School of Pharmacy

Molecular Signaling Pathways Involved in the Glucose-Lowering Effect of *Teucrium polium*

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made. This thesis contains no material which has been accepted for an award of any other degree or diploma in any university.

Signature: ……
Adnan Mannan

Date: 16th May 2017

Acknowledgement

'As we express gratitude, we must never forget that highest appreciation is not to utter words, but to live by them' – John F. Kennedy.

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Publications arising from this thesis

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- IPRS (International Postgraduate Research Scholarship) and Australian Postgraduate Award for PhD study.

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LIST OF ABBREVIATIONS

Abbreviation	Full name
2NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose
2-DG	2-deoxyglucose
ADP	Adenosine diphosphate
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
APH	Acid phosphatase
APS	Ammonium Persulfate
BCA	Bicinchoninic Acid
BK channel	Big Potassium channel
BSA	Bovine serum albumin
CDK1/2	Cyclin-dependent kinase1/2
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECAR	Extracellular acidification rate
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone
FSCA	Forward scatter (Area)
FSCH	Forward scatter (Height)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GRP	Gastrin-releasing peptide
GSK3β	Glycogen synthase kinase 3 beta

Abbreviation	Full name
GSK233447	(3S,6R)-1-[6-(3-Amino-1H-indazol-6-yl)-2-(methylamino)-4- pyrimidinyl]-N-cyclohexyl-6-methyl-3-piperidinecarboxamide
GYS1	Glycogen synthase 1
GYS2	Glycogen synthase 2
H_2O_2	Hydrogen peroxide
HBSS	Hanks' Balanced Salt Solution
HCl	Hydrogen chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1α	Hypoxia inducible factor 1α
IBMX	3-isobutyl-1-methylxanthine
KRB	Krebs Ringer Bicarbonate
K_m	Michaelis-Menten constant
MAPK	Mitogen-activated protein kinase
mM	Millimoles per litre
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
mTOR	Mammalian Target of Rapamycin
OCR	Oxygen consumption rate
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDK-1	Phosphoinositide-dependent protein kinase-1
Pdx1	Pancreatic and duodenal homeobox 1
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein kinase C
pNPP	p-Nitrophenyl phosphate
PTEN	Phosphatase and tension homologue
RIPA	Radioimmunoprecipitation assay
RPMI	Roswell Park Memorial Institute
RSK	p90 ribosomal S6 kinase

Abbreviation	Full name
S6K	S6 kinase
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SOS	Son of sevenless homolog
TBST	Tris buffered saline (TBS) solution with Tween® 20
TEMED	Tetramethylethylenediamine
Tp	*Teucrium polium*
ver	Version
v/v	Volume/volume
Vmax	Maximum velocity
w/v	weight/volume

ABSTRACT

Teucrium polium is a herb that grows in the Mediterranean region and is widely used by the locals in the treatment of a number of ailments including cancer, inflammation, bacterial infection and diabetes. The glucose lowering effect of *Teucrium polium in vivo* has been reported previously from our laboratory (Ireng *et al.*, 2016) and other groups (Esmaeili & Yazdanparast, 2004; Stefkov *et al.*, 2011; Kasabri *et al.*, 2012). The aims of the current study are to investigate whether the constituents of the *Teucrium polium* extract i) lowers blood glucose levels by inducing the release of insulin from pancreatic beta cells *in vitro*, ii) act as an insulin mimetic agent on fat and muscle cells *in vitro* and iii) elucidate the molecular mechanism(s) leading to the demonstrated effect(s).

The constituents of the *Teucrium polium* extract promoted up to a 2.2-fold increase ($p < 0.01$) in insulin secretion in a dose-dependent manner from BRIN-BD11 pancreatic beta cells within 30 minutes. This effect of the *Teucrium polium* extract in promoting insulin secretion was a response exceeding that of glucose alone. The release of insulin from the BRIN-BD11 cells following treatment with the *Teucrium polium* extract could not be explained by some toxic effect of the extract on the cells. Furthermore, the BRIN-BD11 cells used in this study responded as would be expected by secreting insulin in the presence of gliclazide and other known stimuli such as glucose, alanine and potassium chloride.

Glucose uptake in BRIN-BD11 cells treated with the *Teucrium polium* extract increased by about 1.4-fold in a dose-dependent manner within 30 minutes ($p < 0.001$). This effect persisted for up to 24 hours. The increased glucose transport into BRIN-BD11 cells was accompanied by a 1.5-fold increase in the expression of GLUT2 ($p < 0.01$) and with a 1.7-fold increase in the expression of glucokinase ($p < 0.01$) in the cells. This is an important observation as GLUT2 and glucokinase constitute the glucose sensor in beta cells and control the rate limiting step of insulin secretion. As would be expected following increased glucose uptake and commitment to glycolysis by glucokinase, it was observed using a Seahorse XF glycolysis stress test that the *Teucrium polium* extract promoted both the glycolytic processing and mitochondrial metabolism of glucose in BRIN-BD11 cells in 30 minutes. A higher glycolytic rate (1.4-fold, $p < 0.01$), glycolytic capacity (1.3-fold, $p < 0.05$) and increases in basal

respiration (2.1-fold, $p < 0.05$) and respiratory capacity (1.9-fold, $p < 0.05$) were observed in BRIN-BD11 cells when treated with the *Teucrium polium* extract. These observations coincided with a 1.4-fold increase in ATP production ($p < 0.01$) and a 2-fold increase in intracellular calcium influx into BRIN-BD11 treated with the *Teucrium polium* extract. Thus, component(s) within the *Teucrium polium* extract promoted insulin secretion from the BRIN-BD11 cells in accordance with the well-established biochemical pathway for insulin release from pancreatic beta cells.

The *Teucrium polium* extract increased glucose uptake in a concentration dependent manner with an apparent efficacy similar to a maximal dose of insulin in 3T3-L1 adipocytes (1.5-fold, $p < 0.05$), differentiated C2C12 muscle cells (1.4-fold, $p < 0.01$) and differentiated L6 muscle cells (1.6-fold, $p < 0.01$). The ability of the *Teucrium polium* extract to promote glucose uptake in differentiated L6 muscle cells coincided with a 3 to 4-fold increase in the expression of the insulin-regulated glucose transporter, GLUT4 ($p < 0.01$). The glycogen content of the differentiated L6 muscle cells increased about 1.6-fold ($p < 0.01$) in the presence of either insulin or *Teucrium polium* extract.

The effect of the *Teucrium polium* extract in promoting glucose uptake and glycogen synthesis in insulin sensitive cells muscle and fat cells correlated with an increased phosphorylation of several key phosphokinases, including 3-phosphoinositide-dependent protein kinase-1 (PDK-1), protein kinase B (Akt), mammalian target of rapamycin (mTOR), glycogen synthase kinase 3α/β (GSK3α/β), ribosomal protein S6 kinase and extracellular signal–regulated kinase 1/2 (Erk1/2) that are involved in insulin-mediated signalling pathways. These findings are consistent with component(s) within the *Teucrium polium* extract promoting both the PI3K/Akt and the Grb2-SOS-Ras-MAPK pathways that are involved in insulin action.

GSK2334470, a highly specific and potent inhibitor of PDK-1, inhibited the phosphorylation of the ribosomal S6 protein in differentiated L6 muscle cells and also completely ablated the stimulatory effect of the *Teucrium polium* extract in promoting the phosphorylation of this S6 protein to levels indistinguishable to the phosphorylation levels of S6 in the presence of the inhibitor alone. Given that multiple events measured downstream of PDK-1 were promoted in L6 muscle cells treated with the *Teucrium polium* extract, covering both the PI3K/Akt and Grb2-SOS-Ras-MAPK

pathways of insulin action, it seems most likely that component(s) within the extract target either PDK-1 and/or at a step(s) upstream of PDK-1 within the P13K/Akt pathway; this includes, but is not limited to, phosphatidylinositol-4,5-bisphosphate 3-kinase (P13K), the insulin receptor substrates (IRS), the insulin receptor and or various transcriptional and other factors involved in the regulation of their synthesis. A more comprehensive study is now required to identify the actual molecular target(s) conveying the insulin mimetic properties of the *Teucrium polium* extract.

In conclusion, the results from this thesis suggest that, the *Teucrium polium* extract has both insulin secretagogue and insulin mimetic properties. The finding that component(s) within the *Teucrium polium* extract promote the expression of both glucokinase and GLUT2 in beta cells is particularly noteworthy as the activities constitute the glucose sensor of pancreatic beta cells and have a high impact on glucose homeostasis, which is impaired in type 2 diabetes. The *Teucrium polium* extract might thereby prove to be an impactful target for future drug discovery efforts for the treatment of type 2 diabetes and related disorders.

1.0 Introduction

1.1 Diabetes mellitus

Diabetes mellitus presents as a metabolic disorder characterized by high blood glucose levels (hyperglycaemia), of fasting plasma glucose more than 7 mM (126 mg/dL) and plasma glucose more than 11mM (198 mg/dL), 2 hours after an oral glucose tolerance test (American-Diabetes-Association, 2015). This hyperglycaemia is caused by defects in insulin secretion, insulin action, or both (Tuomilehto *et al.*, 2001; Forbes & Cooper, 2013; Ghazarian *et al.*, 2013; Newsholme *et al.*, 2014). There are three main types of diabetes mellitus: Type 1, which is due to no or very little insulin release from pancreatic beta cells; Type 2, which is a result of lack of insulin production capacity or insulin resistance being developed by insulin sensitive cells; and gestational diabetes, which can occur during pregnancy (Klöppel *et al.*, 1985; Gavin III *et al.*, 1997; Kubo *et al.*, 2016; Mayer-Davis *et al.*, 2017).

Type -1 diabetes occurs more commonly in childhood whereas type-2 diabetes is much more common in people over 30 years of age (Patterson *et al.*, 2009). Type-1 diabetes is more frequently associated with higher ketone levels and type-2 diabetes might lead to high blood pressure or cholesterol due to obesity (Sabatier *et al.*, 2002). Insulin is the only mainstay treatment for type-1 diabetes whereas there are several oral medications for type-2 diabetes (Vilsbøll *et al.*, 2003; Cnop *et al.*, 2005).

Hyperglycaemia negatively affects a large number of organs and tissues and may lead to various complications. Hyperglycaemia increases the glycation of proteins in the blood, particularly haemoglobin. Glycation is irreversible, increasing with increased blood glucose levels, which affects the functionality of haemoglobin interfering with the level of oxygen transported to the different tissues and organs. Thus complications arise which include retinopathy, cataract, diabetic glomerulosclerosis, mononeuropathies, distal symmetric polyneuropathy, and autonomic neuropathy (Stratton *et al.*, 2001; Sumner *et al.*, 2003; Wang *et al.*, 2005; Pratt *et al.*, 2016; Pop-Busui *et al.*, 2017). However, the most common complication by far is vascular disease which can result in illnesses distressing the larger arteries that supply the heart (coronary artery disease and heart failure), brain (stroke) and the legs (gangrene and amputations) (McKittrick *et al.*, 1949; Jorgensen *et al.*, 1994; Yagi *et al.*, 1997; Armstrong *et al.*, 2017; Rao & Reddy, 2017). These macrovascular diseases are responsible for 70% of the deaths of people with diabetes. Moreover, small vessel

disease or micro-angiopathy can lead to diabetic retinopathy, neuropathy and nephropathy (Conard, 1967; Savage *et al.*, 1996; Stratton *et al.*, 2000). About 40% of all diabetic patients are affected by diabetic nephropathy. Clinical features and symptoms of diabetes mellitus include elevated blood pressure, higher urinary albumin excretion (300 mg/day) and a persistent decline in renal function (Adler *et al.*, 2000; Stehouwer *et al.*, 2002; Ku *et al.*, 2016; Moriya *et al.*, 2017).

Various hypotheses have been formulated in an attempt to explain the role of hyperglycaemia in the development of diabetes-associated complications. The exact mechanism by which hyperglycaemia causes damage to individual tissues is unknown. However, some underlying initiators have been identified and these include increased activity of the polyol pathway, disturbance in the metabolism of *myo*-inositol and its phospholipid derivatives, abnormal permeability of the small blood vessels, and excessive glycosylation of various protein (Landau & Davis, 1960; Greene & Lattimer, 1984; Asnaghi *et al.*, 2003; Prnova *et al.*, 2015; Jansen *et al.*, 2016; Santamaria *et al.*, 2016). Certainly glycosylated haemoglobin is a reliable predictor of retinopathy (Bunn *et al.*, 1978; Snir *et al.*, 2016). Most mechanisms appear to have similar elements such as increased reactive oxygen species, the inhibition of glycolytic intermediates and decreased mitochondrial biogenesis (Parmeggiani & Bowman, 1963; Laursen *et al.*, 1997; Shah & Brownlee, 2016; Hosokawa *et al.*, 2017).

1.2 Global burden of diabetes mellitus

Diabetes mellitus is increasing in an alarming rate worldwide (Dagenais *et al.*, 2016; Menke *et al.*, 2016; WHO, 2016). Diabetes is currently the fifth most common cause of death in the world (IDF, 2015; WHO, 2016). The number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014 and these numbers are expected to rise dramatically in the future, especially in countries such as China and India as their people adopt Western like lifestyle (WHO, 2016). The global prevalence of diabetes among adults over 18 years of age has risen from 4.7% in 1980 to 8.5% in 2014. If this trend continues, the number of diabetic patients will increase to 592 million by 2035 (WHO, 2016; Diabetes-Australia, 2017a). In Australia alone, 1.7 million Australians are suffering from diabetes and about 100,000 people are diagnosed with diabetes each year (2017b) and diabetes is the 6th leading cause of death. According to recent projections, 3.3 million people in Australia will be living

with diabetes by 2031 (Diabetes-Australia, 2017b). Globally, it has been estimated that the prevalence of diabetes is 8.3% (Atlas, 2015; Da Rocha Fernandes *et al.*, 2016). The International Diabetes Federation currently states that the top 5 countries with the highest amount of people with diabetes are China (109-millions), India (69 millions), USA (29 millions), Brazil (14 millions) and Russia (12 millions) (IDF, 2015; Mendis *et al.*, 2015). All types of diabetes are increasing in prevalence especially type 1 and type 2 which accounts for 10% and 85% of all types respectively (Diabetes in Australia, 2017). Type 2 is increasing at the fastest rate. More than 2 million Australians are at high risk of developing type 2 diabetes (2017b). In addition to these two common types of diabetes, the rate of other type occurring, i.e. gestational diabetes is also increasing (Harding et al., 2014).

It is believed that more than two thirds of people with diabetes are undiagnosed in Africa (IDF, 2015). Although there is progress in diabetes research and therapeutic development, the cost for diabetes treatment is increasing every year. Diabetes costs 10.8% of annual health expenditure worldwide which amounts to 548 billion US dollars. It is projected to rise to $647 billion dollars by 2035 (IDF, 2013). Eighty percent of the people suffering from diabetes are from lower and middle-income countries. In Australia, expenditure for diabetes was AUD $8.11 billion in 2001, AUD $11.57 billion in 2009 and it rose to AUD $14.6 billion in 2010 (Lee *et al.*, 2013).

1.3 Type-1 Diabetes mellitus

Type 1 diabetes is the most severe form of diabetes. Although type 1 diabetes accounts for only 5–10% of all those with diabetes, it remains a serious chronic disorder with important short-term and long-term consequences (Borchers *et al.*, 2010; Stankov *et al.*, 2013). Type-1 Diabetes is generally thought to be due to direct immune-mediated destruction of insulin-producing pancreatic beta cells. In the past, type-1 diabetes was considered as a disorder in children and adolescents, but recent studies have changed this opinion and age is no longer a clear predicting factor. Signs and symptoms of this disease include, along with overt hyperglycaemia remains as the diagnostic hallmark in children and adolescents, and to a lesser extent in adults (Mallare *et al.*, 2003; Roche *et al.*, 2005; Amritanshu *et al.*, 2017). The cause of type-1 diabetes is still unknown; however there appears to be a strong hereditary risk (Atkinson *et al.*, 2014; De Ferranti *et al.*, 2014). An instant need for exogenous insulin replacement is also a hallmark of

type 1 diabetes, for which lifetime treatment is needed.

In the case of type-1 diabetes, two key genes have shown association. The key one is human leukocyte antigen, which codes for the major histocompatibility complex genes, plays an important role in antigen presentation (Lie *et al.*, 1999; Kuric *et al.*, 2017). The second genetic region involved in diabetes susceptibility is the insulin region (Bain *et al.*, 1992; Spielman *et al.*, 1993; Groop & Pociot, 2014). These two genes are most commonly responsible for type-1 diabetes. In addition, several mutations can also cause type-1 diabetes. Several genetic markers confer increased risk for type-1 diabetes, which include HLA DR3, HLA DQA1, HLA DR2, HLA DQB1 and HLA DR4 (Bowden *et al.*, 1997; Schulze *et al.*, 2009; Ali, 2013; Marvel *et al.*, 2017).

The environment also plays a major role in the development of diabetes (Rewers & Ludvigsson, 2016). In the case of type-1 diabetes, it has been identified that the combination of some environmental factors along with the genetic factors predispose or worsen the disease symptoms (Åkerblom *et al.*, 2002). One of the key environmental factors is unhealthy food consumption and some specific proteins such as exposure to foods like cow's milk protein or a dietary antigen similar to gluten in celiac disease (Åkerblom *et al.*, 2002). Viral infection is another factor that is believed to play a role in triggering type-1 diabetes. There are a number of viruses shown to be associated with type 1 diabetes related antibodies, i.e. enterovirus, rubella, mumps, rotavirus, and cytomegalovirus (Banatvala *et al.*, 1985; Dahlquist *et al.*, 1995; Al-Hakami *et al.*, 2016; Oikarinen, 2016; Onal *et al.*, 2016). In addition, there is a large body of evidence pointing towards the important role of Toll like receptor (TLR) activation and vitamin D deficiency in type 1 diabetes mellitus pathogenesis. It seems that TLRs exert their influence on the development of type 1 diabetes through the modulation of immune responses following β-cell destruction as well as to triggering factors, such as enteroviruses (Adamczak *et al.*, 2014). Studies performed with mouse models of type 1 diabetes indicate that TLR1, TLR2, TLR3 and TLR17, are involved in disease development mechanisms (Dasu *et al.*, 2010; Burrows *et al.*, 2015; Pahwa *et al.*, 2016). Upregulation of TLR pathways appeared to reduce the risk of type-1 diabetes development (Mudaliar *et al.*, 2014). Nonetheless, major challenges remain in the development of approaches to the prevention and management of type-1 Diabetes and its complications (Ratner & Kaufman, 2003; Inzucchi & Sherwin, 2011).

1.4 Type-2 Diabetes mellitus

Type-2 diabetes is the most common type of diabetes mellitus and the recent surge in the number of people diagnosed with type-2 diabetes reflects the progressive increase of the diabetic epidemic. Numbers of type-2 diabetes patients have doubled over the last 20 years (WHO, 2016).

Type-2 diabetes is a metabolic disorder where insulin secretion and/or insulin action are impaired (Olokoba *et al.*, 2012). An elevated level of hepatic glucose production is observed with this condition during fasting hyperglycaemia. Type-2 diabetes has various metabolic features which include: defects in insulin action, abnormality in insulin secretion and a rise in endogenous glucose output (Weir & Kahn, 1994; Bogardus, 1996; Chan *et al.*, 2012). Type-2 diabetes is demonstrated by a progressive decline of glucose tolerance over several years. In the early stage, insulin action faults occur which are initially compensated for by increased insulin secretion, then over time the beta cells fail to adequately secrete insulin to meet its demands (Levy *et al.*, 1998; Dugani *et al.*, 2016). Initially type-2 diabetes patients need oral monotherapy but later they need other multiple oral agents and eventually they may become insulin dependent (Turner *et al.*, 1999; Calvert *et al.*, 2007; Miya *et al.*, 2017). Type-2 diabetes patients exhibit resistance to insulin in adipose tissue (Hotta *et al.*, 2001; Miya *et al.*, 2017) and muscle (Esser *et al.*, 2014). It can happen due to inactivity of insulin receptors and defects in their structure, abnormalities in insulin signalling pathway and glucose transportation (DeFronzo, 1999; Floegel *et al.*, 2013). It has been revealed that first-degree relatives have a risk of 20-40 % of developing type-2 diabetes (Ali, 2013). On the other hand, in the case of type-2 diabetes, association of several genes have been found including TCF7L2, ABCC8, CAPN10, GLUT2 and GCGR. These genes are associated with insulin secretion, regulation, glucose production and transport (Thorens *et al.*, 1992; Hanis *et al.*, 1996; Florez *et al.*, 2007; Madubedube, 2015).

The World Health Organization (WHO) states some of the high risk factors of developing type-2 diabetes as: sedentary lifestyle, high calorie intake: (Li *et al.*, 2008; Eaton & Eaton, 2017), smoking, ethnicity, lifestyle, age, hypertension and excessive alcohol consumption (Koppes *et al.*, 2005; Qin *et al.*, 2013).

According to the Centres for Disease Control and Prevention, 1 out of 3 adults are experiencing pre-diabetes; however, 9 out of 10 of those individuals are not aware of the fact that they have pre-diabetes (cdc.gov, 2014). Approximately 1 in 10 adults that are more than 20 years old are living with type 2 diabetes. Moreover, in case of people more than 65 years of age, the rate becomes 1 in 4 people (cdc.gov, 2014).

1.5 Glucose homeostasis

Glucose plays a key role in metabolism and energy production (Greiner *et al.*, 1994; Sibson *et al.*, 1998). Glucose is regulated in the body by several glucoregulatory hormones (such as insulin, glucagon, amylin, glucose-dependent insulinotropic polypeptide, epinephrine, cortisol, and growth hormone) (Armstrong *et al.*, 1985; Hjalmarsen *et al.*, 1996). Briefly, these regulators work at different levels in the body. The different hormones help control the level of entry of glucose from the blood to various cells during food intake or, starvation (Cooper *et al.*, 1988; Cherrington, 1999). The liver cell produces glucose by the breakdown of glycogen during the fasting state (i.e. when insulin: glucagon ratio drops). This glucose is further utilized by insulin independent cells such as smooth muscles, brain and red blood cell (Aronoff *et al.*, 2004; Smith & Steinberg, 2017). As blood glucose levels rise the insulin secretion is promoted. This insulin further accelerates glucose uptake, transportation and metabolism by insulin sensitive cells (Berger *et al.*, 1975; DeFronzo *et al.*, 1985; Pilkis & Granner, 1992).

Glucose works by a sequence of metabolic pathways and steps. Entry of glucose into the cell is facilitated by glucose transporters of the plasma membrane depending on its concentration gradient. Then glucose enters the glycolytic pathway where hexokinase (glucokinase in beta cells) phosphorylates glucose to glucose-6-phosphate which is a rate limiting step and initiator of glycolysis (Gloyn, 2003). Glycolysis results in the production of NADPH, ATP and pyruvate. In the presence of oxygen, pyruvate is converted to acetyl-CoA which enters the tricarboxylic acid (TCA) cycle in mitochondria. The NADH and $FADH_2$ generated by the TCA cycle will feed into the electron transport chain. All these pathways generate ATP (Pelicano *et al.*, 2006). But if the oxygen is limited, pyruvate further produces lactate and glycolysis remains as

the main source of ATP. Mitochondria act as the major organelles for ATP production (Mogensen *et al.*, 2007). Alteration or abnormality in glucose metabolism can lead to disturbances in mitochondrial metabolism(Mookerjee *et al.*, 2015; Sica *et al.*, 2017).

Glucose levels in the blood is a result of: gluconeogenesis (mainly by the liver), absorption from the diets (in the intestine) and glycogenolysis (from deposits in the liver, muscle and fat cells) (Massa *et al.*, 2011; Exton *et al.*, 2013). Glucose is stored in adipose tissues as triglycerides and muscle and liver as glycogen or converted to amino acids (Shaw *et al.*, 2005; Rosen & Spiegelman, 2006). These stores can provide glycerol from triglycerides, lactate and amino acids which all can feed into the gluconeogenic pathway and thus contribute to blood glucose levels (Felig *et al.*, 1970; Cornell *et al.*, 1973; Lundholm *et al.*, 1982; Wei *et al.*, 2015).

1.5.1 Glucose uptake at the cellular level

-Glucose is transported from the circulation to the peripheral tissues of the body for further cellular activities including metabolism, storage and maintenance. This transportation occurs by integral transport proteins. There are two functionally and structurally distinct groups of transport proteins performing this activity (Baker *et al.*, 2014; Brereton *et al.*, 2014; Gilbert, 2014). These transporters are classified as 1) the sodium-dependent glucose co-transporters (SGLT) and 2) the facilitative glucose transporters (GLUT) (Yamamoto *et al.*, 1990; Wood & Trayhurn, 2003; Gilbert, 2014).

1.5.2 Sodium- glucose transporters (SGLT)

SGLTs are cotransporters that use the gradient of sodium ions across the plasma membrane to drive glucose against its concentration gradient into epithelial cells lining the luminal surface of the small intestine and the proximal tubules of the kidney (Hediger *et al.*, 1987). Three members of the SGLTs function as glucose transporters (SGLT1 & SGLT2) or glucose sensors (SGLT3) (Scheepers *et al.*, 2004). SGLT1 has a low expression level but high affinity and is found in intestinal and kidney tissues. SGLT2 has low affinity and is mostly found in the proximal cells of the kidney (Wells *et al.*, 1992; Kanai *et al.*, 1994).

1.5.3 The facilitative glucose transporters (GLUT)

There are 14 different members of the GLUT family (Fukumoto *et al.*, 1989; Bell *et al.*, 1990; Mueckler, 1994; Scheepers *et al.*, 2004; Szablewski, 2017). Although all these transporters allow the movement of glucose down its concentration gradient, they are tissue specific and are activated differently. Their activity is dependent on the distribution of protein on cellular compartments, cellular requirement and substrate supply (Bell *et al.*, 1990). GLUT1 (found in erythrocyte and human pancreatic beta cells) and GLUT3 (found in the brain) are isoforms facilitating basal glucose uptake (Simpson *et al.*, 1994; Nualart *et al.*, 1999). GLUT1 is mostly found in erythrocyte and human pancreatic beta cells (Simpson et al., 1994; Nualart et al., 1999). However, some other studies suggested that it is expressed ubiquitously in tissues but high proportions are expressed in erythrocytes and pancreatic β cells (Wertheimer *et al.*, 1991; Chandler *et al.*, 2003). It is worth noting that rodent pancreatic beta cells express GLUT2 transporters whilst human pancreatic beta cells express GLUT1 (Thorens *et al.*, 2000). The GLUT2 isoform accelerates the bidirectional glucose uptake by hepatocyte and glucose absorption in kidney and small intestine. It is also involved in the glucose sensing mechanism of pancreatic beta cells (Thorens *et al.*, 2000). GLUT4 works in response to insulin and changes subcellular localization in muscle and fat tissues (Bryant *et al.*, 2002; Klip *et al.*, 2014). GLUT5 promotes glucose consumption in small intestine and transportation in epithelial cells. GLUT6 and GLUT10 are found in brain cells. GLUT11 is located in heart and muscle. GLUT9 has not functionally been characterized (Bell *et al.*, 1990; Mueckler, 1994; Wood & Trayhurn, 2003). Most common glucose transporters and their activities have been summarized in Table 1.

GLUT2 activity is dependent on the level of glucose in the blood. It facilitates the entry of glucose into pancreatic beta cells which feeds into the glycolytic pathway to produce ATP. As a result, ATP-sensitive potassium efflux channels close, depolarising the cell causing calcium channels to open and calcium entry propagates a series of events that promoteinsulin secretion. Short and long term supply of dietary sugar influences the expression of GLUT2 (Miyamoto *et al.*, 1993; Schmitt *et al.*, 2017). GLUT2 activity is also dependent on the level of extracellular hormones (eg with stress, epinephrine), and the level of cellular energy. GLUT2 and glucokinase

Table 1: The glucose transporter (GLUT) family in rodents and human body.

Glucose transporter	Location	Function	K_m	Reference
GLUT 1	Erythrocytes, heart cells	Helps in basal glucose uptake	4.3 mM	(Kraegen *et al.*, 1993; Younes *et al.*, 1996)
GLUT 2	Liver cells, pancreatic beta cells (rodents)	Accelerates the bidirectional glucose uptake by hepatocyte and glucose absorption in kidney and small intestine. Glucose sensing mechanism of pancreatic beta cell	15 mM	(Burant & Bell, 1992; Weinstein *et al.*, 1994; De Vos *et al.*, 1995)
GLUT3	Brain (rodents), kidney	Neuronal glucose transport	1.4 mM	(Mantych *et al.*, 1992; Heilig *et al.*, 1995; Simpson *et al.*, 2008)
GLUT 4	Muscle cells, Fat cells	Response towards insulin and promote glucose uptake by muscle and fat cells.	4.6 mM	(Klip & Marette, 1992; Shepherd *et al.*, 1993; Lizunov *et al.*, 2013)
GLUT 5	Small intestine cells, brain, adipose tissue	Triggers glucose utilization in small intestine and transportation in epithelial cells.	Undefined	(Kayano *et al.*, 1990; Shepherd *et al.*, 1992)
GLUT 6-10	Brain cells, liver, kidney, heart.	Promotes fertility, L-dehydroascorbic acid transportation.	Undefined	(Wood & Trayhurn, 2003; Membrez *et al.*, 2006; Lee *et al.*, 2010a)

are considered to be part of the glucose sensor system of pancreatic beta cells. GLUT2 appears to be a unique therapeutic target for glycaemic control (Kellett & Brot-Laroche, 2005). Various mouse and rat pancreatic beta cell lines, namely BRIN-BD11 (from mouse) and MIN6 (from rat) cell lines, are widely used to study the GLUT2 receptors and their activity (McClenaghan *et al.*, 1996b; Evans *et al.*, 2009).

GLUT4 plays a leading role in glucose homeostasis. Expression of GLUT4 is specific to insulin sensitive tissues, namely adipose tissue and striated muscles (skeletal and heart). A pool of GLUT4 transporters are stored in vesicles in the cytosol which are recruited to the cell surface in response to insulin (or acutely stimulated by exercise), independently of transcription or translation (Pessin *et al.*, 1999; Huang & Czech, 2007). GLUT4 has some distinctive sequences in its N and COOH terminal cytoplasmic domains which make it capable of membrane trafficking for facilitation of glucose movement. These contain a) N-terminal sequence having a critical phenylalanine residue and b) COOH terminals containing dileucine and acidic motifs (Piper *et al.*, 1993; Araki *et al.*, 1996; Melvin *et al.*, 1999; Al-Hasani *et al.*, 2002).

Depletion of GLUT4 in adipocytes or skeletal muscles results in insulin resistance (Rossetti *et al.*, 1997; Li *et al.*, 2000). Abnormality in GLUT4 expression can lead to metabolic effects such as obesity (Olefsky *et al.*, 1988; Garvey *et al.*, 1991), thyroid hormone activity (Weinstein *et al.*, 1991; Torrance *et al.*, 1997), muscle atrophy (Didyk *et al.*, 1994; Blakemore *et al.*, 1996) and denervation (Block *et al.*, 1991; Coderre *et al.*, 1992). As GLUT4 can alter the glucose tolerance, the GLUT4 expression is considered to be a target for therapeutic intervention and can be used a biomarker in drug discovery studies (Huang & Czech, 2007). L6, 3T3L1 and C2C12 are some of the cell lines used for GLU4 expression and glucose trafficking studies (Wang *et al.*, 1999; Reed *et al.*, 2013; Yokokawa *et al.*, 2015).

1.6 Insulin

Insulin is a protein hormone produced by beta cells in the pancreas (Fu *et al.*, 2013). Beta cells are the major pancreatic islet cells in mammals and act as the main source of circulating insulin (Orci *et al.*, 1972; Rorsman & Renström, 2003). Beta cells remain as a fundamental part in islets along with other endocrine cells as glucagon secreting α-cells and somatostatin-secreting δ-cells (Rahier *et al.*, 1983). The islet cells are a cluster of cells existing as a folding of a trilaminar sheet containing alpha

cells in the outer layer and beta cells in the inner layer (Rahier *et al.*, 1983; Bosco *et al.*, 2010; Rodriguez-Diaz *et al.*, 2011). Insulin is released into the bloodstream where it promotes glucose uptake by insulin sensitive cells to be used as energy by these cells. Insulin consists of two chains (A and B) containing three α-helices (residues A1–A8, A12–A18 and B9–B19) guarded by one intra and two inter-chain disulphide bonds (Adams *et al.*, 1969; Menting *et al.*, 2013b). In people with type 1 diabetes, the body produces little or no insulin because the insulin-producing cells are destroyed by an autoimmune reaction in the body (J Morgan *et al.*, 2011).

1.6.1 Mechanism of insulin secretion

Insulin secretion from pancreatic beta-cells occurs in response to many factors, most notably in response to blood glucose levels. Insulin secretion is a tightly regulated process that occurs in response to circulating glucose levels as well as various physiological and pharmacological factors (Taniguchi *et al.*, 2006). Glucose and amino acids play significant roles in the regulation of pancreatic beta cell activity (Figure 1). They regulate insulin secretion individually or as a mixture, as observed from both *in vitro* and *in vivo* studies (Krause *et al.*, 2011).

A number of transcription factors, such as hepatocyte nuclear factor (HNF) -4α, HNF-1α, insulin promoter factor (IPF)-1/pancreatic and duodenal homeobox (Pdx)-1, HNF-1β and NeuroD/β-2, are involved in insulin secretion. These transcription factors regulate the expression of genes key to various aspects of beta cell function (Shao *et al.*, 2009).

Insulin exocytosis is coordinated by various processes with regulatory activity including GLUT2 facilitated glucose uptake, glucose utilisation, ATP generation and vesicular movement(Fu *et al.*, 2013). Glucose (mostly derived from carbohydrate containing food) promotes insulin secretion from pancreatic beta cells (Rustenbeck *et al.*, 1994; Fu *et al.*, 2013). Insulin exocytosis occurs as a consequence of enhanced intracellular ATP:ADP ratio from glucose metabolism. Enhanced flux through the glycolytic pathway and tricarboxylic acid cycle (TCA) results in elevated mitochondrial ATP generation, following substrate-level phosphorylation and electron transport in the mitochondria utilising the electron donors NADH and

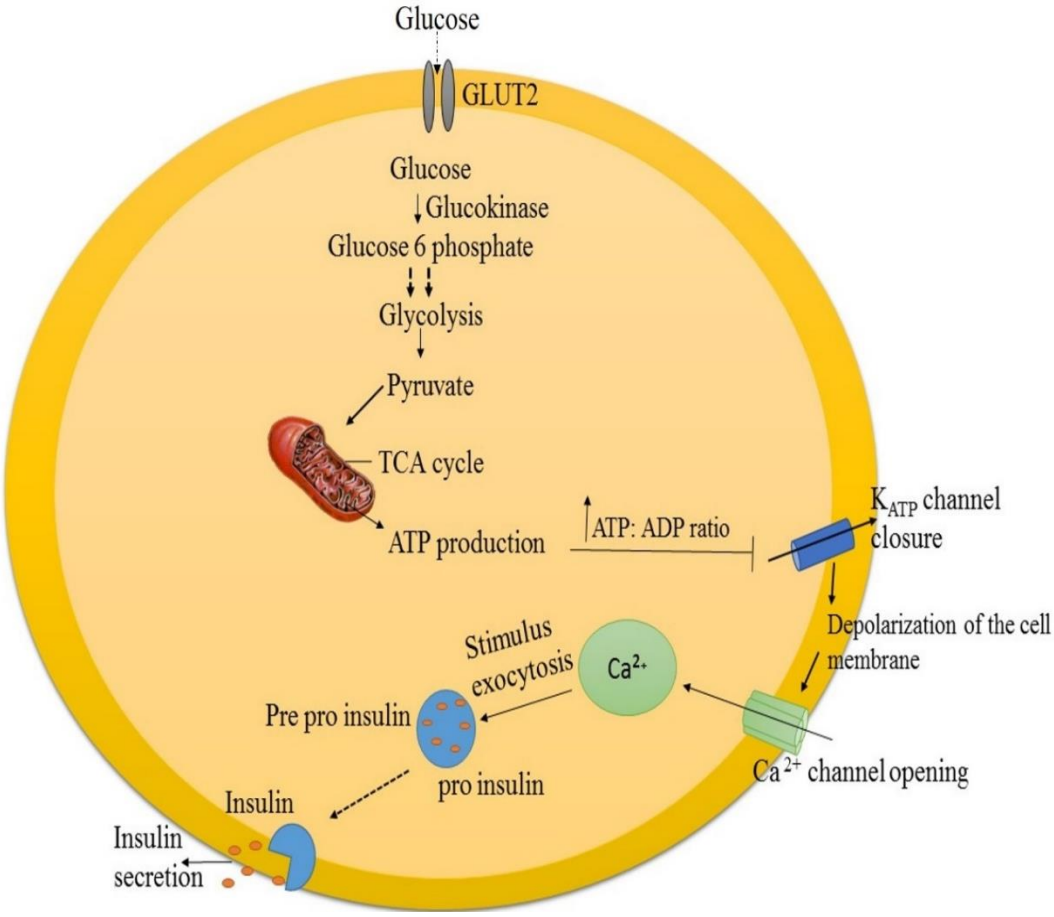


Figure 1: Insulin secretion from pancreatic beta cells. Pancreatic beta cells are stimulated by glucose and glucose uptake occurs by glucose transporter 1 (GLUT1 in humans) or glucose transporter 2 (GLUT2 in rodents). It leads to glycolysis and later TCA cycle occurs in mitochondria. ATP is released from mitochondria which leads to closure of voltage gate K_{ATP} channels and opens calcium channels to promote influx of calcium. Calcium promotes movement of insulin secretory vesicles to plasma membrane to release insulin.

FADH2. The ratio of ATP and ADP enhances closure of the K^+ gated channel and opening of the Ca^{2+} gated voltage channel (Jensen *et al.*, 2008). The resultant influx of Ca^{2+} leads to insulin export through fusion of a readily releasable pool of insulin-containing vesicles with the plasma membrane (Komatsu *et al.*, 2013). This insulin triggering mechanism occurs in the first 5-10 minutes as an initial response, followed by a more prolong phase of insulin release which is mostly dependent on metabolic stimulus–secretion coupling and it happens over a period of 30–60 min (Taniguchi *et al.*, 2006; Newsholme *et al.*, 2014).

Pancreatic beta-cells are equipped with a range of specific amino acid transporters many of which are Na^+ dependent. There are three well studied major modes of insulinotropic action of amino acids (Floyd Jr *et al.*, 1966; Salvucci *et al.*, 2013). Firstly, direct membrane depolarisation which occurs after the transport of cationic amino acids (such as L-Arginine) by beta cells leads to Ca^{2+}-channel opening and insulin release. Secondly, some amino acids that can be metabolised (such as leucine) raise intracellular ATP inducing closure of ATP-sensitive K^+ channels (K_{ATP} channels), membrane depolarization and Ca^{2+} influx ultimately resulting ino insulin release (Newsholme *et al.*, 2014). Thirdly, certain amino acids including alanine may stimulate insulin release through both metabolism and as a direct result of the membrane depolarizing actions of Na^+ co-transport triggering Ca^{2+} influx and ultimately insulin release (Yada, 1994; Brennan *et al.*, 2003).

Besides glucose and amino acids, a range of other fuel secretagogues of insulin also activate the insulin secretion pathway at different points (Rutter *et al.*, 2015). This includes incretin hormones glucagon-like peptide-1 (GLP-1), glucose dependent insulinotropic peptide (GIP), peptide YY (PYY) synthesized from the gut and oxyntomodiun (Campbell & Drucker, 2013). They act via G-protein couple receptors (GPCR) increasing the concentration of intracellular cAMP. This event further activates protein kinase A (PKA) which leads to the activation of molecules involved in releasing ATP and increasing cytosolic Ca^{2+}. Finally the exocytosis of insulin secreting granules occur (Fu *et al.*, 2013; Rutter *et al.*, 2015).

1.6.2 Mechanism of action of insulin

Insulin signalling is important for cellular growth, survival, proliferation, metabolism and glucose uptake (Zierath *et al.*, 1998; Taniguchi *et al.*, 2006). Striated muscle cells, adipocytes and hepatocytes are the most insulin sensitive cells in mammals (Kandror & Pilch, 1996). A series of intracellular messages activated upon insulin binding to the insulin receptor (IR) (Kasuga *et al.*, 1982; Ullrich *et al.*, 1985; Menting *et al.*, 2013a). In striated muscle and fat cells glucose transporters are recruited to internalise glucose (as well as a number of other events) but glucose enters hepatocytes via GLUT2 transporters that are insulin independent (James *et al.*, 1988; Michael *et al.*, 2001; Uldry *et al.*, 2002). The binding of insulin to the IR receptors of hepatocytes triggers events within these cells that include upregulation of glycogen synthesis, lipogenesis and amino acid synthesis.

Insulin and Insulin-like growth factor 1 (IGF-1) regulate a number of biological processes by involving two closely related tyrosine kinase receptors. Activation of the receptors triggers a cascade of phosphorylation events that are followed by the activation of enzymes controlling various metabolic and growth processes (Boucher *et al.*, 2014). The insulin and IGF-1 receptors (IR and IGF-1R) are comprised of two alpha and two beta subunits. The beta subunits have tyrosine kinase activity. When insulin or IGF-1 binds to the alpha subunit, it causes a change in receptor conformation which prompts the auto-phosphorylation of the tyrosine residues of the beta subunit and thus activates the catalytic activity of the kinase (Chang *et al.*, 2004). Then tyrosine residues on intracellular substrates are phosphorylated by activated IR and the best characterized substrates for this receptor are the insulin receptor substrate family (IRS1-6). Other substrates include Grb2-associated binder (Gab-1), adapter protein with a PH and SH2 domain (APS) and Shc (Src and Collagen Homologues) isoforms, and signal regulatory protein (SIRP) family members (Saltiel & Pessin, 2003; Siddle, 2012; Gorgisen *et al.*, 2017).

IRS proteins are linked to critical pathways of metabolic actions of insulin by involving Phosphoinositide 3-Kinase (PI3K) and Protein kinase B (also known as Akt) pathway (Figure 2). PI3-kinases have several heterodimers which can remain in several isoforms (Vadas *et al.*, 2011). Tyrosine phosphorylated IRS proteins bind to

two SH2 domains in the regulatory subunits and that activates PI3K (Myers *et al.*, 1992; Shaw, 2011). Upregulation of PI3K activates the catalytic subunit further, which phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to produce phosphatidylinositol (3,4,5)-trisphospphate (PIP3). Akt binds to PIP3, resulting in translocation Akt to the plasma membrane, thus activating Akt.

Akt Activation is achieved by phosphorylation and promotes downstream signalling (Boucher *et al.*, 2014). Most of the physiological effects of PI3K-generated PIP3 are mediated by a subset of AGC protein kinase family members, which include isoforms of Akt/protein kinase B (PKB), p70 ribosomal S6 kinase (S6K), serum- and glucocorticoid-induced protein kinase (SGK), as well as several isoforms of protein kinase C (PKC), particularly the atypical PKCs. AGCkinase family members share similar structure and mechanisms of activation via phosphorylation of two serine and threonine residues (Figure 2) (Pearce *et al.*, 2010). PDK1 is the key upstream kinase, which facilitates PI3K regulated phosphorylation and activation of the AGC kinase members (Bayascas, 2010). Activation of Akt by PDK1 induces activation of mTORC1 complex followed by phosphorylation and inhibition of 4E binding protein 1 (4E-BP1). Activation of mTORC1 further activates ribosomal protein S6 kinases S6K1 and S6K2 and sterol regulatory element-binding protein 1 (SREBP1), and results in the regulation of a variety of genes regulating protein synthesis, metabolism, and cell growth (Düvel *et al.*, 2010).

Akt also phosphorylates glycogen synthase kinase 3 (GSK3) (Figure 3) which in turn phosphorylates both the isoforms of GSK3: GSK3α and GSK3β. This phosphorylation usually occurs in serine residue 9 and 21 of cytoplasmic GSK3β and GSK3α respectively (Embi *et al.*, 1980; Fang *et al.*, 2000) and blocks the active site of GSK3 partially thereby stopping the enzymatic activity of GSK3 (Hughes *et al.*, 1993; Pap & Cooper, 1998). As a result, it activates some of the downstream signalling molecules such as glycogen synthase which leads to glycogen synthesis and activation of eukaryotic initiation factor elF-2B resulting in protein synthesis (Cross *et al.*, 1995; Mobley *et al.*, 2016).

