively little ATP, further reactions continue to convert pyruvic acid to acetyl CoA and enter the citric acid cycle to form citric acid (Jungermann and Keitzmann 1996). The majority of the ATP is made from oxidations in the citric acid cycle in connection with the electron transport chain (Jungermann and Keitzmann 1996).

During strenuous muscular activity, pyruvic acid is converted into lactic acid rather than acetyl CoA (Jungermann and Keitzmann 1996). During the resting period, the lactic acid is converted back to pyruvic acid. The pyruvic acid in turn is converted back to glucose by the process called gluconeogenesis. If the glucose is not needed at that moment, it is converted into glycogen by glycogenesis (Jungermann and Keitzmann 1996).

1.9.3.1.2 Fat metabolism

The liver plays a central role in the whole body energy homeostasis by its ability to metabolize glucose and fatty acids. When energy intake is abundant, carbohydrates are used to generate ATP and surplus glucose (after replenishing glycogen stores) is converted to fatty acids - the process of lipogenesis. Fatty acids are used for the synthesis and storage of TG in white adipose tissue (Beylot et al. 2004). Although white adipose tissue functions essentially as an unlimited reservoir to accumulate TG, the liver is also able to store significant

quantities of lipids in conditions associated with prolonged excess energy consumption or impaired fatty acid metabolism manifesting as steatosis (Beylot et al. 2004). In fasted states, when glucose availability and insulin levels are low, there is a depletion of hepatic glycogen stores and a reduction in fatty acid production. Under these conditions, TGs stored in adipose tissues are hydrolyzed to free fatty acids and mobilized into plasma to reach the liver. In the liver, they undergo oxidation, converted to ketone bodies to be used as fuel by extrahepatic tissues (Beylot et al. 2004).

Sources of increased TG content in hepatic steatosis include a) excess dietary TG associated with over-eating that reaches the liver as chylomicron particles from the intestine; b) increased TG synthesis in the liver from fatty acids formed from de novo lipogenesis; c) excess fatty acid influx into the liver from lipolysis of adipose tissue in obese and insulin-resistant states and subsequent conversion to TG; d) diminished export of lipids from the liver in very-low-density lipoproteins; and e) reduced oxidation of fatty acids. High insulin suppresses hepatic glucose production, increases hepatic glucose uptake, and enhances lipogenesis in the liver (Reddy and Rao 2005; Beylot et al. 2004). In essence, perturbations affecting fatty acid influx into the liver, their de novo synthesis, and conversion to TG and/or oxidation to generate ATP contribute to disturbances in hepatic lipid homeostasis (Reddy and Rao 2005; Beylot et al. 2004; Garg and Simha 2006).

De novo fatty acid synthesis in the liver is regulated by three known transcription factors: sterol regulatory binding protein 1c, Carbohydrate-responsive element-binding protein (ChREBP), and Peroxisome proliferator-activated receptor gamma (PPAR-γ) (Guillou et al. 2008). Insulin and glucose concentrations regulate fatty acid synthesis in the liver. The

activation of genes responsible for lipogenesis in the liver by insulin is transcriptionally mediated by SREBP-1c (Guillou et al. 2008). These include fatty acid synthase and stearoyl-CoA desaturase (Guillou et al. 2008; Quinn et al. 1983). A few aspects of lipid metabolism are unique to the liver, but many are carried out predominantly by the liver. Major examples of the role of the liver in fat metabolism include:

- The liver is exceedingly active in oxidizing TGs to produce energy. The liver breaks down many more fatty acids than the hepatocytes require, and exports large quantities of acetoacetate into blood where it can be picked up and readily metabolized by other tissues (Reddy and Rao 2005; Beylot et al. 2004).

- A bulk of the lipoproteins is synthesized in the liver.

- The liver is the major site for converting excess carbohydrates and proteins into fatty acids and TGs, which are then exported and stored in adipose tissue.

- The liver synthesizes large quantities of cholesterol and phospholipids. Some of this is packaged with lipoproteins and made available to the rest of the body. The remainder is excreted in bile as cholesterol or after conversion to bile acids (Reddy and Rao 2005; Beylot et al. 2004).

1.9.3.1.3 Protein Metabolism

The most critical aspects of protein metabolism that occur in the liver are:

- Deamination and transamination of amino acids, followed by conversion of the non-nitrogenous part of those molecules to glucose or lipids. Several of the enzymes used in these pathways (such as, alanine and aspartate aminotransferases) are commonly

assayed in serum to assess liver damage (Jungermann and Keitzmann 1996; Tremblay et al. 2007).

- Removal of ammonia from the body by synthesis of urea. Ammonia is very toxic and if not rapidly and efficiently removed from the circulation, will result in central nervous system disease (Jungermann and Keitzmann 1996).

- Synthesis of non-essential amino acids.

Hepatocytes are responsible for synthesis of most of the plasma proteins. Albumin, the major plasma protein, is synthesized almost exclusively by the liver. Also, the liver synthesizes many of the clotting factors necessary for blood coagulation (Jungermann and Keitzmann 1996; Louard et al. 1992).

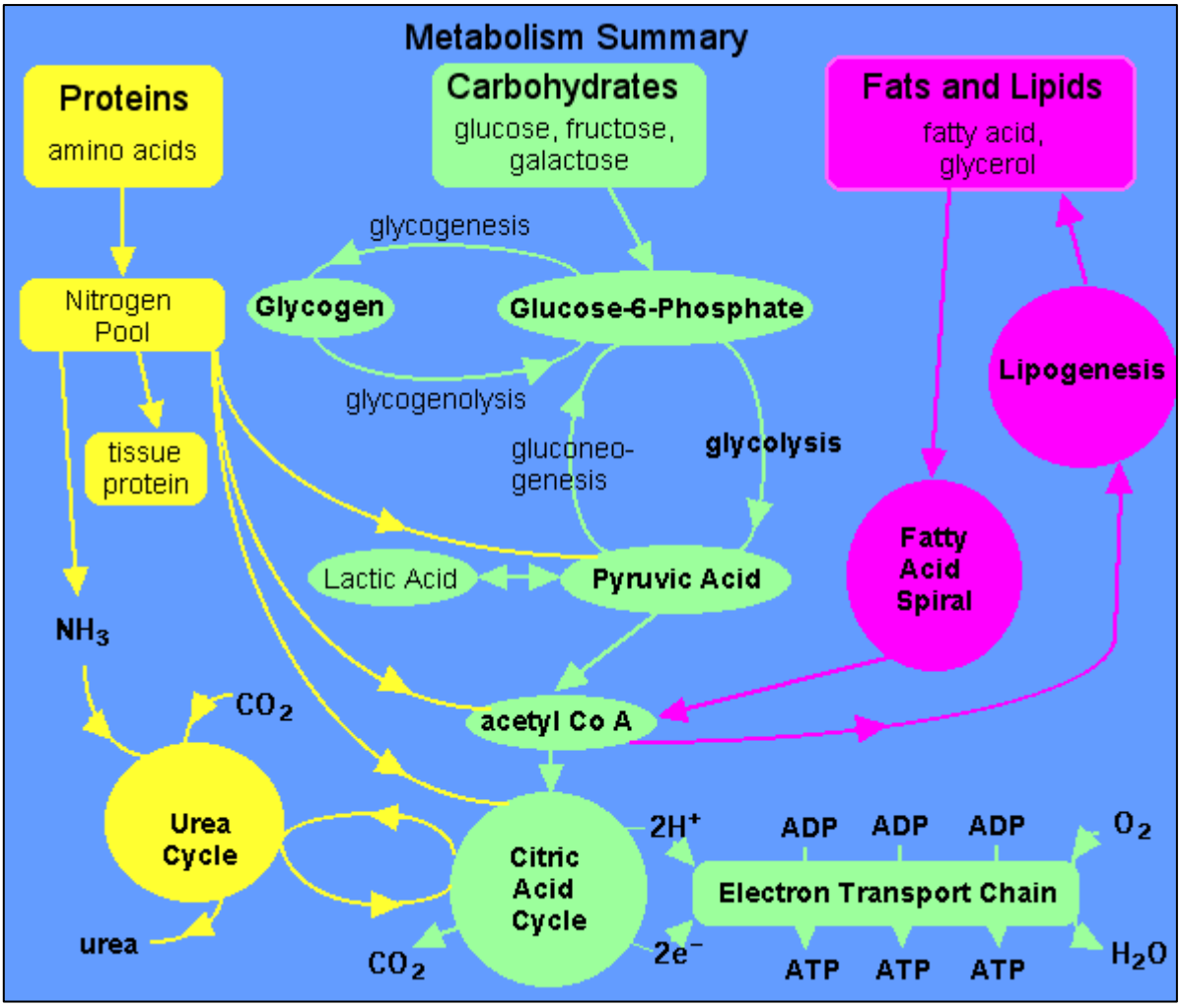

Figure 6 Overview of the metabolic function of the liver

(www.elmhurst.edu/~chm/vchembook/600glycolysis.html)

1.10 Insulin signaling pathway

Insulin is a hormone released by pancreatic beta cells in response to elevated levels of nutrients in the blood. Insulin triggers the uptake of glucose, fatty acids and amino acids into liver, adipose tissue and muscle and promotes the storage of these nutrients in the form of glycogen, lipids and protein respectively. Failure to uptake and store nutrients results in diabetes. Type- I diabetes is characterized by the inability to synthesize insulin, whereas in

T2DM the body becomes resistant to the effects of insulin presumably because of defects in the insulin signaling pathway.

The insulin receptor is composed of two extracellular alpha (α) subunits and two transmembrane beta (β) subunits linked together by disulphide bonds. Binding of insulin to the α subunit induces a conformational change resulting in the autophosphorylation of a number of tyrosine residues present in the β subunit (Johnston et al. 2001; Arner et al. 1991; Hirosumi et al. 2002; Savage et al. 2005; Savage et al. 2007). These residues are recognized by phosphotyrosine-binding (PTB) domains of adaptor proteins such as members of the insulin receptor substrate family (IRS) (Kahn and Saltiel 2001; Alessi and Lizcano 2002). Receptor activation leads to the phosphorylation of key tyrosine residues on IRS proteins, some of which are recognized by the Src homology 2 (SH2) domain of the p85 regulatory subunit of PI-3-kinase (Shulman 2000). The catalytic subunit of PI-3-kinase - p110, then phosphorylates phosphatidylinositol (4, 5) bisphosphate (PtdIns (4, 5) P2) leading to the formation of Ptd (3, 4, 5) P3 (Nolan et al. 1994). A key downstream effector of Ptd (3, 4, 5) P3 is AKT which is recruited to the plasma membrane. Activation of AKT also requires the protein kinase 3-phosphoinositide-dependent protein kinase-1 (PDK1), which leads to the phosphorylation of AKT (Lawlor and Alessi 2001). Once active, AKT enters the cytoplasm where it leads to the phosphorylation and inactivation of GSK-3α/β. A major substrate of GSK-3α/β is GS, an enzyme that catalyses the final step in glycogen synthesis. Phosphorylation of GS by GSK-3α/β inhibits glycogen synthesis; therefore the inactivation of GSK-3α/β by AKT promotes glucose storage as glycogen. Insulin stimulates glucose uptake in muscle and adipocytes via translocation of GLUT4 and in liver via GLUT2 vesicles to the plasma membrane. Glucose transporter4 translocation involves the PI3K/Akt pathway

(Carlson et al. 2003). And GLUT2 translocation involves activation of SREBP1c via insulin (Horton et al. 2002).

In addition to promoting glucose storage, insulin inhibits the production and release of glucose by the liver by blocking gluconeogenesis and glycogenolysis (Kahn and Saltiel 2001; Samuel et al. 2010). Insulin directly controls the activities of a set of metabolic enzymes by phosphorylation and dephosphorylation events and also regulates the expression of genes encoding hepatic enzymes involved in gluconeogenesis. Recent evidence suggests that forkhead transcription factors, which are excluded from the nucleus following phosphorylation by AKT, play a role in hepatic enzyme regulation by insulin (Alessi et al. 2000; Barthel et al. 2001).

Insulin signaling also has growth and mitogenic effects, which are mostly mediated by the Akt cascade as well as by activation of the Ras/MAPK pathway (Pessin and Saltiel 2006). In addition, insulin signaling inhibits gluconeogenesis in the liver, through disruption of CREB/CBP/Torc2 binding. Insulin signaling also promotes fatty acid synthesis through activation of SREBP-1C. A negative feedback signal emanating from Akt/PKB, Protein kinase C, zeta (PKCζ), p70 S6K, and the MAPK cascades results in serine phosphorylation and inactivation of IRS signaling (Morino et al. 2006; Randle 1963).

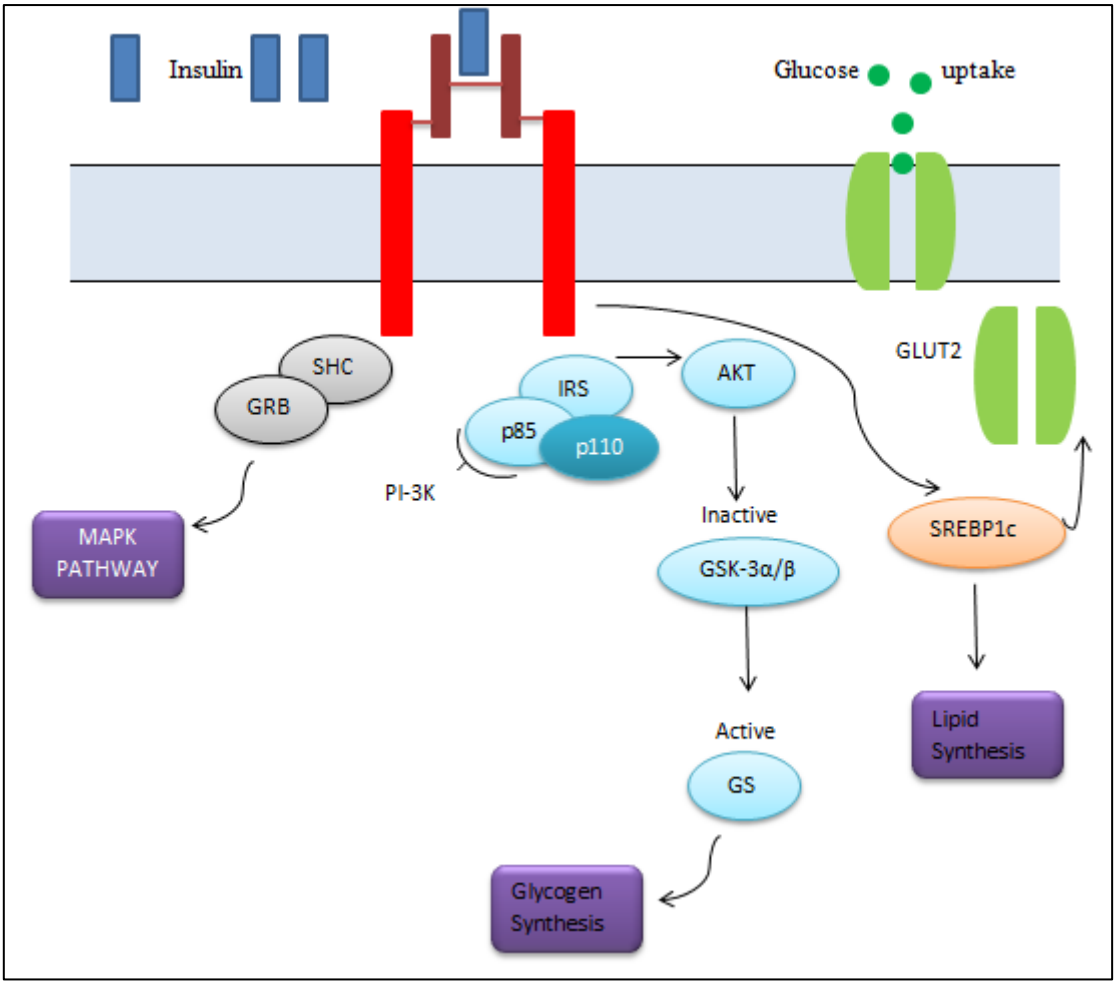

Figure 7: Activation of the insulin receptor evokes increased transcription of SREBP and the phosphorylation of members of the IRS family, SHC and Cbl. Upon tyrosine phosphorylation, these proteins interact with signaling molecules through their SH2 domains, which results in the activation of a variety of signaling pathways, including PI 3-kinase signaling, MAPK activation and the activation of the Cbl/CAP complex. These pathways act in a coordinated manner to regulate glucose, lipid and protein metabolism.

CHAPTER TWO

2. METHODS AND MATERIALS

2.1 Materials

Whole fenugreek seeds were purchased from a local herbal store. 4-hydroxyisoleucine (50118) was purchased from Sigma-Aldrich (Pty.) Ltd (Johannesburg, South Africa). The Hepg2 cell line was purchased from Highveld Biologicals (Johannesburg, South Africa). All other consumables were purchased from Merck unless otherwise stated.

2.2 Preparation of *Trigonella foenum-graecum*

Fenugreek seeds were prepared by grinding in a pestle and mortar and suspended in deionized water, then transferred into a conical tube and centrifuged (3 600 g, 10min) at room temperature (RT) (Bhandari et al. 2014). Supernatant (FSE) was removed, freeze dried and stored at 4^{o}C. Fenugreek seed extract dilutions (10, 50 and 100ng/ml) were prepared using CCM. The total volume for each dilution was 5ml per a flask. For the hyperglycaemic treatment, CCM was supplemented with glucose to obtain a final concentration of 30mM. 4-hydroxyisoleucine dilutions (10 and 100ng/ml) was also prepared using complete culture medium (CCM).

2.3 Preparation of metformin

Tablets containing 500mg of metformin were crushed with a pestle and mortar and suspended in 0.1M PBS. The stock solution was filter sterilized and used to prepare treatments (2mM) in CCM (Anuradha et al. 2009).

2.4 Cell culture

Human liver cells were cultured (37^{o}C, 5% CO_2) to confluency in 25cm^3 flasks in CCM comprising Eagles minimum essential medium, 10% foetal calf serum, 1% L-glutamine and 1% penstrepfungizone. Every second day, the flasks were rinsed three times with phosphate saline buffer (PBS) and fresh CCM (5ml), was added, to allow the cells to grow to 90% confluency. A confluent flask contains approximately a minimum of a million cells and more which is sufficient for treatment. The treatment period was 72 hours.

2.5 Western blotting

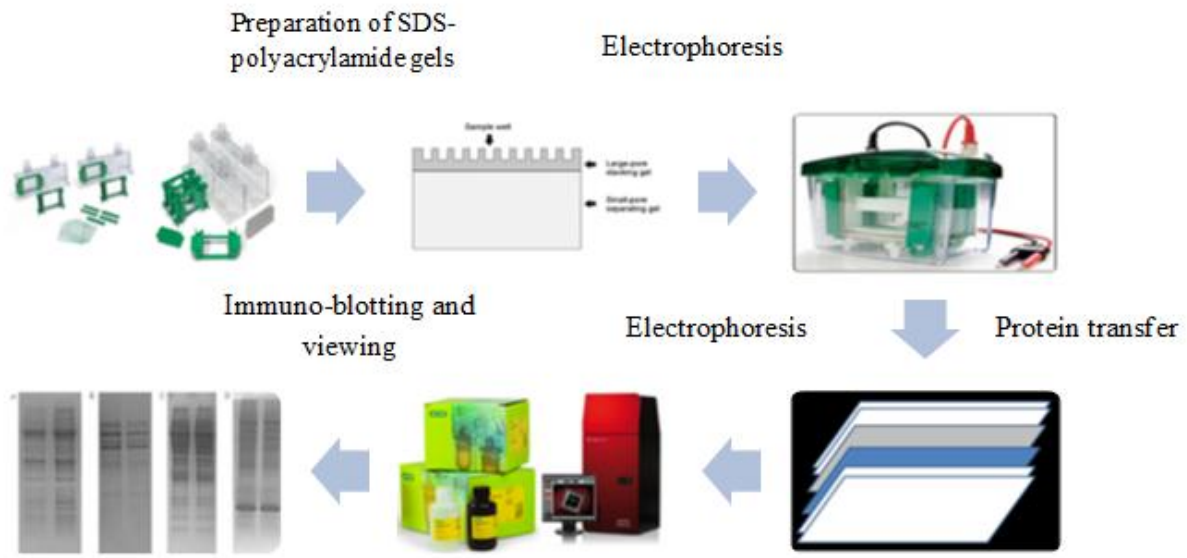

Figure 8: Overview of the western blotting process

2.5.1 Principle

Western blotting is a sensitive assay for detective and characterization of proteins. This technique exploits the inherent specificity by polyclonal or monoclonal antibodies. It is an analytical method wherein a protein sample is electrophoresed on a sodium dodecyl sulphate

polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto a nitrocellulose membrane. The transferred protein is detected using specific primary antibody and secondary enzyme labelled antibody and substrate. A protein sample is subjected to polyacrylamide gel electrophoresis. After this the gel is placed over a sheet of nitrocellulose and the protein in the gel is electrophoretically transferred to the nitrocellulose. The nitrocellulose is then soaked in blocking buffer to "block" the nonspecific binding of proteins. The nitrocellulose is then incubated with the specific antibody for the protein of interest. The nitrocellulose is then incubated with a second antibody, which is specific for the first antibody. The membrane is then viewed via chemilluminescence.

Figure 9: Apparatus utilized in western blotting (1 – Compact power supply, 2 – Plastic combs, 3 – Spacer glass plate, 4 – Glass plates, 5 – Glass plate holder, 6 – Sample loading guides and 7 – Casting stand (http://www.assay-protocol.com/molecular-biology/electrophoresis/denaturing-page)

2.5.2 Protein isolation

HepG2 cells were grown to confluency in a 25cm^2 conical flask and treated with 10, 50 and 100ng/ml of fenugreek seed extract, 4-OH-lle, Insulin and metformin, over a period of 72h. The treatments were replenished every 24h (maintaining the same treatment concentrations previously used). At the end of the treatment period, the supernatants were removed and stored at -80^{o}C and the flasks were then rinsed twice with PBS. Cell lysis buffer (consisting of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 1 % phenoxylpolyethoxyethanol (Tritron X 100), 10 % glycerol, 50 mM sodium chloride (NaCl), protease inhibitor and phosphatase inhibitor) was then added to each flask and placed on ice for 10min. Protease and phosphatase inhibitor tablets had been previously added to the lysis buffer (1 tablet each/10ml of lysis buffer) to protect proteins against dephosphorylation. Specifically, protease inhibitor inhibits a broad spectrum of serine, cysteine, metalloproteases and calpains, while phosphatase inhibitor inhibits a broad spectrum of phosphatases such as acid, alkaline, serine/threonine and tyrosine protein phosphatase. After 10min, a scraper was used to remove the cells from each flask; the dislodged cells were pipetted into a 1ml tube, which was kept on ice. Tubes were then centrifuged at 4 °C and 10 000 rpm for 10 min. The supernatant containing protein from each sample was removed and placed into a new 1ml tube, and kept on ice. The remaining pellet was discarded.

The crude protein extracts were quantified and standardised using the bicinchoninic acid (BCA) assay (Appendix A). During this assay, all samples were kept on ice. Proteins and peptides do not absorb light in the visible region of the spectrum. However, when treated with an alkaline solution of cupric (Cu^{2+}) tartrate complex, a purple colour develops due to the complexing of the Cu^{2+} ions by peptide bonds. This colour reaction is characteristic of the

biuret reaction in which a reduction of Cu^{2+} ion to cuprous (Cu^{+}) ion by protein in an alkaline medium occurs. The Cu^{+} ion is then detected by a reaction with BCA to produce an intense purple colour that absorbs maximally at 562nm. The intensity of the colour produced is proportional to the number of peptides bonds participating in the reaction.

Then, 120 µl of the supernatant were mixed with 30 µl of 5 x Laemmli buffer (25 nM Tris, pH 6.8, 2 % SDS, 0.002 % bromophenol blue, 10 % glycerol and 5 % 2-mercaptoethanol) prior to boiling at 100 ºC for 5 min, and briefly centrifuged to settle all the evaporation from the lid of the micro-centrifuge tube. The cell lysate was stored at -20 °C prior to use, or at -80 °C for extended maintenance.

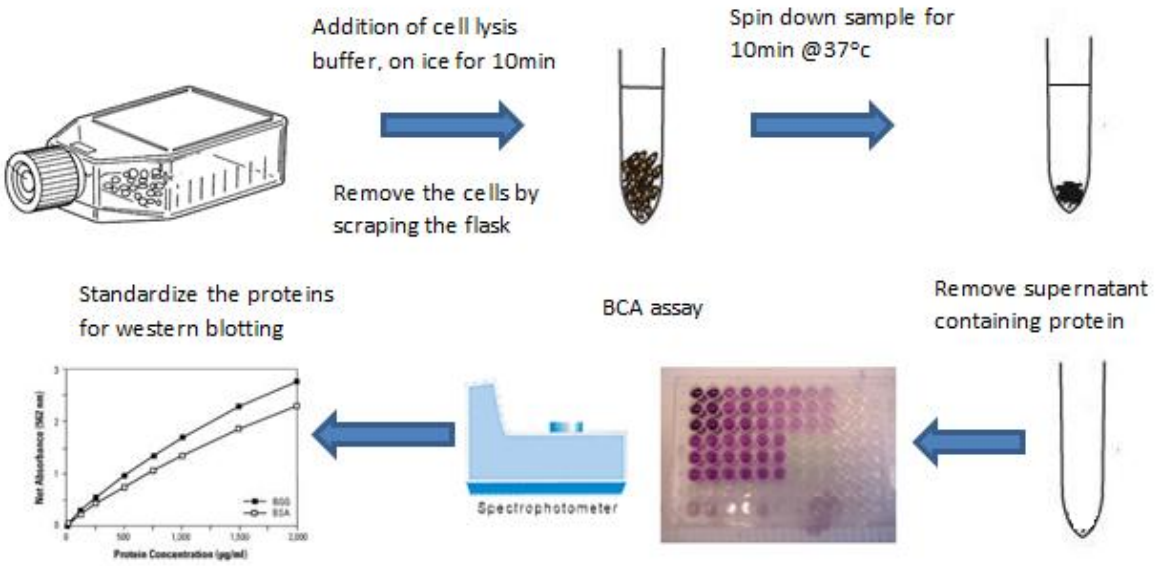

Figure 10: Overview of protein isolation

2.5.3 Preparation of SDS-polyacrylamide gels

The SDS-polyacrylamide gels (9cm x 6cm) were prepared in a multi gel casting chamber (Bio-Rad) according to manufacturer's instructions. Each gel consisted of a 4% stacking gel (upper layer) (0.126M Tris, pH 6.8, 4% acrylamide/bis , 0.1% SDS, 0.05% ammonium persulphate (APS) and 0.1% N, N, N', N',-tetramethylethylenediamine (TEMED) and a 7.8% resolving gel (lower layer) (0.375M Tris, pH 8.8, 7.5% acrylamide/bis , 0.1% SDS, 0.05% APS and 0.05% TEMED). Firstly, smaller glass plate were placed in-front of the bigger one, the plates were levelled out on a flat surface and placed into the glass plate holder. The holder was then placed on a sponge, in the casting stand. The stacking gel was added which requires approximately 1hr to set followed by the resolving gel, which takes 20min to set. A comb is placed in the stacking gel to allow for the formation of 10-wells for sample loading. Once the gels are set, the gel must be removed from the glass plates by placing the plate under cool running water and a gel releaser was used to remove the smaller glass plate and then the gel. The gel releaser was then used to cut away the gel wells (stacking gel) which were no longer needed.