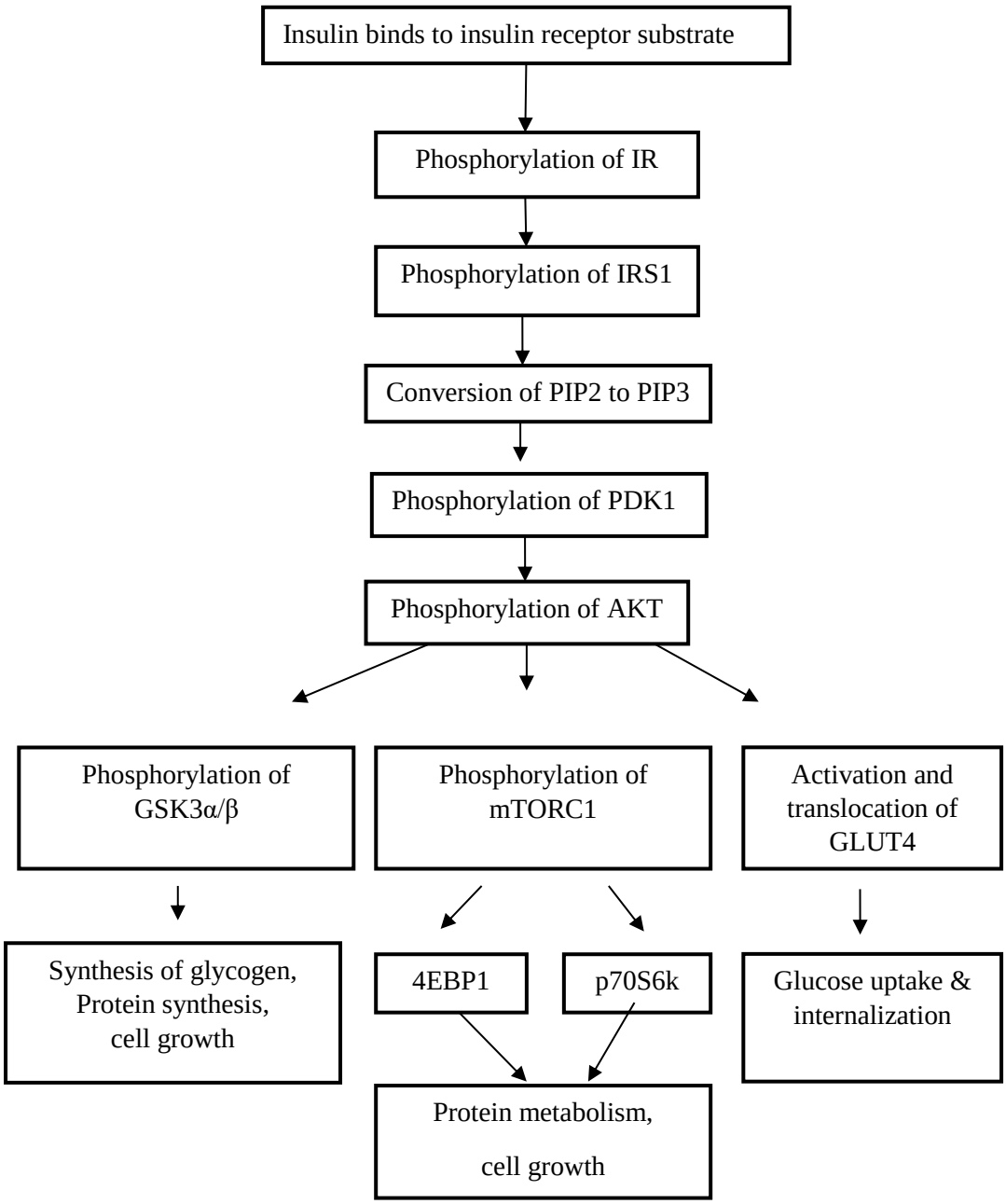


Figure 2: Insulin action on PI3K/Akt pathway. Insulin binds with the insulin receptor (IR) and phosphorylates the insulin receptor substrate (IRS). IRS phosphorylates and activates a catalytic subunit, which immediately phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to generate the phosphatidylinositol (3,4,5)-triphosphate (PIP3). Further it activates Phosphoinositide 3-kinase (PI3K) and 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Protein Kinase C (Akt) and it leads to different actions: glycogen and protein synthesis by phosphorylation and activation of glycogen synthase kinaseα/β (GSK3α/β), protein metabolism by activating mammalian target of rapamycin complex2 (mTORC1) and glucose uptake by activating glucose transporter 4 (GLUT4) translocation.

Akt mediates the phosphorylation of peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α) to initiate gluconeogenesis and fatty acid oxidation (Liu *et al.*, 2007). Moreover, Akt stimulates the phosphorylation and activation of phosphodiesterase 3B (PDE3B) and decreases cAMP levels (Kitamura *et al.*, 1999), which significantly influence insulin to hinder lipolysis in adipocytes and insulin secretion in pancreatic beta cells (Degerman *et al.*, 2011). Insulin is proposed to upregulate the transcription of enzymes involved in lipoprotein uptake (thus cholesterol and fatty acid uptake) by stimulating a transcription factor called steroid regulatory element binding protein (SREBP) in rat liver cells (Shimomura *et al.*, 1999; Najafi-Shoushtari *et al.*, 2010).

Akt plays a key role in facilitating transportation of GLUT4 to the surface of cell membranes in response to insulin (Figure 2) (Sharma *et al.*, 2011). Akt has three isoforms: Akt1 and Akt2 are most abundant in muscle, Akt2 is more responsible for glucose uptake activity (Brozinick *et al.*, 2003; Peng *et al.*, 2003).

A second important branch of the insulin/IGF1-mediated signalling pathway is the Grb2-SOS-Ras-MAPK pathway (Boulton *et al.*, 1991; Boucher *et al.*, 2014) (Figure 3). This pathway transduces signals from the extracellular environment to the nucleuswhere specific genes are activated for cell growth, division, differentiation, cell cycle regulation, wound healing, tissue repair, integrin signalling and is able to stimulate angiogenesis. The Grb2-SOS-Ras-MAPK pathway is activated independently of PI3K/Akt. Activated receptors and phosphorylated IRS proteins have docking sites specific for adaptor molecules (e.g. Grb2 and Shc). The carboxy-terminal SH3 domain of Grb2 binds to proteins such as Gab-1, whereas the amino-terminal SH3 domain binds to proline- rich regions of proteins such as son-ofsevenless (SOS). SOS is a guanine nucleotide exchange factor (GEF) for Ras, catalysing the switch of membrane-bound Ras from an inactive, GDP-bound form (Ras-GDP) to an active, GTP-bound form (Ras-GTP). Ras-GTP then promotes downstream effectors like the Ser/Thr kinase Raf, which in turn enhances its downstream target MEK1 and 2 that phosphorylate and activate the mitogen activated protein kinase (MAP kinases) ERK1 and 2. Activation of ERK1/2 is important for cell proliferation or differentiation. Moreover, ERk1/2 activation controls several gene expression or extra-nuclear events, such as cytoskeletal reorganisation, by inducing phosphorylation and activation of targets in the cytosol and nucleus (Boucher *et al.*, 2014).

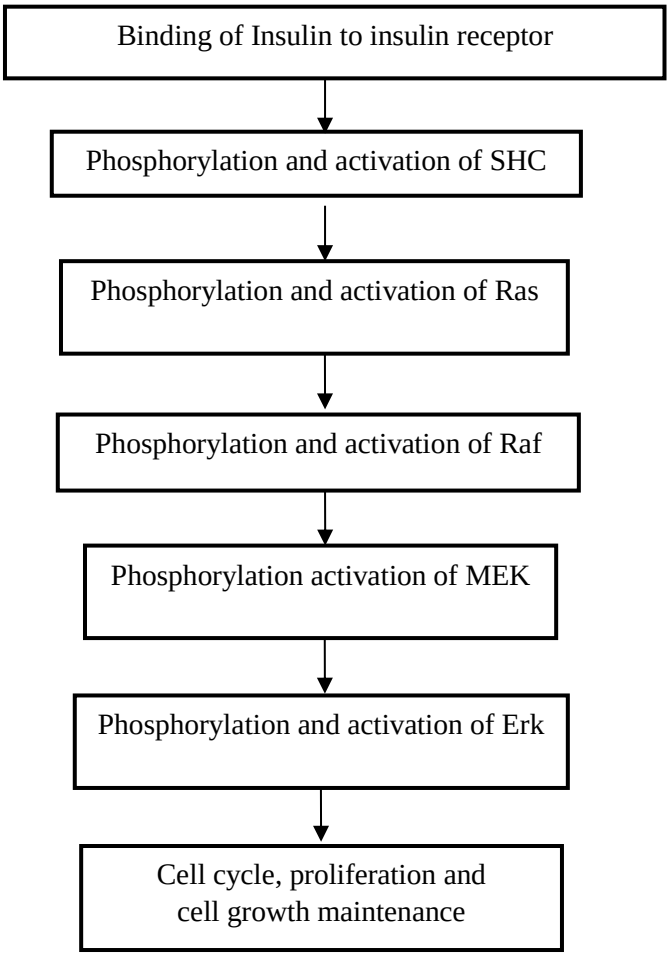


Figure 3: Induction of the Ras/Raf/MEK/Erk Pathway by insulin. Insulin activates the Ras-Raf-MEK pathway for cell cycle maintenance.

There are several transformed insulin sensitive cell lines that can be useful in examining molecular mechanisms and signalling pathways. Various muscle myoblast cells such as C2C12, L6; adipocytes as 3T3L1, liver cells like HEPG2 are used for insulin signalling, metabolic regulation and cellular activity studies. L6 cells are isolated from rat thigh muscle and form multinucleated myotubes and striated fibres (Cross *et al.*, 1994; Meuillet *et al.*, 1999; Schmitz-Peiffer *et al.*, 1999). C2C12 cells originated from the mouse and are able to differentiate to generate contractile myotubes (Dedieu *et al.*, 2002).

1.6.3 Insulin resistance

Insulin resistance may eventually lead to type 2 diabetes mellitus. In insulin resistance, insulin-sensitive cells (striated muscle and fat cells) fail to respond effectively to normal insulin levels released by the pancreatic beta cells, which leads to the pancreas releasing higher amounts of insulin in-order-to compensate for the reduced responsiveness of the IRs on these cells (adipocytes and striated muscle cells) (Shulman, 2000; Cederquist *et al.*, 2017). As a result, not enough glucose is taken up from the blood by the cells, and the level of glucose becomes high in the blood (Shulman, 2000; Yamauchi *et al.*, 2001). In this case, muscle, liver, and fat cells do not respond to insulin properly. Insulin resistance is seen among the type-2 diabetic patients more frequently. It has an impact on various cells and their metabolic activity (Uysal *et al.*, 1997; Weyer *et al.*, 2001; Siwicki *et al.*, 2016). Insulin resistance affects all the metabolic actions of insulin including glucose transport, hexokinase gene expression, synthesis of glycogen, oxidation of glucose. It globally affects glucose metabolism (Cheatham *et al.*, 1994; Dorrestijn *et al.*, 1996).

Deletion or abnormalities in IRS1 and IRS2 genes prevent Akt activation. Kinases such as polo like kinase 1 (PIK1) can phosphorylate the serine residues of IRS (Ser 556 and Ser 1098) and promotes the PI3K pathway activation (Chen 2015). Changes in IRS genes can lead to hyperglycaemia, insulin resistance, and other metabolic abnormalities (Dong *et al.*, 2008). Absence of PDK1 substrate phosphorylation, hepatic inactivation of PI3K and mTORC2 pathway can also lead to insulin resistance resulting in hyperinsulinemia, hypolipidaemia, and hyperglycaemia (Cusi *et al.*, 2000; Bayascas *et al.*, 2008; Zhang *et al.*, 2016).

Insulin resistance has effects on various systems and tissues including hepatic, pancreatic and muscle tissues. Several studies reported that reduced insulin signalling associated with central nervous system activation occurs from either defective secretion or action of contributes to the pathogenesis of common metabolic disorders including obesity (Porte et al. 2005). It stimulates release of the inflammatory mediators in muscle cells and may eventuate in the development of the metabolic syndrome (Kumar *et al.*, 2010; Guo, 2014). In the heart, insulin resistance can result in heart failure (Qi *et al.*, 2013). In the case of beta cells, beta cell regeneration is impaired by insulin resistance (Kulkarni *et al.*, 1999). Moreover, insulin resistance shortens the lifespan of skeletal muscle cells. It promotes hypertension in vascular endothelium and disturbs glucose homeostasis (Duncan *et al.*, 2008; Rask-Madsen *et al.*, 2010; Tsuchiya *et al.*, 2012). Insulin resistance can influence the retinal and glomerular system by raising microvascular complications of diabetes (Kasiske *et al.*, 1992; Groop *et al.*, 2005).

1.7 Existing "anti-diabetic" medications

1.7.1 Oral glucose lowering agents

1.7.1.1 Sulfonylureas

Sulfonylurea drugs are derivatives of sulphonamide and considered as the first line treatment for type 2 diabetes. Sulfonylureas increase pancreatic insulin secretion from beta cells (Aston-Mourney *et al.*, 2008). These drugs work only if there is an adequate amount of beta cell to produce insulin (Eliasson *et al.*, 1996; van Raalte & Verchere, 2017). Sulphonylurea drugs block the K^+ channel and increase intracellular Ca^{2+} concentration. These drugs also reduce hepatic glucose production and peripheral resistance to insulin action (Fleig *et al.*, 1984; Principalli *et al.*, 2015). Several side effects are associated with the drug, which are hypoglycaemia, headache, hypersensitivity reactions, weight gain and gastrointestinal problem (Häring *et al.*, 2013). The first-generation drugs in this class include acetohexamide, carbutamide, chlorpropamide, glycyclamide (tolhexamide), metahexamide, tolazamide and tolbutamide (Lebovitz & Feinglos, 1978; Ballagi-Pordány *et al.*, 1990). Whereas

glibornuride, gliclazide, glipizide, gliquidone, and glyclopyramide are the second-generation drugs (Prendergast, 1983; Lebovitz, 1985). Third generation drug in this group is glimepiride (Home *et al.*, 2015).

1.7.1.2 Biguanides

Biguanide drugs are another class of drugs prescribed for pre-diabetic and type 2 diabetic patients. Metformin, a biguanide drug that has been in use for more than 30 years, is a widely used oral glucose lowering medication (Madiraju *et al.*, 2014). Metformin reduces glucose production from liver cells and increases glucose utilisation by peripheral cells (Penicaud *et al.*, 1989; Bailey *et al.*, 1994; Foretz *et al.*, 2010). The exact mechanism by which metformin delivers these effects is unknown. This drug also activates AMP-activated protein kinase (AMPK) which plays a key role as a master regulator of cellular energy homeostasis (Miller *et al.*, 2013).

1.7.1.3 Thiazolidinedione

Thiazolidinedione is another class of glucose lowering agents. These agents activate the peroxisome proliferator-activated receptor γ (PPARγ) which is a member of ligand-activated receptor family, mostly expressed in adipocytes (Berger & Moller, 2002). PPARγ upregulates a number lipid metabolism related proteins as acyl-CoA synthase, lipoprotein lipase (LPL), fatty acid transport protein 1 (FATP-1) and cluster of differentiation 36 (CD36). It also controls uncoupling protein-1 (UCP-1), uncoupling protein-2 (UCP-2) and leptin expression (Schoonjans *et al.*, 1995; Schoonjans *et al.*, 1996; Martin *et al.*, 1997; Sfeir *et al.*, 1997).

These mechanisms lead to facilitation of the differentiation of adipose tissue and decreases lipolysis and formation of plasma free fatty acids, leptin and TNFα. These events appear to increase the sensitivity of the IRs on adipocytes to insulin. Activation of PPARγ results in adipogenic activity (Ye *et al.*, 2001; Ahmadian *et al.*, 2013). During clinical studies, these drugs show reduced insulin resistance, decreased hyperinsulinemia and reduced hepatic glucose production.(Miles *et al.*, 1997). Adverse effects of this drug include weight gain, congestive heart failure, and osteoporosis (Ahmadian *et al.*, 2013).

1.7.1.4 Alpha glucosidase inhibitor

The alpha glucosidase inhibitor, acarbose has also been used as another option for type-2 diabetes treatment. Acarbose is commercially produced via microbial fermentation with strains from the genera of *Actinoplanes*. It inhibits the α-glucosidase located in the upper intestinal brush border (Van de Laar *et al.*, 2005; Kalra, 2014) and therefore delays the formation of monosaccharides from polysaccharides, decreases glucose uptake by the intestine. As a result, postprandial hyperglycemia is delayed (Kim *et al.*, 2005; Hogan *et al.*, 2010). It is given alone or in combination with other drugs for the treatment of type-2 diabetes. It is also used as an adjunct to insulin therapy (for insulin dependent diabetic patients) or sulfonylureas in some cases (Chiasson *et al.*, 1994); however, this may increase the risk of hypoglycaemia in these individuals. Acarbose is poorly tolerated as it causes gastrointestinal disturbances; adverse effects include diarrhoea, anaemia, flatulence, abdominal pain (Chiasson *et al.*, 1994).

1.7.1.5 Dipeptidyl Peptidase-4 Inhibitors

Dipeptidyl peptidase-4 inhibitors (also known as DPP4 inhibitors or gliptins) are a group of drugs used to treat type 2 diabetes. Dipeptidyl peptidase 4 (DPP4) rapidly deactivates the incretins GLP-1 and GIP (Khan *et al.*, 2010). Gliptins (alogliptin, linagliptin, saxagliptin, sitagliptin and vildagliptin) inhibit the DPP4 thus increasing the half-life of GLP-1 and GIP (Augeri *et al.*, 2005; Mu *et al.*, 2006). This in turn increases insulin secretion and inhibits glucagon release (Marguet *et al.*, 2000; Aaboe *et al.*, 2010).

1.7.2 Parenteral glucose lowering agents

1.7.2.1 Insulin

Insulin is a blood glucose reducing agent used for type-1 diabetes, type-2 diabetes, gestational diabetes and various complications of diabetes including diabetic ketoacidosis (Morris *et al.*, 1997; Callejas *et al.*, 2013; Matias *et al.*, 2014). Insulin is used in an injectable form mostly under the skin. It can also be injected into a vein or muscle (Pickup *et al.*, 2002; Hoogma *et al.*, 2006). However, insulin can cause adverse effects such as hypoglycemic shock, skin changes due to injection and allergic

reaction (Brunkhorst *et al.*, 2005; Strachan & Frier, 2013). Insulin is available in various types, mainly short–acting, intermediate–acting and longer-acting (British-Medical-Association, 2015).

1.7.2.2 GLP-1 analogues

GLP-1 analogues are agents that work like incretin hormones. These agents usually lower blood glucose following certain mechanisms like stimulation of insulin secretion, inhibition of glucagon secretion after a meal by slowing down the absorption of food and reducing calorie intake (Nielsen, 2005; Tushuizen *et al.*, 2006; Nauck *et al.*, 2013). Some of the incretin mimetic agents are Liraglutide , which is a long acting glucagon-like peptide -1 (GLP-1) receptor agonist, Exenatide (Bond, 2006; Nauck *et al.*, 2009), etc. Despite being an incretin mimetic they show adverse effects on the body such as vomiting, diarrhoea and nausea (Nauck *et al.*, 1993).

1.7.3 Drug Combinations

A single drug may not be effective in reducing blood glucose levels, hence a combination of drugs is required. Then a combination of drug is needed. A second drug or even a third drug is necessary in this case. Metformin and sulphonylurea drug is a prescribed combination in some cases of diabetes (Cefalu *et al.*, 2002). When metformin works poorly to control blood glucose, a combination of rosiglitazone and metformin is prescribed (Fonseca *et al.*, 2000).

1.8 The role of natural resources in drug discovery

Compounds derived from plants, animals and microbes have been developed into novel therapeutic medications. However, in the last twenty years there has been a decreased emphasis on drug discover from such natural sources (Newman & Cragg, 2012). The emphasis on natural products changed in the 1990s as a result of the (1) introduction of high throughput screening techniques against clear molecular targets which enabled the screening of libraries of synthetic compounds (Inglese *et al.*, 2006) (2) development of combinatorial chemistry which, at first, promised a quicker and more efficient process in screening of chemical libraries (Rohrer *et al.*, 1998) and (3)

advancements in molecular and genomic biology which increased the number of molecular targets and decreased the drug discovery timelines (Koehn & Carter, 2005; Lamb *et al.*, 2006).

However, the interest in the natural products for drug discovery has been reinvigorated in more recent times especially since combinatorial chemistry have problems regarding greater size, and some of their properties are not suitable for drug development. Natural products have 200-300 times higher hit rate than synthetic compounds (Charifson *et al.*, 1999; Martin & Critchlow, 1999; Hübel *et al.*, 2008). Another interesting feature of the natural product is their unique scaffold structure which can be more convenient for drug design (Lee & Schneider, 2001; Dumpitak *et al.*, 2005; Koch *et al.*, 2005). Availability and diversity is a significant reason behind using natural sources for treatment purpose. More than 800 plants have been documented to have antidiabetic activity (Khemani *et al.*, 2012; Juárez-Rojop *et al.*, 2014; Mohammed *et al.*, 2015). The most frequently used drug for diabetes "Metformin" was developed from the plant *Galega officinalis* (Karakas *et al.*, 2016; Song, 2016). Some plants of reported anti-diabetic potential are listed in Table 2.

Various parts of plants have generally been used in folk medicine and thereafter are being investigated for potential drug discovery (Szychowski *et al.*, 2014; Harvey *et al.*, 2015). These include leaves, aerial part, bark, flower, seeds, fruit, rhizomes, bulb, root, stem and whole plant. Various extraction methods have been reported to be more efficient and suitable for different plants (Azmir *et al.*, 2013; Chua, 2013; Ciriminna *et al.*, 2016). Plant extract preparation and fractionation is necessary for compound-isolation. Mostly used extracts are aqueous, methanol, ethanol, chloroform, hexane extracts of plants. Some plant extracts containing glucose lowering compounds have been listed in Table 2. There is a bulk of secondary metabolites in any plant. Various factors including solubility, toxicity, viscosity, purity of the extraction solvent should be considered during extraction as many compounds can be lost in the process (Maltese *et al.*, 2009; Mohammed *et al.*, 2015). Several different techniques are employed in the process of extracting individual compounds from plants, these include Soxhlet, supercritical, liquid-liquid and solid-liquid extraction techniques (Sun *et al.*, 2008; Chan *et al.*, 2012).

Table 2: Some common natural sources with anti-diabetic properties.

Plant	Used part	Preparation	Reference
Artemisia dracunculus L.	whole plant	ethanolic extract	(Ribnicky *et al.*, 2009)
Capparis spinosa L.	fruits	ethanolic extract	(Huseini *et al.*, 2013)
Curcuma longa	rhizome	ethanolic extract	(Ali Hussain, 2002)
Eurycoma longifolia	bulb	ethanolic extract	(Lahrita *et al.*, 2015)
Galega officinalis	leaves, seeds	aqueous extract	(Shojaee *et al.*, 2015)
Macaranga tanarius	seeds	ethanolic extract	(Patil *et al.*, 2011)
Marrubium vulgare	aerial part	methanolic extract	(Vergara-Galicia *et al.*, 2012)
Morus alba	leaves	ethanolic extract	(Zhang *et al.*, 2009b)
Nigella sativa	seeds	crude aqueous	(Benhaddou-Andaloussi *et al.*, 2010)
Ocimum sanctum	leaves	aqueous extract	(Vats *et al.*, 2002)
Panax japonicus	root	ethanolic extract	(Lee *et al.*, 2010b)
Pinus radiata	bark	Aqueous extract	(Bang & Choung, 2014)
Rhododendron tomentosum	fruit	ethanolic extract	(Harbilas *et al.*, 2009)
Ruta graveolens	leaves	aqueous extract	(Ahmed *et al.*, 2010)
Salacia chinensis	stem, leaves	aqueous extract	(Yoshikawa *et al.*, 2003)
Salacia reticulata	root, stem	aqueous extract	(Oe & Ozaki, 2008)
Stevia rebaudiana	leaves	aqueous extract	(Kujur *et al.*, 2010)
Swertia punicea	whole plant	ethanol extract	(Kshirsagar *et al.*, 2014)
Syzygium cumini	seeds, leaves, flower	ethanolic extract	(Kumar *et al.*, 2013)

Several methods have been used for compound profiling of plant extracts. Thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) are used for crude identification of few compounds or groups of compounds. Various high performance liquid chromatography (HPLC) and gas chromatography (GC) methods with spectroscopic or fluorometric detection have been developed for separation (and semi-identification) of compounds (Mondello *et al.*, 2008; Marriott *et al.*, 2009; Mateus *et al.*, 2010). Techniques that aid in identification of compounds include nuclear magnetic resonance (NMR) and mass spectrometry (MS). These methods help in identifying the known compounds or metabolites within a plant extract (Waksmundzka-Hajnos & Sherma, 2010; Porter *et al.*, 2012).

About 90% of the natural sources have not yet been explored for active constituents (Harvey, 2000; Lee & Schneider, 2001). With biodiversity comes extraordinary biochemical variability and specificity that dictate unique, which is important pharmaceutical research. Drug development of compounds isolated from natural products require optimization of stability, potency and pharmacokinetics (Szychowski *et al.*, 2014). Many different compounds have been identified in plant extracts and some, when examined *in vitro*, may have an interesting array of bioactivities.

However, these activities may diminish when examined *in vivo*. Thus, the majority of these compounds may not survive the "discovery" process and never become drugs. Nonetheless several compounds identified and isolated from natural sources (Table 3) are widely used drugs on the market, for example penicillin, first generation statins, morphine and several glucose-lowering medications (Table 4).

1.8.1 Compounds of plant origin with putative activities that may aid in the treatment of diabetes mellitus

Plants have a large array of compounds (including phytochemicals) with a promise for development of new drugs. About 40% of recently approved new drugs are directly or indirectly derived from natural entities (Newman & Cragg, 2012; Veeresham, 2012; Cragg & Newman, 2013) with a similar percentage of antidiabetic drugs derived from natural products (Newman & Cragg, 2012; Zeng, 2014).

Table 3: Selected phytochemical compounds and the proposed mode of "antidiabetic" activity.

Antidiabetic compound	Activity	References
Apigenin	Increases glucose uptake and insulin secretion.	(Cazarolli *et al.*, 2009a)
Catechin, epicatechin	α-amylase inhibitory activity.	(Hara & Honda, 1990; Yilmazer-Musa *et al.*, 2012)
Chalcomoracin, moracin C	α-glucosidase inhibitory activity.	(Yang *et al.*, 2012)
Ellagitannins (mallotinic acid, chebulagic acid)	α amylase and α-glucosidase inhibitory activity.	(Li *et al.*, 2007a; Yonemoto *et al.*, 2014)
Galegine	Activation of AMPK leading to increased glucose uptake.	(Sangeetha *et al.*, 2013)
Gallotannins (chebulinic acid derivatives)	α-glucosidase inhibitors.	(Gao *et al.*, 2007)
Kaempferol-3-neohesperidoside	Glucose transport to insulin sensitive cells.	(Zanatta *et al.*, 2008)
Methylswertianin	Improves insulin resistance.	(Tian *et al.*, 2010)
Mycaminose	Stimulates pancreatic beta cells.	(Kumar *et al.*, 2013)
p-coumaric acid	Modulates glucose and lipid metabolism by AMPK activation.	(Yoon *et al.*, 2013)
Piperidine alkaloids	Inhibits α-amylase and galactosidase.	(Pawar *et al.*, 2012)
Polyacetylenic glucosides	Prevents Type-1 diabetes by T- cell regulation.	(Chiang *et al.*, 2007)
Quercetin, rutin (quercetin 3-rutinoside)	Increases glucose uptake by cells.	(Enkhmaa *et al.*, 2005)
Sakuranetin	Activation of PPAR-γ and AMPK.	(Saito *et al.*, 2008)
Salacinol	Inhibition of glucosidases.	(Yoshikawa *et al.*, 2002)

1.8.2 Bioavailability of phenolic compounds

A number of phenolic compounds have been reported to have glucose lowering activity. Phenolic compounds are secondary metabolites in plants and are mostly responsible for the sensory or nutritional quality of food (Karakaya, 2004; Crozier *et al.*, 2010). The colon has a significant role in the bioavailability of dietary phenols and a large portion of phenolics is passed to the large intestine. Various studies showed that substantial amounts of phenolic compounds are absorbed and carried to the circulation before excretion (Larrosa *et al.*, 2009; Crozier *et al.*, 2010).

Phenolic compounds have been reported to have antioxidant properties because of their aromatic ring, which can stabilise and delocalise an unpaired electron in its structure (Rice-Evans *et al.*, 1996). Their structure enables donation of the hydrogen atoms or electron from their hydroxyl group (positioned along their aromatic ring) for scavenging of free radicals or for chelation of metals. Phenolic compounds are classified into 6 general groups: flavonoids, phenolic acids, phenolic alcohols, stilbenes, lignans, and tannins (Karakaya, 2004).

After the ingestion of dietary flavonoids which, with the notable exception of flavan-3-ols and proanthocyanidins, exist in planta predominantly as glycoside conjugates, absorption of some but not all components into the circulatory system occurs in the small intestine (Donovan *et al.*, 2006). Usually, the absorption at this site is followed by hydrolysis, with release of the aglycone because of the action of lactase phloridzin hydrolase (LPH) in the brush-border of the small intestine epithelial cells. The released aglycone can then enter the epithelial cells by passive diffusion due to its increased lipophilicity and its proximity to the cellular membrane (Day *et al.*, 2000).

Various factors such as partition coefficient and number of sugar molecules play an effective role in the absorption of phenolics (Scalbert & Williamson, 2000). On the other hand, hydrophilic compounds cannot be absorbed in the upper part of gastrointestinal tract. Phenolics, which have rhamnose in their molecule, cannot be absorbed through the small intestine (Hollman & Katan, 1997). Moreover, individual variations are effective in the absorption and/or transportation of phenolics (Murota *et al.*, 2000; Karakaya, 2004).

Table 4: Drugs used in the treatment of diabetes mellitus that are derived from natural sources (Adapted from Newman & Cragg (2016)).

Generic name	Trade name	Year introduced
Acarbose	Glucoby	1990
Epalrestat	kinedak	1992
Metformin	Glucophage	1995
Troglitazone	Rezulin	1997
Miglitol	Diastabol	1998
Extenatide	Byetta	2005
Triproamylin acetate	Normylin	2005
Liraglutide	Victoza	2009
Lixisenatide	Lyxumia	2013

Phenolic compounds have been found to undergo some structural changes. In many *in vivo* studies, various forms of (−)-epigallocatechin gallate present in plasma were found as sulphate conjugate (65%), free form (20%) and glucuronide (15%) (Sesink *et al.*, 2001). On the other hand, catechin metabolites were mainly found in the form of glucuronidated derivatives in the plasmas of rats fed a 0.25% catechin diet. High methylation of catechin is usually observed in the liver which reflects the intensive catechol O-methyltransferase activity (Manach *et al.*, 1999). Distribution volume of phenolics such as apigenin and quercetin have been found higher in various pre-clinical and clinical trials (de Boer *et al.*, 2005; Cai *et al.*, 2007).

1.9 Teucrium polium

Teucrium polium is a Mediterranean plant which is commonly known as golden germander (Polymeros *et al.*, 2002). It is a chamaephyte that grows to 20-40 cm in length and possesses a tomentose, canescent structure (Alados *et al.*, 1998; Louhaichi *et al.*, 2009; Bukhari *et al.*, 2015). The genus *Teucrium* is a large genus with around 100 species and is widely distributed in Europe, Asia, and North Africa but mainly concentrated in the Mediterranean region (Bahramikia *et al.*, 2009; Bukhari *et al.*, 2015). *Teucrium polium L.* is known by various names, such as j‘ada, germandée tomenteuse, mountain germander, germander, *Teucrium polium*, pouliot de montagne, felty germander, cat thyme, camendrio di montagna, hulwort, polio, berggamander, polio primo, poleigamander, timo bianco (Fayed *et al.*, 2015; Jaradat, 2015). An infusion of the leaves and flowers of the plant is consumed as a refreshing beverage. *Teucrium polium* has been long used in Iran commonly as decoctions or infusions for its diuretic, antipyretic, diaphoretic, antispasmodic, tonic, anti-inflammatory, antihypertensive analgesic, antibacterial, and antidiabetic effects (Movahedi *et al.*, 2014).

Teucrium polium has stems with branches straight, elongate, each ending in a paniculate or corymbose inflorescence (Ozenda, 1991). Leaves range from 1-3 cm and look sessile and oblong (Suleiman *et al.*, 1988), while the flower colour varies from white to pink. It is a hermaphrodite, hence the plant is able to self-fertilize. The flowering period of this plant is from July to September (Capasso *et al.*, 1984; Abdollahi *et al.*, 2003; Sharififar *et al.*, 2009). Growth is enhanced by the dry, moist and alkaline soil.

Figure 4: ***Teucrium polium*** plant. (Reproduced with permission from Sevcik (2010); Appendix 1).

1.9.1 Medicinal properties of *Teucrium polium*

Teucrium polium is a sub-shrub and herb and this plant is brewed for consumption as a tea or condiments (Gordon & Rowe, 1982). Various parts of *Teucrium polium* (mostly leaf) are used for medical purposes (Polymeros *et al.*, 2002) as the plant has folklore reputation for its use for a number of ailments. Many studies report various medicinal effects of *Teucrium polium* (section 1.9.3). The studied therapeutic effects of *Teucrium polium* include anti-diabetic activity (section 1.9.4)

1.9.2 Phytochemicals in *Teucrium polium*

More than 130 compounds have been identified from *Teucrium polium* to date. Table 5 lists the main compounds. Mostly prominent components are monoterpenes, flavonoids (Bruno *et al.*, 1993), polyphenols (Rizk *et al.*, 1986; Kawashty *et al.*, 1999), sesquiterpenes and α–pinene (Bruno *et al.*, 1993).

Among the volatile compounds, α-pinene has been reported to induce oxidative stress and cellular growth inhibition (Singh *et al.*, 2006). Pulegone has hepatotoxic activity (Thomassen *et al.*, 1990). Linalool has anti-nociceptive and anti-bacterial activity (Peana *et al.*, 2003; Park *et al.*, 2012). Studies on menthane present in the *Teucrium polium* extract demonstrated a sedative and physiological cooling effect (Watson *et al.*, 1980; Sousa *et al.*, 2007). Cedrol acts as a platelet activating factor (Jantan *et al.*, 2004). Terpine-4-ol has an anti-bacterial effect (Ebrahimi *et al.*, 2008).

Limonene showed anti-carcinogenic, anti-inflammatory and anxiolytic activity (Crowell *et al.*, 1991; Hirota *et al.*, 2010; Lima *et al.*, 2013). Picropolin has been reported to have an anti-cancer effect (Valcic *et al.*, 2000). Flavonoids identified in *Teucrium polium* have been reported to exhibit a range of activities in different studies. Lauric acid has an antimicrobial effect (Padgett *et al.*, 2000) and palmitic acid induces apoptosis and also is used in infant formulas (Carnielli *et al.*, 1996; Mu *et al.*, 2001). Rutin appears to be predominantly involved in the hepatoprotective, antihyperglycemic and antioxidant effects of *Teucrium polium* (Janbaz *et al.*, 2002; Selloum *et al.*, 2003; Kamalakkannan & Prince, 2006). Some of the main compounds found in *Teucrium polium* have been listed in Table 5.

1.9.3 Various effects of *Teucrium polium*

The medicinal reputation of *Teucrium polium* has been investigated in a number of studies, both *in vitro* and *in vivo.*

Antioxidant effect: *Teucrium polium* is mostly renowned for its antioxidant properties. The methanol, ethanol and chloroform extract of *Teucrium polium* has shown significant free radical scavenging activity (superoxide anion scavenging and hydrogen peroxide scavenging activity) and metal chelating activity. Flavonoids from *Teucrium polium* are proposed to be responsible for this activity (Couladis *et al.*, 2003; Ilhami *et al.*, 2003; Sharififar *et al.*, 2009).

Anti-mutagenic activity: *Teucrium polium* has been reported to have anti-mutagenic effect. The ethanol extract of *Teucrium polium* appears to promote stimulation of detoxifying enzymes and rapid recovery from DNA damage (Khader *et al.*, 2010).

Hepatoprotective activity: Some studies have suggested that *Teucrium polium* exhibits hepato-protective and anti-apoptotic activity. The ethyl acetate and ethanol extract of *Teucrium polium* were reported to have this activity. *Teucrium polium* activates mitogen activated kinase (MAPK), inhibits c-Jun-N-terminal kinase (JNK) and activates extracellular signal-regulated protein kinases 1/2 (Erk1/2) to facilitate this protective activity (Panovska *et al.*, 2007; Amini *et al.*, 2009; Aghazadeh & Yazdanparast, 2010).

Anti-inflammatory effect: The ethanol extract of *Teucrium polium* is reported to deliver a potent anti-inflammatory effect. Flavonoids and steroids have been reported to demonstrate significant anti-inflammatory effect (Capasso *et al.*, 1983; Tariq *et al.*, 1988). Moreover, several studies concluded anti-oxidant activity of this plant extract in their *in vivo* experiments on rats (Ljubuncic *et al.*, 2006; Hasani *et al.*, 2007).

Anti-nociceptive effect: An anti-nociceptive effect has been demonstrated using the essential oil extracted from *Teucrium polium (Abdollahi et al., 2003).* Aqueous extract of *Teucrium polium* also showed the same effect in other studies (Baluchnejadmojarad *et al.*, 2005; Zendehdel *et al.*, 2011).

Table 5: Compounds putatively identified in *Teucrium polium* (modified from Bahramikia & Yazdanparast (2012)).

Volatile compounds/ oil	Flavonoids and other compounds
α-pinene	Lauric acid
pulegone	Palmitic acid
Linalool	Luteolin
Menthane	Cirsimaritin
Menthofuran	Eupatorin
Cedrol	Teucardoside
Guaiol	Apigenin 7 glucoside
Picropolin	Rutin
Terpine-4-ol	Vicenin 2
Teupolin iii, iv, v	stigmasterol
sabinol	clerosterol
Cedrenol	Campesterol
Nerolidol	α- tocopherol
Limonene	Salvigenin
Aromadendrone	Apigenin
Teumicropodine	Brassicasterol
Germacrene D	Obtusifoliol
Teulolin A and B	Teucardoside (Iridoid glycosides)
Imonene	3´,6 Dimethoxy apigenin
Nerolidol	β-Sitosterol
β-Eudesmol	Linoleic
3,6,20-Trideacetyl teupyreinidine	Linolenic
Cadalene	6-Hydroxyluteolin

Hypolipidemic effect: The aqueous extract of *Teucrium polium* appears to deliver a hypo-lipidemic effect as assessed in male Wistar rats. Serum cholesterol and triglyceride levels decreased following treatment of the plant extract for 10 days (Rasekh *et al.*, 2001). It has been reported to lower serum triglyceride and cholesterol in clinical trial (Karimi *et al.*, 2002).

Antiviral and antibacterial activity: The ethanol and aqueous extract of *Teucrium polium* also showed antibacterial activity against gram positive and gram negative bacteria (Capasso *et al.*, 1984). The extract inhibits the growth of bacteria such as *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. Various compounds found in *Teucrium polium* are most likely responsible for this antimicrobial activity. These compounds include essential oils (mainly thymol), triterpenoids, flavonoids and compounds with free hydroxyl group (Essawi & Srour, 2000; Sarac & Ugur, 2007).

Antiulcer activity: Many studies found an antiulcer activity of *Teucrium polium* extracts. It is reported that the crude and ethanol extract of *Teucrium polium* increases healing of ulcer. The *Teucrium polium* extract stimulates mucin secretion and expression of epidermal growth factor receptor and thus reduces the gastric motility (Twaij *et al.*, 1987; Alkofahi & Atta, 1999; Mehrabani *et al.*, 2009).

Anticancer activity: *Teucrium polium* is also reputed for anticancer activity. Oil from *Teucrium polium* displayed anti-proliferative effect on tumour cell lines. Cytotoxicity assays and IC_{50} values show that the extract has significant anticancer properties (Rajabalian, 2008; Menichini *et al.*, 2009).

Cognitive improvement: Since diabetes-related oxidative stress is believed to be a factor to induce cognitive impairment (Studzinski *et al.*, 2009; Morrison *et al.*, 2010), a number of studies investigated whether the extract of *Teucrium polium* can efficiently reduce cognitive impairment in streptozotocin induced diabetic rats (Hasanein & Shahidi, 2012; Mousavi *et al.*, 2015). Both studies reported memory improvement in diabetic rats in response to *Teucrium polium* extract treatment.

1.9.4 Anti- diabetic effect of *Teucrium polium*, as assessed via glucose lowering action

Teucrium polium has been reported to have an anti-diabetic effect. Many studies have investigated the glucose lowering potential of *Teucrium polium* extracts both *in vivo*,

ex vivo and *in vitro* (Table 6).

Teucrium polium has been well studied, predominantly used in streptozotocin-induced diabetic rats (Gharaibeh *et al.*, 1988; Zal *et al.*, 2001; Vessal *et al.*, 2003; Esmaeili & Yazdanparast, 2004; Stefkov *et al.*, 2011; Tatar *et al.*, 2012), some other studies were performed on alloxan induced diabetic rat model (Afifi *et al.*, 2005; Mahjoub *et al.*, 2012) and few in normoglycaemic rats (Kasabri *et al.*, 2012; Ireng *et al*, 2016). However, most of these studies did not use positive drug controls (for example insulin and/or metformin). Two studies reported administration of the extract of *Teucrium polium* demonstrated no significant effect on blood glucose levels: Afifi *et al*., 2005 administered the treatments intranasally and Iriadam *et al*., 2006 used a relatively lower dose of Teucrium polium (85 mg/ kg) than generally used in other studies.

The extract has been assessed in pancreatic beta cells, both *ex vivo* and *in vitro* (Tables 6B and 6C). The results from all these studies indicate that the extract has insulin secretagogue properties. *Tecurium polium* has been reported to have beta cell protective activity, able to regenerate beta cell function (Table 6B). Flavonoids that are also found in *Teucrium polium* extracts have shown significant antioxidant effects. The antioxidant effect of the flavonoids of *Teucrium polium* is speculated to be responsible for this protective action (Esmaeili *et al.*, 2009).

Some insulinotropic mechanisms in beta cells have been investigated in several studies to understand the insulin secretagogue activity of the total extract of *Teucrium polium*. Insulin secretory vesicle formation and exocytosis is stimulated by an increase in intracellular calcium. Calcium influx is stimulated by depolarisation of the cell due to blockade of ATP. There are several K_{ATP} channels for controlling the potassium efflux (Fu *et al.*, 2013). Extracts of *Teucrium polium* were investigated in the presence of either Verapamil, a calcium channel blocker or Digoxin, a K_{ATP} channel opener. Both of them inhibited the activity of *Teucrium polium*. It shows that *Teucrium polium* can promote calcium channel opening and K_{ATP} channel blocking (Mirghazanfari *et al.*, 2010).

Table 6: Anti-diabetic potential of *Teucrium polium*; investigations of effect and putative mechanism(s).

6A) *In vivo* studies

Type of extract	Animal (numbers)	Study design	Result	Reference
Aqueous (decoction powder)	Male fisher rats; N=9 (Normoglycaemic control- water), N=5 (Normoglycemic Tp treated) N=9 (streptozotocin induced control rat) (65mg/kg b/w) N=17 (streptozotocin induced Tp treated rat) (65mg/kg b/w)	**Administration:** once orally, Blood collection-24 hour (ip, oral) 4 hour (iv)	Oral: Decrease in blood glucose level but not significant. i. p: Significant decrease in blood glucose level ($p<0.05$) i.v : Significant decrease in blood glucose level ($p<0.05$)	(Gharaibeh *et al.*, 1988)
Aqueous extract	Sprague-Dawley derived rats; N=5 (Normoglycemic control); N=5 (Normoglycemic Tp treated); N= 5 (streptozotocin induced control rats), N= 5 (streptozotocin induced Tp treated rats),	**Administration:** ad libitum (12 days) Blood collection-0,1,4,8,12 days	Significant decrease in blood glucose level ($p < 0.05$)	(Zal *et al.*, 2001)
Ethanol extract	Male Wistar albino rats, N= 8 (streptozotocin induced rat); N=12 (Tp treated streptozotocin induced rats); N= 8 (Normoglycemic)	**Administration:** Feeding orally (0.5 g powder/ kg body weight) Administration- orally (6 weeks) Blood collection- Day- 7,0,7,14,21,28,35,42.	Significant decrease in blood glucose level ($p<0.05$)	(Esmaeili & Yazdanparast, 2004)

Type of extract	Animal (numbers)	Study design	Result	Reference
Aqueous extract	Sprague Dawley rats, N=10 (streptozotocin induced Control rat); N=10 (streptozotocin induced and Tp treated rat); N=10 (normoglycemic rats), N=10 (normoglycemic Tp treated rats)	**Administration:** ad libitum (12 days) Blood collection-24 hour, 1 ml aliquot (twice a day)	Significant decrease in blood glucose level ($p<0.05$)	(Vessal *et al.*, 2003)
Aqueous extract	White French rabbits. N=10 control rabbits; N=10 Tp treated rabbits	**Administration:** Intranasal Blood collection: At time intervals :0, 15, 30, 45, 60, 120, 180, 240 and 300 min after last Tp treatment	No significant blood glucose decrease.	(Afifi *et al.*, 2005)
Ethanol extract	Male Wistar albino rats, N= 8 (streptozotocin induced rat); N=12 (Tp treated streptozotocin induced rats); N= 8 (Normoglycemic)	**Administration:** orally (6 weeks). Blood collection-0, 7, 14, 21, 28, 35, 42, 45 days (anesthetized).	Significant decrease in blood glucose level ($p<0.05$)	(Yazdanparas *et al.*, 2005)
Aqueous extract	Adult New Zealand rabbits <u>Normal group</u> (N=10 control rabbits, N=10 Tp treated rabbits) <u>Diabetic group</u> (N=10 control rabbits, N=10 Tp treated rabbits).	**Administration:** using a stomach tube Blood collection: 2, 4, 6, 8 hours after Tp administration	No significant blood glucose decrease.	(Iriadam *et al.*, 2006)
Aqueous extract	Sprague Dawley streptozotocin induced diabetic rats, N=7 (control rat); N=10 (Tp treated rats)	**Administration:** orally (4 weeks) Blood collection-after overnight fasting	Significant decrease in blood glucose level ($p<0.05$)	(Shahraki *et al.*, 2007)

Type of extract	Animal (numbers)	Study design	Result	Reference
Ethanol extract	Wistar rats, N=22 (streptozotocin induced rat), N=8 (normoglycemic rats), N=7 (streptozotocin induced Tp treated rats), N=7 (streptozotocin induced rats).	**Administration:** orally (30 days) Blood collection-Every 10 days (till 30 days) 1 ml/ rat (0.5 g powder/ kg body weight)	Significant decrease in blood glucose level ($p<0.05$)	(Ardestani *et al.*, 2008)
Aqueous extract	Male wistar rats, N=5 (streptozotocin induced rats), N=5 (normoglycemic rats); N=5 (2% Tp treated strptozotocin induced rats), N=5 (4% Tp treated streptozotocin induced rats).	**Administration:** ad libitum (2 weeks) Blood collection-2 weeks	Significant decrease in blood glucose level ($p<0.05$)	(Vahidi *et al.*, 2010)
Ethanol solution	Wistar streptozotocin induced diabetic rats, (35 mg/kg body weight), N=5 (control rat), N=10 (Tp treated, i.g.), N=5 (Tp treated; i.p.), N=5 (glibenclamide treated rats).	**Administration-** i.p.(once) Blood collection-4,8,12 hour	Significant decrease in blood glucose level ($p<0.05$)	(Stefkov *et al.*, 2011)
Aqueous extract	Wistar rats, <u>Normoglycemic rats</u> N=8 (control rats); N=8 (Tp aqueous extract); N=7 (Tp ethanolic extract), <u>Alloxan induced diabetic rats</u> N=7 (control rats); N=7 (Tp aqueous extract treated); N=7 (Tp ethanolic extract).	**Administration:** i.p. (once); Blood collection-72 hours	Significant decrease in blood glucose level ($p<0.05$)	(Mahjoub *et al.*, 2012)

Type of extract	Animal (numbers)	Study design	Result	Reference
Aqueous and ethanolic extract	18 streptozotocin induced diabetic rats, N=6 (normoglycemic rats), N=6 (hyperglycemic rats), N=12 (Tp treated hyperglycemic rats).	**Administration:** orally (14 days) Blood collection-0,30, 90, 120 minutes.	Significant decrease in blood glucose level ($p<0.05$)	(Tatar *et al.*, 2012)
Aqueous extract	30 normoglycemic rats, N=8 (Tp treated), N=8 (insulin treated), N=6 (metformin treated), N=8 control rats.	**Administration-** i.v (once) Blood collection: 0, 30, 60, 120, 180, 210 minutes.	Significant decrease in blood glucose level ($p<0.01$).	(Ireng *et al.*, 2016)

Tp denotes Teucrium polium, i.p. denotes intraperitoneal, i.v. denotes intravenous. i.g. denotes intragastric.

6B) Studies on pancreatic beta cells *ex vivo*

Study design (Time interval after treatment and method)		**Outcome**	**Reference**
Duration of *Teucrium polium* (Tp) treatment	**Experiments**		
24 hour after Tp treatment	Histological study of pancreas	Regeneration of islet	(Vessal *et al.*, 2003)
2 hour after Tp treatment	ELISA from serum & Pancreatic islet isolation	Regeneration of islet & Enhanced insulin release	(Yazdanparas *et al.*, 2005)
1 hour after Tp treatment	Pancreatic islet isolation & ELISA of supernatant	Increased insulin secretion significantly	(Esmaeili & Sadeghi, 2009; Mirghazanfari *et al.*, 2010; Monfared & Pournourmohammadi, 2010; Mahjoub *et al.*, 2012)
30-minute treatment with Tp and molybdate	Pancreatic islet isolation & ELISA		
24 hour after feeding Tp	In situ rat pancreas isolation and ELISA		
24 hours after Tp feeding	ELISA of serum		
3 weeks continuous feeding and 1 hour after feeding Tp	ELISA of serum insulin	Reduction in insulin resistance	(Nosrati *et al.*, 2010)

6C) Cell culture studies *in vitro*.

Cell line	**Study duration & control**	**Result**	**Reference**
INS-1 cells	30 mins after Tp treatment **Positive control:** Glucose	Insulin secretion increased by a factor of 2.6 (2.2 ng/cell).	(Stefkov *et al.*, 2011)
MIN6 cell line	1 hour after Tp treatment **Positive control:** Alanine & GLP-1	Significant ($p < 0.01$) increase of insulin secretion (1200 as % control).	(Kasabri *et al.*, 2012)

Another mechanism for blood glucose reduction is inhibition of alpha-glucosidase and inhibition of alpha-amylase. Alpha-glucosidase inhibitors reduce the metabolism and breakdown of carbohydrates and thus lower the postprandial blood glucose level (He, 1998; Evans *et al.*, 2013). *Teucrium polium* was found not to have alpha glucosidase inhibitory activity (Kasabri *et al.*, 2011). Inhibition of alpha amylase is another way to lower blood glucose levels. Alpha amylase is an enzyme that catalyses the breakdown of polysaccharides and formation of glucose. Alpha amylase inhibitors obstruct the activity of this enzyme and slows down the carbohydrate metabolism, thus leads to a reduction in blood glucose level (Bressler & Johnson, 1992; Kasabri *et al.*, 2011). When assessed, *Teucrium polium* did not appear to have any alpha amylase inhibitory effect (Kasabri *et al.*, 2011). The total extract of *Teucrium polium* appears to contain compounds that act on liver cells to reduce glucose production and release. The extract has been reported to reduce glycogenolysis as well as inhibit gluconeogenesis by inhibiting glucose-6-phosphatase and fructose-6-phosphatase. This contributes to the blood glucose lowering action of *Teucrium polium* (Stefkov *et al.*, 2011). The discussed mechanisms are summarized in Figure 5.

Although most of the published studies examined total plant extract for its glucose lowering effect, some of the studies attempted to examine the activity of certain compounds that are putatively identified in *Teucrium polium*. Compounds with glucose lowering potential are listed in Table 7. Rutin, apigenin and quercetin have been putatively found via LCMS study (Ireng *et al.*, 2016). The compounds assessed may have different chemical forms (glycosides or structural isomers) to those identified in *Teucrium polium*. The exact compound(s) that deliver the reported glucose-lowering effect of the total extract are yet to be elucidated.

Table 7: Compounds putatively identified in *Teucrium polium* and possible "anti-diabetic" activities.

Proposed anti-diabetic compounds from *Teucrium polium*	Probable mechanism observed in other experiments	Assays conducted	References
Luteolin	Reduces oxidative stress.	Insulin secretion assay	(Qian *et al.*, 2010; Stefkov *et al.*, 2011)
Apigenin	AMPK pathway activation	Glucose uptake assay Insulin secretion assay	(Zang *et al.*, 2006; Mirghazanfari *et al.*, 2010)
Cirsiliol	Promoting insulin release	Insulin secretion assay	(Rizk *et al.*, 1986; Verykokidou-Vitsaropoulou & Vajias, 1986; Stefkov *et al.*, 2011)
Cirsilineol	Promoting insulin release	Insulin secretion assay	(Carreiras *et al.*, 1989; Stefkov *et al.*, 2011)
Silymarin	Stimulates insulin secretion	Insulin secretion assay	(Lettéron *et al.*, 1990; Soto *et al.*, 2003; Kadifkova Panovska *et al.*, 2005; Meng *et al.*, 2016)
Quercetin-3-rutinoside	Inhibition of alpha glucosidase	Insulin secretion	(Esmaeili *et al.*, 2009)

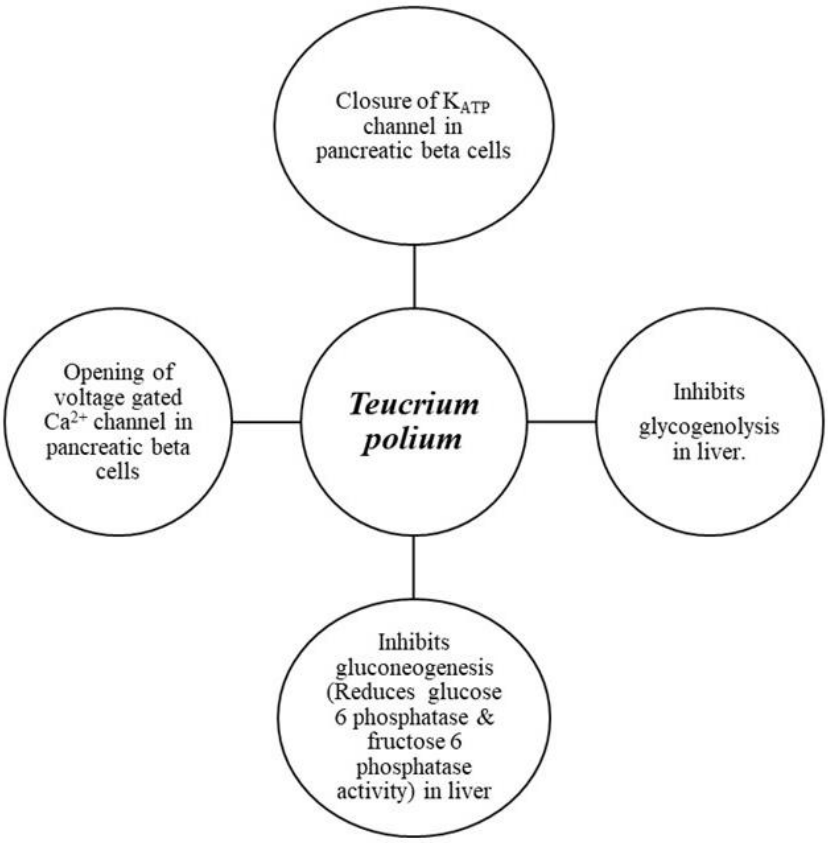


Figure 5: Hypothesized activities of the *Teucrium polium* extract. The *Teucrium polium* extract promotes insulin secretion from pancreatic beta cell by inhibiting K_{ATP} channel and promoting Ca^{2+} voltage gated channel. *Teucrium polium* also influences liver activity by reducing the phosphatase enzymes.

1.10 The present study

The principal aim of this study is to investigate the glucose lowering mechanism of the total *Teucrium polium* extract and molecular signalling pathways involved in the process. As detailed above, compounds in *Teucrium polium* have putative insulin releasing properties. Such studies suggest that it helps resolving insulin resistance. To date, most of the research investigating the glucose lowering effect of *Teucrium polium* has only exhibited changes in blood glucose level and insulin secretion. This study aims to investigate:

1. Whether the *Teucrium polium* extract acts as an insulin secretagogue and promotes insulin secretion from pancreatic beta cells.

2. Whether the *Teucrium polium* extract acts as an insulin mimetic.

3. The possible molecular signalling pathways involved in the action of the *Teucrium polium* extract mentioned in aims 1 or 2 described above.

2.0 Materials and Methods

2.1 Materials

Millex-GS Syringe Filter Unit (0.22 µm) was purchased from Merck Millipore, Bayswater, Australia. Folin & Ciocalteau's phenol, sodium carbonate, tannic acid, ethanol, ninhydrin, sodium chloride, paraformaldehyde, glucose oxidase, horse radish peroxidase, IBMX (3-Isobutyl-1-methylxanthine), dexamethasone (water soluble), insulin solution (from bovine pancreas), newborn calf serum (NCS), dimethyl sulfoxide (DMSO), Oil Red O, collagen (Type I solution from rat tail), Triton® X-100, Tween® 20, gliclazide, glycerol, glycine, bromophenol blue, potassium chloride, potassium hydroxide, sodium phosphate dibasic anhydrous, potassium phosphate monobasic, calcium chloride dihydrate, 2-mercaptoethanol, DAPI (4',6-diamidino-2-phenylindole), gliclazide, Triton X-100, 4-nitrophenyl phosphate disodium salt hexahydrate, propidium iodide, rotenone, antimycin A, 2 deoxy glucose (2DG), adenosine 5´-triphosphate disodim salt hydrate (ATP), N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES), Corning® 96-well plates (polystyrene, TC-treated, black flat bottom wells, sterile, black), Ampliflu™ red (for fluorescence), p-Nitrophenyl phosphate (pNPP), Trypan Blue solution (0.4%), MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Oligomycin, FCCP (carbonyl cyanide ptrifluoromethoxyphenylhydrazone) and rotenone/antimycin A were purchased from Sigma- Aldrich (Castle Hill, Australia). Methanol, TrypLE™ Express enzyme (1X), Gibco Hank's balanced salt solution (HBSS), glucose solution, Hyclone penicillin-streptomycin 100X solution, Pierce BCA protein assay kit, Pierce^{TM} protease inhibitor mini tablets were purchased from Thermo Fisher Scientific (Scoresby, Australia). Vectashield® antifade mounting medium was purchased from Vector Laboratories, Inc. (Burlingame, USA). Foetal Bovine Serum (FBS) was purchased from SerANA (WA) Pty Ltd (Bunbury, Australia). Anti-glucose transporter GLUT2 antibody and anti-glucokinase antibody - N-terminal were bought from Abcam (Melbourne, VIC, Australia). Mercodia insulin rat ultra-sensitive ELISA kit, rosiglitazone and BioVision glycogen colorimetric/fluorometric assay kit were purchased from Sapphire biosciences (Redfern, NSW, Australia). Fluo-4 AM, Nunclon^{TM} delta surface 96-well plate, glucose solution, Gibco- DMEM (high glucose, GlutaMAX$^{(TM)}$, pyruvate) and HyClone™ RPMI 1640 medium were purchased from Life Technologies Australia Pty Ltd. (Mulgrave, VIC, Australia). Hyclone phosphate buffered saline (PBS) (1X) was bought from In Vitro Technologies (Nobel Park North,

VIC, Australia). CellTiter-Glo® Luminescent Cell Viability Assay kit was purchased from Promega (Alexandria, NSW, Australia). Ammonium persulfate (APS), Clarity™ Western ECL Substrate, 4x Laemmli Sample Buffer, protein ladder (Precision plus protein ® standard) and tetramethylethylenediamine (TEMED) were purchased from BIO-RAD Laboratories Pty. Ltd., Life Science (Gladesville, NSW, Australia). CulturPlate™ -96 white plates were purchased from PerkinElmer Pty Ltd. (Springvale road, VIC, Australia).

2.2 Plant Collection

The aerial parts of *Teucrium polium L (Labiatae)* were collected from the Al-Salt region (25 km west of Amman), Jordan. The plant was authenticated by Professor Dawud Al-Eisawi, of the Biological Sciences Department, University of Jordan. The material was brought into Australia under quarantine: AQIS permit 200515757. The leafy parts of the plant material were freeze dried and stored.

2.3 Plant Extraction

Plant extraction was prepared as outlined by Ireng *et al.*, 2016. Five gram of the freeze dried *Teucrium polium* was placed in a Soxhlet apparatus (Electromantle®-EM1000/CE) and refluxed three times (4 hours) at 80-100°C in 400mL of 90% methanol. The extract was then allowed to cool and the residual non-plant material in the thimble was discarded. The extract was then concentrated in a rotary evaporator (Buchi Rotavapor- R -200) at a fixed temperature of 45(±2) °C for 30-40 minutes. The concentrated plant extract was then made up to a 200-mL volume with 90% methanol. Then 2 mL aliquots were pipetted into separate glass vials (light protective) and dried under vacuum at room temperature. Thereafter, the vials were immediately purged under nitrogen (supplied by Comet ™ CIGWELD), sealed and stored at -20°C. Each vial contained extract derived from a 50-mg equivalent of original plant material. All extracts used in this study were generated from the same single batch of extraction. This batch was monitored for total phenolic, tannin and protein content throughout the 3-years of this research period.

2.4 Plant Extract Reconstitution

Dried plant extracts were reconstituted in filter (0.22µm) sterilised phosphate buffered saline (PBS) (10 mM disodium hydrogen phosphate (Na_2HPO_4), 137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 1.8 mM potassium dihydrogen orthophosphate (KH_2PO_4)) (pH 7.4). Insoluble material was sedimented by brief centrifugation (500 x g for 5 minutes). The supernatant was collected and used in the studies. Extracts were reconstituted fresh on the day of each experiment and stored for not more than 7 days at 4°C.

2.5 Total phenolic content of the *Teucrium polium* extract

The phenolic contents have been studied in other studies to monitor the chemical stability of the plant extract. In the Folin-Ciocalteu assay, the reagent reacts with reducing substance and measures the total reducing capacity (Lopes *et al.*, 2012). This is a routine quality control test and measurement of antioxidant stability (Ainsworth & Gillespie, 2007). This colorimetric method depends on the transfer of electrons from phenolics to phosphomolybdic complex in alkaline medium developing a blue complex (Bajaj & Devsharma, 1977; Hagerman, 2002; Von Martius *et al.*, 2012). Phenolic content was evaluated in our study using a modified protocol for Folin-Ciocalteu assay reported by Singleton, Rossi and Von Martius (Singleton & Rossi, 1965; Von Martius *et al.*, 2012). Briefly, dried *Teucrium polium* extract was reconstituted as described in section 2.4 and further diluted in milli –Q water. One mL sodium carbonate (29% (w/v)) solution and 0.5 mL Folin-Ciocalteu reagent was added to the *Teucrium polium* extract solution. The absorbance of the solvent was measured after 30 minutes at 760 nm wavelength against a reagent blank (Folin-Ciocalteu reaction with water). Results were compared against a fresh tannic acid (1-100 µg/mL) standard curve.

2.6 Total tannin content of the *Teucrium polium* extract

Total tannin content was monitored using a modified method of Devsharma and Hagerman (Bajaj & Devsharma, 1977; Hagerman, 2002). In brief, 1 mL of the *Teucrium polium* extract (5 mg/mL), 2.5 mL of acidic sodium chloride solution

(6.1 M sodium chloride (NaCl) in 5% of conc. sulfuric acid solution) and 1.5 mL of 0.25% (w/v) gelatine solution were combined and mixed by shaking for 15 minutes. The solution was then centrifuged at 250 x g for 5 min. The supernatant was used to analyse phenolic content as described in the section 2.5. Total tannin was calculated by subtracting the readings of this assay from those for total phenolics.

2.7 Total protein content of the *Teucrium polium* extract

The measurement of protein content was done using a Bicinchoninic Acid (BCA) (Pierce BCA Protein Assay Kit) to estimate the amount of protein in cell lysates. This procedure relies on a Biuret reaction based method where cupric ions (Cu^{2+}) are reduced to cuprous ions (Cu^{+}) after placing the proteins in alkaline solution. A dark purple colour forms because of the reaction of BCA reagent and Cu^{+} ions.

The BCA kit comprises of BCA Reagent A (containing sodium bicarbonate, bicinchoninic acid, sodium carbonate, and sodium tartrate in 0.1 M sodium hydroxide); BCA Reagent B (comprising 4% cupric sulphate) and albumin standard ampules containing bovine serum albumin in saline and sodium azide (2 mg/mL). Various concentrations of bovine serum albumin (BSA) standards were prepared by diluting it in 1X phosphate buffered saline (PBS) and the subsequent concentrations ranged from 50- 2000 µg/mL. Assay samples were serially diluted in PBS. The samples and standards were transferred to a 96-well microplate in triplicates (25 µL/well) where PBS was used as a blank. A mixture of the two Pierce BCA kit was prepared in the ratio of 50 (Reagent A): 1 (Reagent B). The mixture of reagents was added immediately to the samples, standards, and blanks in the microplate. The microplate was covered with cling film to avoid any loss due to evaporation and incubated at 37°C for 30 minutes. The plate was shaken and then read on an EnSpire® Multimode plate reader (Perkin Elmer, USA) at a wavelength of 562 nm. A standard curve was produced for duplicate BSA protein standard. The absorbance of each sample was corrected against a blank.

2.8 Total alanine content of *Teucrium polium* extract

Alanine was quantified in the *Teucrium polium* extract using a method that was adapted and modifiedfrom Jones *et al.*, Petraco *et al.* and Moore *et al.* (Moore & Stein,

1954; Jones *et al.*, 2002; Petraco *et al.*, 2006). In brief, 100 µL alanine standard (0-200 µM) or the *Teucrium polium* extract (1 mg/mL) was mixed with 75 µL of 3% (v/v) ninhydrin solution. The solution was then heated at 80°C for 30 minutes. The solution was then cooled and 100 µL of 50% ethanol was added. The absorbance of the solution was measured at 570 nm against alanine standards.

2.9 BRIN-BD11 cell culture

The insulin secretagogue effect of the *Teucrium polium* extract was assessed using the rat pancreatic beta cell line BRIN-BD11, which was a kind donation from Professor Philip Newsholme, School of Biomedical Sciences, Curtin University. The cells were grown as previously described (Cunningham *et al.*, 2005). In brief, BRIN-BD11 cells were grown in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% (v/v) fetal bovine serum (FBS), and 100 units/mL penicillin and 0.1 mg/mL streptomycin. The cells were maintained in 5% CO_2 in a NUAIRE Air-Jacketed, DHD autoflow automatic CO_2 incubator NU 5510E at 37°C, 95% air and humidified atmosphere. Cell passages 22-30 were used for all the experiments and the cells were subcultured after becoming 70-80% confluent. Cells were typically grown in T75 flasks.

2.10 Freezing of cells

Cells were grown in tissue culture medium (RPMI) containing 10% FBS as described above and then detached from the flask by adding 3 mL TrypLE™ Express. The action of TrypLE™ Express was stopped using 5 mL medium and the cell suspension was placed in a centrifuge tube followed by centrifugation at 500 x g for 5 minutes using an Allegra® X-12 centrifuge (Beckman Coulter, CA, USA). Medium was discarded by pipetting from the tube containing the cell pellet. The remaining cell pellet was resuspended in 0.5 ml of freeze medium (50% growth medium, 10% DMSO, 40% FBS) and transferred into cryogenic vials (Corning, Tewksbury, MA). The cryogenic vials were then immediately frozen at −80°C for 24 hours before being placed in a liquid nitrogen dewar (5 x10^5 cells/ vial).

2.11 Thawing of cells

Cryopreserved cells were thawed rapidly at 37ºC. One mL of warm tissue culture medium was added to the cells and the cell solution was transferred into a 25 cm^2 tissue culture flask (Nunc, Thermo Fisher Scientific). Then 4 mL of warm tissue culture medium was added to the cells, which was followed by incubation at 37°C, 5% CO_2. The flask was observed every 2 hours and medium was changed after the cells attached to the flask (6-12 hours).

2.12 Mycoplasma test

Mycoplasma is a potential threat for cell culture. It interferes with cellular function and morphology. Mycoplasma should be tested before doing any experiment (Drexler & Uphoff, 2002). Mycoplasma testing was done to detect the presence or absence of mycoplasma in the cell line used for all the experiments in this study (results attached in appendix 2) and only mycoplasma negative cells were used in our experiments. Mycoplasma detection was performed routinely every 3 months in Curtin Health Innovation Research Institute (CHIRI) by Dr Joanna Kelly using polymerase chain reaction (PCR) based modified method published by Cord C. Uphoff and Hans G. Drexler (Uphoff & Drexler, 2014).

2.13 Cell viability assays

Cell viability and proliferation assays are used to investigate cytotoxic effects of any drug or treatment. Viability tests may also be used to study the proliferative effect of a compound or treatment (Yang *et al.*, 1996). We used 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and acid phosphatase (APH) assay for cell viability.

2.13.1 MTT tetrazolium assay

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay relies on formation of the purple coloured formazan crystals which is solubilized in an organic solvent before measuring the absorbance. The present study was done as described by Kauffman and his colleagues (Kauffman *et al.*, 2012). In the present

study, cells were seeded (5000 cells/well in 100 µL medium) in 96-well plates. They were grown overnight to adhere in the well. Culture medium was removed and fresh growth medium (100 µL) was added to all wells. Cells were treated with various concentrations of *Teucrium polium* extract dissolved in culture medium and incubated for 24 hours. After incubation, 20 µL of MTT (5 mg/mL) was added to all wells and incubated for 4 hours. Supernatant were removed after incubation and the formazan crystals of cells were solubilised in 100 µL of neat DMSO. Plates were then read at a wavelength of 550 nm and data was recorded on a plate reader (EnSpire® Multimode plate reader, Perkin Elmer).

2.13.2 Acid phosphatase assay

This method is based on the conversion of the p-Nitrophenyl phosphate (pNPP) to p-nitrophenol catalysed by cytosolic acid phosphatase. The amount of conversion indicates the number of viable cells. Absorption of p-nitophenol occurs at 405 nm wavelengths. This absorption is indicative of cell number (Yang *et al.*, 1996; Friedrich *et al.*, 2007; Patel *et al.*, 2016).

The method was followed as described in Friedrich *et al.* 2007 and Patel *et al.* 2016. Briefly, 5000 cells/well were seeded in a 96-well plate. Medium was discarded on the next day and cells were treated with different doses of *Teucrium polium* extract in growth medium for 24 hours. Supernatant was discarded after 24 hours. Cells were washed once with phosphate buffer saline (PBS). Cells were incubated for 90 minutes at 37°C after the addition of acid phosphatase (APH) assay buffer supplemented with 2 mg/mL para-nitrophenylphosphate (pNPP), 0.1% (v/v) Triton X (dissolved in 0.1 M Citrate buffer). After incubation, 15 µL of 1N NaOH was added. Absorbance was monitored within ten minutes at 405 nm and data was recorded on a plate reader (EnSpire® Multimode plate reader, Perkin Elmer).

2.14 Insulin secretion

BRIN-BD11 cells were grown in 24-well plates at 1.5×10^5 cells/well in 1 mL of medium. Medium was discarded after 24 hours followed by washing twice with PBS. All the treatments were done in Krebs Ringer Bicarbonate (KRB) buffer in two steps relating to acute insulin secretion. Cells were treated with KRB buffer (115 mM

sodium chloride, NaCl; 4.7 mM potassium chloride, KCl; 1.28 mM calcium chloride dihydrate, $CaCl_2.2H_2O$; 1.2 mM potassium dihydrogen phosphate, KH_2PO_4; 1.2 mM magnesium sulfate heptahydrate, $MgSO_4.7H_2O$; 10 mM sodium hydrogen carbonate, $NaHCO_3$; 10 mM N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid), HEPES; 0.5% bovine serum albumin. BSA; pH 7.4) supplemented with 1.1 mM glucose for 40 minutes for glucose starvation. In the first step, cells were subjected to starvation for 40 minutes in low glucose Krebs ringer bicarbonate (KRB) buffer containing 1.1 mM glucose. The glucose concentration was determined based on the previous studies (McClenaghan *et al.*, 1996a). Afterward, stimulation for 30 minute with treatment compound or *Teucrium polium* extract was done in KRB buffer. The supernatant was saved for insulin measurement and cells lysis was done by adding 120 µL ice-cold Radio-immunoprecipitation Assay (RIPA) buffer after a PBS wash.

Insulin ELISA: Insulin release by BRIN-BD11 was measured using the Mercodia Rat Ultrasensitive Insulin ELISA kit. This assay uses a solid two-phase enzyme immunoassay following a sandwich technique. It uses two monoclonal antibodies that target insulin. Further binding occurs between insulin and the anti-insulin antibodies existing in the micro-titration wells and anti-insulin antibodies conjugated with peroxidase in the solution (González-Abuín *et al.*, 2014; Song *et al.*, 2014; Alcarraz-Vizán *et al.*, 2015). This method was followed according to the manufacturer's protocol. Twenty-five µL calibrators, controls and samples were added to an antibody coated 96-well microtiter plate. Afterwards, 100 µL of enzyme conjugate solution was added to each well. The plate was incubated for 2 hours on a Corning® LSE™ digital microplate shaker (700 rpm) at room temperature. The plate was washed 6 times using the washing buffer supplied with the kit. After washing, 200 µL of substrate TMB was added and incubated for 15 minutes at room temperature. Stop solution (50 µL) was then added and the plate was shaken for approximately 5 seconds. The absorbance of each well was measured at 450 nm and data was recorded on a plate reader (EnSpire® Multimode plate reader, Perkin Elmer). Sample readings were normalized by measuring the protein concentration of each sample in a BCA protein quantification assay (section 2.7).

2.15 Measurement of calcium (Ca^{2+}) influx

Calcium was measured with Fluo-4 AM calcium indicator using confocal microscopy

(Ultraview Vox system, Perkin Elmer, USA). Fluo-4 AM is a acetoxymethyl ester probe (Lambert, 1999) with a fluorescent intensity that increases by almost one hundred fold after it binds with Ca^{2+} (Harkins *et al.*, 1993). Imaging using this probe has exposed the spatial dynamics of different initial processes of calcium signalling (Cheng *et al.*, 1996; Bootman *et al.*, 1997).

The present study used a modified method of Maffucci *et al.* (Maffucci *et al.*, 2009). In brief, BRIN-BD11 cells (50,000 cells/ well) were plated in a 6-well plate. The growth medium was removed after 24 hours and replaced with serum-free medium for 6 hours. After 6 hours, supernatant was removed and the cells were incubated for 45 minutes with 4 mM Fluo-4-AM dye (in 2 mL of Hank's balanced salt solution (HBSS) containing 0.5% BSA, 2 mM $CaCl_2$) at 37°C to allow the dye to be uptaken by the cells. Cells were then washed twice in 2 mL of HBSS to remove excess dye which is non-specifically associated with the cell surface. Cells were then incubated for 30 minutes in HBSS (with 1.1 mM glucose added) for de-esterification of the Fluo-4-AM dye which makes the dye capable to bind calcium. Supernatant was removed and the cells were washed once with PBS to eliminate unwanted calcium or dye. The samples were then ready to be analysed using a ultraview spinning disk confocal microscope (using 20X objective) equipped with a chamber for live imaging at 37°C supplied with 5% CO_2. The cells in wells were then treated with Krebs Ringer bicarbonate buffer containing 1.28 mM $CaCl_2.2H_2O$. Calcium entering the cells react with the dye to produce an intensified fluorescent colour which is seen under the microscope. Basal fluorescence was recorded (e.g. time point 0 mins). After recording basal fluorescence, cells were stimulated with 5.5. mM glucose with or without the *Teucrium polium* extract (500 µg/mL)[1] or a sulfonylurea drug gliclazide (5 µM). Fluorescence as a measure of change in calcium intake into cells was measured for 10 min at 37°C. Images were acquired using an Ultraview Vox spinning disk confocal microscope, using the Volocity software (Perkin Elmer). Fluorescence was measured continuously from 0 seconds - 10 minutes, using a Plan Apo 20x objective lens (N.A. 0.75), with 488 nm laser and corresponding 525/50 filter, with a maximum exposure time of 100 ms. ATP (1000 nM) was added as a positive control at the endpoint (10 minutes) to confirm cell labelling and viability. The images were acquired from the

average of eight frames and intensity of Fluo-4 AM calcium fluorescence of captured images from 0 - 10 minutes was measured using ImageJ ® software (Ver. 1.50i).

2.16 Intracellular ATP level measurement using CellTiter-Glo® Luminescent Assay

The concentration of ATP released in response to the *Teucrium polium* extract treatment was measured using a CellTiter-Glo® luminescent assay. This is a homogenous method using a reagent that leads to cell lysis and produces a luminescent signal (Crouch *et al.*, 1993). This glo-type signal is proportional to the amount of ATP present (Kangas *et al.*, 1983; Chen *et al.*, 2016).

ATP measurement was done in accordance with the CellTiter-Glo® luminescent assay kit manufacturer's protocol. In brief, BRIN-BD11 cells were grown in a CulturPlate™ 96-well white plate for 24 hours. The next day, control wells containing KRB buffer without cells were prepared to acquire a value for background luminescence. Cells were treated with alanine, the *Teucrium polium* extract or control KRB buffer and incubated for 30 minutes at room temperature. CellTiter-Glo® reagent (100 µL) was added to each well. The contents of wells were mixed for 2 minutes on an orbital shaker to prompt cell lysis. The plate was incubated at room temperature for 10 minutes to stabilize the luminescent signal. Finally, luminescence was recorded on a plate reader (EnSpire® Multimode plate reader, Perkin Elmer). Data was normalized by measuring protein content in each well using a BCA protein quantification assay (section 2.7).

2.17 Extracellular flux analysis of mitochondrial and glycolytic metabolism

Extracellular flux analysis was done using a Seahorse Bioscience XF analyser. In the test, cells are treated consecutively with: oligomycin which works as ATP synthase inhibitor; carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) which is a protonophoric uncoupler; and rotenone and antimycin A, which function as electron transport inhibitors. These stress conditions give information about basal respiration, maximum respiration rate, non-mitochondrial respiration and proton leak. The oxygen consumption by mitochondria follows a trend illustrated in Figure 7. Oligomycin

treatment leads to a sharp drop in oxygen consumption, whereas FCCP treatment markedly increases the oxygen consumption rate. Finally, Antimycin A and rotenone stop respiration and cause a sharp decline in oxygen consumption.

Glycolysis is measured by the estimation of the extracellular acidification rate (ECAR) of the surrounding medium, which comes from the excretion of lactic acid per unit time (Wu *et al.*, 2007). Moreover, other metabolic processes in cells such as CO_2 production by TCA cycle can change the pH of the medium.

To investigate glycolysis, the glycolysis stress test is often utilized. For this assay, glucose, oligomycin, and 2-DG are added sequentially. Glucose is provided to feed glycolysis, and the change between ECAR before and after addition of glucose is an estimation of the glycolytic rate. Oligomycin prevents ATP synthase in the electron transport chain (ETC), which reduces the ATP/ADP ratio and pushes glycolysis to its maximal capacity. The change between ECAR before and after the addition of oligomycin is equal to the glycolytic reserve capacity. 2-DG hinders glycolysis as it competes with glucose and provides a baseline ECAR measurement. ECAR after 2-DG addition is responsible for the non-glycolytic ECAR of cells (Figure 8).

In the present study, the Seahorse Bioscience XFe96 Flux analyser was used according to the instructions of the manufacturer. The mitochondrial and glycolytic stress test kits used were optimised as described by Krause (Krause *et al.*, 2014) to ensure lowest concentration and maximum effect. In brief, 10,000 BRIN-BD11 cells were seeded into 96-well assay plate in 100 µL of complete RPMI medium and incubated for 24 hours in 5% CO_2 (seeding density of BRIN-BD11 cell was previously optimized to meet the criteria of manufacturer for OCR and ECAR measurement).

During the day of the mitochondrial stress test, the culture medium was replaced with serum free Dulbecco's Modified Eagle's Medium (DMEM**)** medium (pH 7.4) containing 2.5 mM glucose, 1 mM sodium pyruvate in the absence of biocarbonate. The glycolytic stress test was performed in DMEM containing no glucose. Plates were then placed in a non-CO_2 incubator at 37°C for 60 minutes for the equilibration and adaptation of the cells in the new medium.

Thirty minutes prior to the end of the equilibration period, the *Teucrium polium* extract (500 µg/mL) or vehicle control were added to the designated wells and cells were incubated for the remaining 30 minutes. In parallel, the calibration of the XFe96

sensor cartridge was initiated. The cartridge was incubated with callibrant solution for pre-hydration 24 hours before the experiment using a non-CO_2 incubator at 37°C. Drugs were injected to give a final concentration of 2 µM olygomyacin, 0.3 µM FCCP and 1µM Rotanone/Antimycin A.

After the incubation and calibration, the culture plate was transferred to the instrument and basal respiration was measured followed by a series of drug injections. The injection sequence for mitochondrial stress test was followed as outlined in (Figure 6). For glycolytic stress test, the injection strategy was performed as outlined in (Figure 7). Measurements of OCR and ECAR were done using 5 cycles of alternate mixture. Measurements were taken in every 2 minutes. This measurement was done to ensure that there is no notable change in cell numbers after different treatments and injections. The inhibitors were prepared in DMSO. OCR and ECAR of the cells receiving equal concentration of DMSO (in DMEM medium) alone were also measured to check for cell viability and stability.

The data collected was analysed as described in the studies of Chen and Keane (Keane *et al.*, 2015; Chen *et al.*, 2016). Basal respiration was determined by deducting the minimum OCR after adding rotenone/ antimycin A (non-mitochondrial respiration) from the OCR measurement immediate before oligomycin addition. Calculation of proton leak was done by deducting non-mitochondrial respiration from the OCR measurement after oligomycin injection. ATP production linked OCR was determined by the difference between proton leak and basal respiration. Maximum respiration was calculated by deducing the OCR of non-mitochondrial respiration from the maximum OCR after FCCP addition. Reserve capacity was determined from the difference between the basal respiration and maximum respiration. Glycolytic response was calculated by deducting maximum ECAR after the inclusion of glucose from the last ECAR before glucose addition. Glycolytic capacity was calculated by subtracting the minimum ECAR after 2DG addition from the maximum ECAR after oligomycin injection. The glycolytic reserve was calculated from the difference between the glycolytic capacity and the glycolysis. Each experiment was done independently four times in at least triplicate to each group.

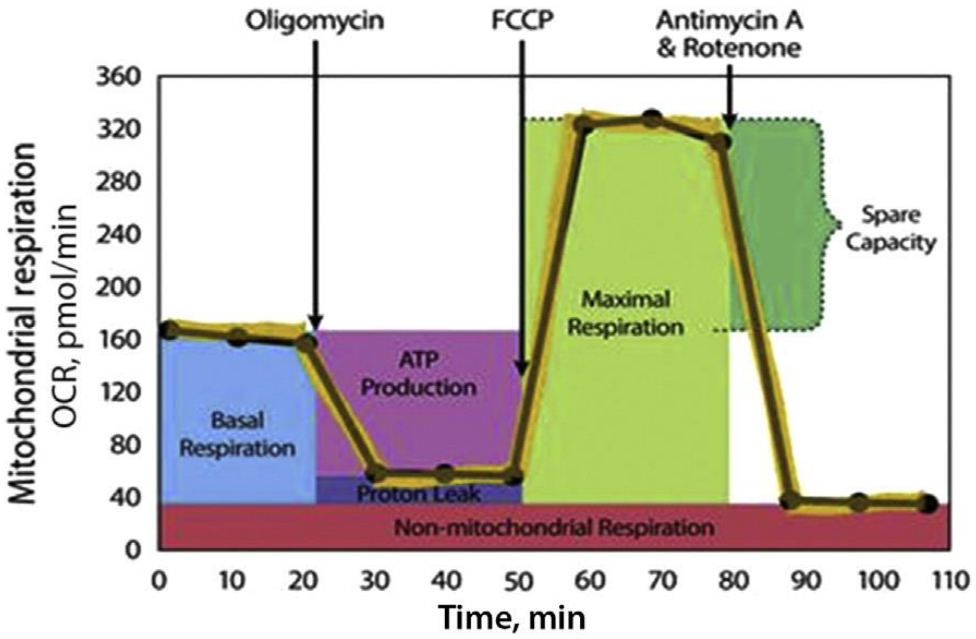


Figure 6: Schematic mitochondrial stress test. Various steps of mitochondrial respiration in response to mitochondrial stress modulator are observed. Oligomycin which works as an ATP synthase inhibitor; carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) which is a protonophoric uncoupler; and rotenone and antimycin A, which function as electron transport inhibitors were added at the specified timepoints. The oxygen consumption rate was measured every 5 minutes capturing time points before and after each addition: Basal respiration was monitored in the first phase. In the successive phases ATP production, maximal respiration and spare capacity were calculated. (Reproduced from Agilent technologies (2016) with permission. Appendix 1B).

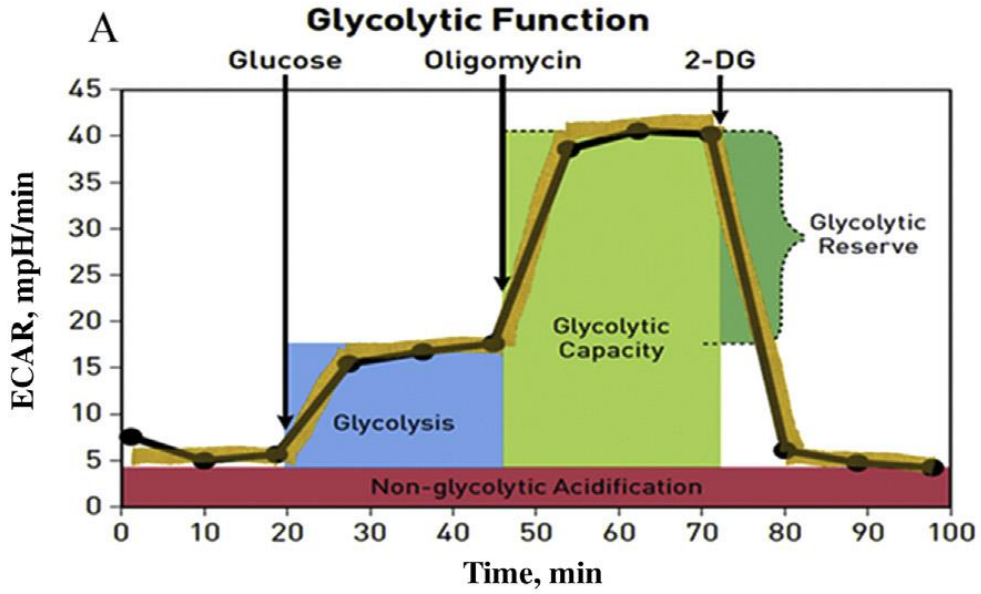


Figure 7: Schematic glycolytic stress test. This test is used to monitor glycolysis, glycolytic capacity and glycolytic reserve. Addition of stress modulator such as glucose, oligomycin and 2DG induces all these effects at various time points. This extracellular acidification rate (ECAR) is monitored to check various effect after the addition of stress modulators. (Reproduced from Agilent Technologies (2016) with permission; Appendix 1B).

2.18 Glucose consumption assay

Glucose consumption was measured using an Amplex red reagent-based glucose/ glucose oxidase detection method. In this experiment, glucose reacts with glucose oxidase and forms D-gluconolactone and hydrogen peroxide (H_2O_2). The H_2O_2 reacts with the Amplex red reagent to form a red fluorescent oxidation product (resorufin) in the presence of horseradish peroxide (Steiner *et al.*, 2011). In the present study, the method described by Chen *et al.* was followed (Chen *et al.*, 2016). In brief, the cells were seeded and grown in 24-well plates. On the day of the experiment, the medium in the wells of plates was discarded. Cells were washed with Hyclone phosphate buffered saline (PBS) and then treated with various concentrations of the *Teucrium polium* extract or drugs dissolved in growth medium. Supernatant was collected after 30 minutes or 24 hours. Supernatant was then diluted in 1X sodium phosphate buffer (34.5 mM monobasic sodium phosphate (NaH_2PO4) and 0.21 M anhydrous dibasic sodium phosphate (Na_2HPO4) in H_2O, pH 7.4) and then incubated with the 1X reaction buffer (composed of 10 mM Amplex red, 10 U/mL horseradish peroxidase, and 100 U/mL glucose oxidase) for 30 minutes at room temperature in the dark. Glucose in the medium was measured to determine the amount of glucose consumption by the cells, before and after 30 minutes or 24-hour incubation in growth medium (RPMI 1640 or DMEM) in the presence or absence of the drug or the *Teucrium polium* extract. The excitation/emission maxima of red fluorescence (571 nm/585 nm) were detected in a fluorometer (Enspire Multimode Plate Reader, USA). Glucose utilization over 30 minutes or 24 hours was calculated by subtracting the glucose concentration at 24 hour or 30 minutes from that at 0 hours. Glucose concentrations were determined against a glucose standard curve (0- 100 µM). The data was normalized against protein using a BCA protein quantification assay as described in section 2.7. For measurement of glucose content of the *Teucrium polium* extract, 500 µg/mL of the *Teucrium polium* extract was dissolved in the 1X reaction buffer and then the protocol was followed as described above.