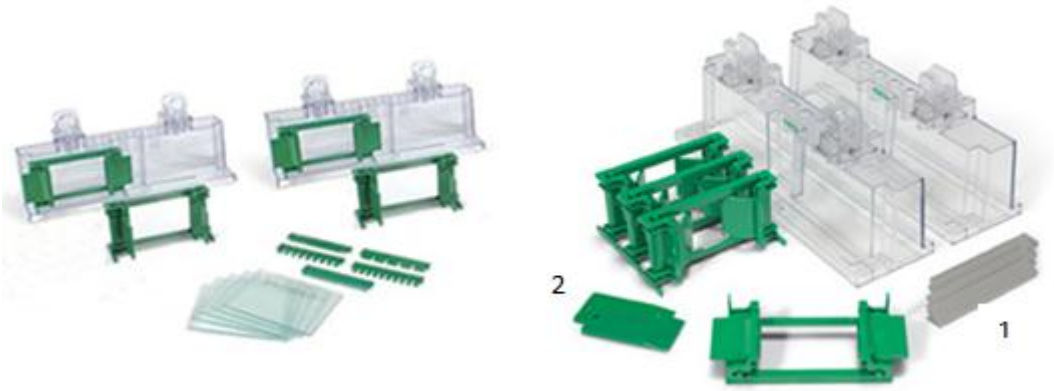

Figure 11: Apparatus utilized in the preparation of SDS polyacrylamide gels (1 – Sponges and 2 – gel releasers) (www.bio-rad.com/en-us/product/nucleic-acid-precast-mini-format-electrophoresis-systems/mini-protean-tetra-cell)

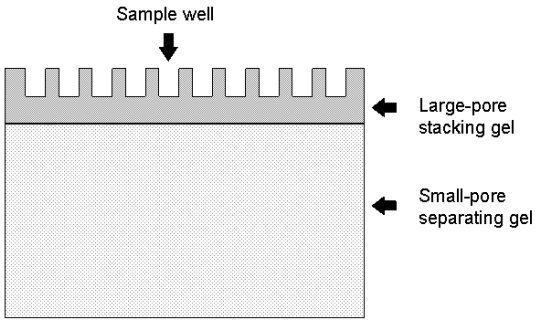

Figure 12: An example of the SDS polyacrylamide gel

2.5.4 Electrophoresis

The protein samples (25µl) and a molecular weight marker (Precision Plus Protein Dual Colour Standard, Bio-Rad) (10µl) were loaded into the wells of the gel. The four gels were clamped into the cassette and placed into the electrode tank (Bio-Rad) filled with running buffer (0.025M Tris, 0.192M glycine and 0.1% SDS). The samples were electrophoresed (150V, 1h) in 7.5% sodium dodecyl sulfate polyacrylamide gels using a Bio-Rad compact power supply.

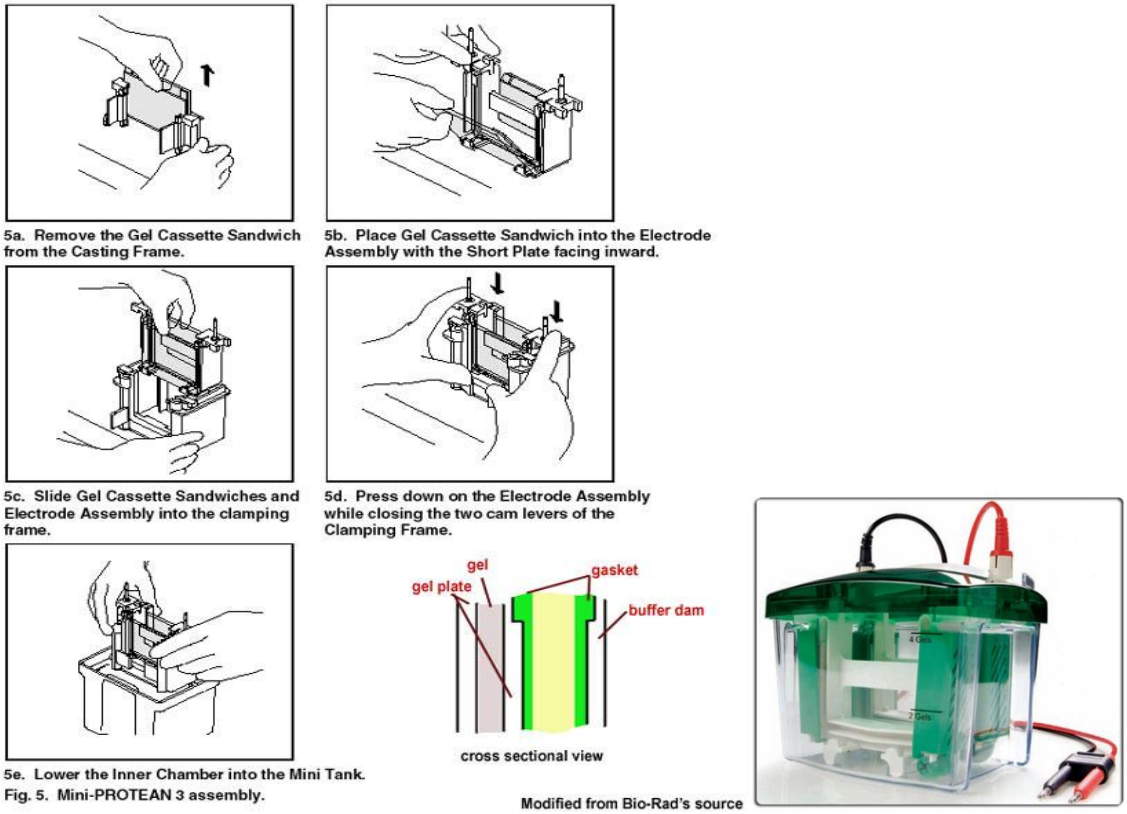

Figure 13: The process of electrophoresis in western blotting (http://stanxterm.aecom.yu.edu/wiki/index.php?page=SDS_PAGE, http://www.bio-rad.com/en-us/sku/165-8004-mini-protean-tetra-cell-for-mini-precast-gels)

2.5.5 Protein transfer

Nitrocellulose membranes and fibre pads were soaked for 10min in transfer buffer (0.025M Tris, 0.192M glycine and 20% methanol). Following electrophoresis, the poly-acrylamide gels were placed in transfer buffer (10min) to equilibrate. The protein transfer sandwich was assembled with the gel placed on the nitrocellulose membrane in the middle of two fibre pads on either side as shown in Figure 14. The separated proteins were electro-transferred to a nitrocellulose membrane (20V, 45min) using the Trans-Blot® Turbo Transfer system (Bio-Rad).

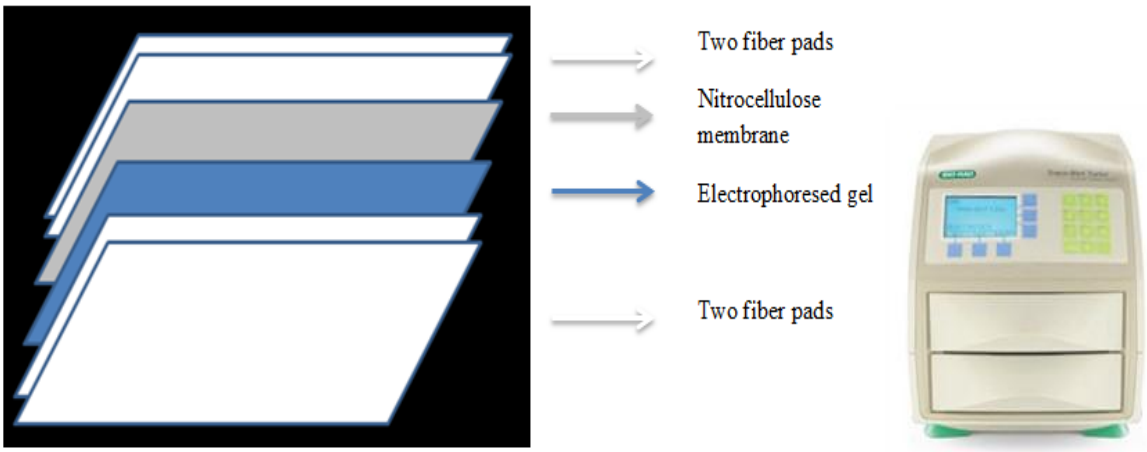

Figure 14: The process of protein transfer

(https://www.research.net/s/ReadersChoiceVoting2012)

2.5.6 Immuno-blotting

The membrane was incubated in a blocking solution of either 5% or 3% BSA in TTBS (0.150M NaCl, KCL, 0.05M Tris, 0.1% Tween 20, dH_2O, pH 7.4) for 1h at room temperature. Thereafter, the membranes were immune-probed with a specific primary antibody (PY20, Akt and GSK-3α/β) at 4°C overnight. The membranes were then washed (5x, 10min) with TTBS and incubated (RT, 1h) with the respective horseradish peroxidase (HRP)-labelled secondary antibody to detect the primary antibody bound to the protein of interest. Lastly, membranes were washed (5x, 10min) with TTBS and then once rinsed with deionized water. Horse radish peroxidase (HRP) chemiluminescence detector and enhancer solution was used for the antigen-antibody complex and the signal was detected with the Alliance 2.7 image documentation system (UViTech). Expression of proteins was analyzed with UViBand Advanced Image Analysis software v12.14 (UViTech). Data was expressed as relative band density. To normalize the expression of the proteins; β-actin (CS1615), 1:2 000 (used for GSK-3α/β); IR-β (CS3025) (used of PY20) and total Akt (CS9272) 1:1000 (used

for Akt) was assessed by quenching each membrane and following the same protocol as per the addition of the primary antibody.

Figure 15: The reagents and equipment utilized for viewing of membranes (http://www.laboratoryequipment.com/product-releases/2012/11/substrate-meets-western-blotting-needs, http://www.hipurebio.com/bbs/board.php?bo_table=menu01_08&wr_id=6)

2.5.7 Re-probing of membranes

The membrane was incubated with hydrogen peroxide solution (for stripping to block the HRP signals) for 30 min at 37 ºC, followed by washes (2x, 10min) and blocked for 1h with respective blocking solution. The membrane was re-probed with a different primary antibody as indicated.

2.6 Quantitative-polymerase chain reaction

2.6.1 Principle

PCR is used to amplify a specific region of a DNA strand (the DNA target). PCR typically amplifies DNA fragments of between 0.1 and 10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40kb in size. The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses. A basic PCR set up requires several components and reagents. These components include: DNA template that contains the DNA region (target) to be amplified, two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target, Taq polymerase or another DNA polymerase with a temperature optimum at around 70 °C, deoxynucleoside triphosphates (dNTPs, sometimes called "deoxynucleotide triphosphates"; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand, buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase, bivalent cations, magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis and monovalent cation potassium ions.

The PCR is commonly carried out in a reaction volume of 10–200µl in small reaction tubes (0.2–0.5ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. There are three defined steps in PCR: 1) denaturation, 2) annealing and 3) extension. Denaturation involves the separation, usually above 90°C, of double stranded (ds) DNA into two single stranded (ss) DNA template strands for accessibility of the target region. In the annealing step, the

temperature is dropped to allow for the hybridisation of oligonucleotide primers to complementary sites flanking the target region. Finally, extension entails the synthesis of DNA (addition of dNTPs) at the free 3' hydroxyl end of the primers by thermostable *Taq* DNA polymerase. This cycle is repeated for 30-40 times, and the result is an exponential amplification of the target DNA. Many modern thermal cyclers make use of the Peltier effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favourable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube. Quantitative-polymerase chain reaction is a technique that enables one to determine the amount of a target gene in a sample.

RNA is first isolated from cells and then reverse transcribed to ss complementary (c) DNA. cDNA is then used as the starting material for Q-PCR. The cycling steps of Q-PCR are similar to conventional PCR. Quantification of DNA is made possible by including a DNA-binding dye called SYBR Green in the reaction. SYBR Green binds to dsDNA amplicons proportionally and the fluorescence is detected after excitation. A curve is plotted with fluorescent emission over time from which the initial amount of DNA in each sample is obtained. A threshold is set at a constant point of exponential increase in the curves and the cycle time value for each sample is determined. Along with the gene of interest, samples are analysed for expression of a house keeping gene, and the amount of target DNA is reported relative to the amount of the house keeping gene for each sample.

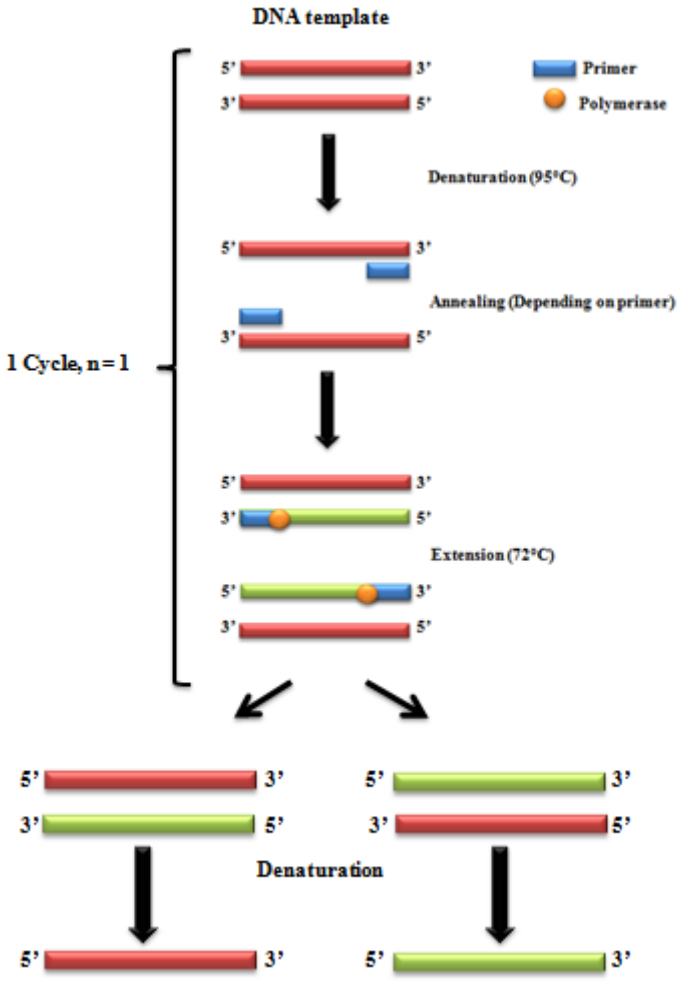

Figure 16: The process of quantitative PCR

2.6.2 RNA Isolation

The level of expression of messenger ribonucleic acid (mRNA) was determined by firstly isolating total ribonucleic acid (RNA) from cells treated in 25cm^3 flasks. After the flasks were rinsed twice with PBS, 500µl Triazol and 500µl 0.1M PBS were added to the flasks (RT, 5min). The cell lysate was removed from the flasks, transferred to 1.5ml tubes and incubated at -80˚C overnight. Following the overnight incubation, 100µl chloroform was added to each sample and centrifuged (12 000 g, 20min, 4˚C). Samples were then washed with 500µl of cold 75% ethanol. Afterwards, samples were centrifuged (7 400 g, 15min, 4˚C), ethanol was removed and the pellet containing RNA was re-suspended in nuclease-free water. The RNA was quantified using a spectrophotometer (Nanodrop2000). All RNA samples were standardized to a concentration of 1500ng/µl.