2.19 Glucose uptake assay

Glucose uptake was measured using the flow cytometry-based method of Deng, Dhanya and Zou. (Zou *et al.*, 2005; Deng *et al.*, 2012; Dhanya *et al.*, 2015). In brief,

the fluorescently labelled glucose analogue 2-[*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxy-d-glucose (2NBDG) was utilized as an indicator to estimate glucose uptake. The glucose uptake by cells was quantified by measuring the shift in median fluorescence compared to control unlabelled cells after suitable gating strategies were implemented to eliminate cell debris, doublets and dead cells.

In brief, BRIN-BD11 cells (50,000 cells/ well) were cultured overnight in 24-well plates. L6 cells (10,000 cells/ well) were cultured for 8-10 days until they are differentiated. Before treatment, BRIN-BD11 cells or L6 myotubes were serum starved for 6 or 24 hours, respectively. The serum starvation was done to minimise the effect of growth factors in serum that could mask the effect of the *Teucrium polium* extract or insulin. Cells were treated for 30 minutes with 20 µM 2NBDG alone or with insulin (1000 nM) or *Teucrium polium* extract (500 µg/mL) in RPMI without glucose and serum. Supernatant was then removed to stop the 2NBDG uptake by the cells after 30 minutes. After treatment, cells were prepared for analysis by flow cytometry. Cells were washed twice with PBS. TrypLE™ Express was added to detach the cells from the wells, and the plates were incubated at 37°C for 5 minutes to aid in detachment. Cells were resuspended in RPMI growth medium and propidium iodide (1 µg/mL) was added to the medium. The mixture was then transferred to a 1.5 mL Eppendorf tubes and centrifuged at 2500 x g for 5 minutes (Eppendorf centrifuge 5417C). Supernatants were discarded. The pellet was resuspended in PBS and maintained on ice for flow cytometry, which was completed within 30 minutes. Background fluorescence was monitored by measuring cells not treated with 2NBDG.

For each measurement, ten thousand single cell events were recorded. Measurements were done using a FACS LSR Fortessa flow cytometer (BD Biosciences, Heidelberg, Germany). FlowLogic FCS analysis software (Inivai Technologies, Melbourne, Australia) was used to analyse the data. Side scatter (SSC) and forward scatter (FSC) and median 2-NBDG fluorescence were attained after gating for PI negative cells. The data from the flow cytometer was plotted for gating in three steps. Firstly, cell debris were excluded by gating for FSC values above a certain threshold. Then doublet cells were excluded [by ratio of area (A) to height (H)] and finally, dead cells (PI positive) were removed. Background was determined by measuring the intensity of cells treated with only growth medium and no 2NBDG. The background measurement was subtracted during data analysis. The gating strategy is illustrated in Figure 8.

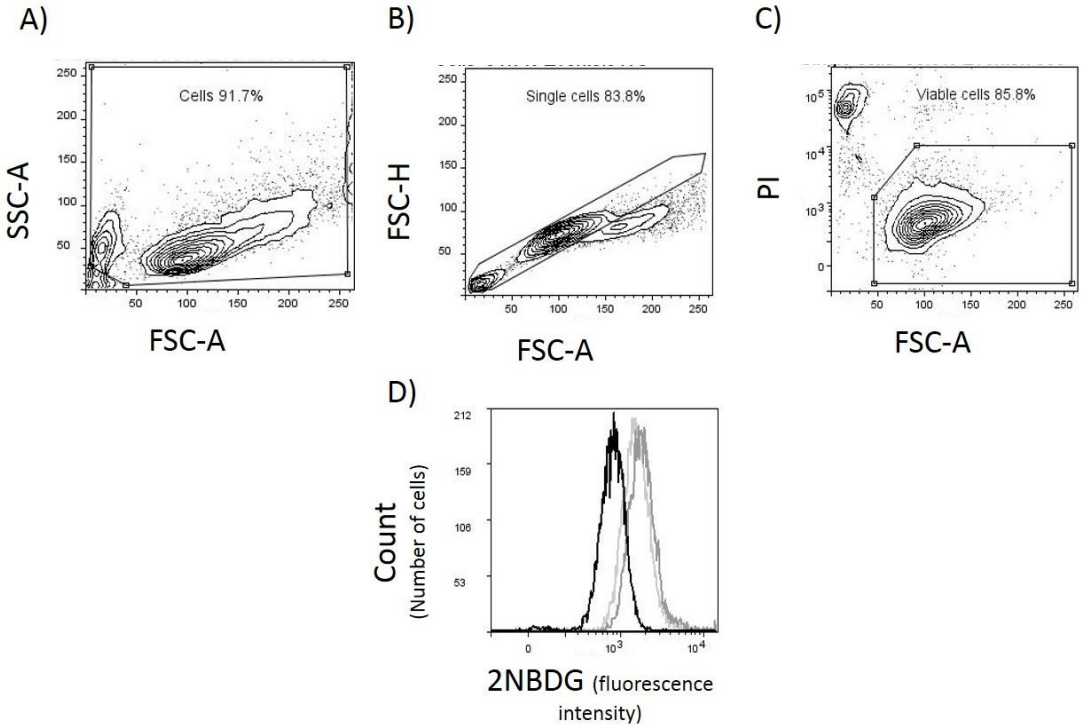


Figure 8: Glucose Uptake Assay: flow cytometry gating strategy. Cellular events were gated through A) the forward scatter (FSC- A) vs side scatter (SSC-A) plot, and further interrogated by B) the ratio of area to height in forward scatter (FSC) to allow for the analysis of single cellular events followed by C) the exclusion of propidium iodide (PI) to gate out dead cells. D) The remaining live cells were assessed for glucose uptake measurement. This figure has been generated from original data of thesis work.

2.20 Immunofluorescence microscopy for GLUT2 expression analysis

BRIN-BD11 cells were grown in 6-well plates. Cells were serum starved for 6 hours on the day of treatment. Cells were then treated for 24 hours with *Teucrium polium* extract. After treatment, the supernatant was removed. The cells were washed 3 times with PBS. The cells were then fixed with 500 µL 1% (v/v) paraformaldehyde (PFA) in phosphate buffer saline (PBS) for 10 minutes at room temperature. Cells were washed twice with PBS and then permeabilized with 500 µL of 0.1 % (v/v) Triton X in PBS solution per well. Cells were kept on ice for 15 minutes. The solution was discarded and cells were washed 3 times. Cells were blocked for 1 hour at room temperature in a blocking buffer (PBS solution containing 10% FBS and 1% BSA). The cells were washed 3 times with PBS and then treated with primary antibody (anti-GLUT2) diluted (1: 250) in blocking solution (10% FBS, 1% bovine serum albumin (BSA), PBS for 2 hours at room temperature. Cells were washed twice with PBS. Secondary antibody (goat anti rabbit alexa –fluor 488) was added (1: 1000 dilution in blocking buffer) for 1 hour at room temperature. Then the cells were washed three times in PBS. DAPI solution (1 µg/mL DAPI in PBS) was added to each well and kept in the dark for 15 minutes. Washing in PBS was repeated 3 times. Olympus BX-51 upright fluorescent microscope was used to capture images. Analysis of fluorescence intensity and detection of GLUT2 was done using cellsens software. Quantification was done using ImageJ ® software (Ver. 1.50i). Similar settings were used for both gain and exposure for image capture.

2.21 Cell culture of 3T3-L1 cell line

3T3-L1 cells were cultured using DMEM GlutaMAX^{TM} medium with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin added to it. The cells were grown at 37°C in 5% CO2 and culture medium was replaced every two days. Experiments were done using cell passages number 5-12. Cells were subcultured when they reached 70%-80% confluence. During subculturing process, the cells were rinsed with PBS and then 1-2 mL of Tryp^{LE} Express was added to detach the cells from the T75 flask (usually within 2-3 min). Cells were then centrifuged at 500 x g for 5 minutes and re-suspended in 5 mL of fresh growth medium. Cells were counted in a hemocytometer and

transferred into a new T75 flask containing fresh growth medium with 10% FBS. 3T3-L1 cells were provided by from Dr. Simon Fox, School of Pharmacy, Curtin University.

2.22 Differentiation of 3T3-L1 cells

The procedure used to differentiate the 3T3L1 cells was a modified protocol of Zebisch (Zebisch *et al*., 2012). The cells (passage 6-12) were revived from −80°C and grown in Hyclone DMEM medium (high glucose) with 10% foetal bovine serum (FBS). Cells were seeded in 6-well plates adding DMEM containing 10% neonatal calf serum (NCS) and 0.1% antibiotics (100 U/ml penicillin and 0.1 mg/mL streptomycin). After 72-98 hours, growth medium was replaced with differentiation medium while cells were 100% confluent. Differentiation medium contains 1 µM Dexamethasone (dissolved in 100% Ethanol), 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) (Dissolved in dimethyl sulfoxide (DMSO)), 10 µg/mL insulin, 2 µM rosiglitazone, 10% FBS and DMEM. After 3-5 days, differentiation medium was replaced by post differentiation medium composed of DMEM (high glucose), 10% FBS, 0.1% (v/v) antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin), and 1 µg/mµ insulin. After 5-7 days, cells were replaced with basal medium DMEM, 1% (v/v) antibiotics and 10% FBS (Figure 9B). Finally, cells were grown in basal medium for 7-14 days until they differentiated as indicated by the rounding up of cells and the presence of lipid droplets in cells (Figure 9C).

2.23 Oil red staining of liquid droplets in 3T3L1 cells

Oil red staining is a method to detect triglycerides and lipids on cells. Oil red O is a fat soluble (lysochrome) dye. It shows the level of adipose conversion in fat cells (Ramirez-Zacarias *et al.*, 1992).

The 3T3-L1 cells were grown and differentiated in 6-well plates. On the day of the experiment, cells were washed with phosphate buffer saline (PBS) and fixed with a 10% paraformaldehyde solution for 60 mins at room temperature. Post fixation, cells were washed with phosphate buffer saline (PBS), the cells were air dried and 1 mL of oil red solution (3:2; Oil red dissolved in 2 mg/mL isopropanol: milli Q water; v/v) was added to each well and the plate was incubated for 1 hour at room temperature.

Supernatant was removed after 60 minute and the cells were washed 3 times with phosphate buffer saline (PBS). Lipid droplets stained red as an indication of cell differentiation (Figure 9D) and was observed under Nikon inverted microscope (Eclipse TS100, Nikon instruments Inc., Tokyo Japan).

2.24 C2C12 cell culture

C2C12 cells were a kind gift from Professor Deirdre Coombe, Curtin University, Australia. Cell culture was performed according to the methods of Chaturvedi and his colleagues (Chaturvedi *et al.*, 2015). C2C12 myoblasts were grown and subcultured in DMEM complete medium containing 10% foetal bovine serum (FBS). C2C12 cells were passaged after reaching 60-70% confluence and cells less than 16 passages were used for all experiments. Briefly, medium was discarded from the T75 flask and cells were washed with 6.7 mM phosphate buffer saline (PBS) before Tryp^{LE} Express was added to the flask and incubated at 37ºC for 5 min or until the cells detached. Cells were pelleted by centrifugation at 500 x g for 5 min in DMEM complete medium. Medium was removed. Cells were resuspended in 1 mL of DMEM complete medium and counted using a haemocytometer. C2C12 cells were seeded in 6 or 24- well plates and were maintained in growth medium DMEM for 3-4 days. Then the growth medium was removed and the cells were grown in 2% FBS for another 10-12 days at to stimulate differentiation. Medium was changed and replaced every 24 hours with DMEM containing 2% FBS after every 24 hours. The cells formed complete myotubes after 8-10 days (Figure 10).

2.25 L6 cell culture

L6 cells were thawed as mentioned in section 2.3.1. Cells were grown and differentiated according to the method described by Hutchinson (Hutchinson & Bengtsson, 2005). In brief, L6 cells were grown in DMEM medium containing 10% FBS. Cells were subcultured when confluency reached to 70-80% and cells were then seeded in 6-well plates. After 3 days, the medium was replaced with DMEM containing 2% FBS. Medium was replaced every 48 hours. Cells were differentiated for 7-10 days (Figure 11). L6 cells were gifted by Dr. Dana S. Hutchinson, Monash University.

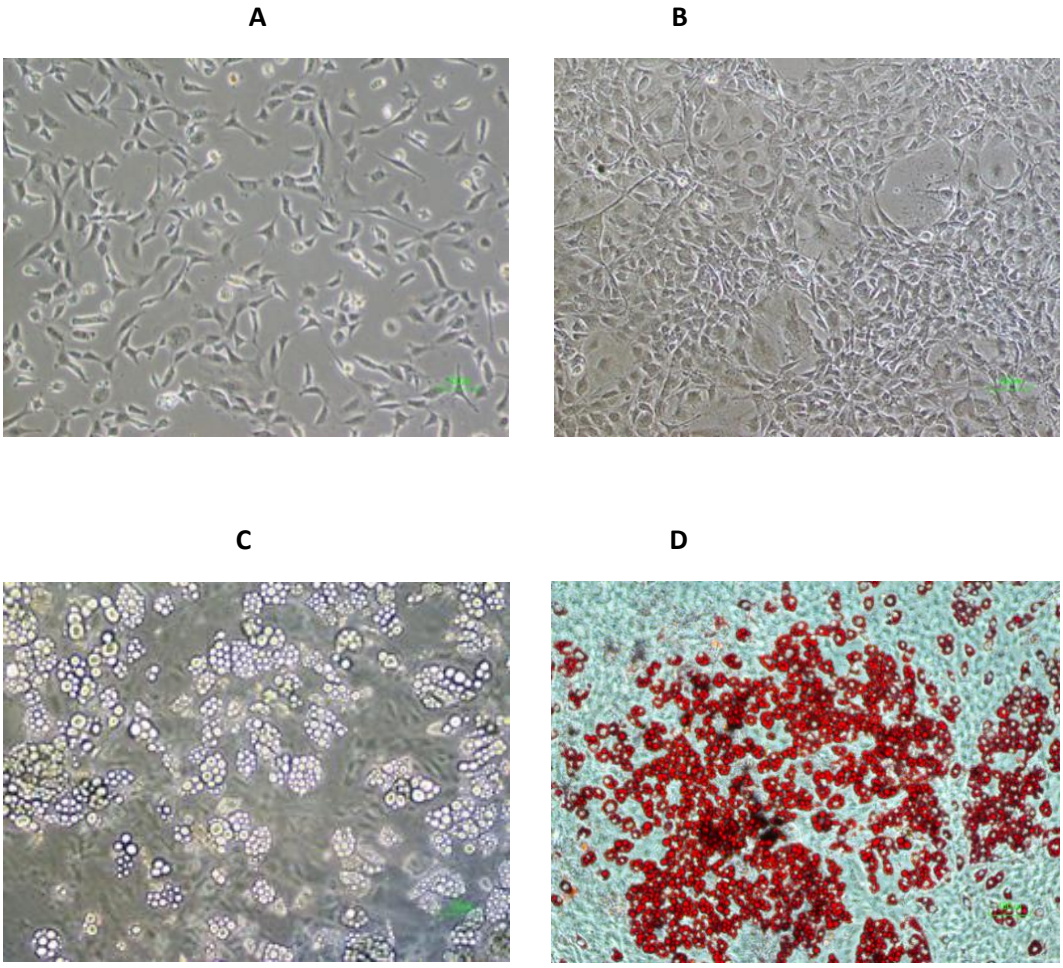


Figure 9: Stages of differentiation of 3T3L1 cells. Images of A) proliferation B) pre-differentiation and C) differentiation of 3T3-L1 cells during different phases of growth and D) The differentiated 3T3-L1 cells used for this study were stained with Oil red as described in section 2.26. Red lipid droplets show the differentiated adipocytes. Cells were grown in 6-well plates and differentiation started from day 8-10. Images were taken at 20x magnification using a Nikon inverted microscope (Eclipse TS100, Nikon instruments Inc., Tokyo Japan).

A B

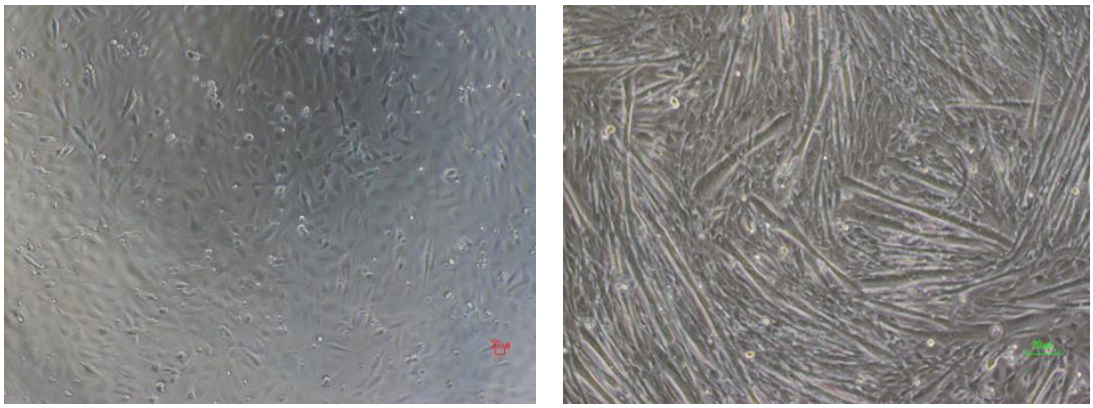


Figure 10: C2C12 cell culture at various stages. A) Cell proliferation B) differentiated C2C12 cells. In 6-well plate, Cell proliferation occurred in the first 3-4 days and differentiation started from day-6. Cell were left for differentiation for 12-14 days. Differentiation medium was replaced from day-4. Pictures were taken at 20x magnification using a Nikon inverted microscope (Eclipse TS100, Nikon instruments Inc., Tokyo Japan).

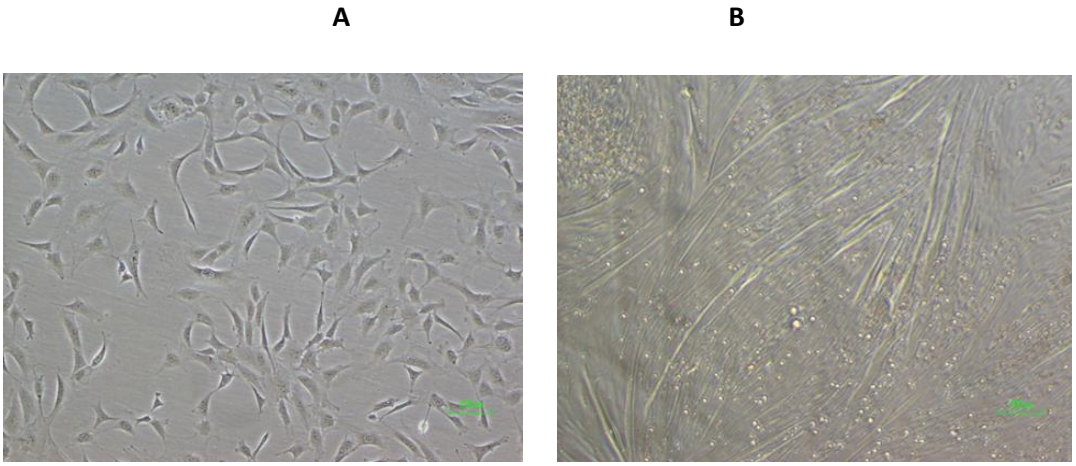


Figure 11: L6 cell culture at various stages. A) Proliferated and B) differentiated, L6 myotubes. Cell proliferation occurred in the first 3-4 days (Picture was taken at day 2) and differentiation started from day-7 (Picture was taken at day 10). Differentiation medium was replaced from day-4. Pictures were taken at 20x magnification using a Nikon inverted microscope (Eclipse TS100, Nikon instruments Inc., Tokyo Japan).

2.26 Proteomic screening of activated kinases using Kinetworks™ KCPS-1.0 (protein kinase screen)

The effect of the *Teucrium polium* extract on key signalling molecules of insulin signalling pathways was analysed by pre-screening using a commercial multi-immunoblot service.

In brief, L6 myotubes were pre-treated with control growth medium DMEM or, insulin or the *Teucrium polium* extract in growth medium for 30 minutes. The cells were lysed with SDS PAGE sample buffer. SDS buffer was prepared using 25% glycerol, 0.25 M Tris-HCl (pH 6.8), 5% SDS, 0.25% bromophenol blue, distilled water and 1.25% β – mercaptoethanol. The rational for homogenising the cells directly in SDS-PAGE sample buffer was to immediately destroy the endogenous protein kinases, phosphatase and proteases by denaturing them, and in doing so preserving the state of phosphorylation of proteins. The cell lysates were immediately sonicated using a Bioruptor® Plus sonication device (Diagenode Inc., USA) for four cycles of 10 seconds each time and centrifuged at 4°C at 14,000 x g for 30 minutes using a Beckman Coulter Microfuge22R (Beckman Coulter Australia Pty Ltd., NSW, Australia). Cell pellet was discarded and the supernatant was stored at 4°C for protein activation/ expression studies. An aliquot of the sample was used to quantitate the amount of protein in the sample using the BCA total protein quantification assay (section 2.7).

The samples from cells treated with control, insulin or the *Teucrium polium* extract were sent to Kinexus Bioinformatics Corporation (Vancouver, Canada) for further proteomic analysis utilizing its Kinetworks™ KCPS-1.0 multi-immunoblotting service.

The Kinetworks immunoblotting analysis was done according to previously published methodology (Pelech *et al.*, 2003) and is also found online at www.kinexus.ca. It is an antibody-based method that relies on sodium dodecyl sulfate (SDS)-polyacrylamide mini gel electrophoresis and multi-lane immunoblotters to permit the specific and quantitative detection of 45 or more protein kinases and other signal transduction proteins at once. The technique also permits resolution of these proteins depending on dissimilarities in their phosphorylation state and other forms of covalent

modification. For the Kinetworks™ Multi-immunoblotting method, a 20-lane Immunetics multi-blotter is used, which features 20 separate slots for creation of effectively 20 separate lanes. In to each slot, about 400 µL of antibody solution were added, and only the exposed region of the nitrocellulose membrane with the proteins transferred from the SDS-PAGE was incubated with the antibodies. Up to three different antibodies may be incubated in the same mixture, provided that the intended target proteins and any cross-reactive proteins are well resolved. A single lysate sample is applied to the SDS-PAGE gel and the sample is electrophoresed across the width and length of the entire gel. After about a 2-hour incubation at room temperature (or overnight with at 4°C), the multi-immunoblotter is removed from the nitrocelluose membrane, and the entire membrane is washed and incubated with secondary antibody (anti-rabbit, anti-goat and anti-mouse IgG) that is coupled to horse radish peroxidase. The final detection of the immunoreactive proteins was performed by Enhanced Chemoluminescence (ECL), scanned in a BIO-RAD Fluoro-S Max Scanner, and quantified with Quantity One software (BIO-RAD).

In the present study, the combination of three pooled samples was initially screened to determine which kinases could be successfully detected in further studies with individual samples. In the initial pre-screen, antibodies for 25 kinases involved in various insulin signalling pathways, including protein metabolism, glucose uptake, lipogenesis, glycogen synthesis and cell proliferation were tested. These included glycogen synthase kinase β (GSK3β) (Ser 9), phosphatase and tension homologue (PTEN), protein kinase C (PKC), ribosomal S6 protein-serine kinase 1 [S221], ribosomal protein serine S6 kinase beta 1 [T412], cyclin-dependent protein-serine kinase 1/2 [Y15], extracellular regulated protein-serine kinase 1 (p44 MAP kinase) [T202+Y204], extracellular regulated protein-serine kinase 2 (p42 MAP kinase) [T183+Y185], protein-serine kinase B α [T308] (Akt1), protein-serine kinase B β (Akt2), insulin receptor [Y999] (IR), insulin receptor substrate 1 [S312] (IRS1), insulin-like growth factor 1 receptor protein-tyrosine kinase [Y1161+T1163] (IGF1), mammalian target of rapamycin (mTOR), AMP-activated protein-serine kinase alpha 2 [S377] (AMPKα) and phosphatidylinositol 3-kinase regulatory subunit alpha [Y467/Y199] (PI3K) (Ma *et al.*, 2007). Among them, detected kinases and other relevant proteins such as their targets were further checked in the final pre-screening of individual samples.

2.27 Western Blot

L6 myotubes were cultured for 8-10 days for differentiation in DMEM medium until the differentiated cells reached 60-70% confluence. Time response for each antibody was optimized. The cells were then treated for 10 minutes (for p-PDK-1), 15 minutes (for pAkt), 30 minutes (for p-mTOR, p-S6, p-GSK3α/β) and 24 hours (p-ERK and GLUT4) with either medium alone, 1000 nM insulin or 50 µg/mL *Teucrium polium* extract. The wells were then washed for 3 times in PBS. Radioimmunoprecipitation assay (RIPA) buffer (150 mM sodium chloride (NaCl), 1.0% (v/v) IGEPAL® CA-630, 0.5 g/L sodium deoxycholate, 1g/L SDS, 50 mM Tris, pH 8.0.) mixed with Pierce™ protease inhibitor mini tablets (1 in 10 mL) was added to each well to lyse the cells. Cells were sonicated with the aid of four 10 second cycles in a Bioruptor® Plus sonication device (Diagenode Inc., USA). Cells were then centrifuged at 4°C for 20 minutes at 14000 x g using a Beckman Coulter microfuge22R centrifuge (Beckman Coulter Australia Pty Ltd., NSW, Australia). The supernatant was removed and stored at −80°C. An aliquot of each sample was used to determine the total protein content using a BCA protein quantification assay as described in section 2.7.

Samples were denatured for 5 minutes at 95°C in Laemmli buffer (1X, pH 8.4) and an equivalent of 40 µg protein loaded for each sample onto an acrylamide gel [6% stacking gel containing 0.5 M Tris HCL (pH 6.8), acrylamide (0.3 g/mL bis acrylamide, 10% SDS, 10% APS, TEMED, milli-Q H_2O]. Precision plus (10-250 KDa) protein ® standards were run on each gel to enable the molecular size of protein in samples to be determined. Samples were run onto an acrylamide gel (10% running gel containing 30% acrylamide, 1.5 M Tris HCl (pH 8.8), 10% SDS, 10% APS, TEMED, milli-Q H_2O) using 1X running buffer [3 g/L Tris, 14.4 g/L glycine, 1 g/L SDS in H_2O (pH 8.8)] with a continuous 200V for 40 minutes until the dye front reached the bottom of the gel.

Proteins were transferred from the gel to a PVDF membrane after separation by SDS PAGE. The membrane was activated with 20% methanol and the transfer was done at 34V for 60 minutes using 1X transfer buffer (3 g/L Tris base, 14.4 g/L glycine in mili-Q H_2O). The membrane was blocked using blocking buffer (3% (w/v) bovine serum albumin in 0.05% (v/v) Tris Buffered Saline Tween 20) for 60 minutes. The membrane was then incubated overnight with primary antibody at 4°C. The membrane

was then washed with 1% (w/v) Tris-buffered saline tween 20 (TBST) three times (10 minutes each) to remove unbound antibodies. After the wash, appropriate anti-rabbit secondary antibody (anti-rabbit IgG, HRP-linked Antibody) was incubated for 1 hour at room temperature added to membrane at a ratio of 1: 40000 in blocking buffer. The washing step was repeated three times after incubation with the secondary antibody. Finally, a Clarity™ Western ECL Substrate (BIO-RAD) kit was used to detect proteins. Images were captured using Molecular Imager® Gel Doc™ XR System (BIO-RAD, USA). Densitometric analysis was done by using the imagelab (BIO-RAD) software (version 5.2.1). All the antibodies used are listed in Table 6.

2.28 Immunofluorescence microscopy for GLUT4 expression analysis in L6 myotubes

L6 cells were grown on collagen coated cover slips in 6-well plates for 8-10 days to promote myotube formation. The coating of cover slips was carried out as per manufacturer's protocol. Briefly, 0.1 mg/mL filter sterilized collagen solution was added to coat the coverslips at 37°C for 2 hours. The coverslips were then washed twice with Hyclone phosphate buffer saline (PBS) and then dried under UV light.

Immunofluorescence and microscopy was performed according to the procedure of Chaturvedi (Chaturvedi *et al*., 2015). In brief, L6 myotubes were serum starved for 24 hours. Cells were treated for 24 hours with control DMEM growth medium, insulin or *Teucrium polium* extract in DMEM growth medium. The cells were washed 3 times with PBS. The cells were then fixed with 500 µL 1% (v/v) paraformaldehyde (PFA) in phosphate buffer saline (PBS) for 10 minutes at room temperature. Cells were washed twice with PBS and then permeabilised with 500 µL of 0.1 % (v/v) Triton X in PBS solution per well. Cells were kept on ice for 15 minutes. The solution was discarded and cells were washed 3 times. Cells were blocked for 1 hour at room temperature in a blocking buffer (PBS solution containing 10% FBS and 1% BSA). It was incubated. The cells were washed 3 times with PBS and then treated with primary antibody (anti- GLUT2) diluted (1: 250) in blocking solution (10% FBS, 1% BSA), PBS for 2 hour at room temperature. Cells were washed twice with PBS. Secondary

Table 8: List of antibodies used in the present study

Name	Manufacturer
pAKT (phospho-ser-473)	Cell Signaling Technology
pAKT (phospoho-ser-308)	Cell Signaling Technology
Alpha actinin	Cell Signaling Technology
pERK1/2	Cell Signaling Technology
GAPDH	Cell Signaling Technology
GLUT4	Abcam
pGSK-3α/β (Ser21/9)	Cell Signaling Technology
pmTOR (Ser2448)	Cell Signaling Technology
pS6 (Ser240/244)	Cell Signaling Technology
pPDK-1 (Ser241)	Cell Signaling Technology
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling Technology
GSK233447	Glaxo Smithkline

antibody (goat anti rabbit alexa –fluor 488) was added (1: 1000 dilution in blocking buffer) for 1 hour at room temperature. Then the cells were washed three times in PBS. DAPI solution (1 µg/mL DAPI in PBS) was added to each well and kept in the dark for 15 minutes. Washing in PBS was repeated 3 times. The collagen coated coverslips were removed from the plate and placed in slides by adding Vectashield® antifade mounting medium.

An Olympus IX-51 inverted microscope (20x objective lens) with Cellsens software was used for capturing images. Similar settings were used for both gain and exposure for image capture. Quantification was done using ImageJ ® software (Ver. 1.50i).

2.29 Glycogen content measurement in L6 myotubes

The glycogen content measurement in L6 myotubes was done according to the Biovision glycogen colorimetric/ fluorometric assay kit manufacturer's protocol and as described in the study of Elsner (Elsner *et al.*, 1998).

In the present study, L6 cells were grown and differentiated in 24-well plates as described in the section 2.25. L6 myotubes were serum starved for 24 hours before treatment. On the day of treatment, Cells were washed twice with Hyclone phosphate buffered saline (PBS) and then treated with control medium, the *Teucrium polium* extract or insulin for 1 hour at 37°C. Cells were washed twice with PBS and then lysed with potassium hydroxide (KOH) (1 mol/L) and cells were then centrifuged at 4°C for 20 minutes at 14000 x g using a Beckman Coulter microfuge22R centrifuge (Beckman Coulter Australia Pty Ltd., NSW, Australia). The supernatant was removed and stored at −80°C. On the day of experiment, 1 µL hydrolysis enzyme mix (provided by the manufacturer) was added to glycogen standards and samples. To avoid any glucose background, a glucose control was used where hydrolysis buffer was not added to the samples. Glucose background was subtracted from the final glycogen readings. Samples and glycogen standards (0 - 0.2 µg/mL) were incubated for 30 minutes with hydrolysis buffer at room temperature on the day of experiment. Next, samples were treated with 50 µL reaction mix (mixture of development buffer, development enzyme and oxired probe) (provided by the manufacturer). This reaction was light protected and done for 30 minutes at room temperature. Finally, glycogen content measurement was done by detecting fluorescence at an excitation wavelength of 535 nm and

emission wavelength of 587 nm. The experiment was done using Corning® 96-well flat bottom black polystyrene assay plate for fluorescence measurement. Background levels were corrected for by subtracting the blank or, zero glycogen standard from all readings.

2.30 Statistical analysis

The data have been presented as means (± SD, standard deviation) in the current thesis. Each individual experiment was performed with (at least) 3 replicates. In the case that any one of the replicates was more than 2 standard deviations from the mean of the other two (with the standard deviation being based on those remaining 2 replicates), then it was considered to be an outlier and was removed from analyses. Differences in the response between the different treatments were examined using one-way ANOVA or t-tests as appropriate. The analysis was performed using GraphPad prism software (6.0, GraphPad prism Software Inc, CA, USA). A General Linear Model (GLM) was used to explore the relationship between dose of *Teucrium polium* extract and insulin release or glucose uptake by the cells. A p-value < 0.05 was taken to indicate a statistically significant difference between the specific data analysed.

3.0 Results

3.1 Phenolic, glucose, alanine and protein composition of the *Teucrium polium* extract

Total phenolic, tannin and protein content of the extract (sections 2.2, 3.2.3, 2.4, 2.5) were assessed over the duration of the study in order to monitor the stability of the plant extract (Figure12), as conducted by others in the field (Katalinic *et al.*, 2006). The total phenolic content of the *Teucrium polium* extract was evaluated using the Folin–Ciocalteu method, which is a colorimetric assay. This assay depicts the phenolic compounds in their un-oxidised form (Ainsworth & Gillespie, 2007) and was used to estimate the stability of the constituents of the plant extract over time (Figure 12A). Total tannin were measured from the estimate of phenolic content in a solution (Figure 12B) (Von Martius *et al.*, 2012). Essentially, the tannins were precipitated-out and the non-tannins phenolics were quantitated. Therefore, the difference in absorbance readings between the two assays (i.e. total phenolics and non-tannins) directly reflects the amount of tannin present in the sample. Total phenolic compounds and tannins were quantified in parallel. Total protein was measured using a bicinchoninic acid (BCA) method which is a formulation based on BCA for the colorimetric detection and quantitation of total protein (Smith *et al.*, 1985). The total phenolic and tannin contents of the *Teucrium polium* extract decreased slowly over time, with a significant ($p < 0.05$) decline detected from 24 months of storage (Figure 12A, 12B). There was no significant reduction in the amount of total protein over the 36-month time-period (Figure 12 C).

Alanine and glucose are well known insulin secretagogues (Newsholme *et al.*, 2014; Kaufman *et al.*, 2015) and have been used as positive controls in the ensuing studies described in this thesis. Thus, the concentration of alanine and glucose were quantitated in the plant extract (section 2.8, 2.18). The plant extract contained 22.7 micromoles of alanine and 15.4 millimoles of glucose derived per gram of the original extract. Thus, when experiments were undertaken using extract derived from 500 µg of extract dissolved in 1 mL of culture media (a typical concentration of *Teucrium polium* extract used in the subsequent studies of this thesis), the concentrations of alanine and glucose would be about 9 µM and 7.7 µM, respectively. As discussed in the discussion chapter of the thesis, these concentrations would have no impact on insulin secretion by beta cells used in the study.

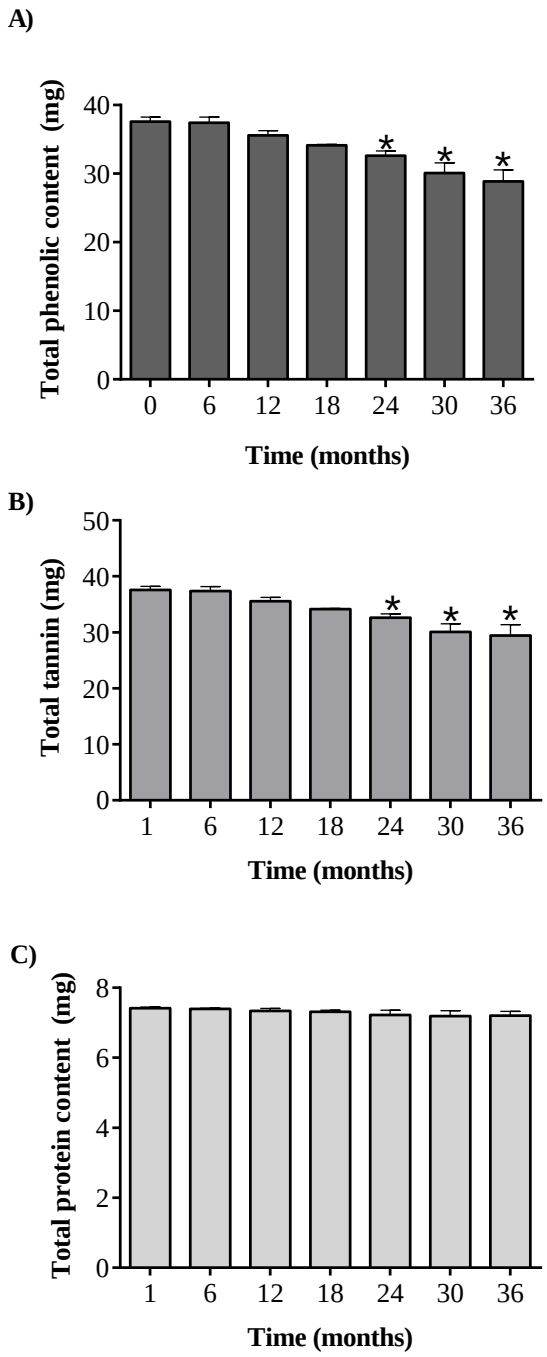


Figure 12: Total phenolic, tannin and protein content in the Tp [ǂ]extract. The A) phenolic B) tannin and C) protein content of the Tp extract was measured as described in section 2.5, 2.6 and 2.7 after every six months over three years of the study period. The total amount of phenolics, tannin and protein was expressed as mg of phenolics/ tannins/ protein in 5.0-gram plant powder. Statistical differences between the treatments were assessed as per section 2.30. All values are reported as the mean (± SD) of three separate experiments, * denotes $p < 0.05$ compared to 0 month (A) or 1 month (B, C).

[ǂ] *Throughout this result section, Teucrium polium has been abbreviated as Tp in the figures and legends*.

3.2 Does the *Teucrium polium* extract have insulin secretagogue properties?

The cytotoxicity of the extract of *Teucrium polium* was assessed by measuring cell viability in two independent assays. In the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Figure 13), the *Teucrium polium* extract did not significantly decrease cell viability at concentrations up to 1 mg/mL[1]. The cell viability was also investigated using an acid phosphatase (APH) assay. The *Teucrium polium* extract did not demonstrate cytotoxic effects on BRIN-BD11 cells at concentrations up to 2 mg/mL[1]. However, the cell viability was significantly ($p < 0.05$) reduced with doses exceeding 5 mg/mL[1] of the *Teucrium polium* extract. Less than 30% of cells were viable in the presence of 25 mg/mL[1] of the *Teucrium polium* extract (Figure 14).

The release of insulin by BRIN-BD11 cells increased in response to various known insulin secretagogues (Figure 15). Alanine, an amino acid well-known for increasing ATP release to promote insulin secretion (Newsholme *et al.*, 2007) in the presence of high concentration of glucose (16.7 mM) strongly promoted insulin secretion ($p < 0.001$) in BRIN-BD11 cells (Figure 15 A). Potassium chloride (KCl), which is required to block the K_{ATP} channel and promote calcium influx, also significantly ($p < 0.0001$) increased insulin release, about ten-fold relative to the control (2.5 mM glucose) (Figure 15 B).

The combination of *Teucrium polium* and glucose was investigated to perform further experiments. Insulin release by BRIN-BD11 cells was several folds greater at glucose concentrations above 1.1 mM, as was the effect of the *Teucrium polium* extract (500 µg/mL) (Figure 16). On examining the glucose alone data, glucose concentrations up to 5.5 mM promoted a significant ($p < 0.01$) increase in insulin release with increasing glucose with no significant difference in insulin release between 5.5 or 16.7 mM of glucose. Insulin release was significantly ($p < 0.01$) increased in the presence of the *Teucrium polium* extract and glucose compared to glucose alone (Figure 16), which was apparent at all the glucose concentrations (1.1 mM, 5.5 mM and 16.7 mM). This data indicates that a combination of 5.5 mM glucose and *Teucrium polium* is most effective for insulin secretion.

[1] *Throughout this thesis, the amount of Tp is expressed as the extract obtained from a weight of original plant material. Where this extract is used in culture/buffer, the amount is then stated as an extract obtained from an original weight of plant material per mL of culture medium.*

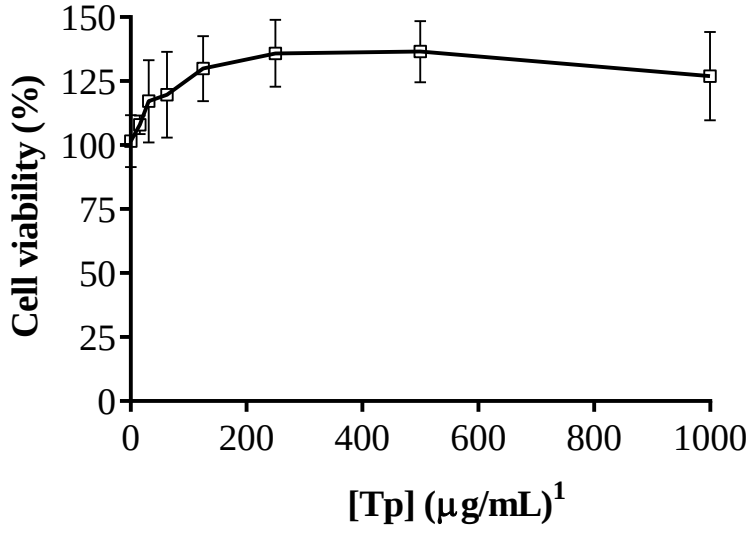


Figure 13: Viability of BRIN-BD11 cells in response to various doses of the Tp extract. BRIN-BD11 cells were cultured as described in section 2.9. The cells were then treated for 24 hours with Tp extract (0-1000 µg/mL)[1]. The viability of cells following the treatment was assessed using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) in a cell viability assay as described in section 2.13.1. All values are reported as the mean (± SD) of three separate experiments, with each point done in triplicate for each experiment.

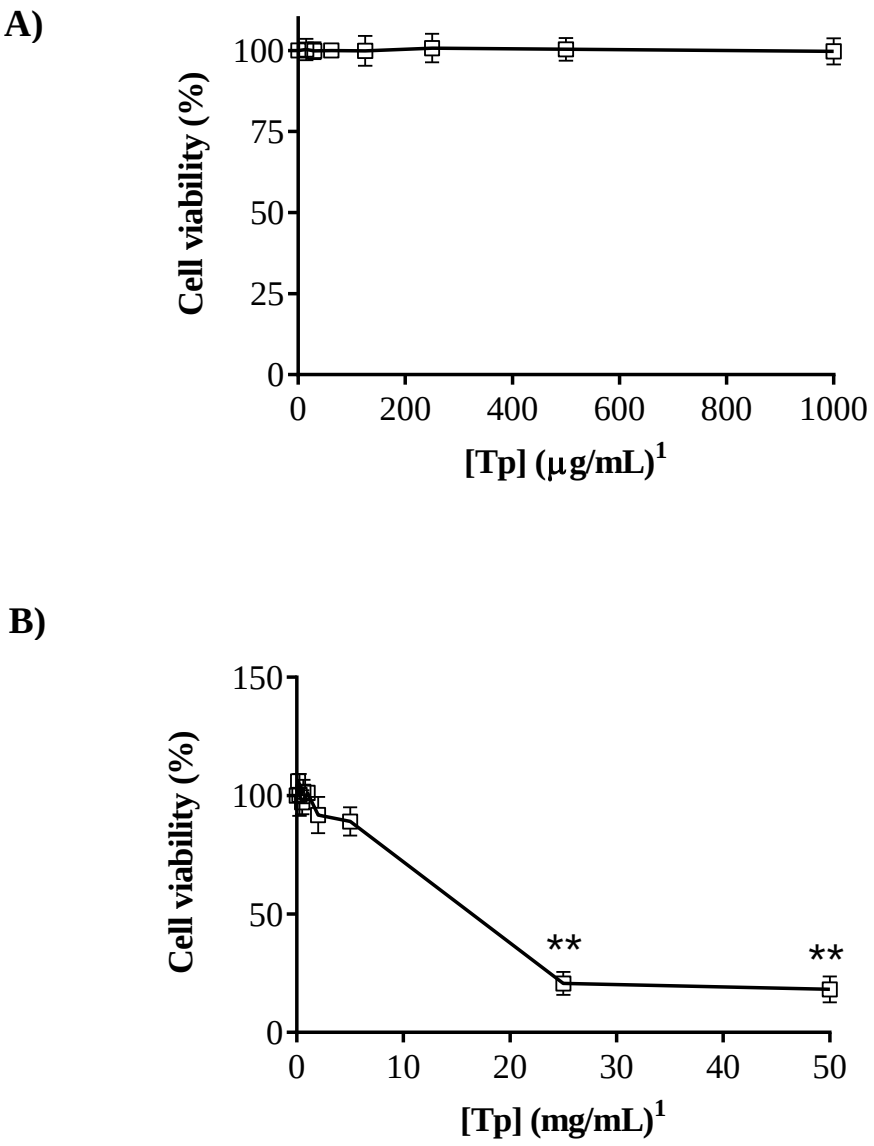
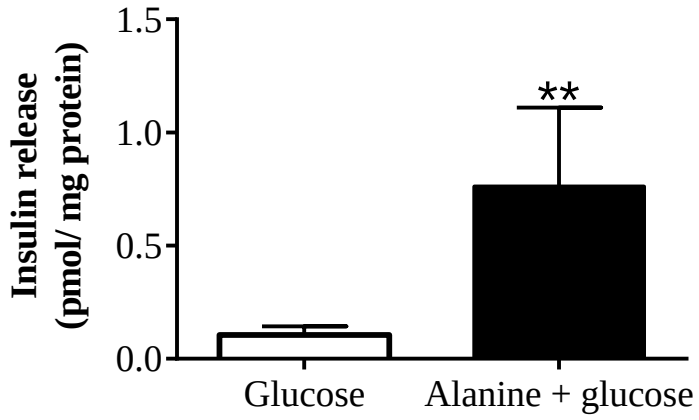


Figure 14: Viability of BRIN-BD11 cells in response to various doses of the Tp extract. BRIN-BD11 cells were cultured as described in section 2.9. The cells were treated for 24 hours with A) 0-1 mg/mL or B) 1-50 mg/mL of the Tp extract. The viability of cells following the treatment was assessed using an acid phosphatase (APH) assay as described in section 2.13.2. All values are reported as the mean (± SD) of three separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30 and ** denotes $p < 0.01$ in comparison with control.

A)



B)

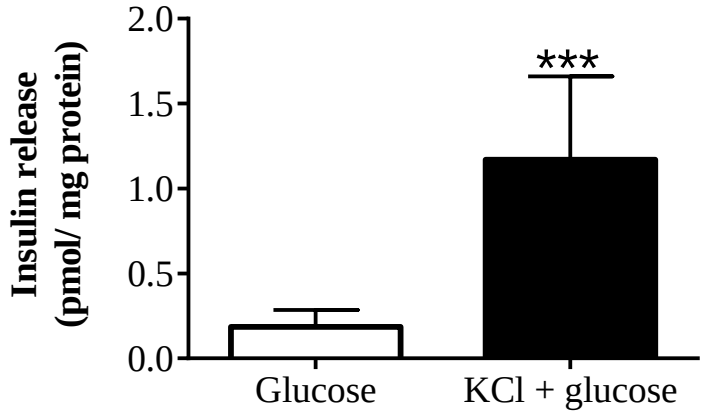


Figure 15: Insulin release from BRIN-BD11 cells stimulated with various insulin secretagogues. BRIN-BD11 cells were cultured as described in section 2.9. Cells were glucose starved for 40 minutes in Krebs Ringer Bicarbonate (KRB) buffer containing 1.1 mM glucose. Cells were then treated for 30 minutes with either A) 16.7 mM glucose in the presence or absence of 10 mM alanine in KRB buffer; B) 2.5 mM glucose in the presence or absence of 30 mM KCl in KRB buffer. Insulin released by the cells was then measured as described in section 2.14. Data was normalized to amount of protein using a BCA protein quantification assay as described in the section 2.7. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30 and ** depicts $p < 0.01$, *** $p < 0.001$ compared to control (glucose alone).

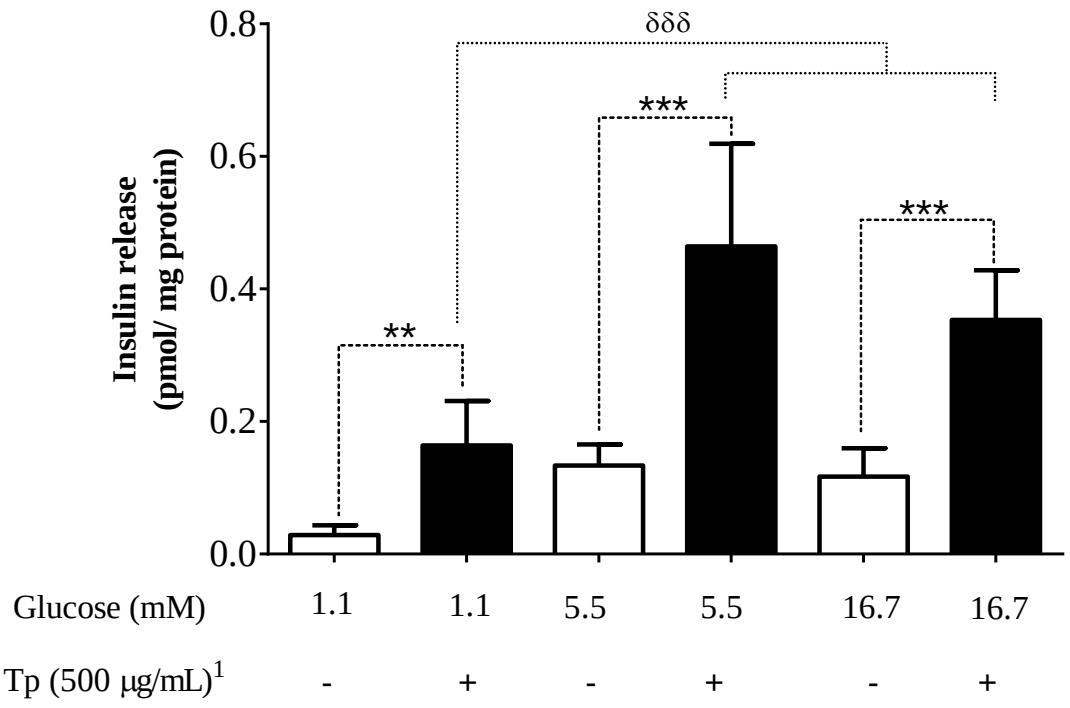


Figure 16: Insulin secretion from BRIN-BD11 cells stimulated with different concentrations of glucose in the presence and absence of the Tp extract. BRIN-BD11 cells were cultured as described in section 2.9. The cells were initially glucose starved for 40 minutes in KRB buffer containing 1.1 mM glucose. The cells were then treated for 30 minutes with the various concentrations of glucose (1.1. mM, 5.5 mM and 16.7 mM) in KRB buffer in the presence or absence of Tp extract. Insulin released by the cells was then measured as described in section 2.14. The data were normalised to the amount of protein in the cells using a BCA protein quantification assay as described in section 2.7. The data of three individual experiments has been presented as the mean (± SD) of individual experiments with each point done in triplicate. ** denotes $p < 0.01$, *** $p < 0.001$ compared to respective glucose control (i.e. relative to same glucose concentration) and δδδ denotes $p < 0.001$ compared to the Tp extract with 1.1 mM glucose treatment.

Gliclazide is a widely used hypoglycaemic agent that belongs to the sulfonylurea group. It blocks potassium efflux ion channels thus facilitating calcium influx in beta cells. This leads to increased insulin release from insulin secretory vesicles (Lawrence *et al.*, 2001). As would be expected, gliclazide promoted insulin release in a dose-dependent manner in BRIN-BD11 cells (Figure 17). The *Teucrium polium* extract also significantly ($p < 0.001$) promoted insulin secretion in a dose-dependent manner giving a similar insulin release profile to that demonstrated by gliclazide (Figure 17).

Calcium influx in BRIN-BD11 cells was monitored using confocal microscopy (Figure 18A, 18B). Live cell imaging was used to detect the calcium transported within the cells over 10 min. Intracellular Ca^{2+} binding to the internalised Fluo-4 AM dye was detected at set time points (0-600 sec) using the Vox software (Ultraview Vox system, Perkin Elmer, USA). To assess interferences by the extract and the dye, the cells incubated with (1) the *Teucrium polium* extract in the absence of the Fluo-AM dye and, separately (2) the Fluo-AM dye alone, were monitored using the same procedure. Figure 18A shows a sharp increase in the calcium influx after treating with *Teucrium polium* extract (Caption iii of Figure 18A) and a trend of increase after gliclazide treatment (Caption ii of Figure 18A). In the immunofluorescent microscopy images, a significant increase in the intracellular calcium (green dots) was observed after treatment with 5 µM gliclazide or the *Teucrium polium* extract (500 µg/mL)[1] in the presence of 5.5 mM glucose solution, respectively (Figure 18B). Both gliclazide and the *Teucrium polium* extract caused an almost two-fold increase in calcium influx in BRIN-BD11 cells (caption i, ii and iii of Figure 18A).

ATP is one of the major stimulatory factors for insulin release as it promotes plasma membrane depolarization and calcium uptake by beta cells (Sato & Henquin, 1998). Intracellular ATP levels in BRIN-BD11 cells were measured after exposure to the *Teucrium polium* extract (500 µg/mL)[1] or alanine (10 mM) for 30 minutes by using a Celltiter-Glo luminescent assay (Figure 19). The *Teucrium polium* extract significantly ($p < 0.01$) promoted intracellular ATP generation from BRIN-BD11 cells compared to the control (Figure 19). Alanine, another reputed insulin secretagogue also promoted significant ($p < 0.01$) increase in intracellular ATP production in the presence of 16.7 mM glucose (Figure 19) which is consistent with a previous finding (Hannan *et al.*, 2006).

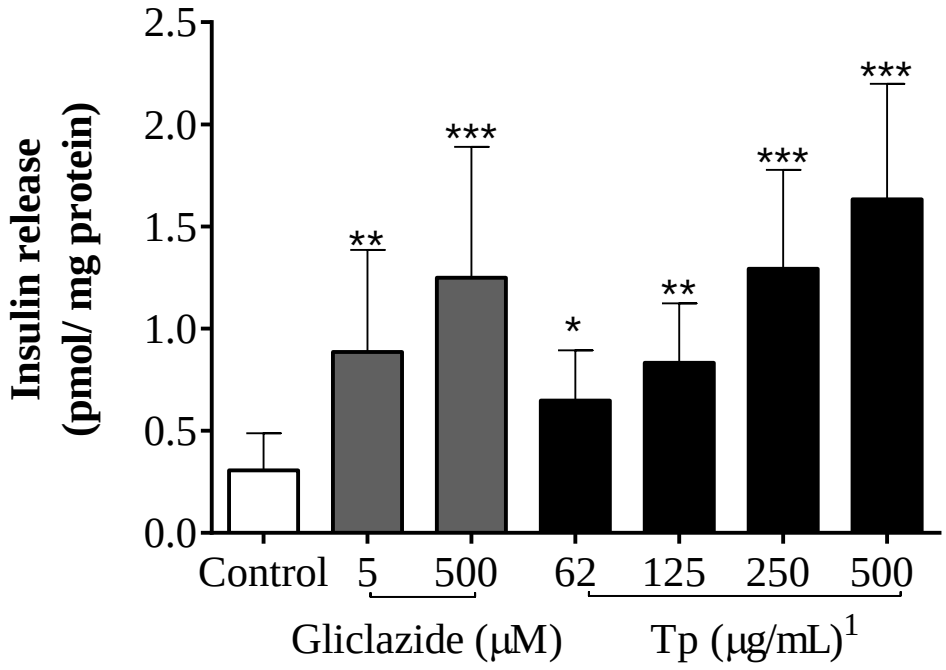


Figure 17: Insulin secretion from BRIN-BD11 cells in response to various concentration of either Tp extract or gliclazide. BRIN-BD11 cells were cultured as described in section 2.9. The cells were glucose starved for 40 minutes in KRB buffer containing 1.1 mM glucose. The cells were then treated for 30 minutes in KRB buffer containing control glucose (5.5 mM), gliclazide (5.0, 500 μM in 5.5 mM glucose) or Tp extract (62, 125, 250, 500 μg/mL^{1} in 5.5 mM glucose). Insulin released by the cells was then measured as described in section 2.17. The data was normalized to the amount of protein in the cells using a BCA protein quantification assay as described in section 2.7. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control (glucose alone).

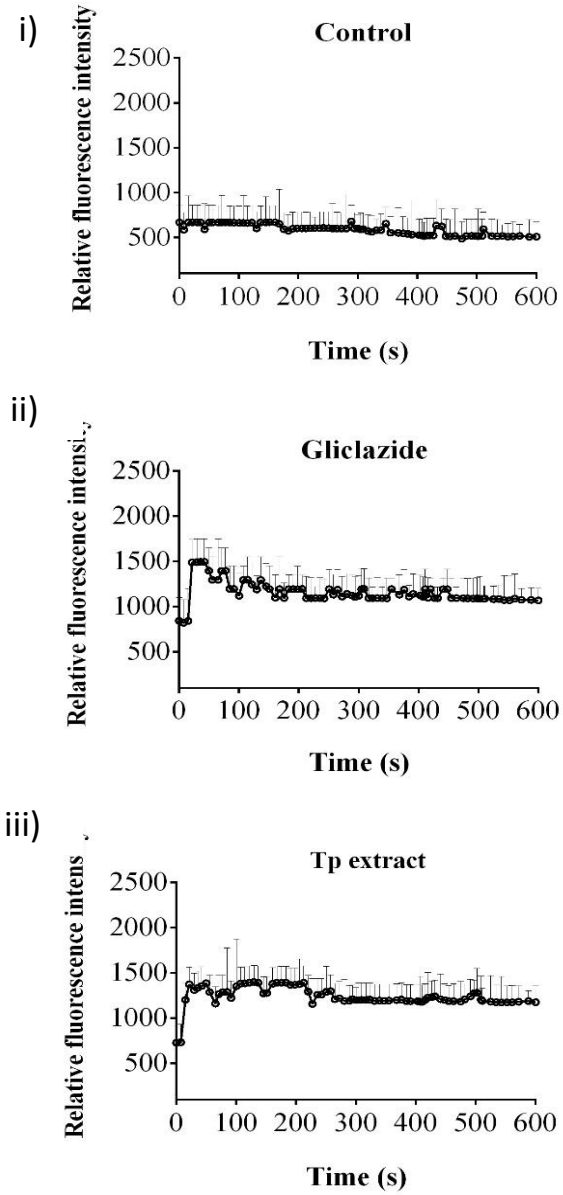


Figure 18A: The effect of the Tp extract on intracellular Ca^{2+} in BRIN-BD11 cells. BRIN-BD11 cells were cultured as described in section 2.9. BRIN-BD11 cells were stained with the Fluo-4 AM dye for 40 minutes and then washed twice. Cells were treated with HBSS buffer for 30 minutes for glucose starvation. After washing with PBS, cells were stimulated with 5 µM gliclazide or Tp (500 µg/mL)[1] or control KRBB buffer alone (as described in section 2.15). The time course of intracellular calcium binding with internalised Fluo4-AM dye was monitored using a Perkin-Elmer confocal microscope as described in the section 2.15. Fluorescence of calcium at various time points (0- 600 sec) was observed and measured using the Vox software (Ultraview Vox system, Perkin Elmer, USA) (caption i, ii, iii). All values are reported as the mean (± SD) of three separate experiments, with each point done in triplicate for each experiment.

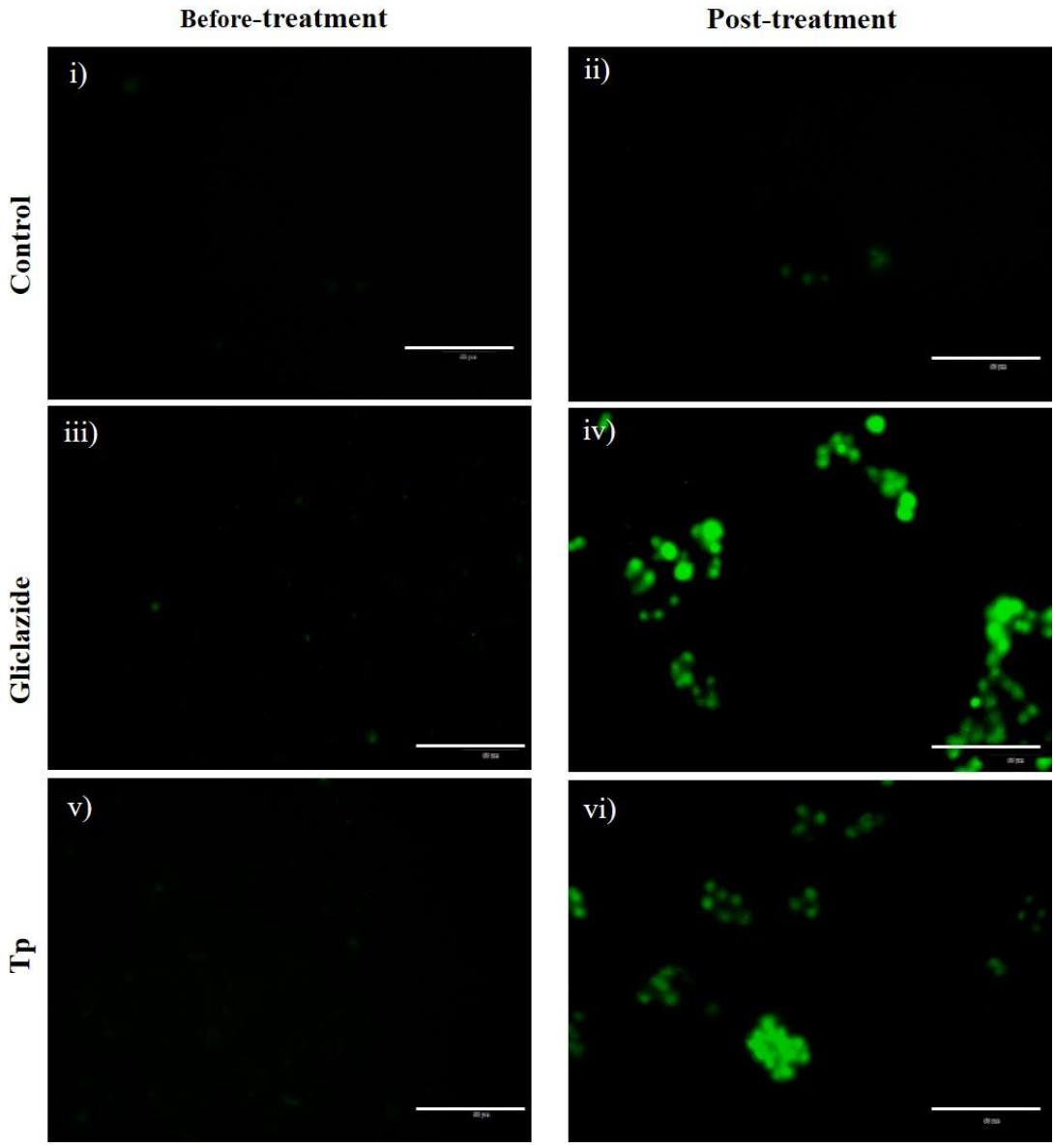


Figure 18B: The effect of the Tp extract on Ca^{2+} influx in BRIN-BD11 cells imaged by confocal microscopy. BRIN-BD11 cells were cultured as described in section 2.9. Cells were stained with the Fluo-4 AM dye and treated as per figure 18A. Images were taken before (i, iii, iv) and after (ii, iv, vi) the addition of 5.0 µm gliclazide or 500 µg/ mL^{1} Tp extract using Perkin-Elmer confocal microscope as described in the section 2.15. Green fluorescence indicates the presence of calcium inside the cells. The present images (ii, iv, vi) were captured after 30 seconds of the Tp extract or gliclazide treatment. Scale bar corresponds to 50 µm. The experiment was repeated three times with similar results. A representative image of one experiment is shown here.

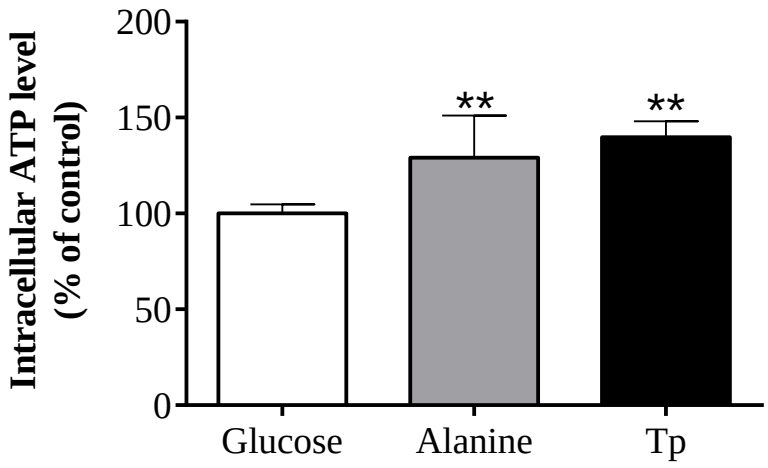


Figure 19: Intracellular ATP level of BRIN-BD11 cells in a Cell-Titer Glo Luminescent assay. BRIN-BD11 cells were cultured as described in section 2.9. The cells were glucose starved for 40 minutes in KRB buffer containing 1.1 mM glucose. The cells were then treated in KRB buffer for 30 minutes with 5.5. mM glucose (control), 10 mM alanine containing 16.7 mM glucose (positive control) or Tp extract (500 µg/mL^{1} in 5.5 mM glucose). ATP was then measured as described in section 2.16. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30 and ** denotes $p < 0.01$ compared to control 5.5 mM glucose.

A seahorse mitochondrial stress test was employed to measure the oxygen consumption rate following the addition of various modulators of mitochondrial function. Three modulators of mitochondrial respiration Oligomycin, FCCP, and the combination of Antimycin A and Rotenone were sequentially added to observe the effect of the *Teucrium polium* extract on the electron transport chain (ETC) and several mitochondrial parameters (Figure 20A). As was observed with the Celltiter-Glo luminescent assay (Figure 19), the amount of ATP produced, as independently measured in a sea horse mitochondrial stress test (last rate measurement before oligomycin injection – minimum rate measurement after oligomycin injection) was also significant ($p < 0.05$) (Figure 20B). Other mitochondrial parameters measured included basal respiration (20C, caption i), maximal respiration (20C, caption ii) and spare respiratory capacity (Figure 20C, Caption iii). The oxygen consumption rate measurements indicated that all the mitochondrial bioenergetics parameters were significantly ($p < 0.05$) increased in the presence of the *Teucrium polium* extract (Figure 20C).

There was also a rise in the extracellular acidification rate of BRIN-BD11 cells after pre-treatment with *Teucrium polium* extract for 30 minutes (Figure 21A). The extracellular acidification rate (ECAR) is a measurement of glycolytic and respiratory function. It is significant in terms of speedy ATP production and is used by many cell types when rapid ATP generation is required (Kogot-Levin *et al.*, 2016). The *Teucrium polium* extract treatment substantially enhanced the response of BRIN-BD11 cells to both glucose and oligomycin by increasing extracellular acidification rate compared to control cells. Therefore, ECAR measurements indicated glycolysis (Figure 21B, caption i), glycolytic capacity (Figure 21B, caption ii) and non-glycolytic acidification (21B, caption iii) were increased by the *Teucrium polium* extract treatment.

Given the result, the expression of glucokinase, as a rate-limiting step in glycolysis was also measured. Western Blot measurement of the enzyme revealed a 2-fold increase in the expression of glucokinase in BRIN-BD11 cells in response to the *Teucrium polium* extract treatment compared to control (Figure 22A, 22B).

GLUT2 facilitates the entry of glucose into beta cells. This leads to glucose-stimulated insulin secretion (Thorens, 2015). The level of GLUT2 protein expressed after the -

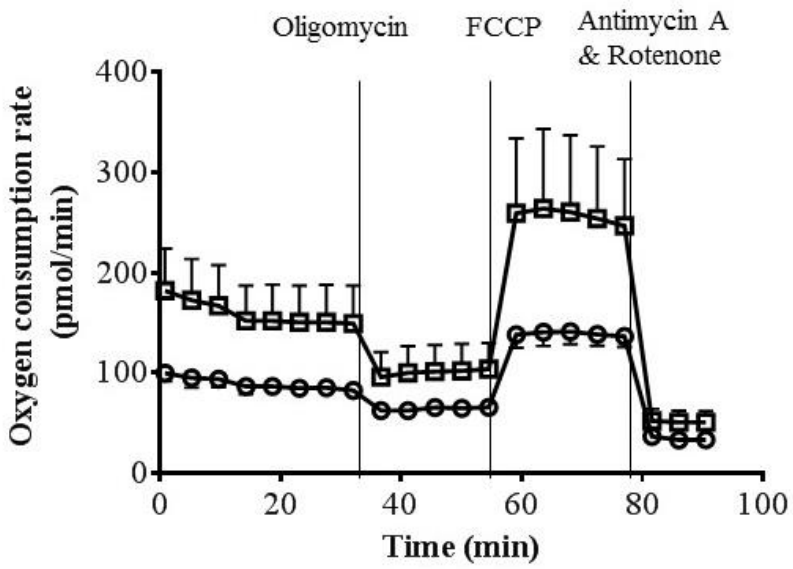


Figure 20A: Effects of the Tp extract on mitochondrial metabolism in BRIN-BD11 cells. BRIN-BD11 cells were seeded and cultured for 24 hours as described in section 2.9. On the day of experiment, BRIN-BD11 cells were glucose starved (0 mM glucose) for 30 minutes and then pre-treated for 30 minutes with the Tp extract □ (500 µg/mL)[1] or control ○ KRB buffer. After pre-treatment, the oxygen consumption rate was measured under basal conditions and followed by the sequential addition of oligomycin (0.5 mM), FCCP (1 mM), rotenone and antimycin A (1 mM), as indicated. The oxygen consumption rate (OCR) was monitored as described in section 2.17. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment.

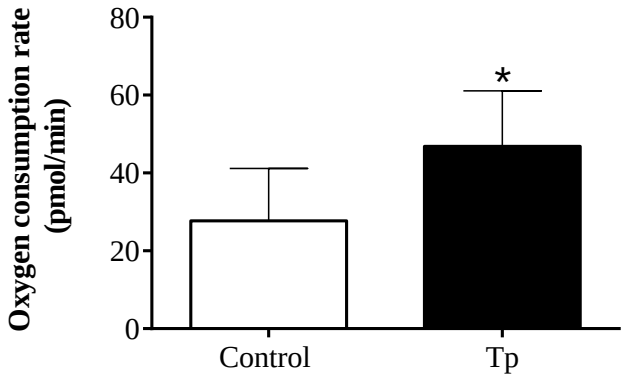


Figure 20B: Effect of the Tp extract on mitochondrial ATP production in BRIN-BD11 cells. Oxygen consumption rate (OCR) of mitochondria after the Tp extract or vehicle control treatment was monitored as described in section 2.9. ATP released by the mitochondria was calculated as mentioned in section 2. 17. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment; Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$ versus control.

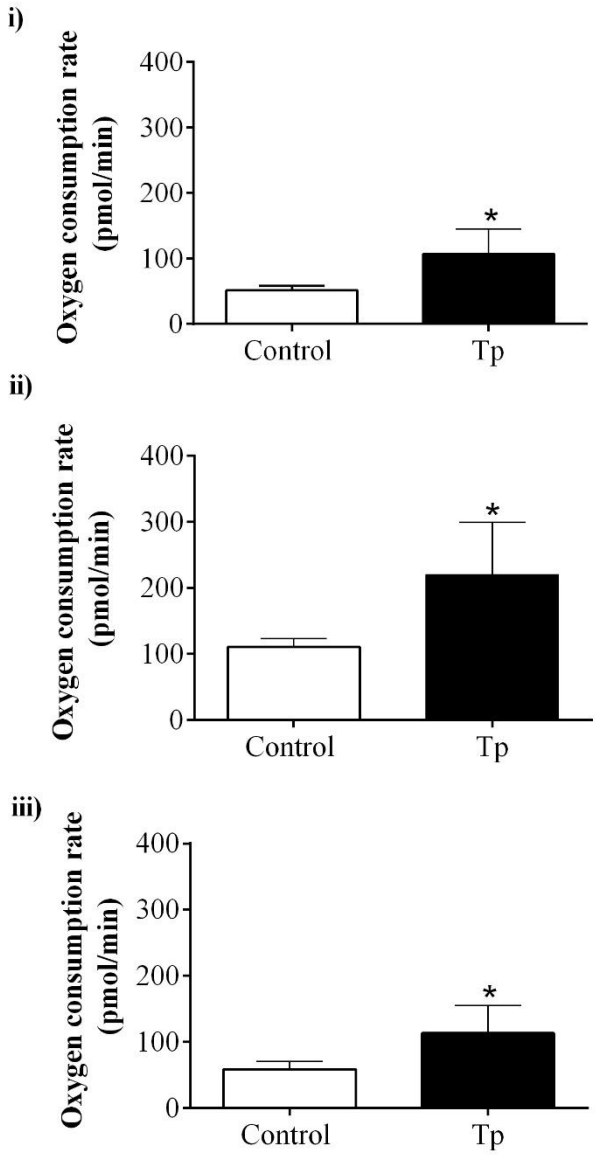


Figure 20C: Effect of the Tp extract on bioenergetics and mitochondrial metabolism in BRIN-BD11 cells. Oxygen consumption rate (OCR) of mitochondria after Tp extract or vehicle control treatment was monitored as described in section 2.9. Individual parameters for i) basal respiration, ii) maximal respiration, and iii) spare respiratory capacity were calculated as mentioned in section 2.17. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment; Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$ compared to control.

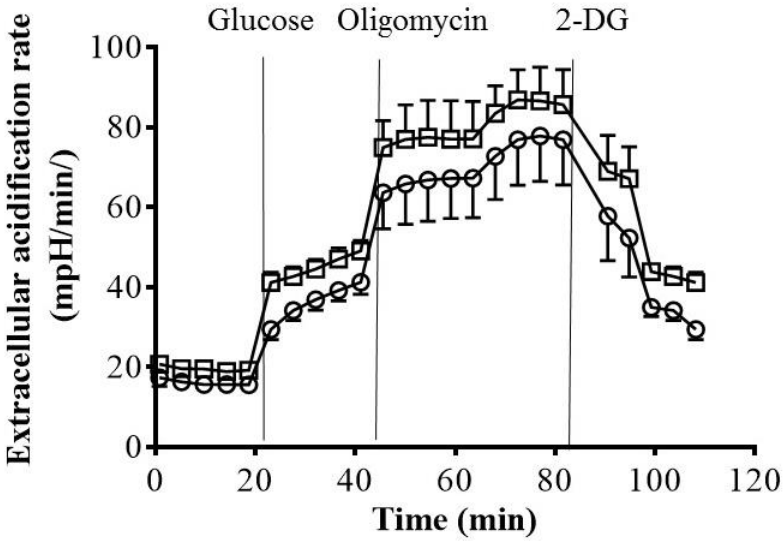


Figure 21A: The effect of the Tp extract on a glycolysis stress test in BRIN-BD11 cells. BRIN-BD11 cells were seeded and cultured for 24 hours as described in section 2.9. On the day of experiment, BRIN-BD11 cells were pre-treated for 30 minutes with control KRB buffer ○ or Tp extract □ (500 µg/mL)[1] for 30 minutes. The extracellular acidification rate (ECAR) was measured under basal conditions and later sequentially 25mM glucose, oligomycin and 2 Deoxyglucose (2DG) was added and Extracellular acidification rate was monitored as described in section 2.17. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment.

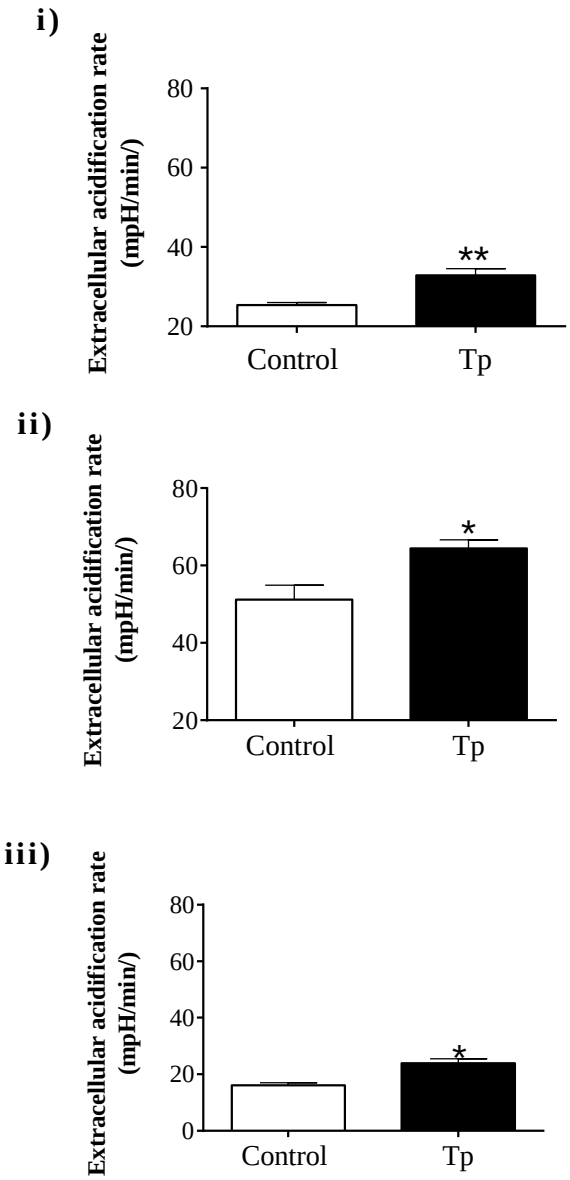


Figure 21B: Effect of the Tp extract on glycolysis in BRIN-BD11 cells. Extracellular acidification rate was monitored as described in section 2.9. Individual parameters for glycolytic stress test. i) Glycolysis ii) Glycolytic capacity and iii) Non-glycolytic acidification was calculated as mentioned in section 2.17. Each data point represents an Extracellular acidification rate (ECAR) measurement. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$, ** $p < 0.01$ compared to control.

Teucrium polium extract treatment was significantly ($p < 0.05$) increased compared to control (Figure 22 A, C). The level of GLUT2 present after the *Teucrium polium* extract treatment was 1.4-times higher than the untreated control in the Western Blot (Figure 22, caption A, C). GLUT2 expression was also visualized by immunofluorescent staining of BRIN-BD11 cells after the *Teucrium polium* extract treatment (Figure 23A, 23B). Figure 23A depicts the staining pattern of control and the *Teucrium polium* extract-treated cells. The fluorescent staining of GLUT2 increased significantly in the presence of the *Teucrium polium* extract (Figure 23A, caption ii and vi). The merged figures (Figure 23A, caption v and vi) represent that this staining coincides with DAPI staining (Figure 23A, caption iii and iv) and confirms that the localization of the GLUT2 was cell specific. The quantification suggests that fluorescence intensity of GLUT2 was increased by 2.4-fold in the presence of the *Teucrium polium* extract compared to control treatment (Figure 23B). However, treatment of primary antibody or secondary antibody or Thet *Teucrium polium* extract alone did not exhibit any auto-fluorescence or interference. GLUT2 was found to be expressed after 30-minute treatment of *Teucrium polium* extract in the present study.

Glucose, which is necessary for the survival of the β-cell, provides the main stimulus to drive insulin exocytosis via glycolysis and mitochondrial oxidation (Keane and Newsholme, 2014). Glucose uptake by β-cells after 24 h incubation was measured in the presence of the *Teucrium polium* extract to determine if the extract impacted on glucose metabolism. Glucose levels in the cell culture media before and after the *Teucrium polium* extract treatment were measured. Glucose consumption in the extract-treated group was not significantly ($p > 0.05$) different in comparison to the control group. After 24 hours of treatment, the *Teucrium polium* extract (500 µg/mL) increased glucose consumption by 1.4-fold (Figure 24). The effect of the *Teucrium polium* extract on glucose consumption by BRIN-BD11 cells was further investigated in another independent assay, monitoring the uptake of a fluorescent 2NBDG (Figure 25, 26). The *Teucrium polium* extract (500 µg/mL)1 significantly ($p < 0.001$) promoted 2NBDG uptake into BRIN-BD11 cells within 30 minutes of treatment (Figure 25). The effect of the *Teucrium polium* extract in promoting 2NBDG uptake persisted in a concentration-dependent manner following 24 hours (Figure 26).

A)

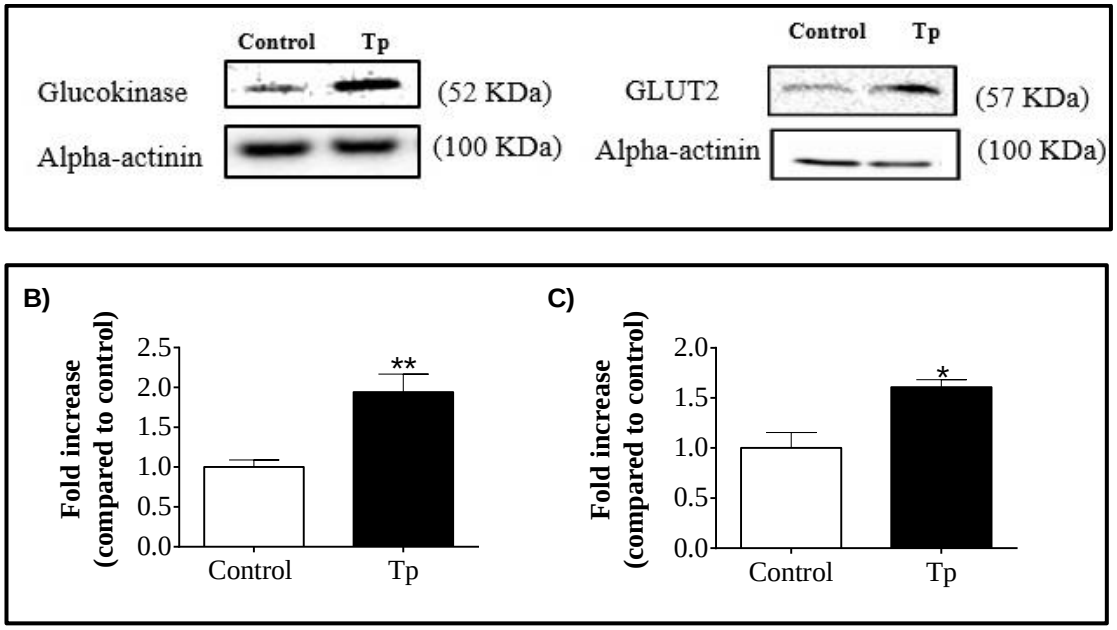


Figure 22: Effect of the Tp extract on glucokinase and glucose transporter 2 (GLUT2) expression in BRIN-BD11 cells. BRIN-BD11 cells were cultured as described in section 2.9. Cells were glucose starved for 40 minutes in KRB buffer containing 1.1 mM glucose. Cells were then treated for 30 minutes with control glucose (5.5mM), or, Tp extract (500 µg/mL[1] in 5.5 mM glucose). Cell lysates were collected and used for Western Blot as mentioned in section 2.27. A) Glucokinase and GLUT2 present in BRIN-BD11 after treating with Tp extract and control media. B) Level of glucokinase upregulation and C) GLUT2 upregulation by Tp extract. α-Actinin was used as a housekeeping control. All data are presented after normalising with α-actinin expression and are expressed relative to the control. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment, Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$ and ** $p < 0.01$ compared to control.

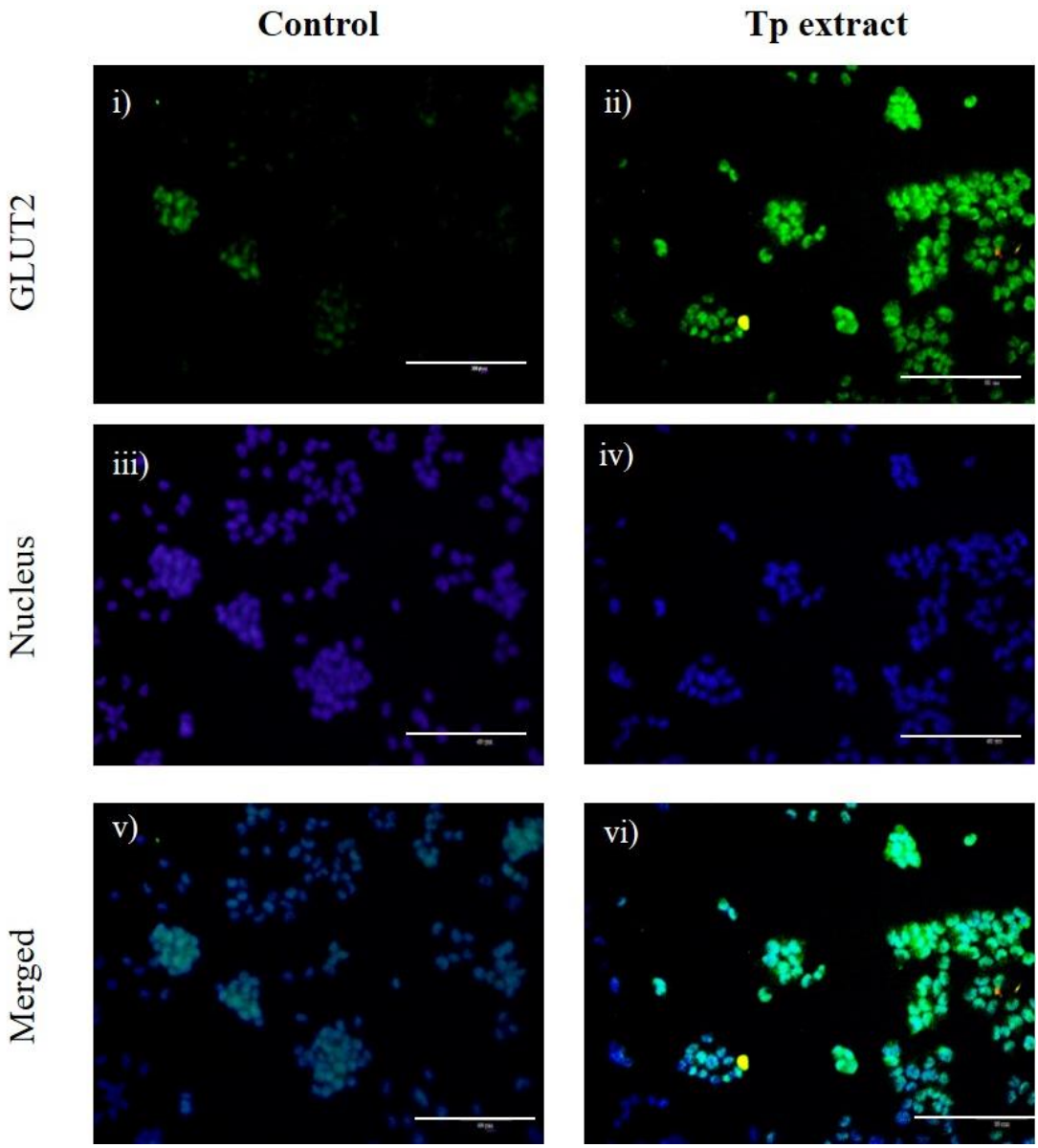


Figure 23A: Confocal microscopy imaging of GLUT2 in BRIND-BD11 cells treated with the Tp extract. BRIN-BD11 cells were cultured as described in section 2.9. Cells were glucose starved for 40 minutes in KRB buffer containing 1.1 mM glucose. Cells were then treated for 30 minutes with 5.5 mM glucose (control) or Tp extract (500 µg/mL^{1} in 5.5 mM glucose) in KRB buffer. Cells were stained with antibodies as described in section 2.20 and observed in an Olympus upright microscope to obtain images of GLUT2 (i, ii) and DAPI nuclear staining (iii, iv). Merged pictures of both GLUT2 and nuclei in control and the Tp extract (500 µg/mL)^{1} treated cells are shown in caption v and vi, respectively. The scale bar corresponds to 50 µm. The experiment was repeated three times with similar results. A representative result of one experiment is shown here.