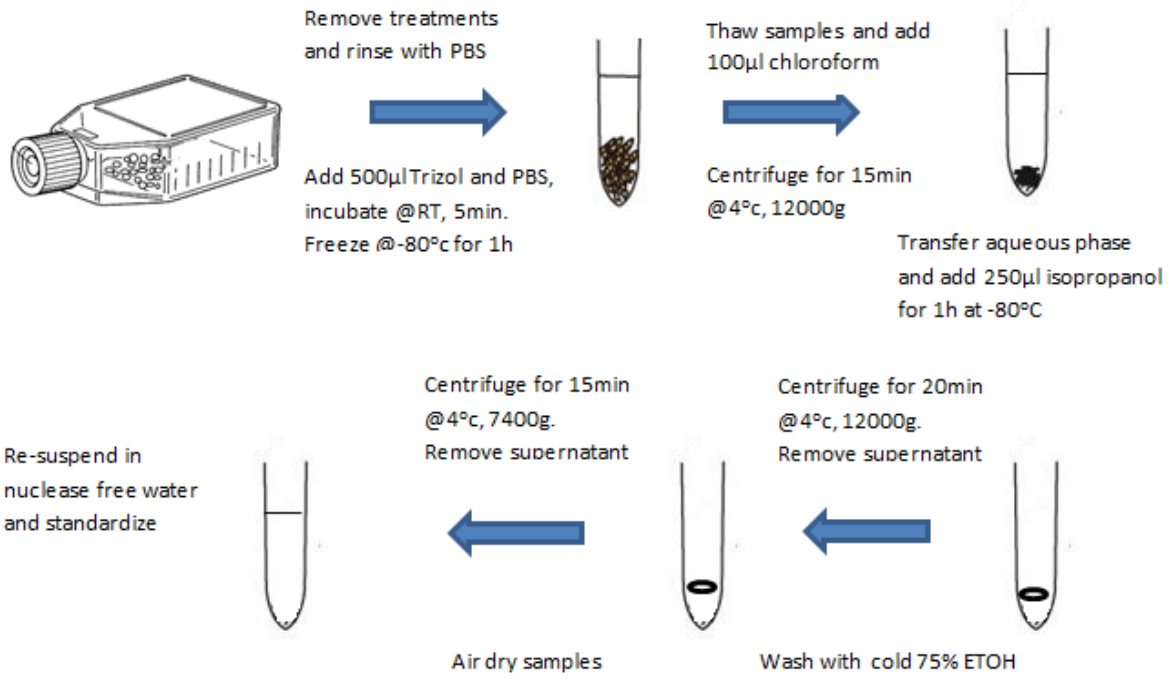

Figure 17: The process of RNA isolation

2.6.3 cDNA synthesis

Standardized RNA was reverse transcribed by reverse transcriptase into copy deoxyribonuclease acid (cDNA) using Script II RT kit (Qiagen) according to the manufacturer's instructions. The reaction was then subjected to 42°C (15min) and 95°C (5min) (GeneAmp® PCR System 9700, Applied Biosystems) to obtain cDNA.

RNA was reverse transcribed to cDNA as per manufacturers' guidelines using the RT2 First Strand Kit (SA Biosciences™). Firstly, genomic DNA elimination mixture (10µl) was prepared with 2µl (200ng) of total RNA, 2µl of 5X cDNA elimination buffer and 6µl of RNase-free water. Secondly, reverse transcriptase cocktail (10µl) was prepared with 4µl of reverse transcriptase buffer 3, 1µl of primer and external control mix, 2µl of reverse transcriptase enzyme mix and 3µl of RNase-free water. For the cDNA synthesis reaction, a 20µl reaction containing 10µl each of genomic DNA elimination buffer and reverse transcriptase cocktail for each sample was prepared and subjected to incubation at 42°C (15min) followed by heating at 95°C (5min) (GeneAmp® PCR System 9700, Applied Biosciences). Subsequently, 80µl of RNase-free water was added to each reaction mixture and stored at -70°C for further use.

Figure 18: The process of cDNA synthesis

2.6.4 Quantitative-polymerase chain reaction

Quantitative PCR was used to determine mRNA expression using the iScript SYBR Green PCR kit (Qiagen). A reaction of 12.5µl was prepared consisting of 6.25µl IQ™ SYBR® green supermix (cat. no. 170–8880), 3.75µl nuclease free water, 1µl cDNA, and 0.75µl sense and anti-sense primer (10mM, inqaba biotec™) were used. The mRNA expression was compared and normalized to a housekeeping gene, β-Actin and 18S. The reaction was subjected to an initial denaturation (95°C, 10min), followed by 40 cycles of denaturation (95°C, 15s), annealing (specific temperatures) and extension (72°C, 30s) (CFX and CFX analysis software) and mRNA expression was determined using the method described by Livak and Schmittgen (Livak et al., 2001) to calculate relative fold change. Primer sequences and annealing temperatures were as follows: SREBP1c– Sense 5'-GTGGCGGCTGCAT TGAGAGTGAAG-3'; SREBP1c– Antisense 5'-AGGTACCCGAGGGCATCCGAGAAT-3'(58˚C); GLUT2- Sense 5'-GGAGTCCTGTCAATTCCAGG-3'; GLUT2– Antisense 5'-CAAGTCTAATCTTCTCAGCG-3'(56˚C); GS– Sense 5'- GAAAGCCAAGAAACTGT TGTGATG-3'; GS– Antisense 5'- GAGGACTGGAGGCCTGAGAC-3'(60˚C); GK– Sense 5'-TCCACTTCAGAAGCCTACTG-3'; GK–Antisense 5'-TCAGATTCTGAGGCTCAAAC -3'(60˚C); β-Actin– Sense 5'-TGACGGGTCACCCACTGTGCCCAT-'3 and β-Actin– Antisense 5'- CTAGAAGCATTTGCGGTGGACGATGGAGGG-'3.

Figure 19: Reagents utilized for the qPCR run (http://www.bio-rad.com/en-us/category/high-fidelity-standard-pcr-reagents)

2.7 Measurement of glucose concentration

Glucose concentration in each sample supernatant was measured using our in-house piccolo express protocol.

Figure 20: Equipment utilized for the measurement of glucose (http://www.medwow.com/med/blood-gas-analyzer-poc/abaxis/piccolo-xpress/42563.model-spec)

2.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism v5.0 software (Graph Pad Software Inc., La Jolla, USA). The data were expressed as relative fold change and the mean standard deviation of the samples in the experiments. Statistical comparisons were made using a ONE-WAY ANOVA, non-parametric test and a Dunn's post test. The data were considered statistically significant with a value of $p < 0.05$.

CHAPTER THREE

3. RESULTS

3.1 Western blotting

To determine the effect of FSE, 4-OH-lle, insulin and metformin treatments on protein expression, the levels of IR-β, pAkt and pGSK-3α/β were assessed using western blot. The concentration of FSE and 4-OH-lle used for the treatments were determined from a range of concentrations including 0, 10, 50 and 100ng/ml (figure 21). The treatments included an untreated control (0ng/ml) and a treatment of 100ng/ml of FSE and 4-OH-lle coupled with the use of insulin (100ng/ml) and metformin (2mM) which served as positive controls. Western blotting experiment was performed twice

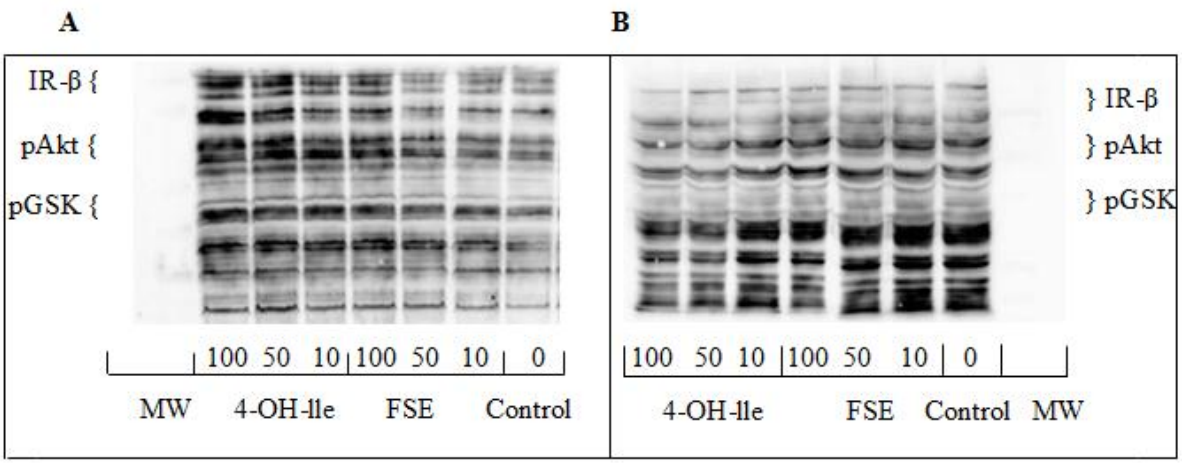

Figure 21: Range of fenugreek seed (FSE) (10, 50, 100ng/ml), 4-hydroxyisoleucine (4-OH-lle) (10, 50, 100ng/ml) treatment and control (0ng/ml) on phosphorylation of PY20, on HepG2 cells. A - Normoglycaemic conditions. B - Hyperglycaemic conditions. MW – Molecular weight marker.

Normoglycaemic conditions

FSE: Induced a significant 2.29-fold increase in PY20/IR-β expression (*p<0.0729); a 1.89-fold increase in pAkt/total Akt expression (**p<0.0046) and a 1.73-fold increase in pGSK-3α/β/total GSK-3αβ expression (*p<0.0128) (figure 22C, 23E, 24G).

4-OH-lle: Induced a significant 2.91-fold increase in PY20/IR-β expression (*p<0.0729); a 2.06-fold increase in pAkt/total Akt expression (**p<0.0046) and a 2.69-fold increase in pGSK-3α/β/total GSK-3αβ expression (*p<0.0128) (figure 22C, 23E, 24G).

Insulin: Induced a significant 2.47-fold increase in PY20/IR-β expression (*p<0.0729); a 2.09-fold increase in pAkt/total Akt expression (**p<0.0046) and a 2.57-fold increase in pGSK-3α/β/total GSK-3αβ expression (*p<0.0128) (figure 22C, 23E, 24G).

Metformin: Induced a significant 1.57-fold increase in PY20/IR-β expression (*p<0.0729); a 1.59-fold increase in pAkt/total Akt expression (**p<0.0046) and a 1.47-fold increase in pGSK-3α/β/total GSK-3αβ expression (*p<0.0128) (figure 22C, 23E, 23G).

Hyperglycaemic conditions

FSE: Induced a significant 1.83-fold increase in PY20/IR-β expression (*p<0.0121); a 2.32-fold increase in pAkt/total Akt expression (***p<0.0005) and a 1.70-fold increase in pGSK-3α/β/total GSK-3αβ expression (**p<0.0048) (figure 22 D, 23F, 24H).

4-OH-lle: Induced a significant 1.84 -fold increase in PY20/IR-β expression (*p<0.0121); a 2.42 -fold increase in pAkt/total Akt expression (***p<0.0005) and a 1.75 -fold increase in pGSK-3α/β/total GSK-3αβ expression (**p<0.0048) (figure 22D, 23F, 24H).

Insulin: Induced a significant 2.13 -fold increase in PY20/IR-β expression (*p<0.0121); a 2.09 -fold increase in pAkt/total Akt expression (***p<0.0005) and a -fold increase in pGSK1.50 -3α/β/total GSK-3αβ expression (**p<0.0048) (figure 22D, 23F, 24H).

Metformin: Induced a significant 1.34 -fold increase in PY20/IR-β expression (*p<0.0121); a 1.26 -fold increase in pAkt/total Akt expression (***p<0.0005) and a 2.50 -fold increase in pGSK-3α/β/total GSK-3αβ expression (**p<0.0048) (figure 22D, 23F, 24H).