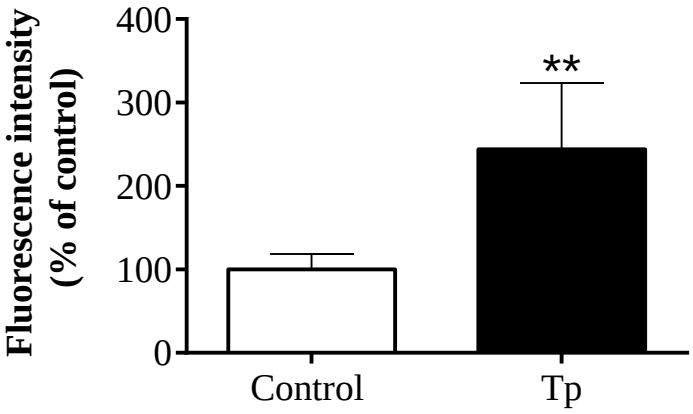


Figure 23B: Quantification of GLUT2 from confocal microscope imaging in BRIND-BD11 cells treated with the Tp extract. GLUT2 was observed as described in Figure 23A. Fluorescence intensity of GLUT2 observed (green fluorescence) in BRIN-BD11 cells shown in caption A and B of Figure 23A were measured using ImageJ ® software (Ver. 1.50i) as described in section 2.20. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment; Statistical differences between the treatments were assessed as per section 2.30 and ** denotes $p < 0.01$ compared to control.

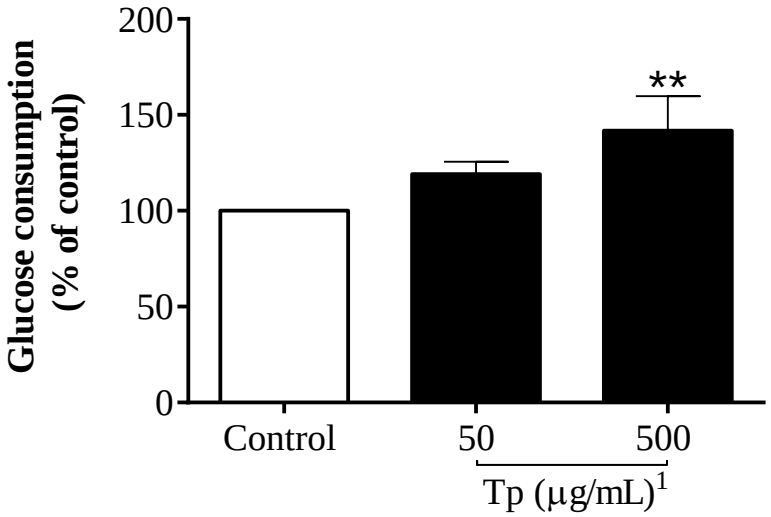


Figure 24: Glucose consumption by BRIN-BD11 cells in response to the Tp extract treatment. BRINBD-11 cells were cultured as described in section 2.9. BRIN-BD11 cells were incubated for 24 hours with control media or Tp extract. The supernatant was then diluted in 1X sodium phosphate buffer and finally, the Amplex red glucose oxidase assay was done as described in section 2.23. Fluorescence was measured as described in section 2.18. Glucose concentration in the media was measured before and after 24 hours of treatment. Glucose values were calculated from a glucose standard curve and data was normalized to the amount of protein using a BCA protein quantification assay as described in the section 2.7. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30 and ** denotes $p < 0.01$ compared to control.

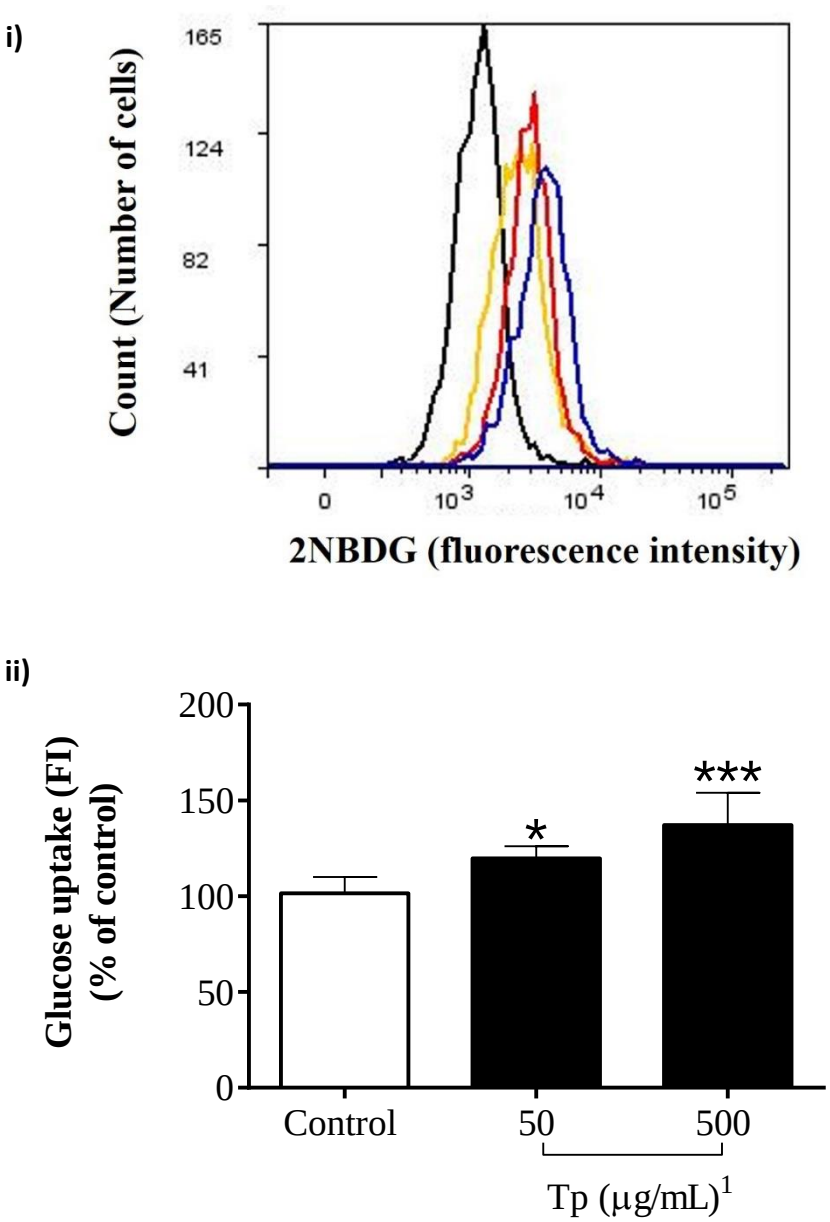
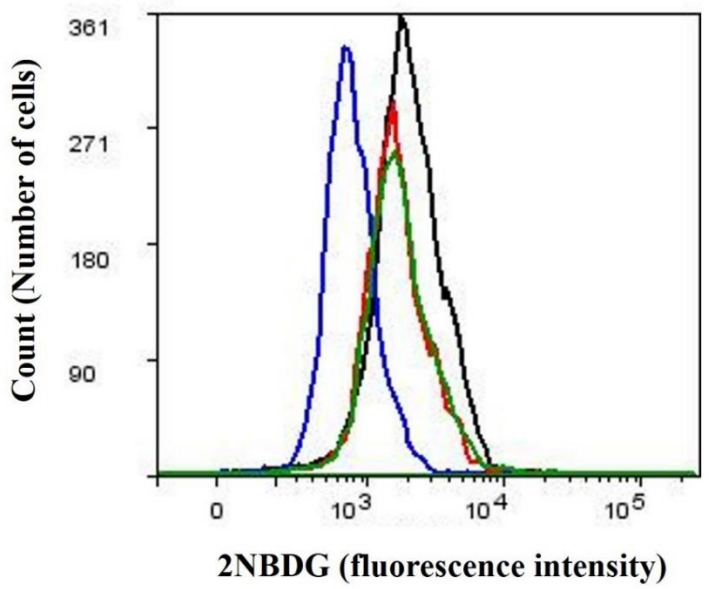


Figure 25: Flow cytometric determination of glucose uptake using 2NBDG in BRIN-BD11 cells treated with the Tp extract for 30 minutes. BRINBD-11 cells were cultured as described in section 2.9. BRIN-BD11 cells were serum starved for 6 hours and then treated for 30 minutes with control DMEM media ■ or the Tp extract (5 ■, 500 ■ µg/mL[1]) supplemented with 2NBDG or control without (■) 2NBDG. Cells were then washed, detached with trypsin and stained with propioneiodide (PI). 2NBDG uptake was measured using flow cytometer as described in section 2.19. The peak shift of fluorescence intensity after various treatments is shown in caption (i) and quantitated in caption (ii). All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment, Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$ and *** $p < 0.001$ compared to control.

i)



ii)

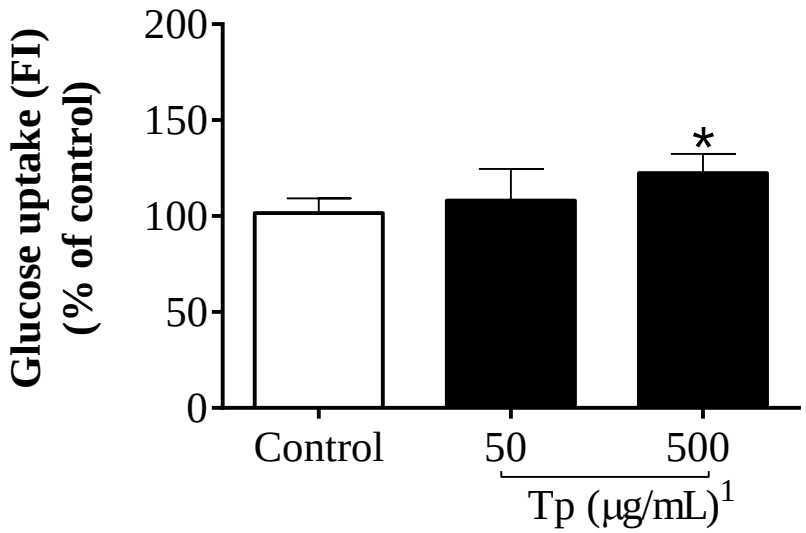


Figure 26: Flow cytometric determination of glucose uptake using 2NBDG in BRIN-BD11 cells treated with the Tp extract for 24 hours. BRINBD-11 cells were cultured as described in section 2.9. BRIN-BD11 cells were serum starved for 6 hours and then treated for 24 hours with control DMEM media ■ or Tp extract (50 ■, 500 ■ µg/mL[1]) supplemented with 2NBDG or control without (■) 2NBDG. 2NBDG uptake was measured using flow cytometer as described in section 2.19. The peak shift of fluorescence intensity after various treatments is shown in caption (i) and quantitated in caption (ii). All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment, Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$ compared to control.

3.3 Does the *Teucrium polium* extract have insulin mimetic properties?

Before any experiments were undertaken in cultured cell lines to evaluate whether the *Teucrium polium* extract might act as an insulin mimetic, its potential toxicity to the cell lines to be used in subsequent studies was first determined. The *Teucrium polium* extract did not significantly affect the viability of the 3T3-L1 cells up to a dose of 1 mg/mL in the APH assay (Figure 27). However, a 2 mg/mL dose of the *Teucrium polium* extract did have a significant ($p < 0.05$) impact on cell viability. At this dose of the *Teucrium polium* extract, cell viability decreased to less than 90% (Figure 27). Similarly, the levels of the *Teucrium polium* extract used in the current studies did not appear to significantly decrease the cell viability of C2C12 and L6 cells at doses up to 1 mg/mL of the *Teucrium polium* extract (Figure 28 and 29).

Glucose plays a significant role in the metabolism, survival and proliferation of fat cells (Rodbell, 1964) and its consumption or uptake is a key indicator of insulin action in these cells (Kono *et al.*, 1982). We measured glucose consumption by the 3T3-L1 adipocytes after a 24-hour incubation with different doses of insulin or the *Teucrium polium* extract (Figure 30) to determine if the *Teucrium polium* extract impacted glucose metabolism. The constituents of the *Teucrium polium* extract appear to promote glucose consumption by the 3T3-L1 cells after 24 hour of treatment. Figure 30 illustrates that glucose consumption of the 3T3-L1 cells treated with the *Teucrium polium* extract was significantly ($p < 0.05$) different in comparison to the control group. The efficacy of response was similar to a maximal dose of insulin. Indeed, there was no significant difference between glucose consumption by the *Teucrium polium* extract (500 µg/mL)[1] compared to insulin (100 nM).

Insulin performs important metabolic and cellular activities in the muscle cells (Samuel & Shulman, 2012). Glucose consumption was evaluated in the C2C12 myotubes (derived from the thigh muscle of C3H mice (Burattini *et al.*, 2004)) before and after the *Teucrium polium* extract treatment (Figure 31). The *Teucrium polium* extract (500 µg/mL)[1] significantly ($p < 0.05$) promoted glucose consumption 1.5-fold over 24 hours in C2C12 myotubes in a dose-dependent manner. These observations were further supported in L6 myotubes treated with the *Teucrium polium* extract or insulin for either 30 min or 24 hour (Figure 32 i, ii).

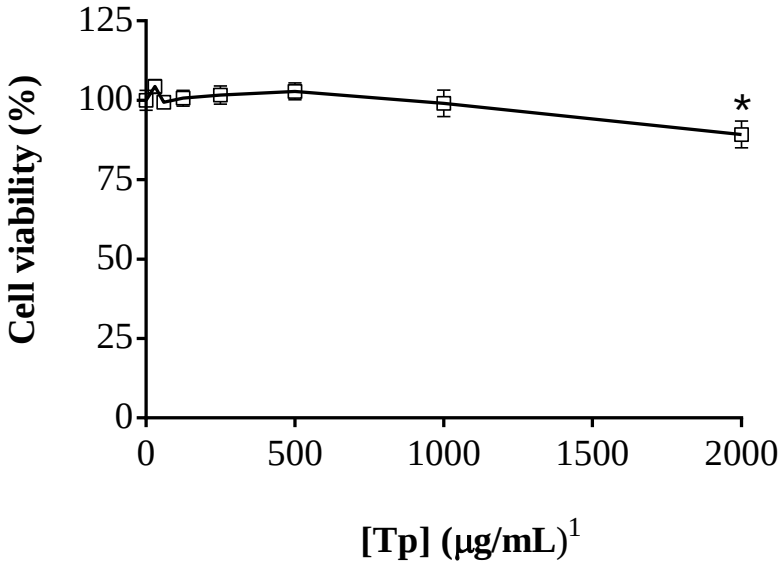


Figure 27: The effect of Tp extract on viability of 3T3-L1 cells. 3T3-L1 cells were cultured as described in section 2.21. Cells were treated with various doses of the Tp extract (0 - 2000 µg/mL[1]) for 24 hours. The viability of cells following the treatment was assessed using an acid phosphatase (APH) assay as described in section 2.13.2. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment, Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$ in comparison with control.

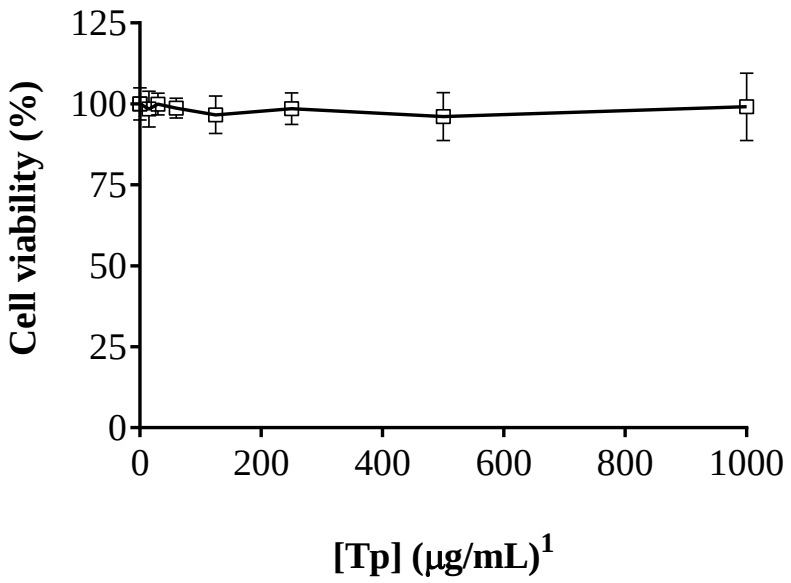


Figure 28: The effect of Tp extract on viability of C2C12 cells. C2C12 cells were cultured as described in section 2.24. Cells were treated with various doses of the Tp extract (0-1000 μg/mL^{1}) for 24 hours. The viability of cells following the treatment was assessed using an acid phosphatase (APH) assay as described in section 2.13.2. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30.

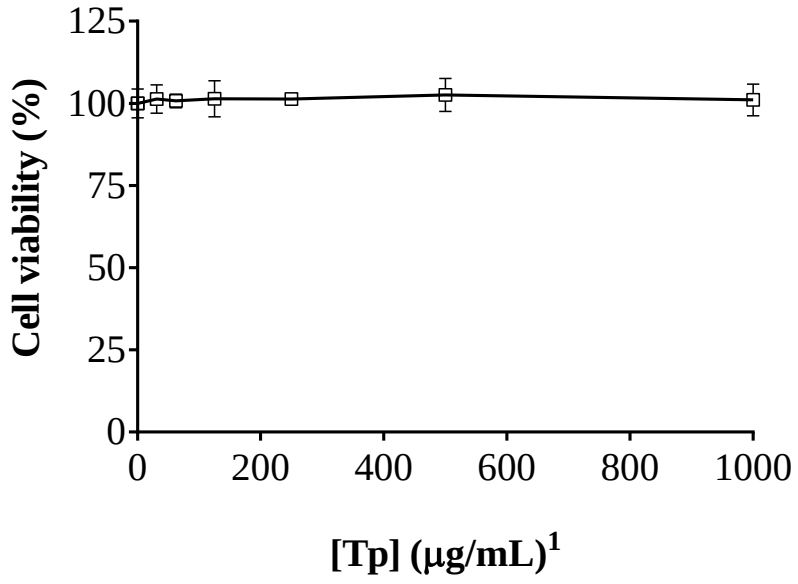


Figure 29: The effect of Tp extract on viability of L6 cells. L6 cells were cultured as described in section 2.25. Cells were treated with various doses of the Tp extract (0-1000 μg/mL[1]) for 24 hours. The viability of cells following the treatment was assessed using an acid phosphatase (APH) assay as described in section 2.13.2. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30.

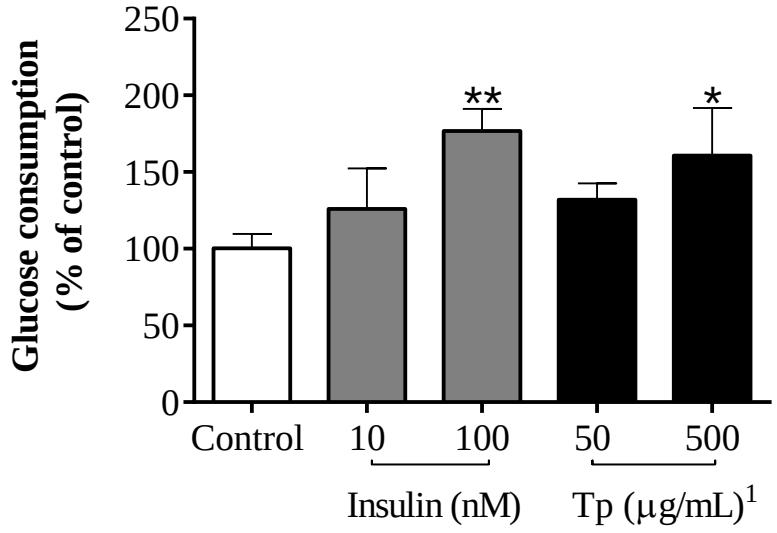


Figure 30: The effect of Tp extract on glucose consumption by 3T3-L1 cells. 3T3-L1 cells were cultured and differentiated as described in section 2.21 and 2.22. Cells were serum starved for 24 hours and then treated with insulin (10, 100 nM) or Tp extract (50, or 500 µg/mL[1]) for 24 hours. The supernatant was then diluted in 1X sodium phosphate buffer and finally, the Amplex red glucose oxidase assay was done as described in section 2.18. Glucose concentration in the media was measured before and after 24 hour of treatment. Glucose values were calculated from a glucose standard curve and the data was normalized to the amount of protein using a BCA protein quantification assay as described in the section 2.7. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$, ** $p < 0.01$ compared to control.

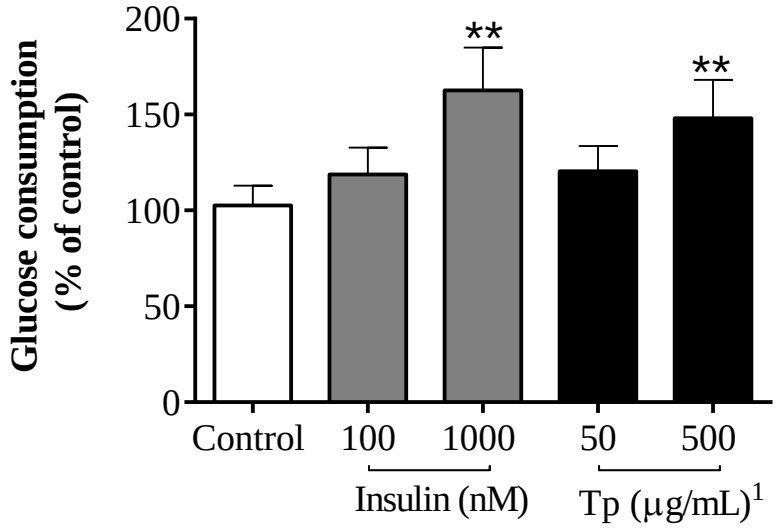
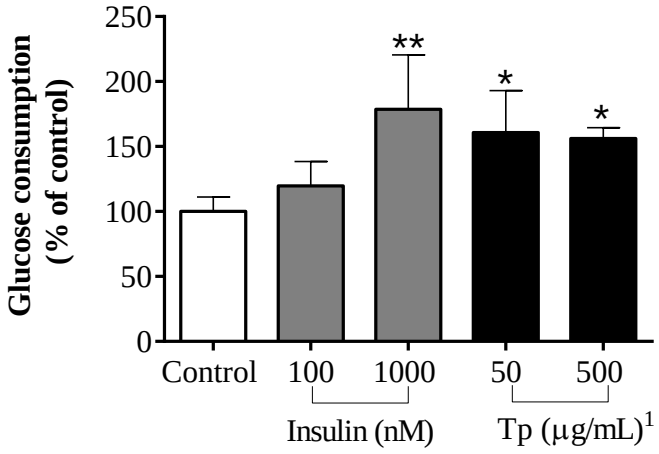


Figure 31: The effect of Tp extract on glucose consumption by C2C12 myotubes. C2C12 cells were cultured as described in section 2.24. Cells were serum starved for 24 hours and then treated with insulin (10, 100 nM) or Tp extract (50, or 500 µg/mL[1]) for 24 hours. The supernatant was then diluted in 1X sodium phosphate buffer and finally, the Amplex red glucose oxidase assay was done as described in section 2.18. Glucose concentration in the media was measured before and after 24 hour of treatment. Glucose values were calculated from a glucose standard curve and the data was normalized to the amount of protein using a BCA protein quantification assay as described in the section 2.7. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$, ** $p < 0.01$ compared to control.

i)



ii)

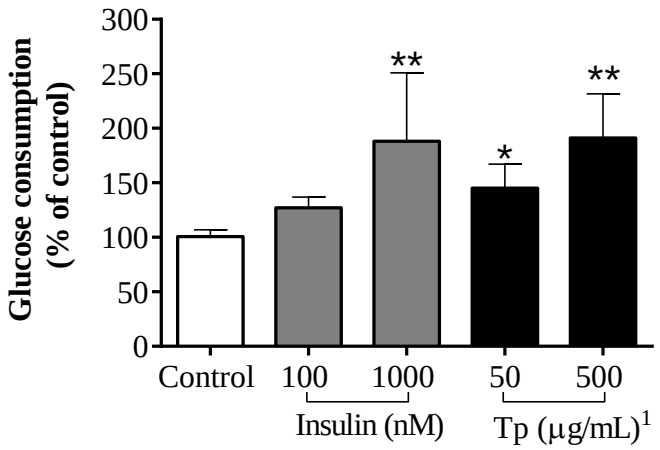


Figure 32: The effect of Tp extract on glucose consumption by L6 myotubes. L6 cells were cultured as described in section 2.25. Cells were serum starved for 24 hours and then treated with insulin (10, 100 nM) or Tp extract (50, or 500 µg/mL^{1}) for i) 30 minutes ii) 24 hours thereafter. The supernatant was then diluted in 1X sodium phosphate buffer and finally, the Amplex red glucose oxidase assay was done as described in section 2.18. Glucose concentration in the media was measured before and after 30 minutes or, 24 hour of treatment. Glucose values were calculated from a glucose standard curve and data was normalized to the amount of protein using a BCA protein quantification assay as described in the section 2.7. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$, ** $p < 0.01$ compared to control.

Again, as was observed in the 3T3-L1 and C2C12 cells, the action of the *Teucrium polium* extract was as effective as a maximal dose of insulin (1000 nM) in promoting glucose uptake in L6 myotubes.

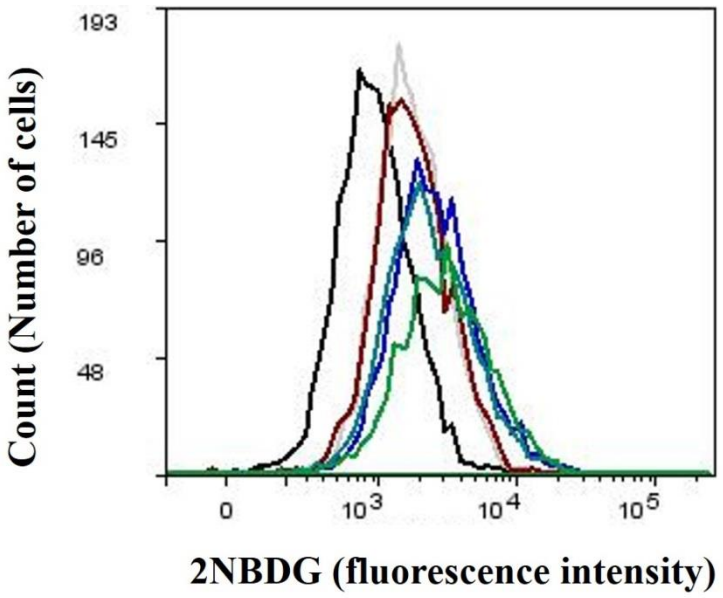
This stimulatory effect of the *Teucrium polium* extract on promoting glucose consumption in muscle cell culture, was also confirmed using a 2NBDG glucose uptake assay. This assay is a more sensitive fluorescent assay (Zou *et al.*, 2005) than the Amplex red assay used in the foregoing studies. The effect of the *Teucrium polium* extract on promoting glucose uptake by L6 myotubes was evident following 30 minutes of treatment (Figure 33) and persisted following 24 hours of treatment (Figure 34). The effect of the *Teucrium polium* extract on glucose uptake was equivalent to a maximal dose of insulin in all cell lines. The effect of the *Teucrium polium* extract was dose-dependent (Figure 33, 34), however, a 50 $\mu g/mL^{1}$ dose was required to achieve a statistically significant response relative to control treated cells.

3.4 Does the *Teucrium polium* extract influence insulin signalling pathways?

Insulin signalling is involved in a number of cellular processes including glucose uptake, protein metabolism, lipid metabolism, glycogen synthesis, cellular growth and proliferation (Boucher *et al.*, 2014). Key insulin signalling effector molecules (such as kinases) mediate metabolic activities of insulin in muscle and fat cells (Pessin *et al.*, 1999). Several key signalling molecules within insulin signalling pathways in L6 myotubes were investigated in this study.

As an initial exploratory step, 32 kinases were pre-screened by Kinexus Bioinformatics Corporation, Canada to determine which kinases could be detected in L6 myotubes using their proprietary antibodies (Figure 35 A, B, C, D). Fifteen kinases could be detected in pooled lysates from control, insulin stimulated and the *Teucrium polium* extract stimulated L6 myotubes. The individual effect of control media, insulin and the *Teucrium polium* extract was then evaluated by Kinexus for their effect on these 15 kinases (Figure 35 A, B, C). Both insulin and the *Teucrium polium* extract promoted a large increase in the phosphorylation of the p70 ribosomal S6 kinase beta (S6Kb) protein (Figure 35 D). In addition, insulin and the *Teucrium polium* extract appeared to both increase the phosphorylation of glycogen synthase kinase β (GSK3β), and ribosomal S6 protein-serine kinase 1 (RSK1) proteins in L6 myotubes.

i)



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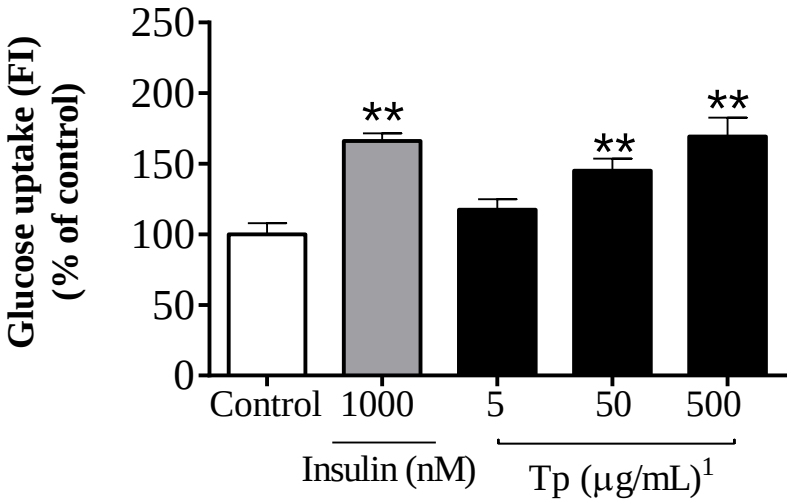
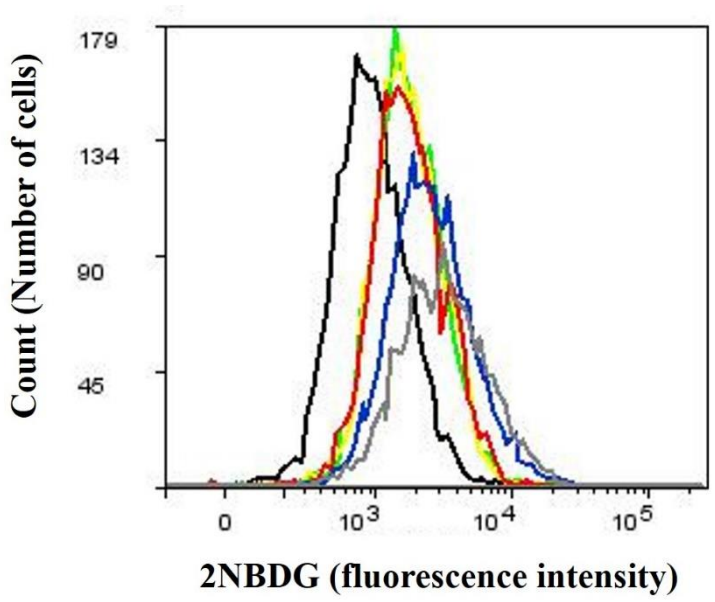


Figure 33: Flow cytometric determination of glucose uptake using 2NBDG in L6 myotubes treated with the Tp extract for 30 minutes. L6 cells were cultured as described in section 2.25. L6 myotubes were serum starved for 24 hours and then treated for 30 minutes with control DMEM media ■ or the Tp extract (5 ■, 50 ■ , 500 ■ µg/mL)[1] or insulin ■ (1000 nM) supplemented with 2NBDG or control without 2NBDG (■). 2NBDG uptake was measured using flow cytometer as described in section 2.19. The peak shift of fluorescence intensity after various treatments is shown in caption (i) and quantitated in caption (ii). All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30 and ** denotes $p < 0.01$ compared to control.

i)



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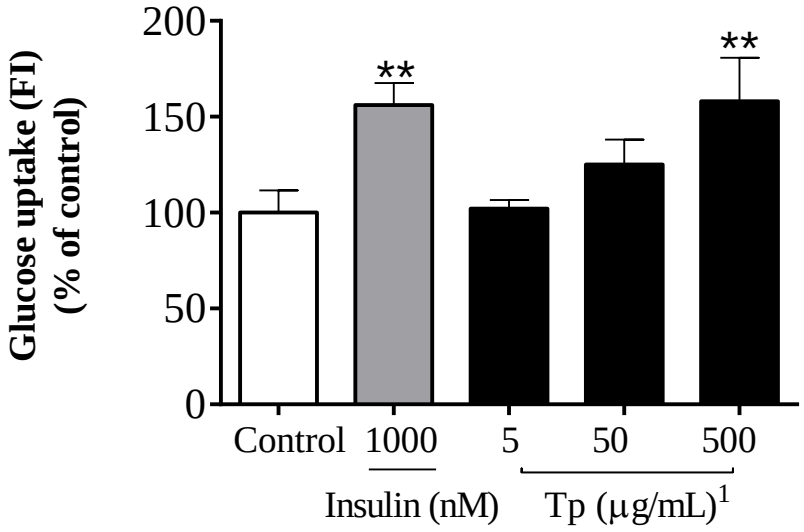


Figure 34: Flow cytometric determination of glucose uptake using 2NBDG in L6 myotubes treated with the Tp extract for 24 hours. L6 cells were cultured as described in section 2.25. L6 myotubes were serum starved for 6 hours and then treated for 24 hours with control DMEM media ■ or the Tp extract (5 ■ , 50 ■ , 500 ■ µg/mL)[1] or insulin ■ (1000 nM). After 24 hours, cells were supplemented with 2NBDG or control without 2NBDG (■) solution for 30 minutes. 2NBDG uptake was measured using flow cytometer as mentioned in section 2.19. The peak shift of fluorescence intensity after various treatments is shown in caption i and quantitated in caption ii. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30 and ** denotes $p < 0.01$ compared to control.

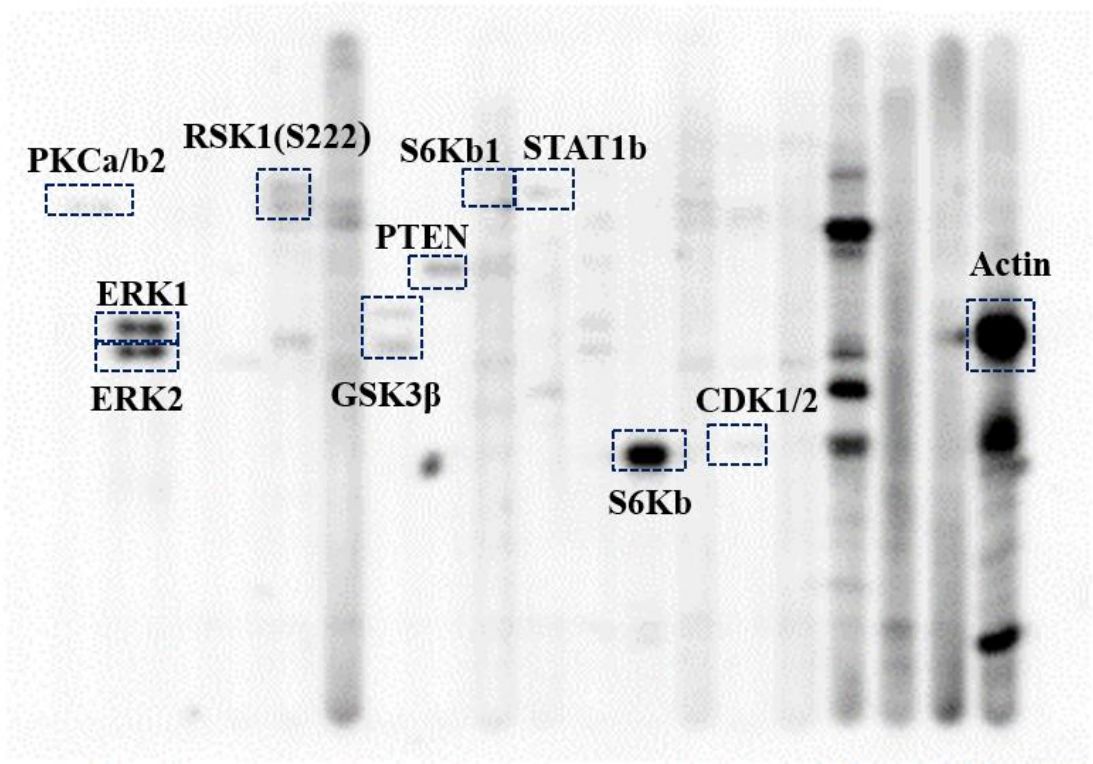


Figure 35A: Multi-immunoblot proteomic measurement of selected phospho-kinases within insulin signalling pathways in L6 myotubes in the absence of any treatment. L6 cells were cultured as described in section 2.25. L6 myotubes were serum starved for 24 hours and then treated for 30 minutes with control DMEM media. Cell lysates were prepared and sent to Kinexus Bioinformatics Corporation (Vancouver, Canada) for multi-immunoblot using Kinetworks KCPS-1.0 service as described in section 2.26. Phosphorylation of cyclin dependent kinase (CDK1/2), extracellular signal-regulated kinase1 (ERK1), extracellular signal-regulated kinase 2 (ERK2), glycogen synthase kinase β (GSK3b), phosphatase and tension homologue (PTEN), protein kinase C (PKC), p70 ribosomal S6 kinase beta (S6kb), ribosomal protein serine S6 kinase beta-1 (S6Kb1), ribosomal S6 protein-serine kinase 1 (RSK1) were detected. All the screens performed have used multi-immunoblotting with pre-validated antibodies against the signalling molecules. Actin was used as a housekeeping control. Data represents one experiment (n=1).

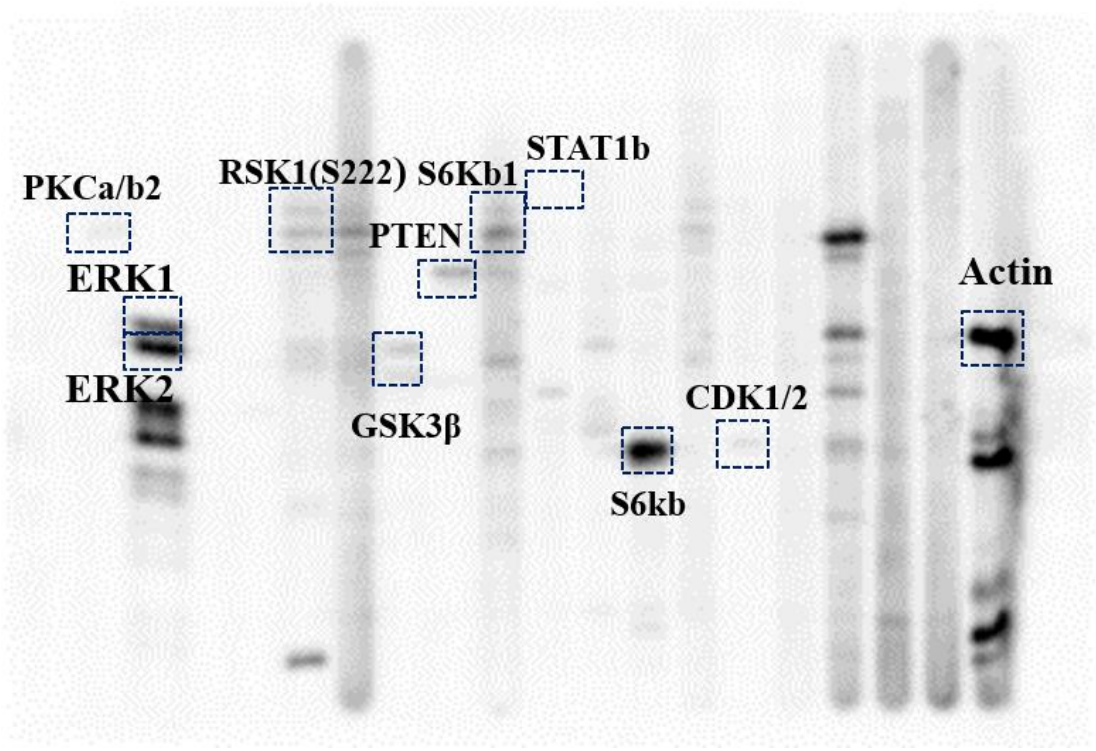


Figure 35B: Multi-immunoblot proteomic measurement of selected phospho-kinases within insulin signalling pathways in L6 myotubes treated with insulin. L6 cells were cultured as described in section 2.25. L6 myotubes were serum starved for 24 hours and then treated for 30 minutes with insulin (1000 nM). Cell lysates were prepared and sent to Kinexus Bioinformatics Corporation (Vancouver, Canada) for multi-immunoblot using Kinetworks KCPS-1.0 service as described in section 2.26. Phosphorylation of cyclin dependent kinase (CDK1/2), extracellular signal-regulated kinase1 (ERK1), extracellular signal-regulated kinase 2 (ERK2), glycogen synthase kinase β (GSK3b), phosphatase and tension homologue (PTEN), protein kinase C (PKC), p70 ribosomal S6 kinase beta (S6kb), ribosomal protein serine S6 kinase beta-1 (S6Kb1), ribosomal S6 protein-serine kinase 1 (RSK1) were detected. All the screens performed have used multi-immunoblotting with pre-validated antibodies against the signalling molecules. Actin was used as a housekeeping control. Data represents one experiment (n=1).

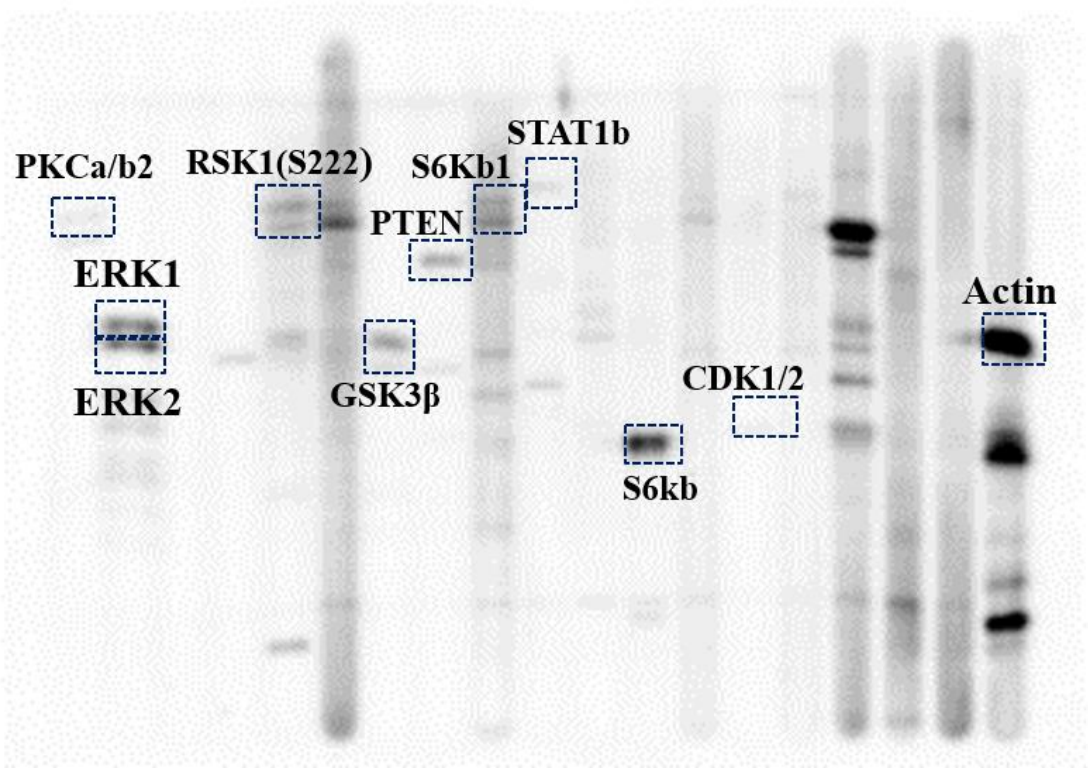


Figure 35C: Multi-immunoblot proteomic measurement of selected phospho-kinases within insulin signalling pathways in L6 myotubes treated with the Tp extract. L6 cells were cultured as described in section 2.25. L6 myotubes were serum starved for 24 hours and then treated for 30 minutes with Tp extract (50 µg/mL)[1]. Cell lysates were prepared and sent to Kinexus Bioinformatics Corporation (Vancouver, Canada) for multi-immunoblot using Kinetworks KCPS-1.0 service as described in section 2.26. Phosphorylation of cyclin dependent kinase (CDK1/2), extracellular signal-regulated kinase1 (ERK1), extracellular signal-regulated kinase 2 (ERK2), glycogen synthase kinase β (GSK3β), phosphatase and tension homologue (PTEN), protein kinase C (PKC), p70 ribosomal S6 kinase beta (S6kb), ribosomal protein serine S6 kinase beta-1 (S6Kb1), ribosomal S6 protein-serine kinase 1 (RSK1) were detected. All the screens performed have used multi-immunoblotting with pre-validated antibodies against the signalling molecules. Actin was used as a housekeeping control. Data represent one experiment (n=1).

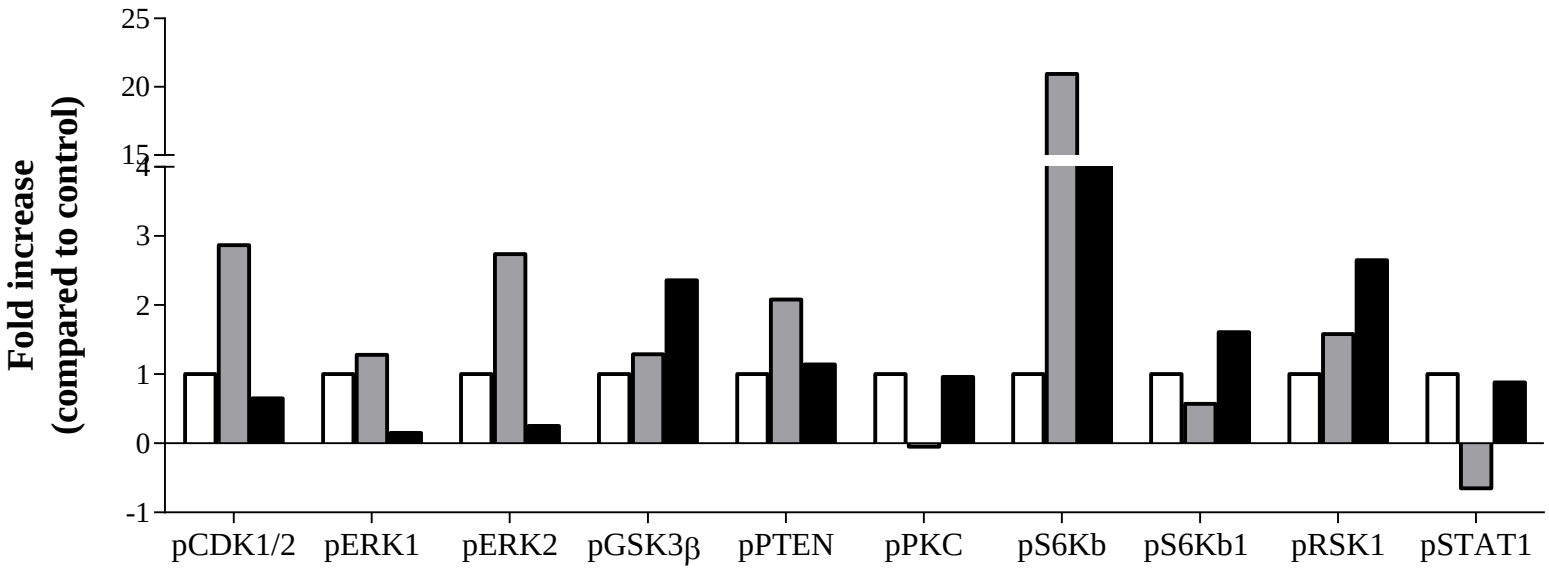


Figure 35D: Quantification of selected phospho-kinases within insulin signalling pathways in L6 myotubes treated with either insulin or the Tp extract. L6 cells were grown as described in section 2.25. L6 myotubes were serum starved for 24 hours and then treated for 30 minutes with control □ (DMEM media) or insulin ■ (1000 nM) or the Tp extract ■ (50 µg/mL)[1]. Cell lysates were prepared and sent to Kinexus Bioinformatics Corporation (Vancouver, Canada) who performed multi-immunoblot using Kinetworks KCPS-1.0 screen of selected kinases as described in section 2.26. Phosphorylation of cyclin dependent kinase (CDK1/2), extracellular signal-regulated kinase1 (ERK1), extracellular signal-regulated kinase 2 (ERK2), glycogen synthase kinase β (GSK3β), phosphatase and tension homologue (PTEN), protein kinase C (PKC), p70 ribosomal S6 kinase beta (S6kb), ribosomal protein serine S6 kinase beta-1 (S6Kb1), ribosomal S6 protein-serine kinase 1 (RSK1) were detected. The screens performed used pre-validated antibodies against the signalling molecules. Actin was used as a housekeeping control. Data represents one experiment (n=1).

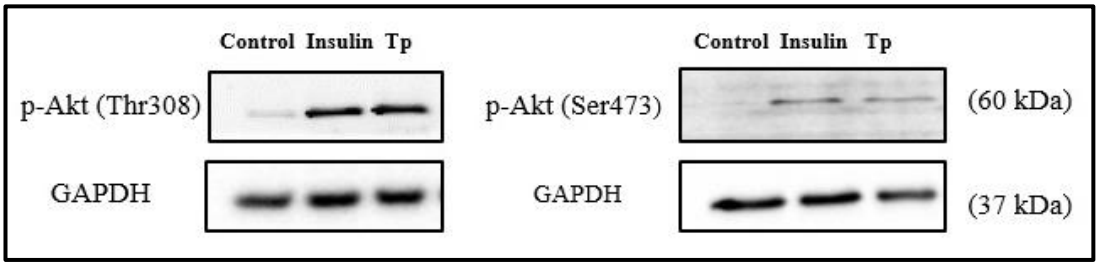
However, whereas insulin appeared to promote extracellular signal-regulated kinase 2 (ERK2) phosphorylation by several-fold, the *Teucrium polium* extract seemed to dramatically inhibit phosphorylation of both extracellular signal-regulated kinase 1 and 2 (ERK-1 and ERK-2). Furthermore, whereas insulin promoted and inhibited cyclin dependent kinase (CDK1/2) and protein kinase C (PKC) phosphorylation respectively, the *Teucrium polium* extract had little apparent effect on the activation of these kinases (Figure 35 D).

Whilst these observations may suggest some clear similarities in the action between insulin and the *Teucrium polium* extract, they also appear to highlight several contrasts or disparities in their mode of action. A note of caution should, however, be observed in consideration of these results. In particular, it must be noted that the screen detailed in Figure 35 D represents just one experiment. Validation of these observations, therefore, requires a mere detailed analysis of individual kinases, some of which are discussed in the following paragraphs of this thesis.

Other key kinases and proteins involved in insulin signalling pathways that were undetected in L6 myotubes using the kinexus screen, were also followed up in this study. This included optimisation and measurement of the phosphorylation of phosphoinositide-dependent kinase-1 (PDK-1) (Tyr 373/376), protein-serine kinase B α [T308] (Akt1) (Thr 308 and Ser 473), mammalian target of rapamycin (mTOR), glycogen synthase kinase 3α/β (GSK3α/β), ribosomal S6 protein, extracellular signal-regulated kinase1 (ERK) and glucose transporter 4 (GLUT4). Finally, glycogen content of L6 myotubes in the presence or absence of insulin or the *Teucrium polium* extract was evaluated.

Akt is one of the key regulators of insulin triggered pathways involving glucose uptake, protein synthesis and glycogen synthesis (Mackenzie & Elliot, 2014). The effect of the *Teucrium polium* extract (50 µg/mL) [1] on two different phosphorylation sites of phospho-Akt (Ser 473 and Thr 308) were monitored (Figure 36). The *Teucrium polium* extract significantly ($p < 0.01$) increased the phosphorylation of p-Akt-Thr 308 and p-Akt-Ser 473 compared to the control. Insulin also promoted activation of pAkt-308 and pAkt-473 as expected.

A)



B)

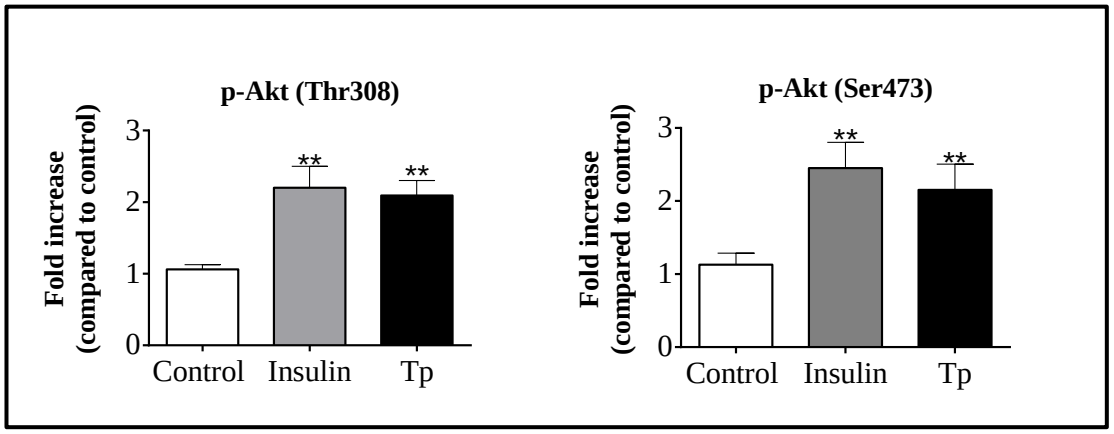


Figure 36: Effect of the Tp extract on phosphorylation of p-Akt in differentiated L6 muscle cells. L6 cells were cultured as described in section 2.25. L6 myotubes were serum starved for 24 hours and treated with insulin (1000 nM) or the Tp extract (50 µg/mL)[1] or DMEM control media for 15 minutes thereafter. Whole cell lysate protein was used in a Western Blot. Cell lysate preparation, SDS PAGE and immune-blotting was done as described in section 2.27. Both phospho Akt-thr308 and phospho Akt-ser 473 were immunoblotted and GAPDH was used as a housekeeping control. A) Representative Western Blot images of phopshorylated Akt (Thr-308 and Ser-473); B) Quantification of phospho Akt-308/control and Akt-473/control. All data are presented after normalising with GAPDH expression and are expressed relative to the control. This is a representative result of three independent experiments. Statistical differences between the treatments were assessed as per section 2.30. Values are expressed as mean (±SD) and ** denotes $p < 0.01$ compared to control.

PDK-1 is a "master" kinase that is centrally involved in the activation of of Akt, PKB, PKC and S6. It is thereby involved in insulin and growth factor signalling pathways. 3-Phosphoinositide-dependent protein kinase-1 (PDK1) appears to play a central regulatory role in many cell signalling between phosphoinositide-3 kinase and various intracellular serine/threonine kinases. PDK1 is known to be constitutively active and is further activated by tyrosine phosphorylation (Tyr (9) and Tyr (373/376)) following the treatment of the cell with insulin. The activation of p-PDK-1 pathways in response to insulin or the *Teucrium polium* extract is illustrated in Figure 37. Insulin promoted a significant ($p < 0.05$) increase in the expression of p-PDK-1. The effect of *Teucrium polium* extract on p-PDK-1 expression was even more pronounced, being about 3-fold greater than the control ($p < 0.01$). The role of the *Teucrium polium* extract on insulin mediated protein synthesis was checked in immunonoblots by investigating the phosphorylation level of p-mTOR (ser2448) and p-S6 (Figure 38).

The *Teucrium polium* extract activated the downstream signalling molecule p-mTOR (Ser2448) 2.8-fold and phospho-S6 ribosomal protein (pS6) (Ser240/244) 2-fold (Figure 39). Both these results were highly significant ($p < 0.01$) compared to the controls. The level of activation by insulin was 1.9-fold more for S6 and 1.4-fold more for mTOR compared to control treatments, respectively. Interestingly, a PDK-1 inhibitor inhibited the phosphorylation of ribosomal protein S6 in control cells (Figure 39). Moreover, this inhibitor (GSK2334470) (GlaxoSmithKline) ablated the effect of the *Teucrium polium* extract on promoting the phosphorylation of S6 to phosphorylation levels equivalent to the presence of the inhibitor alone. There was no significant difference between the level of S6 phosphorylation by PDK-1 inhibitor plus the *Teucrium polium* extract and the PDK-1 inhibitor alone (Figure 39).

The *Teucrium polium* extract (50 µg/mL)[1] did not phosphorylate p-ERK in the present study after 30 min of treatment, whereas insulin phosphorylated p-ERK significantly (data not shown). However, the *Teucrium polium* extract significantly ($p < 0.01$) phosphorylated and activated p-ERK after 24-hour treatment (Figure 40). Insulin also activated p-ERK after 24-hour treatment and this result was highly significant ($p < 0.01$).

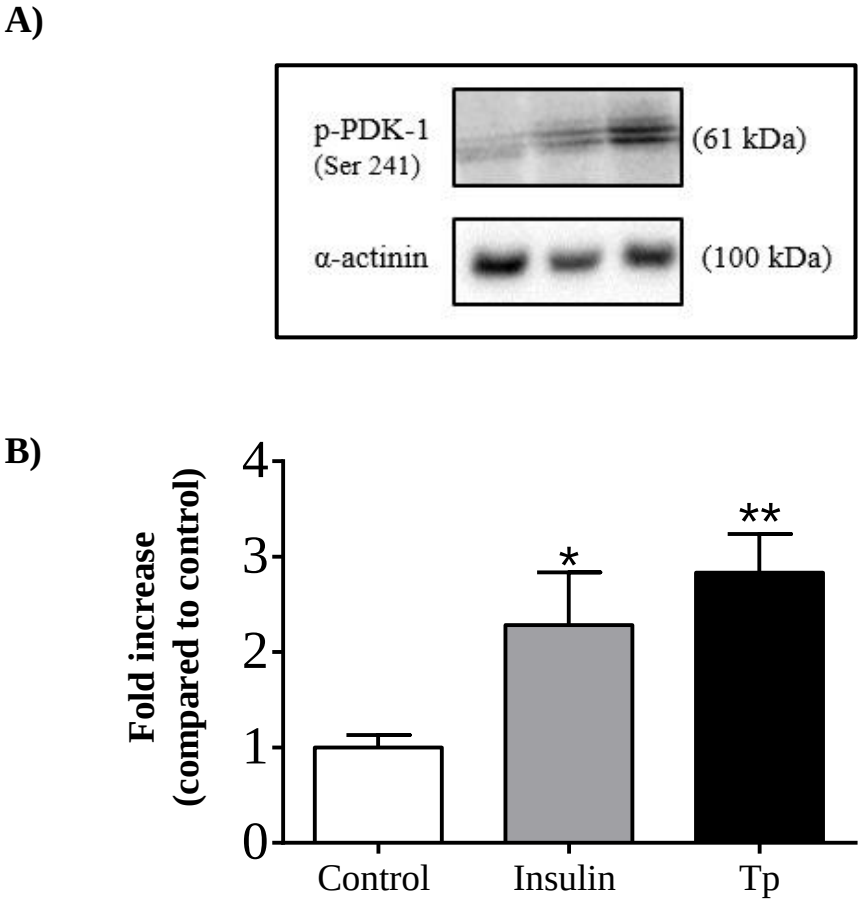
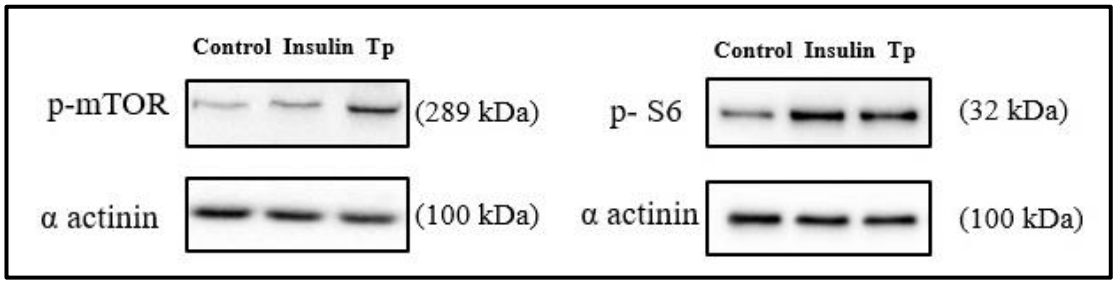


Figure 37: Effect of the Tp extract on phosphorylation of p-PDK-1 (Tyr 373/376) in differentiated L6 muscle cells. L6 cells were cultured and differentiated as described in section 2.25. L6 myotubes were serum starved for 24 hours and treated with insulin (1000 nM) or the Tp extract (50 µg/mL)[1] or DMEM control media for 10 minutes thereafter. Whole cell lysate protein was used in a Western Blot. Cell lysate preparation, SDS PAGE and immune-blotting was done as described in section 2.27. The phospho-PDK-1 (Tyr 373/376) was immunoblotted and α-actinin was used as a housekeeping control. A) Representative Western Blot images of phopshorylated PDK-1; B) Quantification of phospho PDK-1/control. All data are presented after normalising with α-actinin expression and are expressed relative to the control. This is a representative result of three independent experiments. Values are expressed as mean (±SD). Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$ and ** $p < 0.01$ compared to control.

A)



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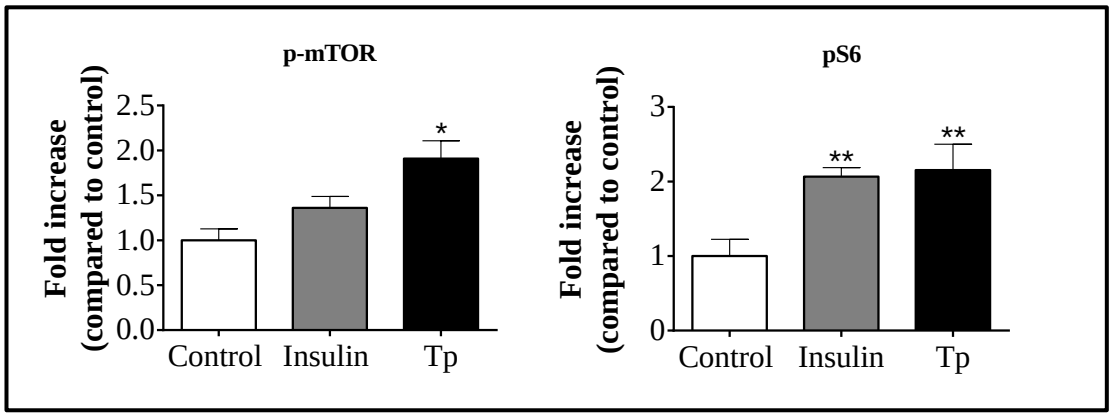


Figure 38: Effect of the Tp extract on phosphorylation of p-mTOR (Ser2448) and p-S6 (Ser240/244) in differentiated L6 muscle cells. L6 cells were cultured and differentiated as described in section 2.25. L6 myotubes were serum starved for 24 hours and treated with insulin (1000 nM) or the Tp extract (50 µg/mL)[1] or DMEM control media for 30 minutes thereafter. Whole cell lysate protein was used in a Western Blot. Cell lysate preparation, SDS PAGE and immune-blotting was done as described in section 2.27. The signalling molecules S6 and mTOR which are activated by insulin, were investigated by Western Blot and α-actinin was used as a housekeeping control. A) Representative Western Blot images of phopshorylated mTOR and S6; B) Quantification of of p-mTOR/control and p-S6/ control. All data are presented after normalising with α-actinin expression and are expressed relative to the control. This is a representative result of three independent experiments. Values are expressed as mean (±SD). Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$, ** $p < 0.01$ compared to control.

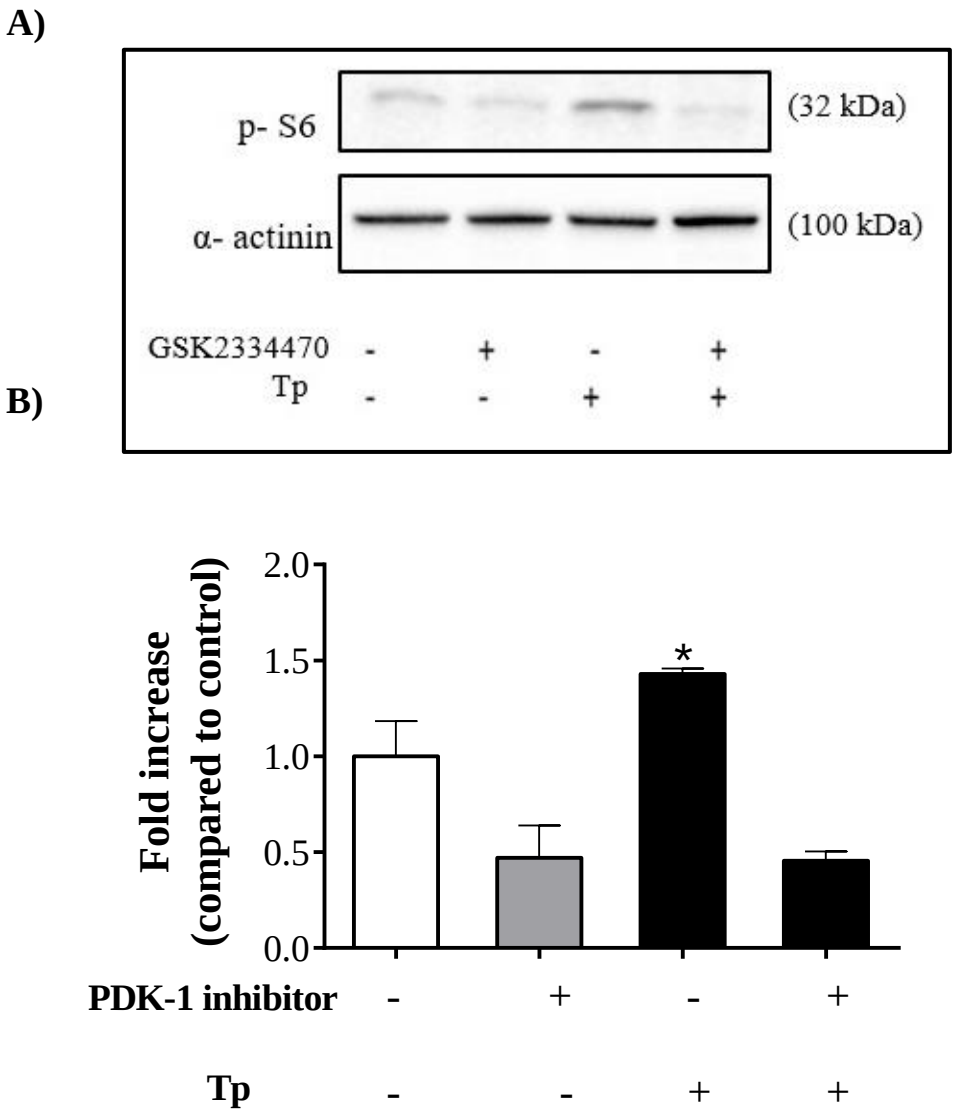
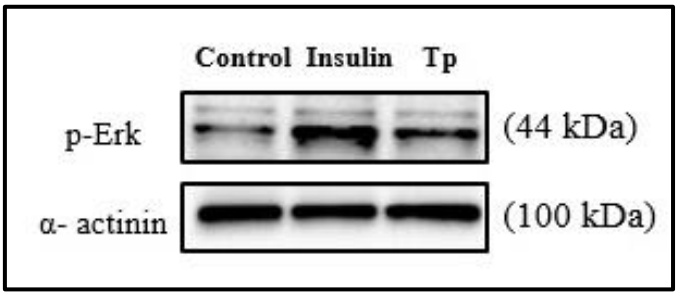


Figure 39: Effect of a selective PDK-1 inhibitor (GSK2334470) on phosphorylation of phospho-S6 ribosomal protein (Ser240/244) in L6 myotubes treated with the Tp extract. L6 cells were cultured as described in section 2.25. L6 myotubes were serum starved for 24 hours and treated with the Tp extract (50 µg/mL)[1] and /or PDK-1 inhibitor (GSK2334470) (5 µM) or DMEM control media for 15 minutes thereafter. Whole cell lysate protein was used in a Western Blot. Cell lysate preparation, SDS PAGE and immune-blotting was done as described in section 2.27 and α actinin was used as a housekeeping control. A) Representative western blot images of phosphorylated S6; B) Quantification of p-S6/control. All data are presented after normalising with α-actinin expression and are expressed relative to the control. Values are of three independent experiments are expressed as mean (± SD). Statistical differences between the treatments were assessed as per section 2.30 and * denotes $p < 0.05$ compared to control

A)



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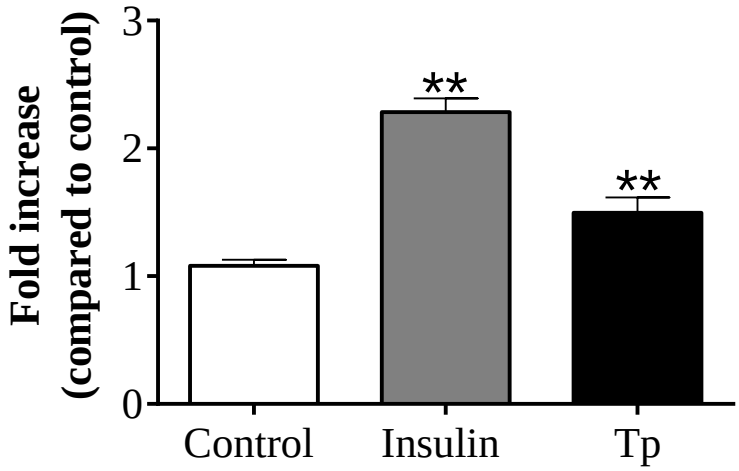


Figure 40: Effect of the Tp extract on phosphorylation of p-ERK in differentiated L6 muscle cells. L6 cells were cultured as described in section 2.25. L6 myotubes were serum starved for 24 hours and treated with insulin (1000 nM) or the Tp extract (50 µg/mL)[1] or DMEM control media for 24 hours. Whole cell lysate protein was used in a Western Blot. Cell lysate preparation, SDS PAGE and immune-blotting was done as described in section 2.27. The signalling molecule p-ERK was identified by Western Blot and α-actinin was used as a housekeeping control. A) Representative Western Blot images of phopshorylated ERK; B) Quantification of phospho ERK/control. All data are presented after normalising with α-actinin expression and are expressed relative to the control. This is a representative result of three independent experiments. Values are expressed as mean (±SD). Statistical differences between the treatments were assessed as per section 2.30 and ** denotes $p < 0.01$ compared to control.

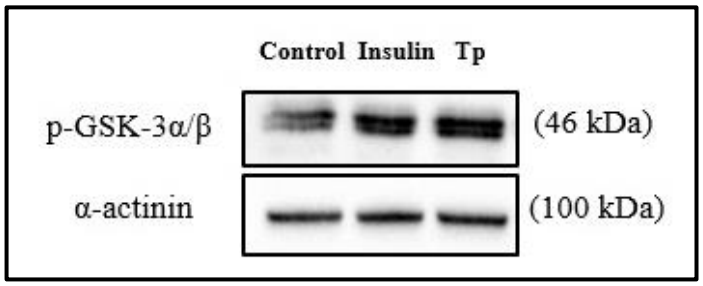
GSK3β is a protein kinase involved in phosphorylation of glycogen synthase and promotes a number of pathways including glycogen synthesis (Beurel *et al.*, 2015). In the present study, the *Teucrium polium* extract (50 µg/mL) [1] phosphorylated the p-GSk3α/β (Ser21/9) molecule 2.2-fold more than the control treatment, which was highly significant ($p < 0.01$) (Figure 41). Insulin also increased the phosphorylation of GSk3α/β 2.1-fold more than the control treatment.

The level of GLUT4 present was measured to determine whether the increase in glucose uptake by L6 myotubes in response to the *Teucrium polium* extract (50 µg/mL) [1] treatment was a result of GLUT4 upregulation and glucose transportation (Figure 42). As expected, insulin promoted GLUT4 expression in L6 myotubes (Figure 42). The level of GLUT4 present in response to *Teucrium polium* extract was highly significant ($p < 0.01$) relative to control treated cells.

The effect of the *Teucrium polium* extract on GLUT4 expression was further checked using immunofluorescence microscopy and quantified by ImageJ software (Figure 43). GLUT4 expression in L6 myotubes increased 3.2-fold (compared to control) after treatment with the *Teucrium polium* extract ($p < 0.01$) (Figure 43). As anticipated, insulin also promoted a 3.6-fold increase of GLUT4 in these L6 myotubes (Figure 43). Collectively, our findings suggest that the *Teucrium polium* extract stimulates GLUT4 expression and increases glucose uptake in L6 myotubes via GLUT4 translocation.

Finally, given that the *Teucrium polium* extract promoted glucose uptake and GLUT4 expression in, the glycogen content of L6 myotubes was evaluated using a Biovision glycogen content measurement kit. Like insulin, the *Teucrium polium* extract significantly ($p < 0.01$) increased the glycogen content in L6 myotubes (Figure 44).

A)



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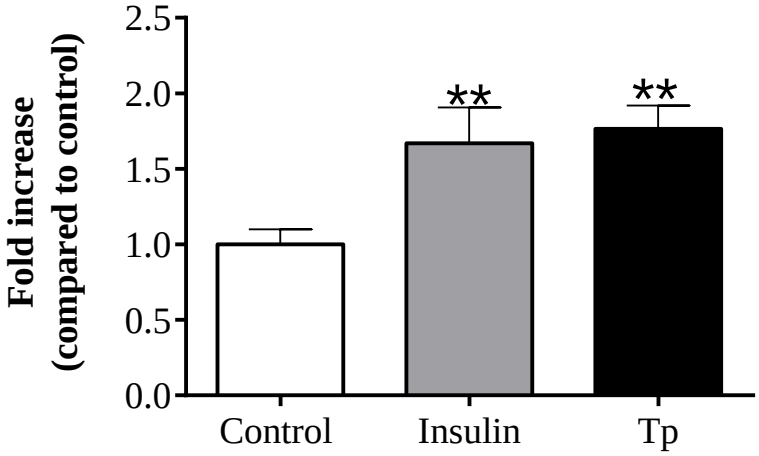
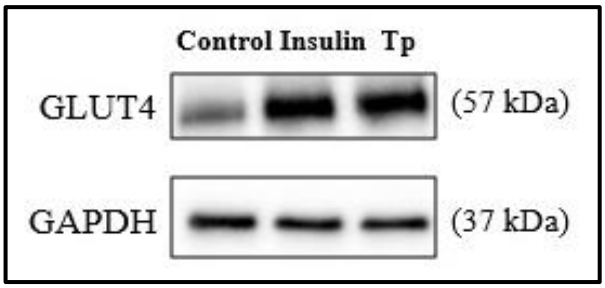


Figure 41: Effect of the Tp extract on phosphorylation of p-GSK3α/β (Ser21/9) in differentiated L6 muscle cells. L6 cells were cultured as described in section 2.25. L6 myotubes were serum starved for 24 hours and treated with insulin (1000 nM) or the Tp extract (50 µg/mL)[1] or DMEM control media for 30 minutes thereafter. Whole cell lysate protein was used in a Western Blot. Cell lysate preparation, SDS PAGE and immune-blotting was done as described in section 2.27. The signalling molecule p-GSK3 α/β (Ser 21/9) which is activated by insulin was investigated by Western Blot and α actinin was used as housekeeping control. A) Representative Western Blot images of phosphorylated GSK-3 α/β. B) Quantification of GSK/ control. All data are presented after normalising with α-actinin expression and are expressed relative to the control. This is a representative result of three independent experiments. Values are of three independent experiments are expressed as mean (± SD). Statistical differences between the treatments were assessed as per section 2.30 and ** denotes $p < 0.01$ compared to control.

A)



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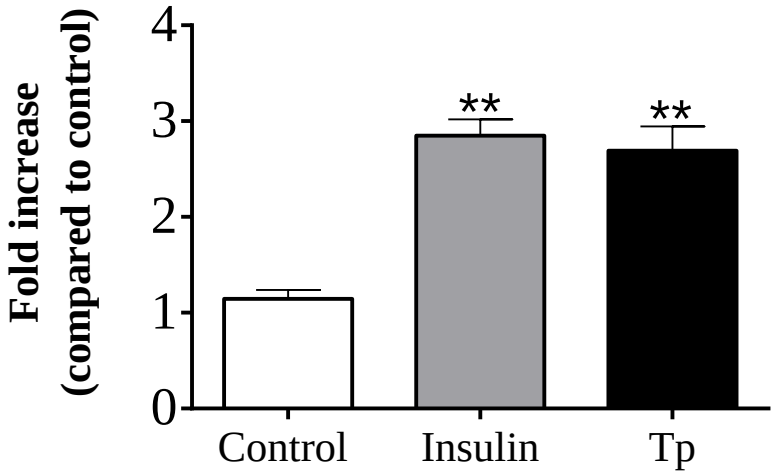

Figure 42: Effect of the Tp extract on GLUT4 expression. L6 cells were cultured and differentiated as described in section 2.25. L6 myotubes were serum starved for 24 hours and treated with insulin (1000 nM) or Tp extract (50 µg/mL)[1] or DMEM control media for 24 hours. Whole cell lysate protein was used in a Western Blot. Cell lysate preparation, SDS PAGE and immune-blotting was done as described in section 2.27. The signalling molecule GLUT4 which is activated by insulin was investigated by Western Blot and GAPDH was used as a housekeeping control. A) Representative Western Blot images of total GLUT4. B) Quantification of GLUT4/ control. All data are presented after normalising with GAPDH expression and are expressed relative to the control. This is a representative result of three independent experiments. Values are of three independent experiments are expressed as mean (± SD). Statistical differences between the treatments were assessed as per section 2.30 and ** denotes $p < 0.01$ compared to control.

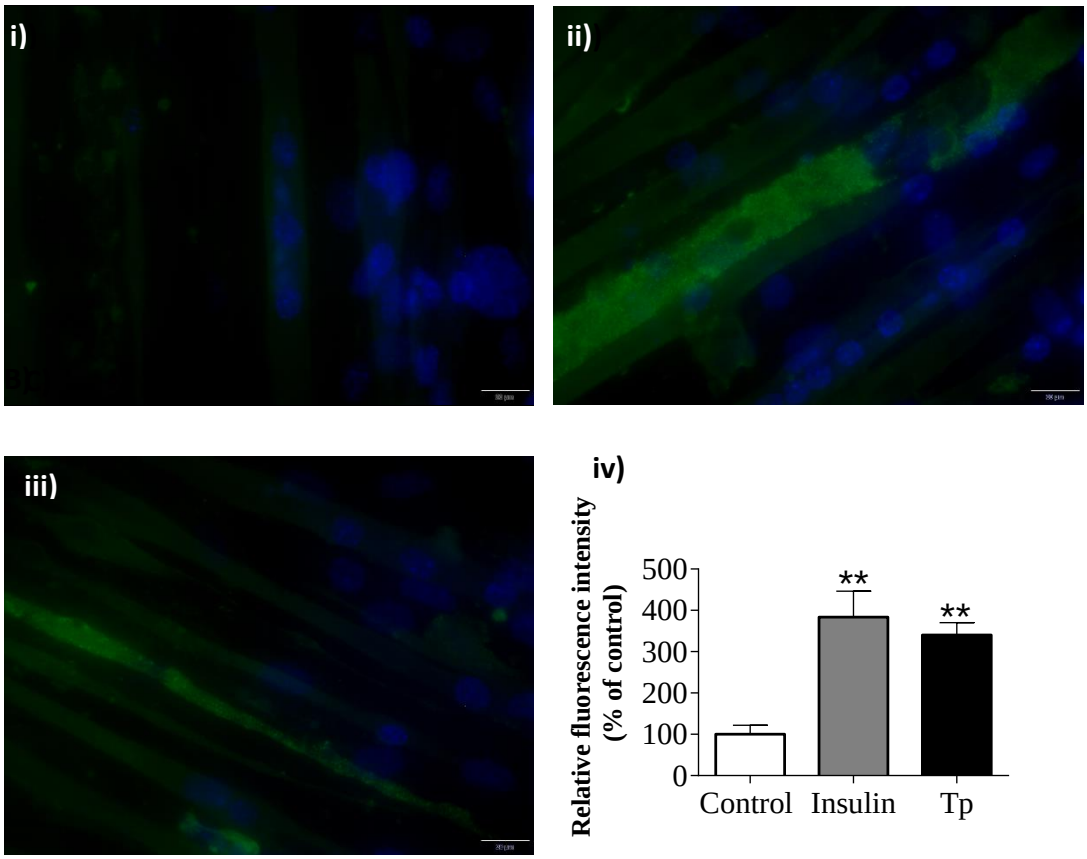

Figure 43: GLUT4 expression in differentiated L6 myotubes following treatment with the Tp extract. L6 cells were cultured and differentiated as described in section 2.25. L6 myotubes were treated with i) control growth media ii) insulin (1000 nM) and iii) the Tp extract (500 µg/mL)[1] for 24 hour and further prepared for microscopic observation as described in 2.28. Cells were imaged using an Olympus IX-51 inverted microscope. The antibody recognising GLUT4 (green) was a rabbit polyclonal antibody. The secondary antibody used was a goat anti-rabbit alexafluor 488 conjugated antibody. Nuclei were stained with DAPI (blue). Scale bar corresponds to 20 µm and images were captured at 40x magnification. iv) Quantification of fluorescent intensity of GLUT4 present in the cell. The fluorescence intensity of expressed GLUT4 was measured using ImageJ ® software (Ver. 1.50i). The experiment was repeated three times with similar results. Representative images of one experiment is shown here. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30 and ** denotes $p < 0.01$ versus control.

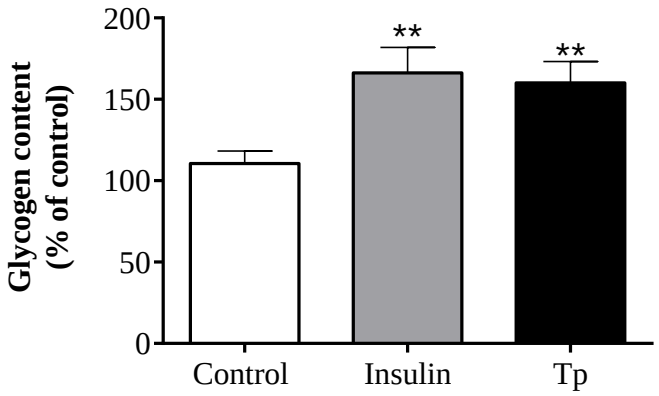

Figure 44: Glycogen content in L6 myotubes following treatment with the Tp extract. L6 cells were cultured and differentiated as described in section 2.25. L6 myotubes were serum starved for 24 hours and treated for 30 minutes with control media (DMEM) or insulin (1000 nM) or the Tp extract (50 µg/mL)[1]. Cells were lysed and glycogen content was estimated using a Biovision kit as described in section 2.29. All values are reported as the mean (± SD) of four separate experiments, with each point done in triplicate for each experiment. Statistical differences between the treatments were assessed as per section 2.30 and ** denotes $p < 0.01$ versus control.

4.0 Discussion

4.1 Does the *Teucrium polium* extract have insulin secretagogue properties?

In the current study, the constituents of the *Teucrium polium* extract promoted insulin secretion from BRIN-BD11 pancreatic beta cells in a dose-dependent manner (Figure 17). Moreover, this effect of the *Teucrium polium* extract in promoting insulin secretion was a response exceeding that of glucose alone in BRIN-BD11 cells (Figure 16). That the *Teucrium polium* extract promotes insulin secretion is supported by three other key studies (Mirghazanfari *et al.*, 2010, Stefkov *et al*., 2011, and Kasabri *et al*., 2012). Stefkov *et al.* (2011) reported that an ethanol extract of *Teucrium polium* promoted a 2.6-fold, dose-dependent increase in insulin release by MIN6 pancreatic beta cells in the presence of 20 mM glucose. Kasabri *et al.* (2012) reported that an aqueous extract of *Teucrium polium* promoted a 19-fold increase in insulin release by MIN6 in the presence of 5.6 mM glucose. Mirghazanfari *et al*. (2010) reported that a methanolic extract of *Teucrium polium* promoted a significant ($p < 0.0001$) 23 and 2.9-fold increase in insulin release by *in situ* rat pancreas in the presence of 2.8 and 16.7 mM glucose, respectively. Collectively, these previous studies and the present study provide compelling evidence that constituent(s) within the *Teucrium polium* extract promote insulin secretion.

The observation that insulin is released from BRIN-BD11 cells following treatment with the *Teucrium polium* extract could possibly arise as a consequence of beta cells in our study, or cells used in other studies (Mirghazanfari *et al.*, 2010; Stefkov *et al.*, 2011; Kasabri *et al.*, 2012), became "leaky" or, non-viable during the course of the experiment, thereby non-specifically releasing insulin into the surrounding media. The toxicity of some polyphenolics (luteolin-7-O-glucoside quercetin, apigenin) has been reviewed previously (Kwak *et al.*, 2009; Song & Park, 2014). However, at the concentrations used in this study, the constituents of the *Teucrium polium* extract did not appear to be toxic to the BRIN-BD11 cells used in this study. This observation was confirmed using two independent assays (Figure 13, 14). The constituents of the *Teucrium polium* extract only showed some toxicity towards BRIN-BD11 cells when used at concentrations exceeding 2000 $\mu g/mL^{1}$, which is about 20 times higher than the concentration needed to demonstrate insulin release from these cells. In addition, the constituents of the *Teucrium polium* extract were not toxic at the concentration used to elicit responses in other cell types used in this study (Figure 27, 28, 29). It is

also notable that the BRIN-BD11 cells used in this study responded as would be expected in the presence of gliclazide (Figure 17) and other known stimuli for insulin secretion (e.g. alanine and KCl) (Figure 15).

Given that the release of insulin from BRIN-BD11 cells could not be reasonably explained by some toxic effect of the *Teucrium polium* extract on beta cells, it might be expected that insulin is specifically secreted from beta cells in accordance with the well-established biochemical pathway for this process. Figure 45 highlights this biochemical process. In brief, glucose is the primary stimuli for insulin secretion in human and rodent beta cells (Ashcroft *et al.*, 1984; Rutter *et al.*, 2015). The amount of insulin secretion in the presence of glucose exceeds that in the presence of either fat or proteins. GLUT2 is the first glucose sensor in the beta cell that equilibrates glucose by facilitated diffusion (Fu *et al.*, 2013). After the entry of glucose into the beta cells, glucose is phosphorylated by glucokinase. Glucokinase catalyses the rate limiting step by glycolysis in beta cells (Matschinsky, 1990; German, 1993; Ferre *et al.*, 1996). The formation of pyruvate is the endpoint of glycolysis, where it is further metabolised by the tricarboxylic acid cycle in the mitochondria of beta cells to produce ATP (Suckale & Solimena, 2008). As a result of higher ATP production, the intracellular ATP/ADP ratio is increased. This further facilitates closure of K_{ATP} channels (Maechler & Wollheim, 2000; Markworth *et al.*, 2000). This depolarizes the plasma membrane and opens the voltage-gated Ca^{2+} channel, thereby facilitating Ca^{2+} entry into beta cells (Maechler & Wollheim, 1999, 2000). This activates the exocytosis of insulin from insulin secretory granules as they move towards the plasma membrane (Fu *et al.*, 2013).

The effect of the *Teucrium polium* extract on each of the sequential steps illustrated in Figure 45 that leads to insulin secretion was carefully assessed in the current study. Firstly, it was confirmed in two independent assays that the constituents of the *Teucrium polium* extract increased glucose uptake into BRIN-BD11 cells (Figure 25, 26). The constituents of the *Teucrium polium* extract significantly ($p < 0.05$) promoted glucose uptake into BRIN-BD11 cells within 30 minutes (Figure 25). This effect persisted for up to 24 hours (Figures 24, 26). That the *Teucrium polium* extract promotes increased glucose transport into BRIN-BD11 cells coincides with the observation that there is increased expression of GLUT2 following treatment of these cells with the *Teucrium polium* extract (Figure 22, 23). This observation is in keeping

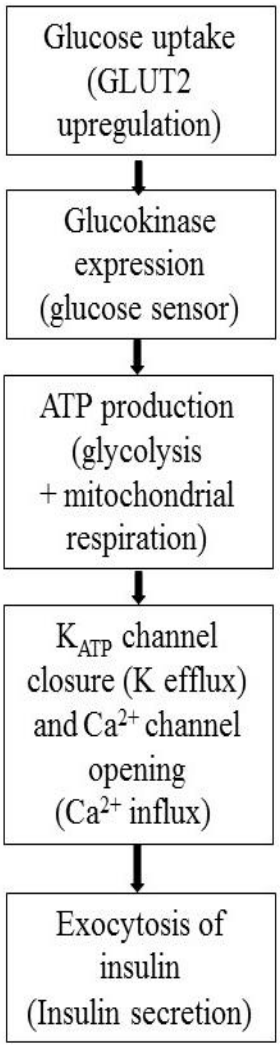

Figure 45: Established biochemical pathway for insulin release from pancreatic beta cells. The glucose transporter 2 (GLUT2) facilitates the entry of glucose into pancreatic beta cells. Following entry into the beta cell, glucose is phosphorylated by glucokinase. This step commits the resulting glucose-6-phosphate to glycolysis and mitochondrial metabolism. This leads to ATP production, which closes the K_{ATP} channel and leads to opening of the voltage with the key role that GLUT2 plays in glucose uptake and transport in the beta cells gated calcium channel. This promotes the fusion of insulin secretory granules with the cell membrane and the secretion of insulin from beta cells.

with the key role that GLUT2 plays in glucose uptake and transport in the beta cells of the rodents (Fu *et al.*, 2013). Once glucose is transported into beta cells by GLUT2, it is rapidly phosphorylated to glucose-6-phosphate by glucokinase. In addition to promoting GLUT2 expression, the constituents of the *Teucrium polium* extract significantly ($p < 0.01$) increased the expression of glucokinase in the current study (Figure 22). The observation that the expression of glucokinase is increased in BRIN-BD11 beta cells following treatment with the *Teucrium polium* extract is supported by the finding that the *Teucrium polium* extract promotes the activity of glucokinase in the liver of diabetic rats (Vessal *et al.*, 2003). The increased expression and activity of glucokinase in the beta cells in the presence of the *Teucrium polium* extract would prime the cells for increased glycolytic activity. Glucose uptake might happen due to overexpression of glycolytic genes to increase glucose utilisation (Zhao et al 2003). In future, it would be interesting to check the effect of the *Teucrium polium* extract on glycolytic genes.