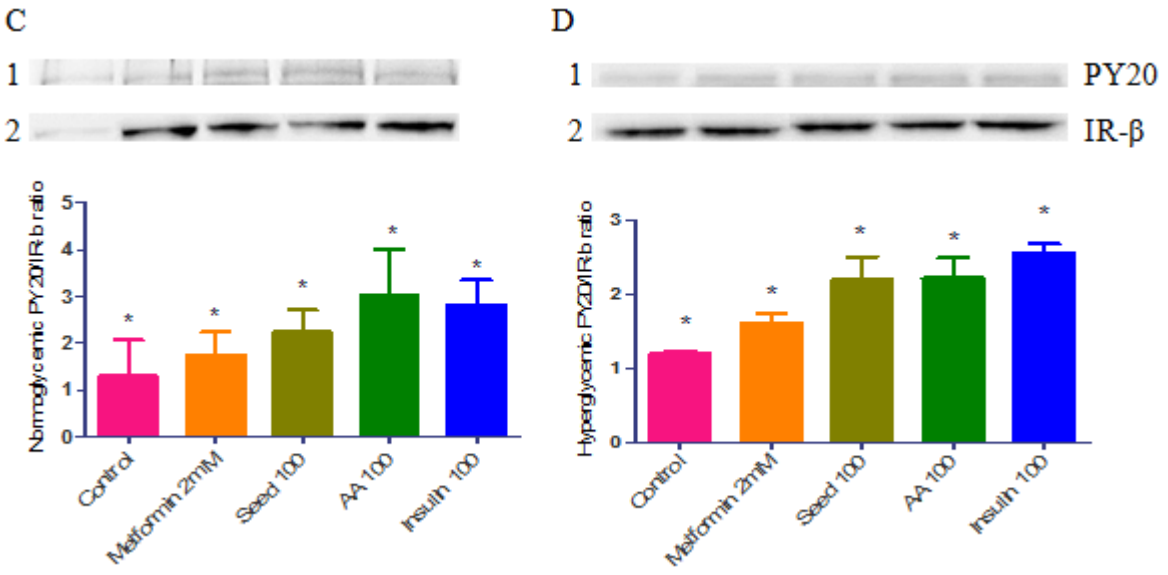

Figure 22: Fenugreek seed extract (FSE), 4-hydroxyisoleucine (4-OH-lle), metformin and insulin treatments on phosphorylation of IR-β, on HepG2 cells. C – Normal conditions (*P<0.0729) and D – Hyperglycaemic conditions (*p<0.0121).

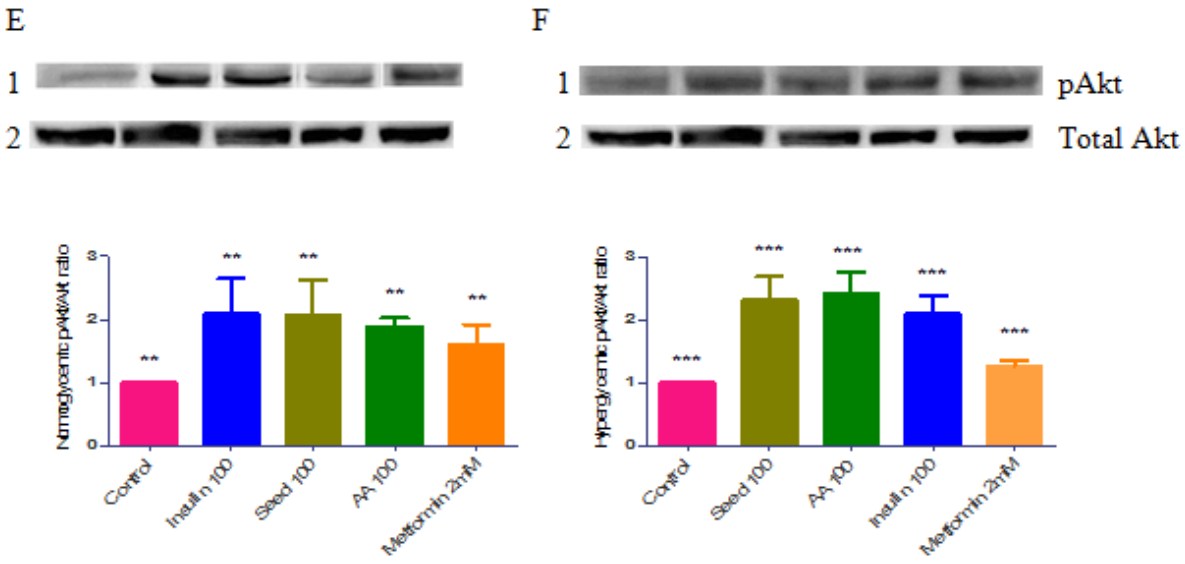

Figure 23: Fenugreek seed extract (FSE), 4-hydroxyisoleucine (4-OH-lle), metformin and insulin treatments on phosphorylation of Akt, on HepG2 cells. E – Normal conditions (P<0.0046) and F – Hyperglycaemic conditions (***p<0.0005).**

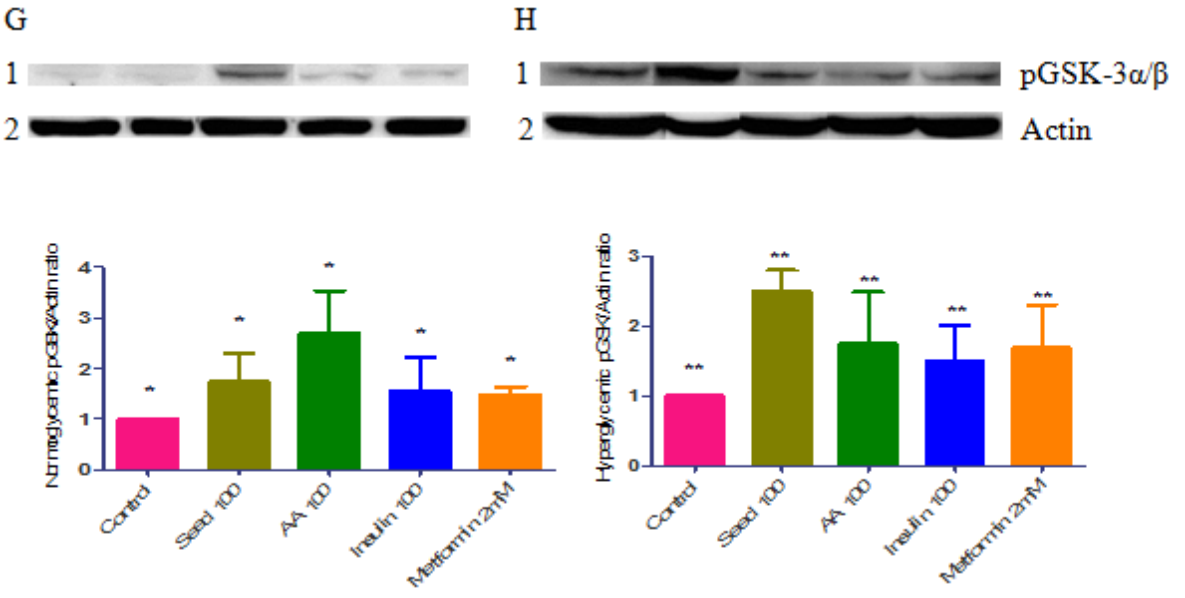

Figure 24: Fenugreek seed extract (FSE), 4-hydroxyisoleucine (4-OH-lle), metformin and insulin treatments on phosphorylation of GSK-3α/β, on HepG2 cells. G – Normal conditions (*P<0.0128) and H – Hyperglycaemic conditions (p<0.0048).**

3.2 Quantification of mRNA

The mRNA expression of SREBP1c, GLUT2, GS and GK in HepG2 cells was determined using qPCR. The samples were run in triplicate.

Normoglycaemic conditions

SREBP1c: Fenugreek seed extract induced a significant 1.47-fold increase; 4-OH-lle a 5.08-fold increase; insulin a 2.09-fold increase and metformin caused a significant 0.03-fold decrease in expression (*$p<0.0157$) (figure 25).

GLUT2: Expression increased 3.79-fold by FSE; a 1.37-fold increase by 4-OH-lle; a 1.51-fold increase by insulin and metformin reduced expression 0.9-fold (*$p<0.0330$) (figure 26).

GS: Fenugreek seed extract induced a significant 1.37-fold increase and a 0.04-fold decrease by 4-OH-lle, 0.2-fold by insulin and 0.18-fold by metformin (*$p<0.0262$) (figure 27).

GK: Increased expression 3.99-fold by FSE and decreased expression 0.19-fold by 4-OH-lle, 0.86-fold by metformin and 0.23-fold by insulin (*$p<0.345$) (figure 28).

Hyperglycaemic conditions

SREBP1c: Expression was reduced by FSE 0.82-fold; 0.76-fold by 4-OH-lle; 0.19-fold by metformin and insulin increased expression 3.91-fold (**$p<0.0012$) (figure 25).

GLUT2: Fenugreek seed extract caused an elevation of 9.87-fold; 2-fold by 4-OH-lle; 2.47-fold by insulin and a 0.63-fold decrease by metformin (*$p<0.0417$) (figure 26).

GS: Expression was increased 2-fold by FSE and 1.07-fold by insulin and decreased 0.29-fold by metformin and 0.5-fold by 4-OH-lle (*p<0.0333) (figure27).

GK: Fenugreek seed extract caused an elevation of 23.92-fold; 2.11-fold by 4-OH-lle; 15.85-fold by insulin and 3.35-fold by metformin (*p<0.0213) (figure 28).

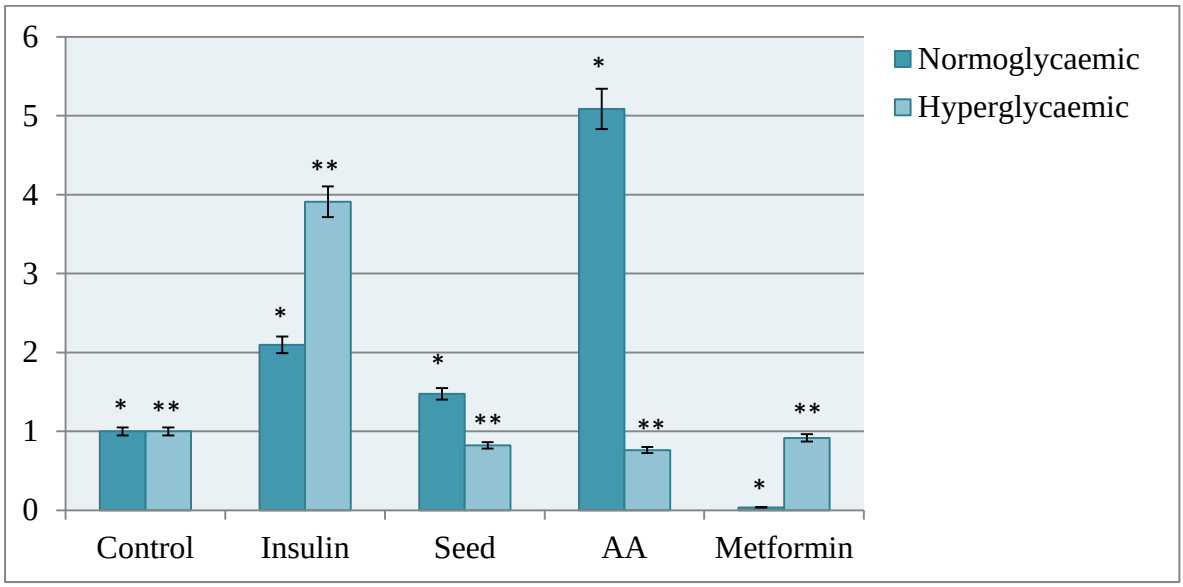

Figure 25: The effect of fenugreek seed extract (FSE), 4-hydroxyisolecuine (4-OH-lle) and insulin treatment on SREBP1c expression, on Hepg2 cells under normoglycaemic (*p<0.0157) and hyperglycaemic conditions (p<0.0012).**

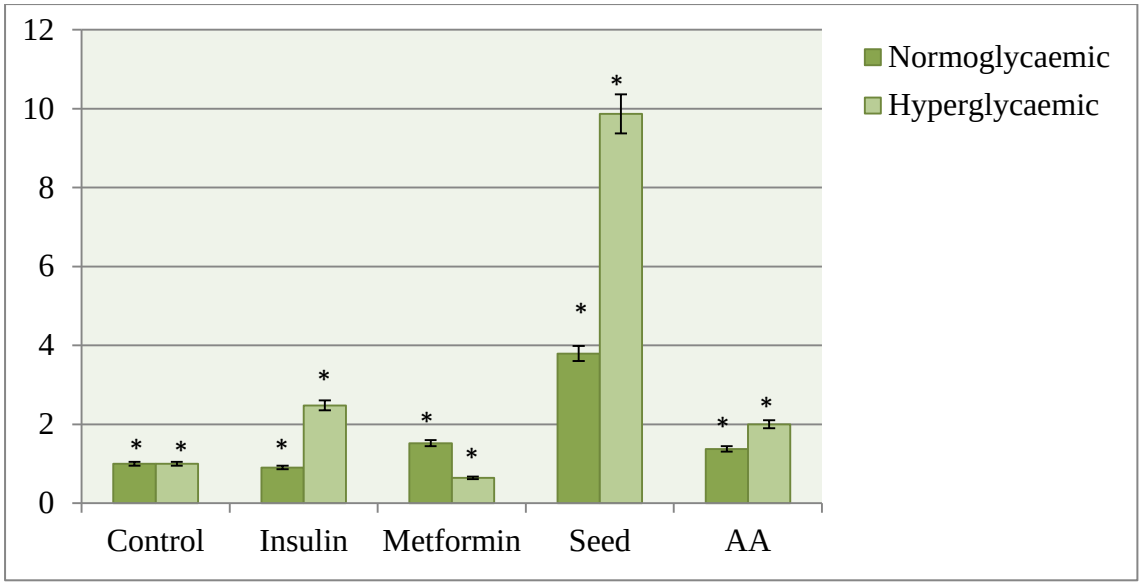

Figure 26: The effect of fenugreek seed extract (FSE), 4-hydroxyisolecuine (4-OH-lle) and insulin treatment on GLUT2 expression, on Hepg2 cells under normoglycaemic (*p<0.0330) and hyperglycaemic conditions (*p<0.0417).

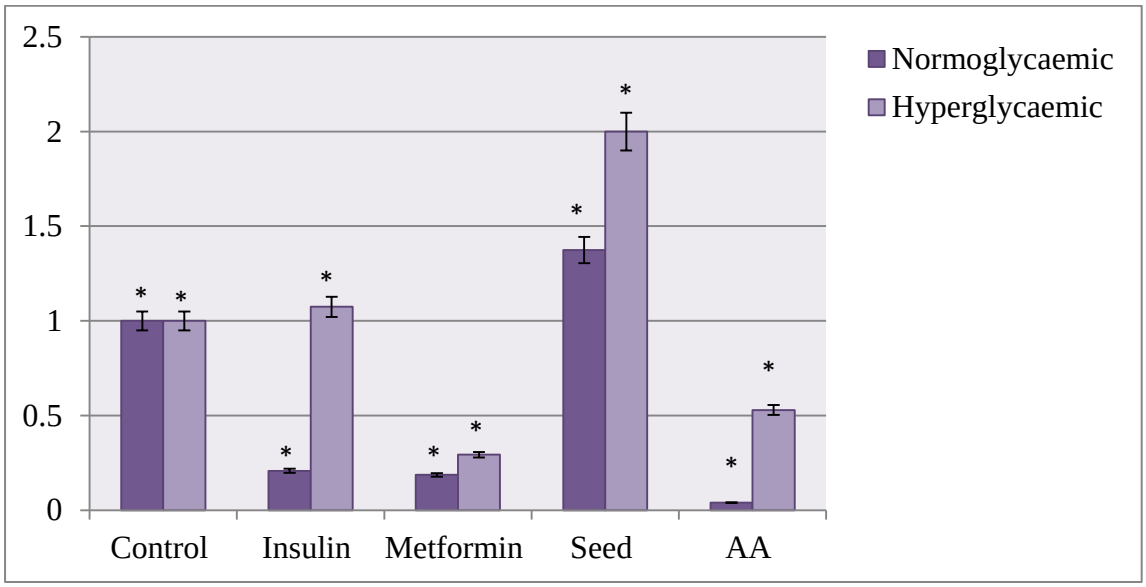

Figure 27 The effect of fenugreek seed extract (FSE), 4-hydroxyisolecuine (4-OH-lle) and insulin treatment on GS expression, on Hepg2 cells under normoglycaemic (*p<0.0262) and hyperglycaemic conditions (*p<0.0333).

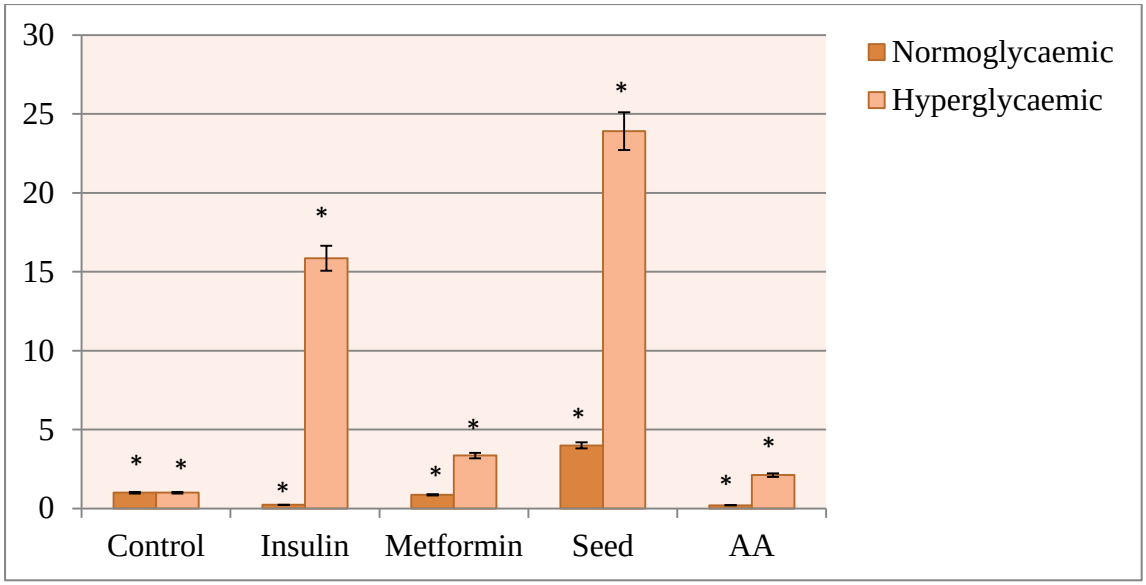

Figure 28 The effect of fenugreek seed extract (FSE), 4-hydroxyisolecuine (4-OH-lle) and insulin treatment on GK expression, on Hepg2 cells under normoglycaemic (*p<0.0345) and hyperglycaemic conditions (*p<0.0213).

Table 1 Supernatant glucose concentrations following fenugreek seed extract (FSE), 4-hydroxyisoleucine (4-OH-lle), insulin and metformin treatment

Normoglycaemic state		**Hyperglycaemic state**	
Treatment (ng/ml)	**Glucose mMol/L)**	**Treatment (ng/ml)**	**Glucose (mMol/L)**
Control (0)	5.1	Control (0)	>28 (read too high)
Insulin (100)	1.9	Insulin (100)	18.0
Metformin (100)	2.8	Metformin (100)	21.0
FSE (100)	< 1.7 (read too low)	FSE (100)	19.5
4-OH-lle (100)	2.5	4-OH-lle (100)	23.4

CHAPTER FOUR

4. DISCUSSION

Fenugreek has been reported to aid in the reduction of glucose concentrations during hyperglycaemic conditions. Previous studies have reported this glucose lowering effect owing to the seed, more specifically 4-OH-lle (Bhat et al. 2005; Breil et al. 2004). However these studies showed no cellular or metabolic action of the seed and amino acid in exerting their glucose lowering effect. Our study investigated the molecular effects of FSE and 4-OH-lle on both proximal and distal insulin signaling proteins, entry of glucose into the cell and glycogen storage, *in vitro*.

In the present study, the results revealed FSE, 4-OH-lle, insulin and metformin increased activation of IR-β (figure 21, 22). This activation is important in initiating the signal transduction of internal cellular mechanisms that directly affect glucose uptake (Cheatham and Kahn 2005). High circulating blood glucose triggers the release of insulin from pancreatic beta cells. Insulin binds to the extracellular alpha subunits of the insulin receptor. This causes a conformational change in the beta subunit, activating the kinase domain of the insulin receptor (Cheatham and Kahn 2005). In order of stimulation; metformin, FSE, insulin and 4-OH-lle, caused a significant elevation in tyrosine phosphorylation of IR-β (figure 21, 22). Activated kinase domain of IR-β is imperative in auto-phosphorylation of Akt via the phosphoinositide 3-kinase (PI3K) pathway (Kim et al. 1999; Lawler et al. 2001). Fenugreek seed extract displayed the most significant increase in tyrosine phosphorylation, following insulin, 4-OH-lle and metformin with the least effect (figure 23 E, F). Phosphorylated Akt plays a critical role in regulating glycogen synthesis via the auto-phosphorylation of GSK-3α/β (Cheatham and Kahn 2005). Phosphorylation of a protein such as GS by GSK-3α/β regularly inhibits the activity of its downstream target (Cheatham and Kahn 2005; Bhat et al.,

2005). Therefore, when pAkt auto-phosphorylates GSK-3α/β, its activity is reduced. A reduction in GSK-3α/β activity loses its ability to inhibit GS, causing an increase in the active form of GS. Fenugreek seed extract, 4-OH-lle, insulin and metformin cause significant elevation in tyrosine phosphorylation of GSK-3α/β (figure 24 G, H). These findings correlate with the 1.37 (normoglycaemic) and 2.00 (hyperglycaemic) fold increase by FSE of GS (Figure 27). Insulin caused a 0.2 (normoglycaemic) fold decrease and 1.07 (hyperglycaemic) fold increase in GS expression. However, 4-OH-lle and metformin caused a decrease in expression of GS (Figure 27). Glycogen is a polysaccharide that is the principal storage form of glucose. Glycogen is not as reduced as fatty acids are and consequently not as energy rich however glycogen is an important fuel reserve for several reasons. The controlled breakdown of glycogen and release of glucose increase the amount of glucose that is available between meals. Hence, glycogen serves as a buffer to maintain blood-glucose levels. Glycogen's role in maintaining blood-glucose levels is especially important because glucose is virtually the only fuel used by the brain, except during prolonged starvation. Moreover, the glucose from glycogen is readily mobilized and is therefore a good source of energy for sudden, strenuous activity. Unlike fatty acids, the released glucose can provide energy in the absence of oxygen and can thus supply energy for anaerobic activity. With the liver serving as a major site of glycogen storage, Fenugreek seed extract is more efficient than insulin and metformin in allowing for the storage of glycogen via the up-regulation of GS (Figure 27). Resulting in the maintenance of blood-glucose levels as required to meet the needs of the organism as a whole.

Phosphorylated sugar molecules do not readily penetrate cell membranes, because there are no specific trans-membrane carriers for these compounds, and since they are too polar to diffuse through the lipid core of membranes (Francini et al. 2011). The irreversible phosphorylation of glucose, therefore, effectively traps the sugar as cytosolic glucose 6-

phosphate (G-6-P), consequently committing it to further metabolism in the cell (Francini et al. 2011; Matschinsky and Sweet 1996). Mammals have several isozymes of the enzyme hexokinase (HK) that catalyze the phosphorylation of glucose to G-6-P. In most tissues, the phosphorylation of glucose is catalyzed by HK, one of three regulatory enzymes of glycolysis. Hexokinase has broad substrate specificity and is able to phosphorylate several hexoses in addition to glucose. Hexokinase is inhibited by the reaction product, G-6-P, which accumulates when further metabolism of this hexose phosphate is reduced. Hexokinase has a low Km (and high affinity) for glucose (Francini et al. 2011). This permits the efficient phosphorylation and subsequent metabolism of glucose even when tissue concentrations of glucose are low. Hexokinase, however, has a low Vmax for glucose and, therefore, cannot sequester cellular phosphate in the form of phosphorylated hexoses, or phosphorylate more sugars than the cell can use (Francini et al. 2011).

In liver parenchymal cells and beta cells of the pancreas, GK (also called HK D, or type IV) is the predominant enzyme responsible for the phosphorylation of glucose. In beta cells, GK functions as the glucose sensor, determining the threshold for insulin secretion (Francini et al. 2011). In the liver, the enzyme facilitates glucose phosphorylation during hyperglycaemia. Like HK, GK catalyzes the ATP-dependent phosphorylation of glucose to form G-6-P and ADP. This is the first step of glycolysis. The enzyme will act on a variety of 6-carbon sugars, producing moieties phosphorylated at position six. Glucokinase differs from HK in several important properties. For example, it has a much higher Km (10mM vs. 1mM), requiring a higher glucose concentration for half-saturation (Francini et al. 2011). The higher Km for GK means that less glucose will be phosphorylated, allowing more glucose to be exported to the bloodstream, where it can be delivered to other tissues that do not make their own glucose (Cárdenas 1995). Also, GK is not inhibited by G-6-P therefore glucose can be shunted into glycogen synthesis. Hence the activity of GK is increased when blood-glucose concentrations

are high. Similarly, insulin release is dependent on GK in pancreatic beta cells. Thus, GK functions only when the intracellular concentration of glucose in the hepatocyte is elevated, such as during the brief period following consumption of a carbohydrate-rich meal, when high levels of glucose are delivered to the liver via the portal vein. Glucokinase has a high Vmax, allowing the liver to effectively remove the flood of glucose delivered by the portal blood (Francini et al. 2011). This prevents large amounts of glucose from entering the systemic circulation following a carbohydrate-rich meal, and thus minimizes hyperglycaemia during the absorptive period (Francini et al. 2011). Glucose receptor 2 ensures that blood glucose equilibrates rapidly across the membrane of the hepatocyte. Glucokinase activity rises and falls with available glucose (Buhler et al. 1995; Cheatham et al. 2005). Concomitant rise of insulin, amplifies this effect by induction of GK synthesis (Buhler et al. 1995; Cheatham and Kahn 2005).

Fenugreek seed extract caused an elevation in GK activity by 3.99 fold (normoglycaemic) and 23.92 fold (hyperglycaemic) (Figure 28). However, under normoglycaemic conditions 4-OH-lle (0.19 fold), insulin (0.23 fold) and metformin (0.86 fold) reduced the expression but under hyperglycaemic conditions 4-OH-lle (2.11 fold), insulin (15.86 fold) and metformin (3.36 fold) increased the expression of GK (Figure 28). Fenugreek seed extract effectively up-regulated the activity of GK, under both conditions. Fenugreek was as successful or if not more successful in controlling hyperglycaemia as compared to insulin and metformin. Fenugreek seed extract has displayed its effect efficiently by regulating glycogen synthesis via GSK-3α/β and regulating the expression of GK.