The increased expression of both GLUT2 and glucokinase in BRIN-BD11 cells following treatment with *Teucrium polium* in the present study are observations that are consistent with the effect of *Teucrium polium* on promoting insulin secretion from these beta cells. An increased transport of glucose into cells via GLUT2 and commitment of glucose to glycolysis by the action of glucokinase, should lead to increased insulin secretion as illustrated by the sequence of events in Figure45. This conclusion assumes that the GLUT2/glucokinase sensor system in BRIN-BD11 cells is the rate-limiting step in this process, an assumption which is supported by several studies (Tal *et al.*, 1992; Matschinsky *et al.*, 1993; Kellett & Brot-Laroche, 2005; Muller *et al.*, 2014).

It might be informative to know more about whether the *Teucrium polium* extract promotes increased expression of GLUT2 at the plasma membrane. It should be noted that whilst the current study shows that GLUT2 expression is increased, this does not necessarily guarantee that the increased expression translates to increased translocation at the plasma membrane where it is needed to facilitate glucose transport into a beta cell. Although Figure 23 illustrates fluorescently tagged GLUT2 in cells, these experiments did not enable actual cellular localisation of the transporter molecules. Future studies might focus on microscopy experiments on transfected beta cells using subcellular compartment reporters (Ait-Omar *et al.*, 2011; Cohen *et al.*,

2014), endosome markers or studies on loss of function of GLUT2 (e.g. siRNA knockdown) (Ohtsubo *et al.*, 2011) to enable localisation of the transporters to be determined.

The observation in the present study that insulin secretion appears to be limited by glucose concentration in BRIN-BD11 beta cells, with a maximal secretion of insulin apparent below or around 5 mM glucose (Figure 16), deserves further comment. This observation is also consistent with several other published studies (McClenaghan *et al.*, 1996c; O'Harte *et al.*, 1998), some of the relevant data of which has been replotted on an appropriate linear scale in Appendix 3.[2] GLUT2 and glucokinase in beta cells have Michaelis constants (K_m) in the order of 15-20 mM (Burant & Bell, 1992; Thorens *et al.*, 1992) and 6 mM (Matschinsky *et al.*, 1993), respectively. Thus, their activity (initial velocity) should be highly sensitive to changes in blood glucose concentration (Lenzen *et al.*, 1987; Alkaladi *et al.*, 2014) to help ensure that the insulin release is matched to glucose concentration to enable glucose homeostasis (Agius, 2008). However, glucokinase has a different localisation and specificity than hexokinase. Glucokianse is much more specific in beta cells than hexokinase (Liu 2012). Given these K_m values and assuming the glucose sensor is the rate limiting step for insulin secretion (Burant & Bell, 1992; Thorens *et al.*, 1992), the concentration of glucose stimulating the maximal rate (V_{max}) of insulin secretion (about 10 times the K_m value) should well exceed even the highest physiological concentration of glucose. But as stated above, insulin secretion is maximal at below or around 5 mM glucose in the present study and as reported by others (Gray & Flatt, 1997). It may be that the capacity of GLUT2 and or glucokinase is limiting in the BRIN-BD11 beta cells used in the present study. This conclusion is supported by the observation that the *Teucrium polium* extract promotes insulin secretion above the effect of glucose alone (irrespective of glucose concentration) (Figure 16). This is an observation that could be explained by the effect of the *Teucrium polium* extract on increasing GLUT2 and glucokinase expression (V_{max} increases proportionately with increasing enzyme concentration; given that substrate is not limiting). However, GLUT2 is reported to have a very high capacity for glucose (Johnson *et al.*, 1990; Matschinsky *et al.*, 1993; Thorens, 2015). Indeed, Tal *et al*. (1992) concluded that glucose transport capacity in beta cells far exceeds (by over 100-fold) the needs of glycolysis. Interestingly, the capacity/expression of glucokinase has been reported to vary quite markedly in

[2] *The glucose dependent nature of insulin secretion is difficult to evaluate in the column graphs or in non-linear x-axis. Thus, the data have been presented in a linear graph.*

various beta cell types (Heimberg *et al.*, 1995; Raimondo *et al.*, 2015; Edghill *et al.*, 2016) and this could perhaps explain why insulin secretion does not increase as the glucose concentration is increased above 5 mM. In future, it would be interesting to evaluate the capacity of BRIN-BD11 cells to express glucokinase under various conditions, including those used in the present study. A more carefully orchestrated study ensuring that assumptions inherent in enzyme kinetics studies are valid would be desirable. For example, it is important to ensure that measurements genuinely evaluate initial rates of reaction. A single end time (30 min) point for insulin secretion was used in this and other studies (McClenaghan *et al.*, 1996b; O'Harte *et al.*, 1998; Hannan *et al.*, 2007). It would also be informative to ensure that substrates in the cell culture were not limiting over the course of the experiment. Substrates for measurement should include glucose, calcium and potassium, as a starting point, as these nutrients are central to the process of insulin secretion.

In keeping with the observation that the *Teucrium polium* extract promotes glucose transport through increased expression of GLUT2 and glucokinase, it might be expected that this leads to the increased phosphorylation of glucose, which thereby commits glucose to glycolytic processing within these beta cells. This commitment is made as there is no gluconeogenic capacity in these cells (Mithieux *et al.*, 2004). The speculation that the *Teucrium polium* extract does indeed promote glycolytic processing of glucose is clearly supported by a Seahorse XF glycolysis stress test (Figure 21A, 21B). The constituents of the *Teucrium polium* extract increased the extracellular acidification rate and glycolysis via glucose uptake (Figure 21A), which is important for energy metabolism. A higher glycolytic rate and glycolytic capacity were observed in BRIN-BD11 cells when treated with the *Teucrium polium* extract (Figure 21B). Further studies on mRNA and protein expression of other key glycolytic genes are necessary to explore the detailed mechanism of the constituents of the *Teucrium polium* extract on the glycolytic pathway. For example, the role of this plant extract on a transcriptional factor- hypoxia- inducible factor 1α (HIF-1α) and mTOR could be examined. These factors regulate glycolytic genes and pyruvate kinase (Carmeliet *et al.*, 1998; Fraenkel *et al.*, 2008; Van de Velde *et al.*, 2011).

It would be expected that if the glycolytic capacity of beta cells is enhanced by the *Teucrium polium* extract, that there is either an increased production of lactate, as an end-point of glycolysis, or that pyruvate enters the mitochondrial pathways

(tricarboxylic acid cycle and oxidative phosphorylation). Lactate measurements were not performed in the current study. However, beta cells typically express very low level of lactate dehydrogenase (Sekine *et al.*, 1994; Ainscow *et al.*, 2000). This low level of lactate dehydrogenase activity in insulin secreting cells is important to enable pyruvate to be preferentially channelled towards mitochondrial respiration (Ainscow *et al.*, 2000). The sea horse mitochondrial stress test data support the conclusion that the *Teucrium polium* extract enhances mitochondrial respiration (Figure 20 A, B, C). The constituents of the *Teucrium polium* extract promoted basal respiration in mitochondria, which indicates that ATP turnover in the cells and non-mitochondrial respiration were increased (Figure 20C). The pyruvate and NADH from glycolytic and mitochondrial metabolism are important for glucose stimulated insulin secretion and its regulation (Hutton *et al.*, 1980; Berg *et al.*, 2002; Rutter *et al.*, 2015). Abnormalities in mitochondrial gene expression can lead to impaired insulin secretion and diabetes (Reardon *et al.*, 1992; Van den Ouweland *et al.*, 1992). There was a significant ($p < 0.05$) increase in maximal mitochondrial respiration after the *Teucrium polium* extract treatment (Figure 20C), which in turn enhances substrate availability for the respiratory chain (Brand & Nicholls, 2011; Ruas *et al.*, 2016). Previously, Baali *et al.* reported an increase in mitochondrial respiration of the rat liver after the chronic *Teucrium polium* extract treatment *in vivo* (Baali *et al.*, 2016). Although Baali and his group measured the hepatic mitochondrial bioenergetics using high resolution Oxygraph-2K, their data is in line with the findings of the current study reporting an upregulation of mitochondrial respiration in pancreatic beta cells following treatment with the *Teucrium polium* extract.

Upregulation of glycolytic activity and mitochondrial respiration should lead to an increase in oxidative phosphorylation and ATP production in the cells. Indeed, as would be expected, the treatment of BRIN-BD11 cells with the *Teucrium polium* extract promoted ATP production in BRIN-BD11 cells (Figure 19, 20 B). This finding was verified in two independent assays; a seahorse mitochondrial stress test and a Celltiter-Glo luminescent assay. In support of this finding, the *Teucrium polium* extract increases AMP activated protein kinase (AMPK) in the rat pancreas (Tatar *et al.*, 2011; Qujeq *et al.*, 2013). AMPK is one of the key known factors that promote the generation of ATP (Hardie, 2008).

The increased production of ATP in the presence of the *Teucrium polium* extract would increase the ATP:ADP ratio to facilitate closure of the ATP-sensitive K^+ channel (Miguel *et al.*, 2004; Ruas *et al.*, 2016). This should lead to membrane depolarization and the opening of Ca^{2+} channels (Tarasov *et al.*, 2004). In the current study, although the effect of the *Teucrium polium* extract on K_{ATP} channel closure was not investigated, calcium influx into the beta cell, which is a consequence of K_{ATP} channel closure, was observed. As would be expected with increased intracellular ATP production, the constituents of the *Teucrium polium* extract increased intracellular calcium influx in BRIN-BD11 (Figure 19, 18A, 18B). This result is in agreement with a previous study which found that diazoxide, a K_{ATP} channel opening agent, and verapamil, a Ca^{2+} channel blocking agent, inhibit the *Teucrium polium* extract promoted insulin secretion in perfused rat pancreas (Mirghazanfari *et al.*, 2010). The authors of this study concluded that the *Teucrium polium* extract acts on K_{ATP} and Ca^{2+} channels. However, the *Teucrium polium* extract may not act directly on these channels. As demonstrated in the present study, the *Teucrium polium* extract promotes GLUT2 (Figure 22, 23) and glucokinase (Figure 22) expression, which leads to increased glycolytic activity (Figure 21) and mitochondrial respiration (Figure 20). This in turn results in increased ATP production (Fig 19, 20B), which would lead to the presumed action of the *Teucrium polium* extract in inhibiting potassium efflux and thereby promoting calcium influx (Figure 18) into the BRIN-BD11 cells. The actions of diazoxide or verapamil in inhibiting the *Teucrium polium* extract promoted insulin secretion may therefore have nothing to do with the action of *Teucrium polium* directly on ion channels per se but rather reflect its action on the upstream events of GLUT2 and glucokinase expression. Studies evaluating the action of the *Teucrium polium* extract on isolated ion channels (such as BK channel, K_V 2.1/2.2 channels, Na_V1.6 and Na_V1.7, T- and P/Q-type Ca^{2+} channels) (Braun *et al.*, 2008) may be needed in future to definitely confirm or refute whether constituent(s) of the *Teucrium polium* extract interact directly with various ion channels.

In summary, it can be concluded that the constituent(s) of the *Teucrium polium* extract act as an insulin secretagogue. The *Teucrium polium* extract stimulates pancreatic beta cells to promote glucose uptake. The mechanism of action includes upregulation of GLUT2 to facilitate entry of glucose, enhanced glucokinase expression, which commits glucose to glycolysis and mitochondrial metabolism. These metabolic

activities, in turn, increase ATP production and leads to calcium channel opening. Opening of the calcium channels promotes calcium influx into the beta cell and this in turn leads to the secretion of insulin from the beta cells.

4.2 Does the *Teucrium polium* extract have insulin mimetic properties?

Several independent analyses were conducted on cells in culture to assess if the *Teucrium polium* extract was capable of upregulating insulin signalling pathways in typical insulin sensitive tissues (reviewed in section 1.6.2). Firstly, attention was given to evaluating the potential of the *Teucrium polium* extract to promote glucose uptake in muscle and adipose cells, which respond to insulin by promoting GLUT4 translocation to the cell membrane. The *Teucrium polium* extract increased glucose uptake in a concentration dependent manner with an apparent efficacy similar to a maximal dose of insulin in three different insulin sensitive cell lines in 30-minute treatment (3T3-L1 adipocytes, differentiated C2C12 and L6 myotubes) (Figure 30, 31, 32, 33, 34). This observation was verified using two independent assays (section 2.18, 2.19). The ability of the *Teucrium polium* extract to promote glucose uptake in muscle and fat cells is likely explained by the effect of the *Teucrium polium* extract in upregulating glucose transporter 4 (GLUT4) expression. GLUT4 expression was increased three to four-fold in L6 myotubes in the presence of the *Teucrium polium* extract (Figure 41, 42, 43). The localisation of these glucose transporter molecules at surface of the cells is necessary for glucose uptake in muscle cells (Cushman & Wardzala, 1980; Klip & Marette, 1992). Further studies need to be conducted to evaluate the translocation of the glucose transporters in response to treatment with the *Teucrium polium* extract.

Whilst this is the first report of the ability of a *Teucrium polium* extract to promote glucose uptake and GLUT4 expression in cell culture, the glucose lowering properties of *Teucrium polium in vivo* has been reported previously by this laboratory (Ireng *et al.*, 2016) and by others (Gharaibeh *et al.*, 1988; Esmaeili & Yazdanparast, 2004; Shahraki *et al.*, 2007). These previous studies are reviewed in detail in Table 5 of the literature review of this thesis. Whilst muscle and adipose cells play a key role in carbohydrate, fat and protein metabolism *in vivo* (Saltiel & Kahn, 2001; Kahn *et al.*, 2005; Wolfe, 2006), liver and other cells also form part of the "orchestra" controlling metabolism and glucose homeostasis *in vivo* (Rosen & Spiegelman, 2006; Zhang *et al.*, 2009a). Future studies should evaluate the effect of the *Teucrium polium* extract in liver cells, and in particular on whether the *Teucrium polium* extract, like insulin, promotes glycogen synthesis and inhibits gluconeogenesis in these cells. However,

The *Teucrium polium* extract has been reported to have hypolipidemic effects in rats (Rasekh *et al.*, 2001). The effect of the *Teucrium polium* extract on lipid metabolism in liver, fat and muscle cells should also be explored so that the orchestration of events relating to carbohydrate and lipid metabolism can be better understood at a holistic level.

In order to further explore the mechanisms involved in how the constituent(s) of the *Teucrium polium* extract promotes glucose uptake, the phosphorylated forms of some key molecules involved in insulin signalling pathways were measured. The levels of phosphorylated Akt and PDK-1, which play key roles in insulin mediated cell signalling and help regulate glucose transport in cells (Cohen *et al.*, 1997; Czech & Corvera, 1999; Lawlor & Alessi, 2001; Sarbassov *et al.*, 2005) were upregulated in response to the *Teucrium polium* extract or insulin (Figures 36 and 37). This finding is supported by a report that the administration of the *Teucrium polium* extract to rats, resulted in a 2-fold increase in hepatic Akt phosphorylation (Aghazadeh & Yazdanparast, 2013). Thus, the *Teucrium polium* extract appears to upregulate Akt phosphorylation in both muscle (current study) and liver (Aghazadeh & Yazdanparast, 2013).

As a consequence of Akt phosphorylation and activation by either the *Teucrium polium* extract or insulin, it would be expected that Akt would further promote the phosphorylation and activation of glycogen synthase kinase (GSK) which would lead to glycogen synthesis, protein synthesis and cell growth (Kitamura *et al.*, 1998; Pap & Cooper, 1998; Shioi *et al.*, 2002; Doble & Woodgett, 2003). Notably, treatment of L6 myotubes with either the *Teucrium polium* extract or insulin promoted about a 2-fold increase in the level of phosphorylated GSK3α/β (Figure 41). Not unexpectedly then, the glycogen content of the myotubes was significantly ($p < 0.01$) increased by either insulin or the *Teucrium polium* extract (Figure 44), which is consistent with the observed elevation of phosphorylated GSK3α/β. In an apparent contradiction to this finding, however, two *in vivo* studies on the effect of the *Teucrium polium* extract in rats reported that the *Teucrium polium* extract does not increase liver glycogen content (Stefkov *et al.*, 2011; Mousavi *et al.*, 2012). Given that there are two different genes, GYS1 and GYS2, encoding two different isoenzymes of glycogen synthase in mammalian muscle and liver, respectively (McCue *et al.*, 2008; Cameron *et al.*, 2009; Pescador *et al.*, 2010), it could be possible that the *Teucrium polium* extract affects

this enzyme differently in different tissues. However, it is also possible that the ethyl acetate extract of *Teucrium polium*, which was used in these *in vivo* studies, comprises components and thereby properties, that differ from the extract used in the present study. It is also notable that the current study involved measurement of glycogen content in L6 myotubes following a 30-minute acute administration, whereas the *in vivo* studies described above evaluated a chronic 10-day administration regime of their ethyl acetate - derived *Teucrium polium* extract in liver tissue samples.

Given that the level of phosphorylated Akt was elevated following treatment with the *Teucrium polium* extract in L6 myotubes, it might also be expected that there would be increased expression of phosphorylated mTOR and that this would coincide with an increase in expression of phosphorylated ribosomal protein S6. Indeed, the phosphorylation of p70 ribosomal S6 kinase beta (S6Kb) was quite dramatically increased by both insulin and the *Teucrium polium* extract in the L6 myotubes in the Kinexus pre-screening (Figure 35D). On follow up using an optimised (and appropriately duplicated) in-house assay, the phosphorylation of mTOR and ribosomal protein S6 was similarly increased in the presence of both insulin and the *Teucrium polium* extract (Figure 38). Collectively, the activation of Akt, mTOR, and S6 kinases would be expected to lead to increased protein synthesis and growth in muscle cells (Cantley, 2002; Jacinto *et al.*, 2006; Gingras *et al.*, 2007). Thus, the *Teucrium polium* extract seems to act in a manner similar to insulin in this regard.

A second essential branch of insulin and IGF-1-signaling pathways is the Grb2-SOS-Ras-MAPK pathway (Boucher *et al.*, 2014), wherein the MAP Kinases ERK1 and 2 play a direct role in cell proliferation and growth. This pathway is activated independently of the PI3K/Akt pathway. The effect of *Teucrium polium* on the phosphorylation of ERK1 and ERK2 was evaluated in more detail as the Kinexus pre-screen suggested that whilst insulin promoted ERK phosphorylation (as would be expected; (Boulton *et al.*, 1991; Wilden *et al.*, 1998)), the *Teucrium polium* extract appeared to inhibit the phosphorylation of both ERK1 and ERK2 (Figure 35). This result was surprising and in apparent contradiction to an *in vivo* study conducted in rat livers (Aghazadeh & Yazdanparast, 2010), which concluded that the *Teucrium polium* extract promotes the phosphorylation of ERK. On follow up using an optimised assay in our laboratory, like insulin, the *Teucrium polium* extract reproducibly and significantly increased ERK phosphorylation in our in-house optimised

assay (Figure 40). Clearly, the data emanating from the Kinexus phosphoprotein pre-screens, although often published, must be cautiously interpreted, especially given that the assays are not necessarily optimised for each cell type and are not typically duplicated to enable statistical significance to be determined.

ERK1/2 activation should lead to the phosphorylation of ribosomal S6 kinase (RSK1) and promote cell proliferation, growth, motility and development control (Anjum & Blenis, 2008; Smadja-Lamère *et al*., 2013). As RSK can also further accelerate the mTOR pathway, its activation should lead to increasing protein synthesis in cells (Smadja-Lamère *et al*., 2013). The observation that RSK-1 is activated in L6 muscle cells following treatment with insulin or the *Teucrium polium* extract is tentatively supported by the results of the Kinexus pre-screen. (Figure 35D). However, more detailed studies are now needed to statistically evaluate the observation that *Teucrium polium* promotes the phosphorylation of RSK-1.

In conclusion, the action of the *Teucrium polium* extract promotes glucose uptake in muscle and fat cells and glycogen storage in muscle cells by a mechanism that appears to mimic, at least in part, both the PI3K/Akt pathway and the Grb2-SOS-Ras-MAPK pathway (Boucher *et al.*, 2014) (Figure 46). Although it is not possible to exclude that the compounds of the *Teucrium polium* extract targets an event or events downstream of PDK-1, given that all events measured downstream of PDK-1 were promoted in L6 muscle cells, covering both the PI3K/Akt and Grb2-SOS-Rasw-MAPK pathways of insulin action, it seems most likely that the *Teucrium polium* extract acts either through PDK-1 and/or at a step(s) upstream of PDK-1 within the P13K/Akt pathway; this includes, but is not limited to, phosphatidylinositol-4,5-bisphosphate 3-kinase (P13K), the insulin receptor substrates (IRS), the insulin receptor and or various transcriptional and other factors involved in the regulation of their synthesis. This conclusion is supported by our finding that GSK2334470, a highly specific and potent inhibitor of PDK-1 (Raimondi & Falasca, 2011; Medina, 2013), which expectedly inhibited the phosphorylation of the ribosomal S6 protein in L6 muscles cells, also completely ablated the stimulatory effect of the *Teucrium polium* extract in promoting the phosphorylation of this S6 protein to levels indistinguishable to the phosphorylation levels of S6 in presence of the inhibitor alone (Figure 39). A more comprehensive study is now required to identify the actual molecular target(s) conveying the insulin mimetic properties of the *Teucrium polium* extract.

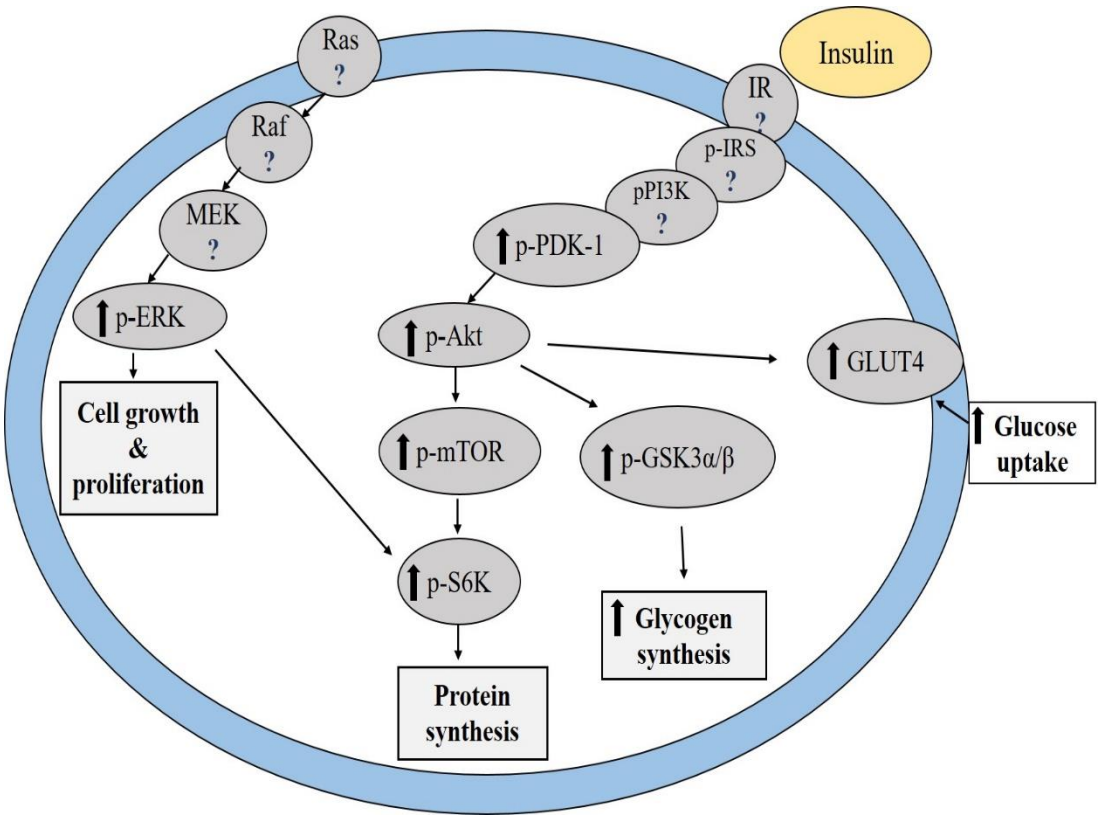

Figure 46: Summary of events within insulin signalling pathways influenced by the constituents of the Tp extract. Upward arrows highlight those activities that were increased following treatment with Tp. Question marks highlight those activities still to be determined.

4.3 Future directions

The increased expression of GLUT2 and glucokinase, which form the glucose sensor and form the rate limiting step for the commitment of glucose to glycolysis and mitochondrial metabolism within beta cells, likely explains the sequence of events leading to the enhanced secretion of insulin by BRIN-BD11 cells following treatment with the *Teucrium polium* extract. Future studies should focus on evaluating the mechanism(s) by which the *Teucrium polium* extract promotes the increased expression of both GLUT2 and glucokinase. The effect of the *Teucrium polium* extract on the expression of the glucokinase regulatory protein (GKRP) (Grimsby *et al.*, 2000; Orho-Melander *et al.*, 2008) and transcription factors such as Rfx3, Pdx1 and MafA (Brissova *et al.*, 2002; Ait-Lounis *et al.*, 2010) might provide insight into how component(s) within the *Teucrium polium* extract promote glucokinase expression. The transcription factor Pdx-1 and another regulatory factor, Nkx6.1, which play a regulatory role in the expression of GLUT2 (Taylor *et al.*, 2015; Dai *et al.*, 2016), should be studied to provide insight into how component(s) in the *Teucrium polium* extract promote the expression of GLUT2.

The molecular target(s) of component(s) within the *Teucrium polium* extract that deliver its insulin mimetic properties should also be explored in greater detail. Events related to PDK-1 phosphorylation, and upstream steps including the activation of P13-kinase, insulin receptor substrates (IRS) and the insulin receptor itself should be evaluated. The effect of the *Teucrium polium* extract in other insulin sensitive tissues such as liver cells should also be explored. In particular, it will be valuable to determine whether component(s) within the *Teucrium polium* extract, like insulin, promotes glycogen synthesis and inhibits gluconeogenesis. The effect of the *Teucrium polium* extract on lipid metabolism in liver, fat and muscle cells should also be explored so that the orchestration of events relating to carbohydrate and lipid metabolism can be better understood at a holistic level in the presence of the *Teucrium polium* extract.

The effect of component(s) within the extract on promoting GLUT4 expression in L6 muscle cells also needs to be investigated. The expression of GLUT4 is dependent on many factors including the myocyte enhancer factor 2 (MEF2) and the GLUT4 enhancer factor (GEF) protein (Thai *et al.*, 1998; Oshel *et al.*, 2000; Holmes & Dohm,

2004). The transcriptional co-activator PGC-1 can also control GLUT4 expression in muscle cells (Michael *et al.*, 2001). In addition, GLUT4 gene expression is regulated by the transcription factor C/EBP (Kaestner *et al.*, 1989; Stephens & Pekala, 1991) whereas, tethering and fusion of GLUT4 secretory vesicles (GSV) is mediated by SNAP-associated receptor (SNARE) proteins and unconventional myosin-Ic (MYO1C) in muscle (Klip, 2009; Foley *et al.*, 2011; Rowland *et al.*, 2011; Boguslavsky *et al.*, 2012; Esteves *et al.*, 2017). Each of these factors affecting GLUT4 expression could be considered in future studies to understand how GLUT4 expression is promoted by component(s) within the *Teucrium polium* extract.

Given that both GLUT2 and GLUT4 expression are both promoted by component(s) within the *Teucrium polium* extract, might suggest the involvement of common factor(s). AMP-Activated Protein Kinase (AMPK) has a regulatory role in both GLUT2 and GLUT4 mediated glucose uptake in muscle and other cells (Ojuka, 2004; Walker *et al.*, 2005; Zhang *et al.*, 2009c). In addition, the carbohydrate-response element binding protein (ChREBP) is a glucose responsive transcription factor for GLUT2 and GLUT4 expression (Ma *et al.*, 2006). It will be interesting to determine if either one or both of AMPK or ChREBP might influence GLUT expression in different cell types in response to treatment with the *Teucrium polium* extract.

Identification and characterisation of the actual compound(s) within the *Teucrium polium* extract, which deliver its insulin secretagogue and or insulin mimetic properties, must be a focus of future research. However, despite the availability of a number of new techniques and methods for compound identification, this process will likely be a challenge (Silva *et al.*, 1998; Bucar *et al.*, 2013). Isolation of any active compound from a complex mixture of compounds, as would comprise the *Teucrium polium* extract, will require careful observation and "trial and error" attempts using multiple chromatographic techniques. Whatever, the preferred separation methods, purification of any "active" compound(s) is critical to ensure that the presence of interfering substances are removed; however, complete elimination of the interfering compounds during this process cannot be guaranteed (Silva *et al.*, 1998). Elucidation of a full chemical structure also requires highly purified compounds (Bucar *et al.*, 2013). Reliable and sensitive assays to measure the activities of various fractions also need to be carefully considered. The insulin ELISA immunoassay used in this study proved to be highly reliable and sensitive and would be a useful measure of the ability

of various fractions to promote insulin secretion in BRIND-BD11 cells. The 2-NBDG uptake assay also proved to be a reliable and sensitive method that could be utilised to screen for the active compounds acting as either insulin secretagogues or insulin mimetics.

Whilst it is not possible to confidently predict the identify or type of compounds that promote insulin secretagogue or insulin mimetic activity, the active components in the *Teucrium polium* extract could be phenolic-based compounds as there are numerous studies reporting phenolic compounds with insulin secretagogue potential (Iwamoto *et al.*, 1991; Zhang *et al.*, 2004; Sriplang *et al.*, 2007; Hamden *et al.*, 2009; Johnson & de Mejia, 2016). It is particularly noteworthy that apigenin (4′,5,7-trihydroxyflavone) and various conjugates of apigenin (glycosylated and other), which are found in *Tecrium polium* and some other plants, promote insulin secretion (Panda & Kar, 2007; Cazarolli *et al.*, 2009b; Cazarolli *et al.*, 2009a; Jung *et al.*, 2016), GLUT 4 expression and translocation (Li *et al.*, 2007b; Hossain *et al.*, 2014), glucose uptake (Cazarolli *et al.*, 2009b; Cazarolli *et al.*, 2012) and glycogen synthesis (Cazarolli *et al.*, 2009b; Johnson *et al.*, 2011). Apigenin also affects many other activities *in vitro* and *in vivo*, however, its effects on the insulin mediated signalling pathways are unclear. Some studies report that apigenin promotes the PI3K (Cazarolli *et al.*, 2012) and MAPK (Cazarolli *et al.*, 2012) pathways, whereas other studies report that apigenin inhibits the PI3K (Fang *et al.*, 2005; Erdogan *et al.*, 2016) and MAPK (Yin *et al.*, 2000; Aroui *et al.*, 2016) pathways. There are also reports that apigenin inhibits the expression of human pancreatic and lung GLUT1 (Melstrom *et al.*, 2008; Lee *et al.*, 2016). Further studies are now needed to more comprehensively evaluate the effect of apigenin and its various conjugates on activities within the insulin mediated signalling pathways. Attention will need to be paid to quantitate the potencies of apigenin and its conjugates in the same study, as this is lacking in most studies to date. Given the findings in the present study regarding the increased expression of GLUT2 and glucokinase in BRIN-BD11 cells treated with the *Teucrium polium* extract, it will also be important in future to evaluate the effect of apigenin on the expression of both GLUT2 and glucokinase in these beta cells.

Apigenin, quercetin and its glycoside, rutin, have been putatitively identified in our Teucrium polium extract (Ireng et al., 2016). The concentrations of these compounds in the extract are believed to be much lower than what has been used in activity

experiments (Panda & Kar, 2007; Cazarolli *et al.*, 2009a). However, their exact structures and concentrations are yet to be elucidated and confirmed.

If apigenin is indeed a component in the *Teucrium polium* extract in sufficient concentration that, at least in part, provides it with its insulin secretagogue and/or insulin mimetic properties, then it would be reasonable to ask how it might confer these activities? Apigenin is recognised as a specific inhibitor targeting protein kinase CK2 (formerly casein kinase II) (Liu *et al.*, 2015; Nelson *et al.*, 2017). CK2 is an highly conserved serine-threonine kinase that is expressed ubiquitously in cells where it plays a regulatory role in the metabolism and proliferation of cells (Boldyreff *et al.*, 1993; Allende & Allende, 1995; Dobrowolska *et al.*, 1999; Guerra & Issinger, 1999; Channavajhala & Seldin, 2002). Notably, CK2 is part of the glucose induced signalling cascade of pancreatic beta cell (Zhang & Kim, 1997). It negatively regulates insulin secretion by phosphorylating Pdx-1 (pancreatic and duodenal homeobox-1), a transcription factor within pancreatic beta cells that regulates the GLUT2 promoter (Waeber *et al.*, 1996). Phosphorylation of Pdx-1 targets it for degradation and this leads to a reduction in insulin secretion (Meng *et al.*, 2010). Inhibition of CK2 by apigenin, would thereby be expected to lead to increased GLUT2 expression and increased glucose transport into a beta cell. This in turn would be predicted to lead to the cascade of events illustrated in Figure 45. Moreover, CK2 inhibits the expression of the glucose transporter 4 (GLUT4) in glucose sensitive cells such as 3T3-L1 cells (Ma *et al.*, 2014). CK2 has been also reported to prime the phosphorylation of Ser657 of GSK3, a specific phosphate recognition motif. Thus, it maintains the glycogen level in muscle and downregulates insulin action (McManus *et al.*, 2005). Thus, I postulate that the action of apigenin on inhibiting CK2 activity, leads to both insulin secretagogue action in beta cells and insulin mimetic like activity in insulin sensitive cells in a manner that is entirely consistent with the observations of the present study. It might be worthwhile to investigate the effect of the *Teucrium polium* extract on beta cells using RNAi based knockdown of CK2 (Maier *et al.*, 2009) and other physiological inhibitors of CK2 (Allende & Allende, 1995) to better understand the effect of the constituents of the *Teucrium polium* extract on CK2.

Extracts of *Teucrium polium* are complex and also contain other constituents that have been reported to have glucose lowering properties. For example, quercetin is a common flavonol aglycone found in many plants and has also been identified in

Teucrium polium extracts (Panovska *et al.*, 2005). It has been reported to increase liver glycogen content and regeneration of beta cells in diabetic rats (Nuraliev & Avezov, 1991; Coskun *et al.*, 2005). Quercetin has also been reported to enhance insulin release by promoting Ca^{2+} influx in beta cells (Hii & Howell, 1984). In addition, quercetin increases GLUT4 expression and glucose uptake by muscle cells (Aguirre *et al.*, 2011). These actions of quercetin appear to be through an insulin-independent mechanism involving adenosine monophosphate-activated protein kinase (AMPK) (Mehta *et al.*, 2017; Rezvan *et al.*, 2017). Quercetin also and inhibits hepatic glycogenolysis and gluconeogenesis (Jakobs *et al.*, 2006). Thus, quercetin is a compound of interest that needs to be also studied further. Rutin, a glycoside conjugate of quercetin and rutinose (α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranose), is another compound of interest that has been identified in *Teucrium polium* extracts (Sharififar *et al.*, 2009). It increases plasma insulin and reduces blood glucose *in vivo* in rats (Prince & Kamalakkannan, 2006). Given the foregoing discussion, it may be that multiple compounds elicit the responses seen using the *Teucrium polium* extract and that isolation of any one compound may not in itself give the same response in either secretagogue or insulin mimetic activity.

The finding in the present study that component(s) within the *Teucrium polium* extract promote the expression of both glucokinase and GLUT2 in beta cells is particular noteworthy as these activities constitute the glucose sensor of pancreatic beta cells and have a high impact on glucose homeostasis, which is impaired in type 2 diabetes. Not surprisingly, there has been substantial effort to discover glucokinase "activators" that activate the glucose sensor of pancreatic beta cells (McKerrecher *et al.*, 2006; Pfefferkorn *et al.*, 2012; Katz *et al.*, 2016). The *Teucrium polium* extract might thereby prove to be an impactful target for future drug discovery efforts for the treatment of type 2 diabetes and related disorders.

5.0 References

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6.0 Appendices

Appendix-1A

Correspondence for permission of Figure 4

5/6/2017 Re: Regarding permission for including your photography - Adnan Mannan

Re: Regarding permission for including your photography

Jan Sevcik <jan.sevcik86@centrum.cz>

Wed 3/08/2016 5:46 PM

To:Adnan Mannan <adnan.mannan@postgrad.curtin.edu.au>;

Hi,
include to school thesis is possible.
Regards, JS.

> Od: Adnan Mannan <adnan.mannan@postgrad.curtin.edu.au>
> Komu: "jan.sevcik86@centrum.cz" <jan.sevcik86@centrum.cz>
> Datum: 02.08.2016 07:00
> Předmět: Regarding permission for including your photography
>

Dear Jan,
Hello.
I am a researcher of Curtin University, Australia. I am working on the anti-diabetic effect of teucrium polium. We are publishing our research work soon. We found a beautiful photograph captured by you in this link- http://www.biolib.cz/en/image/id136137/
We would like to include this photo in our thesis mentioning your name and reference.
Would you kindly allow us to include this photo in our research thesis for promoting the good effects of teucrium polium?

Regards,

Adnan Mannan
PhD Candidate
School of Pharmacy
Curtin University
Perth, Australia

Appendix 1B

Correspondence for permission of Figure 6 and Figure 7

5/8/2017 RE: Request for permission: using figure - Adnan Mannan

RE: Request for permission: using figure

seahorse.support@agilent.com

Tue 21/03/2017 8:38 PM

To:Adnan Mannan <adnan.mannan@postgrad.curtin.edu.au>;

Hi Adnan,

I checked with our marketing group and yes, Agilent will allow you to use these figures as long as the proper attribution is included with each figure.

Please let me know if you have any further questions.

Best regards,

Nikhat

Nikhat Zaidi, PhD
Technical Support Scientist
Seahorse XF Products
Email: Seahorse.support@agilent.com
Ph: 800-227-9770, option 3, option 8

Agilent Technologies

From: Adnan Mannan [mailto:adnan.mannan@postgrad.curtin.edu.au]
Sent: Monday, March 20, 2017 10:15 PM
To: SH,SUPPORT (A-Americas,exgen1) <seahorse.support@agilent.com>
Subject: Request for permission: using figure

Hi,
I am a PhD student at Curtin University.
I am doing PhD on- "Molecular signalling pathways involved in the glucose-lowering effect of Teucrium polium". I have used Seahorse XF Stress Test for my experiments in the laboratory of Curtin Health Innovation Research Institute.
I want to use two figure from "Seahorse XF Stress Test Report Generator-User Guide".
Title of the figures are-
1) Seahorse XF Cell Mito Stress Test Profile
2) Seahorse XF Glycolysis Stress Test Profile

Would you please allow me to use these figures from the user guide published by your company?
It would be great to get permission granted.

Appendix-2

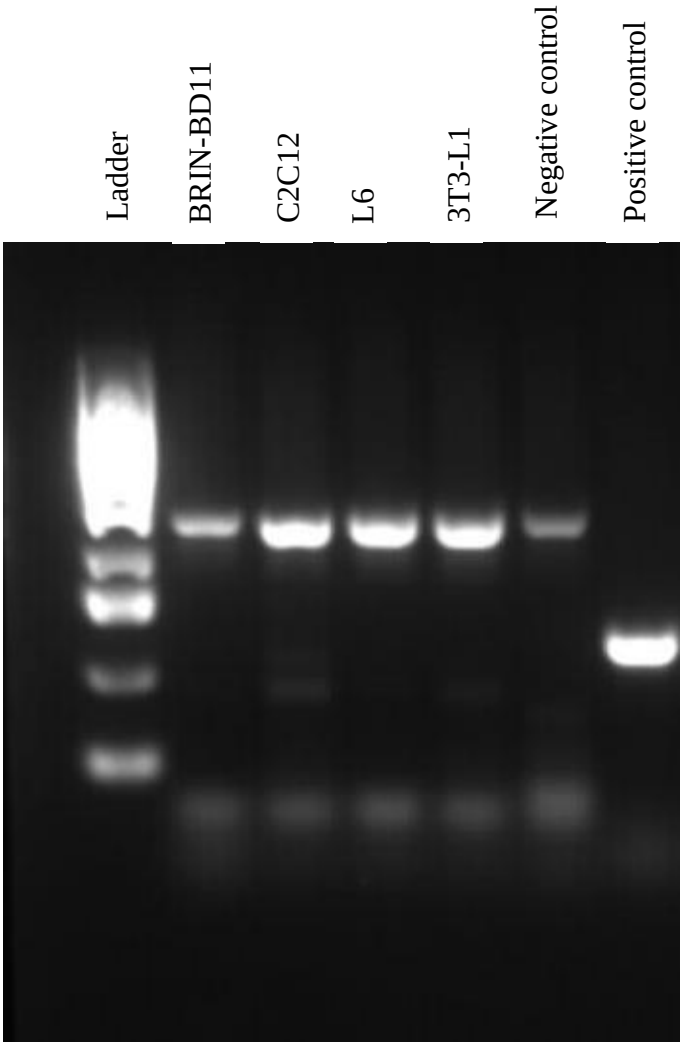

Figure 47: PCR screen for mycoplasma in various cell lines used in the present study. The ethidium bromide-stained bands of the reaction products run in an agarose gel are shown as mentioned in section 2.12. Cell culture supernatant of BRIN-BD11, C2C12, L6 and 3T3-L1 cell lines were used for the assay.

Appendix-3

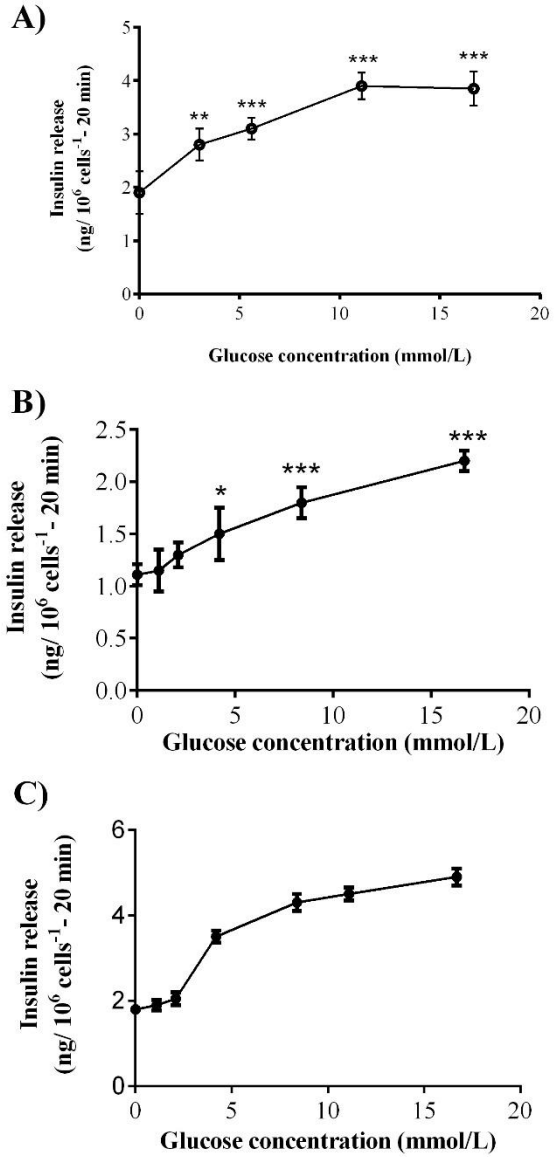

Figure 48: Insulin secretion by BRIN-BD11 cells in various studies. A) Insulin release by BRIN-BD11 cells in response to D-glucose (McClenaghan *et al.*, 1996c). B) Insulin release from BRIN-BD11 cells in response to various concentration of glucose (O'Harte *et al.*, 1998). C) Effect of glucose on Insulin release as a percentage of the cellular insulin content (McClenaghan & Flatt, 1999).