Increased circulating blood glucose requires entry into the cell, in order to efficiently utilize the glucose for metabolic activity and storage and ultimately reduce blood glucose levels (Ahn et al. 2005; Enjoji et al. 2008). Entry into the cell requires the transcriptional activation of GLUT2 via SREBP1c (Ahn et al. 2005; Enjoji et al. 2008). Glucose transporter 2 has a

high capacity for glucose but low affinity thus serving as an efficient carrier for blood glucose in the liver (Ahn et al. 2005). Insulin activates the hepatic expression of SREBP1c (Enjoji et al. 2008). Sterol regulatory element binding protein 1c plays a key role in glucose stimulated GLUT2 gene expression (Enjoji et al. 2007). The GLUT2 promoter region is activated by SREBP1c (Enjoji et al. 2008, Caro et al. 1987). Fenugreek seed extract up-regulated the expression of SREBP1c 2.36-fold (normoglycaemic) and 5.03-fold (hyperglycaemic) (figure 25). This correlated with the increase in GLUT2 expression 3.79-fold (normoglycaemic) and 9.87-fold (hyperglycaemic) (figure 26). 4-Hydroxyisoleucine reduced the expression of SREBP1c 0.82-fold (normoglycaemic) and 0.9-fold (hyperglycaemic) (figure 25). Whereas the expression of GLUT2 is slightly up-regulated 1.37-fold (normoglycaemic) and 2-fold (hyperglycaemic) (figure 26). Insulin increased the expression of SREBP1c 2.09-fold (normoglycaemic) and 3.91-fold (hyperglycaemic) (figure 25). The expression of GLUT2 was up-regulated 1.5-fold (normoglycaemic) and 2.4-fold (normoglycaemic) (figure 26). Sterol regulatory element binding protein 1c was decreased 0.03-fold (normoglycaemic) and 0.91-fold (hyperglycaemic) by metformin (figure 25). Accompanied by a decrease in GLUT2 expression, 0.19 (normoglycaemic) and 0.64 (hyperglycaemic) fold were observed (figure 246). Fenugreek seed extract displayed a greater ability in increasing the expression of GLUT2 following SREBP1c, than insulin – which is the cells natural response to increased glucose and metformin – a first line choice of drug for the treatment of T2DM. This would be beneficial to the cell, allowing maximum clearance of glucose from circulation, into storage, therefore effectively reducing the increased circulating blood glucose.

In T2DM the post-prandial glucose level is approximately 9mMol/l. Under a hyperglycaemic state, 100ng/ml insulin reduced the level of glucose by 50% (table 1). Whereas FSE reduced the glucose concentrations by 46% (table 1). In comparison to *in vivo* studies, the use of

40ng/ml and 10ng/ml insulin displayed significant reductions in glucose levels (Cignarelli et al. 2006; Bolder et al. 2008). When analyzing insulin and FSE these treatments did not bring the levels back to normal, however a decrease was noted. When comparing our study to *in vivo* studies, the concentration used is double the current treatment concentration. Therefore one can conclude that an increase in the treatment concentration used could effectively reduce increased glucose levels back to or near normal.

Metformin is the first line drug of choice for treatment of T2DM. It has been effective in its treatment via the control of energy homeostasis (Pearson et al. 2013; Viollet and Foretz 2013). In our study, FSE and 4-OH-lle has effectively caused an increase in IR-β, Akt and GSK-3α/β protein expression, under both normoglycaemic and hyperglycaemic conditions. The genes imperative in the metabolism of glucose – SREBP1c, GLUT2, GS and GK were significantly up-regulated under both conditions by FSE and 4-OH-lle greater than metformin. It can be concluded that FSE and 4-OH-lle has successfully accomplished its role in glucose metabolism by contending with metformin, the common drug used for the treatment of T2DM.

Insulin is the natural regulator of glucose. A rise in glucose is required for the release of insulin. A low insulin level predisposes the cell to reduced glucose availability, producing an extracellular hyperglycaemic state. Hyperglycaemia is characteristic of T2DM caused by insulin resistance. Our data strongly suggests the mechanism of action of FSE is similar to or even better than that of insulin and metformin. Fenugreek seed extract effectively and efficiently displayed its glucose lowering effect by increasing IR-β and phosphorylation of Akt, GSK-3α/β, GS and GK, furthermore an increase in SREBP1c, GLUT2, GS and GK.

CHAPTER FIVE

5. CONCLUSION

Natural herbal products have been and are still a major source in supporting the primary health care systems. Their activity is mainly associated with biologically active compounds present within the plant. Some of these active compounds aid in the treatment of diseases which is overwhelming the population, such as diabetes mellitus. Therefore many plants are targets for drug prospecting diabetic - associated programs. Fenugreek is a source of a group of active compounds where 4-OH-lle has been labelled as the most effective in combating hyperglycaemia – a chronic symptom in T2DM.

Fenugreek seed extract and 4-OH-lle have displayed their effects at effectively reducing important proteins and genes involved in the metabolism of glucose. Their effect has shown to be greater than that of the natural glucose reducer – insulin and the common drug – metformin. Therefore fenugreek possesses great potential in combating the high-blood glucose present in T2DM. However the lack in substantial scientific data regarding the use of this plant as an anti-diabetic compound, serves as an open avenue to a new discovery.

REFERENCES

1. Abdel-Barry, J. A., Abdel-Hassan, I. A., Al-Hakiem, M. H and Jawad, A. M. (2000) Hypoglycaemic effect of aqueous extract of the leaves of Trigonella foenum-graecum in healthy volunteers. East Mediterranean Health Journal **6**:83-88.

2. Abdo, S. A., Althwab, F. M., Hasson, S and Mothana, R. A. (2008). Antimicrobial, Antioxidant and Cytotoxic Activities and Phytochemical Screening of Some Yemeni Medicinal Plants. Journal Evidence Based Complementary Alternative Medicine.

3. Abel, R., Arad, J., Madar, Z and Samish, S. (1988). Glucose lowering effect of fenugreek in non-insulin dependent diabetics. Europe Journal of Clinical Nutrition **42**:51-54.

4. Abubakar, M. G., Agaie, B. M., Hassan, S. W and Ukwuani, A. N. (2013). Antinociceptive activity of Hydromethanolic Extract of Some Medicinal Plants in Mice. International Journal of Pharmacy. Photon **104**, 120-125.

5. Ahn, Y., Im, S., Kang, S., Kim, S., Kim, H., Kim, J and, Kim K. (2005). Glucose-Stimulated Upregulation of GLUT2 Gene Is Mediated by Sterol Response Element–Binding Protein-1c in the Hepatocytes. Diabetes **54**.

6. Alberti, K. G, Zimmet, P, and Shaw, J. (2006). Metabolic syndrome - a new world-wide definition. A Consensus Statement from the International Diabetes Federation. Diabetes Medicine **23**(5), 469-480.

7. Alcock, N. W., Crout. D. H. G., Gregorio, M. V. M., Lee, E., Pike, G and Samuel, C.J. (1989). Stereochemistry of the 4-hydroxyisoleucine from Trigonella foenum graecum. Phytochemistry **28:** 1835–1841.

8. Alessi, D. R and Lizcano, J. M. The insulin signalling pathway. (2002) Curr Biology **12**:R236–R238.

9. Alessi, D. R., Burchell, A., Grempler, R., Guo, S., Schmoll, D., Unterman, T. G., Walker, K. S and Walther, R. (2000). Regulation of glucose-6-phosphatase gene expression by protein kinase Balpha and the forkhead transcription factor FKHR. Evidence for insulin response unit-dependent and-independent effects of insulin on promoter activity. Journal of Biol. Chemistry **275**, 36324–36333.

10. Anuradha, C. V and Kannappan, S. (2009) Insulin sensitizing actions of fenugreek seed polyphenols, quercetin & metformin in a rat model. Indian J Med Res **129**, 401-408.

11. Ara, N., Asadi, A. Z. S., Ferdaus, A., Moosa, A. S. M., Rashid, M. U and Uddin, M. (2006). Hypolipidemic effects of fenugreek seed powder. **1**: 64-67.

12. Ardekani, M. R. S., Haeri, M., Izaddoost, M., Nobar, M. R and White, K. N. (2009). The effect of fenugreek 4-hydroxyleucine on liver function biomarkers and glucose in diabetic and fructose-fed rats. **23**: 61-64.

13. Arner, P., Pollare, T., Lithell, H and Livingston, J., N. (1991) Defective insulin receptor tyrosine kinase in human skeletal muscle in obesity and type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia **30**(6), 437-440.

14. Assad, S and Morse, J. K. (2013). A review on the efficacy of four popular herbs used for the treatment of diabetes. **Review Article** **3**(1):13-22.

15. Au, W. S., Kung, H., F and Lin, M. C. (2003). Regulation of microsomal triglyceride transfer protein gene by insulin in Hepg2 cells. Diabetes **52** (5), 1073-1080.

16. Baissac, Y., Broca, C., Manteghetti, M., Petit, P and Sauvaire, Y. (1998). 4-hydroxyisoleucine: A novel amino acid potentiator of insulin secretion. Diabetes **47**: 206-210.

17. Bapat, M. M., Niphadkar, P. V and Patil, S. P. (1997). Allergy to fenugreek (Trigonella foenum graecum). Ann Allergy Asthma Immunology **78**:297-300.

18. Baquer, N. Z., Cowsik, S. M., Kale, R. K., Kumar, P., Mclean, P and Taha, A. (2011). Metabolic and molecular action of Trigonella foenum-graecum (fenugreek) and trace metals in experimental diabetic tissues. **36**.

19. Bar Dayan, Y., Boaz, M, Leibovitz, E and Wainstein, J. (2011). "Functional foods in the treatment of type 2 diabetes: olive leaf extract, turmeric and fenugreek, a qualitative review". Function of Foods Health and Disease **1**(1), 472–481.

20. Barthel,A., Schmoll, D., Kruger, K. D., Bahrenberg, G., Walther, R., Roth, R. A., and Joost, H. G. (2001). Differential regulation of endogenous glucose-6-phosphatase and phosphoenolpyruvate Carboxykinase gene expression by the forkhead transcription factor FKHR in H4IIE-hepatoma cells. Biochem. Biophys. Res. Commun. **285**, 897–902.

21. Basch, E., Ulbricht, C., Kuo, G., Szapary, P and Smith M. (2003). Therapeutic applications of fenugreek. **8**.

22. Basu, T. K and Sricjamroen, A. (2010). Health benefits of fenugreek (Trigonella foenum-graecum). **28:** 425 – 435.

23. Belahcen, A., Mdaghri, A. A., Rhouda, H., Smires, N., Taloubi, L. M and Thimou, A. (2013). An overview of plants causing teratogenicity: Fenugreek (Trigonella foenum graecum). **4**(2), 516-519.

24. Bessler, R and Jackson, A. (1981). Clinical pharmacology of sulfonylurea hypoglycaemic agents. Drugs **22**: 211-245.

25. Berthiaume, F., Chan1, C., Lee, K and Yarmush, M. L. (2004). Metabolic flux analysis of hepatocyte function in hormone- and amino acid-supplemented plasma. **5**.

26. Beylot, M., Large, V., Letexier, D., Peroni, O and Ray, H. (2004). Metabolism of lipids in human white adipocyte. Diabetes Metabolism **30**(4), 294-309.

27. Bhandari, U., Kumar, P and Jamadagni, S. (2014). Fenugreek seed extract inhibits fat accumulation and ameliorates dyslipidemia in high fat diet-induced obese rats.

28. Bhat, M. K., Chhipa, R. R., Singh, S and Vijayakumar, M. V. (2005). The hypoglycaemic activity of fenugreek seed extract is mediated through the stimulation of an insulin signalling pathway. British Journal of Pharmacology **146**, 41–48.

29. Bhat, M. K., Mishra, G. C., Pandey, V and Vijayakumar, M. V. (2010). Hypolipidemic Effect of Fenugreek Seeds Is Mediated Through Inhibition of Fat Accumulation and Upregulation of LDL Receptor. Obesity **18**, 667–674.

30. Bhattacharya, S., Dey, D and Roy, S. S. (2007). Molecular mechanism of insulin resistance. J Bioscience **32**(2), 405-413.

31. Bolder, U., Jauch, K., Jeschke, M. J., Klein, D., Schlitt, H., Thasler, W. E and Weiss, T. (2008). Insulin Decreases Inflammatory Signal Transcription Factor Expression in Primary Human Liver Cells after LPS Challenge. Mol Medicine **14** (1-2).

32. Bordia, A., Verma, S. K., Srivastava, K. C. (1997). Effect of ginger (Zingiber officinale Rosc.) and fenugreek (Trigonella foenumgraecum L.) on blood lipids, blood sugar and platelet aggregation in patients with coronary artery disease. Prostaglandins Leukot Essent Fatty Acids **56**:379-384.

33. Breil, V. (2004). The insulinotrophic agent ID1101 (4-hydroxyisoleucine) activates insulin signaling in rats.

34. Briskin, D. (2000). Medicinal plants and Phytomedicines. Linking plant biochemistry and physiology to human health. Plant Physiology **124**: 507-514.

35. Broca, C., Gross, R., Le Manteghetti, M., Gomis, R., Masiello, P., Tournire, M., Petit, P., Sauvaire, Y and Ribes, G. (1999). 4-Hydroxyisoleucine: experimental evidence of its insulinotropic and antidiabetic properties. American Physiological Society.

36. Buhler, L., Deng, S., Gjinovci, A., Genin, B., lynedjian, P., Marie, S., Morel, P and Mentha, G. (1995). Glucokinase and Cytosolic Phosphoenolpyruvate Carboxykinase (GTP) in the Human Liver . Regulation of Gene Expression in Cultured Hepatocytes. **95**.

37. Cárdenas, M. L. (1995). Glucokinase: its regulation and role in liver metabolism.

38. Carlson, C. J., Koterski, S., Poccard, G. B., Sciotti, R and Rondinone, C. M. (2003). Enhanced Basal Activation of Mitogen-Activated Protein Kinases in Adipocytes From Type 2 Diabetes. Potential Role of p38 in the Downregulation of GLUT4 Expression. Diabetes **52**.

39. Caro, J. F., Sinha, M. K., Raju, S. M., Ittoop, O., Pories, W. J., Flickinger, E. G., Dohm, G. L. (1987). Insulin receptor kinase in human skeletal muscle from obese subjects with and without noninsulin dependent diabetes. J Clin Invest **79**(5).

40. Causevic-Ramosevac, A. (2013). International Journal of Traditional and Herbal Medicine. **1** (6), 159-167.

41. Cheatham, B and Kahn, S. R. (2005). Insulin action and the insulin signaling network. **16**:2, 117 – 142.

42. Chempakam, B., Parthasarathy, V and Zachariah, T. J. (2008). Chemistry of spices. Institute of spices research, Cailcut, Kerala, India **244**.

43. Chiba, H., Hayashi, Y., Kasono, K., Muraki, E and Tsunoda, N. (2011). Dose-dependent effects, safety and tolerability of fenugreek in diet-induced metabolic disorders in rats. Lipids in Health and Disease **0**:240.

44. Cignarelli, A., Cuscito, M., Cignarelli, M., De Stefano, F., De Fazi, Ao, M., Giorgino, R., Giorgino, F., Laviola, L., Leonardini, A., Memeo, V., Natalicchio, A., Neri, V and Perrini, S. (2006). Insulin Signaling in Human Visceral and Subcutaneous Adipose Tissue In Vivo. Diabetes **55**.

45. Danielsson, A., Ost, A., Lystedt, E., Kjolhede, P., Gustavsson, J., Nystrom, F. H and Stralfors, P. (2005). Insulin resistance in human adipocytes occurs downstream of IRS1 after surgical cell isolation but at the level of phosphorylation of IRS1 in type 2 diabetes. FEBS J **272**(1), 141-151.

46. Decker, K. (1990). Biologically active products of stimulated liver macrophages (Kupffer cells).

47. DeFronzo, R. A. Tobin, J. D and Andres, R. (1979). Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiology **237**(3), E214-223.

48. de Souza, R. J., Neelakantan, N., Narayanan, M and van Dam, R. M. (2014). Effect of fenugreek (Trigonella foenum-graecum L.) intake on glycemia: a meta-analysis of clinical trials. Nutr Journal 1475-2891.

49. Desvergne, B., Michalik, L and Wahli, W. (2006). Transcriptional regulation of metabolism. Physiol Rev **86**: 465-514.

50. Doble, M and Prabhakar, P. K. (2011). Mechanism of action of natural products used in the treatment of diabetes mellitus. Chinese Journal of Integrative Medicine 17**,** Issue 8, pp 563-57.

51. Enjoji, M., Fujino, T., Higucchi, N., Harada, N., Kohjima, M., Kato, M., Kotoh, K., Nakamuta, M., Takayanagi, R., Yoshimoto, T., Yada, M and Yada. R. (2008). SREBP-1c, regulated by the insulin and AMPK signaling pathways, plays a role in nonalcoholic fatty liver disease. International journal of molecular medicine **21**: 507-511.

52. Felig, P. (1975). Amino acid metabolism in man. Annual Rev Biochemistry **44**, 933-955.

53. Fowden, L., Pratt, H. M., Smith, A. (1973). 4-Hydroxyisoleucine from seed of Trigonella foenum-graecum. Phytochemistry **12**: 1707-1711.

54. Francini, F., Gagliardino, J. J and Massa, M. L. (2011). Liver Glucokinase: An Overview on the Regulatory Mechanisms of its Activity. **63**(1):1–6.

55. Garg, A and Simha, V. (2006). Lipodystrophy: lessons in lipid and energy metabolism. Curr Opin Lipidol 17(2), 162-169.

56. Greyber, E., Pasternak, G.A., Salpeter, E. E and Salpeter, S. R. (2010). Risk of fatal and nonfatal lactic acidosis with metformin use in type 2 diabetes mellitus (Review). Cochrane review, **4**.

57. Guillou, H. 1., Martin, P. G., and Pineau, T. (2008). Transcriptional regulation of hepatic fatty acid metabolism. **49**:3-47.

58. Gupta, D. D., Khosla, P and Nagpal, R. K. (1995). Effect of *Trigonella foenum graecum* (fenugreek) on blood glucose in normal and diabetic rats. Indian J Physiology Pharmacology **39**(2): 173-174.

59. Hazara, D. K., Maheshwari, B. B., Maheshwari, P. K., Mishra, B., Sarkar, A., Sharma, S. K., Sharma, R. D and Singh, J. B. (1996). Use of Fenuqreek seed powder in the management of non-insulin dependent diabetes mellitus.

60. Hazra, D. K., Sarkar, A and Sharma, R. D. (1996). Toxicological evaluation of fenugreek seeds: a long term feeding experiment in diabetic patients. Phytother Res **10**:519-520.

61. Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C. Z., Uysal, K. T., Maeda, K., Hotamisligil, G. S. (2002). A central role for JNK in obesity and insulin resistance. Nature **420**(6913), 333-336.

62. Horton, J. D., Goldstein, J. L and Brown, M. S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest **109**(9), 1125-1131.

63. Hotamisligil, G.S. (2010). Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell **140**(6), 900-917.

64. Houghton, P and Raman, A. (1998). Laboratory Handbook for the Fractionation of Natural Extracts.

65. Huie, C. W. (2002). A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. Analytical and Bioanalytical Chemistry **373**, 23-30.

66. Jennings, A., Ward, R and Wilson, R. (1989). Symptomatic hypoglycemia in NIDDM patients treated with oral hypoglycemic agents. Diabetes Care **12**: 203–208

67. Johnston, E., Pirola, L and Van Obberghen, A. M. (2004). Modulation of insulin action. Diabetologia **47**:170–184.

68. Jungermann, K and Keitzmann, T. (1996). Zonation of Parenchymal and Nonparenchymal Metabolism in Liver. Annual Review of Nutrition **16**: 179-203.

69. Kahn, C. R and Salhel, A. R. (2001). Insulin signaling and the regulation of glucose and lipid metabolism. Nature **44**.

70. Kar, A., Panda, S and Tahiliani, P. (1999). Inhibition of triiodothyronine production by fenugreek seed extract in mice and rats. Pharmacol Res **40**:405-409.

71. Kavishik, G., Khandelwal, R. K., Naik, S. N and Satya, S. (2010). Commonly consumed Indian plant food materials in the management of diabetes mellitus. Diabetes and metabolic syndrome: clinical research and reviews **4:** 21 – 40.

72. Kim, Y. B., Nikoulina, S. E., Ciaraldi, T. P., Henry, R. R and Kahn, B. B. (1999). Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. J Clin Invest **104**(6), 733-741.

73. Laffel, L. (1999). Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. Diabetes Metabolism Res Rev **15**(6), 412-426.

74. Lawlor, M. A and Alessi, D. R. (2001). PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? J Cell Sci **114**(16), 2903-291.

75. Leng, Y., Karlsson, H. K and Zierath, J. R. (2004). Insulin signaling defects in type 2 diabetes. Rev Endocrine Metabolism Disorder **5**(2), 111-117.

76. Li, G., Barrett, E. J., Wang, H., Chai, W and Liu, Z. (2005). Insulin at physiological concentrations selectively activates insulin but not insulin-like growth factor I (IGF-I) or insulin/IGF-I hybrid receptors in endothelial cells. Endocrinology 146(11), 4690-4696.

77. Livak, K and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method. Methods **25**, 402-408.

78. Louard, R. J., Fryburg, D. A., Gelfand, R.A and Barrett, E. J. (1992). Insulin sensitivity of protein and glucose metabolism in human forearm skeletal muscle. J Clin Invest **90**(6), 2348-2354.

79. Matschinsky, F. M and Sweet, I. R. (1996). Annotated questions about glucose metabolism and insulin secretion of beta-cells. Diabetes Rev **4**, 130–144.

80. Meyer, M. M., Levin, K., Grimmsmann, T., Beck-Nielsen, H and Klein, H. H. (2002). Insulin signalling in skeletal muscle of subjects with or without Type II-diabetes and first degree relatives of patients with the disease. Diabetologia **45**(6), 813-822.

81. Morino, K., Petersen, K. F and Shulman, G. I. (2006). Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction. Diabetes **55** Suppl 2, S9-S15.

82. Muralidhara, Narasimhamurthy, K., Ramesh, B. S and Viswanatha, S. (1999). Acute and subchronic toxicity assessment of debitterized fenugreek powder in the mouse and rat. Food Chem Toxicology **37**:831-838.

83. Mwaniki, C. G., Mwendia, C. M and Ngoci, S. N. (2011). Phytochemical and cytotoxicity testing of Indigofera lupatana. Baker F.J Anim. Plant Science **11**: 1364-1373.

84. Nepomuceno, D. C., Paciencia, M. L. B., Suffredini, I. B., Varella, A. D and Younes, R. N. (2006). Antibacterial and cytotoxic activity of Brazilian plant extracts Clusiaceae. Mem Inst Oswaldo Cruz **101**: 287-290.

85. Nolan, J. J, Freidenberg, G., Henry, R.., Reichart, D and Olefsky, J. M. (1994). Role of human skeletal muscle insulin receptor kinase in the in vivo insulin resistance of noninsulin-dependent diabetes mellitus and obesity. J Clin Endocrinol Metabolism 78(2), 471-477.

86. Pandey, S. K and Srivastava, A. K. (1998). Potential mechanism(s) involved in the regulation of glycogen synthesis by insulin. Mol Cell Biochemistry **182**(1-2), 135-141.

87. Pearson, E. R., Rena, G and Sakamoto, K. (2013). Molecular mechanism of action of metformin: old or new insights? Diabetologia **56**:1898–1906.

88. Pessin, J. E and Saltiel, A. R. (2006). Signaling pathways in insulin action: molecular targets of insulin resistance. J Clin Invest **106**(2), 165-169.

89. Prakash, V and Sandhu, P. (2012). In vitro antimycobioticand antibacterial action of seed extract of Celastruspaniculatus Willd. (Jyotismati). Journal of Antimicrobials. Photon **127** (2012) 123-132.

90. Quinn, D., Shirai, K and Jackson, R. L. (1983). Lipoprotein lipase: mechanism of action and role in lipoprotein metabolism. Prog Lipid Res **22**(1), 35-78.

91. Randle, P. J. (1963). Endocrine control of metabolism. Annu Rev Physiology **25** 291-324.

92. Rao, P. S., Rao, P. U and Sesikeran, B. (1996). Short term nutritional and safety evaluation of fenugreek. Nutr Res **16**:1495-1505.

93. Reddy, J. K and Rao, M. S. (2005). Lipid Metabolism and Liver Inflammation. II. Fatty liver disease and fatty acid oxidation. American Journal of Physiology - Gastrointestinal and Liver Physiology **290**.

94. Samuel, V. T, Petersen, K. F and Shulman, G. I. (2010). Lipid-induced insulin resistance: unraveling the mechanism. Lancet **375**(9733), 2267-2277.

95. Savage, D. B., Petersen, K. F and Shulman, G.I. (2005). Mechanisms of insulin resistance in humans and possible links with inflammation. Hypertension. **45**(5), 828-833.

96. Savage, D. B., Petersen, K. F and Shulman, G.I. (2007). Disordered lipid metabolism and the pathogenesis of insulin resistance. Physiol Rev **87**(2), 507-520.

97. Sharma, R. D., Raghuram, T. C., Rao, N. S. (1990). Effect of fenugreek seeds on blood glucose and serum lipids in type I diabetes. Eur J Clin Nutritional **44**:301-306.

98. Sharma, R. D and Raghuram, T. C. (1990). Hypoglycaemic effect of fenugreek seeds in non-insulin dependent diabetic subjects. Nutr Res **10**:731-739.

99. Shulman, G. I. (2000). Cellular mechanisms of insulin resistance. J Clin Invest **106**(2), 171-176.

100. Sowmya, P and Rajyalakshmi, P. (1999). Hypocholesterolemic effect of germinated fenugreek seeds in human subjects. Plant Foods Human Nutritional **53**:359-365.

101. Tran, L. T. H. (2003). Fenugreek.

102. Tremblay, F., Lavigne, C., Jacques, H and Marette, A. (2007). Role of dietary proteins and amino acids in the pathogenesis of insulin resistance. Annu Rev Nutr **27**, 293-310.

103. Viollet, B and Foretz ,M. (2013). Revisiting the mechanisms of metformin action in the liver. **74**(2):123-9.

104. WHO, world health statistics, 2008.

105. WHO, world health statistics, 2012.

APPENDIX A

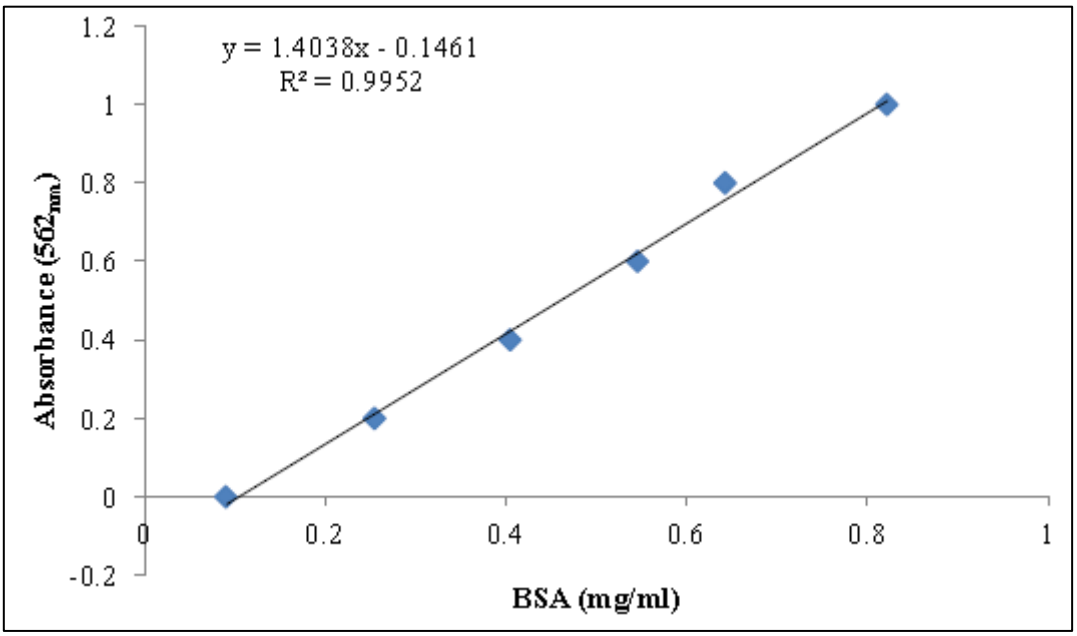

Figure 1: Calibration curve using known concentrations of BSA for the determination of protein concentration in samples using BCA assay.