CONCURRENT TREATMENT OF *IN VITRO* CELL LINES WITH *UTHULI LWEZICHWE*™, AN AFRICAN TRADITIONAL MEDICINE USED IN THE MANAGEMENT OF DIABETES MELLITUS IN KWAZULU-NATAL, WITH CONVENTIONAL TREATMENTS AND THEIR EFFECTS ON GLUCOSE UPTAKE AND INSULIN SECRETION

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Submitted in fulfilment of the requirements for the Master of Medical

Sciences in Public Health degree in the Discipline of Traditional Medicine, School of Nursing and Public Health, College of Health Sciences

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2022

PLAGIARISM DECLARATION

I, Sphamandla Hlatshwayo (210505551) declare that

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I, Sphamandla Hlatshwayo (210505551), hereby declare that the dissertation entitled

Concurrent treatment of *in vitro* cell lines with *Uthuli Lwezichwe*™ plant extracts used by an African traditional healer in the management of diabetes mellitus in KwaZulu-Natal, South Africa; with conventional treatments and their effects on glucose uptake and insulin secretion

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ACKNOWLEDGEMENTS

Ngithanda ukubonga Omkhulu kakhulu, Ongumnini mandla onke, Okunguye owazi isiqalo kanye nesiphetho sakho konke ngokungidala aphinge namandla okuthi ngize ngifinyelele lapho ngikhona njengamanje. Ngiphinde futhi ngedlulise ukubonga ulibo lonke engaqhuma kulo kanye neZilo zakwaMthwakazi nakwaZulu abebekhuleka ngaphansi kwazo kuze kufike emndenini engiphuma kuwo njengolibo engiqhume kulo.

*Ngibonga ku-*Dr M. Ngcobo *ngokungincintisa odengezini lwakhe kanye nokuthi angilonde njalo-njalo kuloluhambo lwami lwezemfundo. Izandla zedlula ikhanda ngokungifukamela Fuze, ungipha iziyalo, ungibonisa ukuthi impilo endaweni yokusebenzela kanye nangaphandle iphathwa kanjani.*

*Ngibonga u-*Prof. N Gqaleni *ngamaqhinga-su abengivezela wona uma sethula izinkulumo zocwaningo kanye namasu nje onkana ukuthi iyiyphi indlela okuyiyona-yona ekumele ngiphathe ngayo lomsebenzi.*

Ngibonga ku Mr S. Ndlovu *ngokuhlezi enza okusemandleni akhe ukuthi sithole izinsiza kusebenza ukuze sikwazi ukuqhuba umsebenzi nokuthi siwuphothule ngesikhathi.*

Ebese ngibonge u-Mr. P. Cele *kanye nawo wonke umndeni wakwa*Cele *ngekhambi Uthuli Lwezichwe™ okuyilo engisebenze ngalo kulouhambo lwezemfundo lokuthola leziqu.*

Ngibonga i-Moses Kotane Institute *kanye ne-*College of Health (CHS) *ngoxhaso-mali lokuthi ngikwazi ukuqhuba futhi ngephothule izifundo zami.*

Ebese ngedluse ukubonga kuzozonke izintanga zami ngokwezifundo esinabo ngaphansi kweso nesandla sika Dr Ngcobo *kanye no* Prof Gqaleni *ngokungibambisa lapho ngidinga isandla khona okanye umngani.*

LOMSEBENZI NGIWUBHEKISE KUBAZALI BAMI ASEBENDELA KWELAMATHONGO, NGITHI LOMSEBENZI UYISIHLUTHULELO ENGIKHONZA NGASO KINI UKUTHI NJENGENDODANA YENU MINA NGIBE NENHLANHLA NGAPHILA NGAZE NGAFIKA EMINYANI ENINGAKAZE NAFIKA KUYO.

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LIST OF ABBREVIATIONS

ADP	Adenine diphosphate
AGE	Advanced glycation end-product
AGEs	Advanced glycosylation end products
Akt	Protein kinase B
ALT	Alanine aminotransferase
AM	*Abelmoschus manihot* (L.) medic
AMPK	Adenosine-5′-monophosphate-activated protein kinase
ANOVA	One-way analysis of variance
AR	Aldose reductase
AST	Aspartic transaminase
ATM	African traditional medicine
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CE	Cinnamon extracts
CP	Cinnamon polyphenols
CSQ	Combination of swertiamarin and quercetin
DAG	Diacylglycerol
DHPAA	Dihydroxyphenylacetic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide

EMEM	Eagle’s Minimal Essential Medium
EMT	Epithelial-mesenchymal transformation
Enos	Endothelial nitric oxide synthase
FBG	Fasting blood glucose
FBP	Fructose-6-phosphate kinase-2
FCS	Foetal calf serum
FFA	Free fatty acid
FOXO1	Forkhead Box protein O1
Fpase	Fructose 1,6 bisphosphatase
G6P	Glucose-6-phosphatase
G6pase	Glucose-6-phosphatase
G6Pase	Glucose 6 phosphatase
GBI	Glibenclamide
GDM	Gestational diabetes mellitus
GFAT	Glutamine fructose-6-phosphate amidotransferase
GK	Glucokinase
GLUT1	Glucose transporter subtype-1
GLUT2	Glucose transporter subtype-2
GLUT3	Glucose transporter subtype-3
GLUT4	Glucose transporter subtype-4
GPx,	Glutathione peroxidase
GS	Glycogen synthase
GSH	Glutathione

GSI	Glomerulo-sclerosing index
GSIS	Glucose-stimulated insulin secretion
GSK3	Glycogen synthase kinase 3
GSP	Glycated serum protein
h	Hour
H_2O_2	Hydrogen peroxide
HbA1c	Glycated haemoglobin
HDL	High density lipoprotein
HFD	High fat diet
HKC	*Huangkui* capsule
HLA	Human leukocyte antigen
HOMA	Homeostatic model assessment of beta cell
HOMA-IR	Homeostatic model assessment of insulin resistance
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
InsR	Insulin receptor
IR	Insulin receptor
IRS	Insulin receptor substrate
IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate 2
JNK	C-Jun N-terminal kinase
K^+	Potassium
K^+-ATP channel	ATP-sensitive potassium channel

L	Litre
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LDLs	Low density lipoproteins
M	Molar
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
mg	Milligrams
min	Minutes
mL	Millilitre
mM	Millimolar
mmol/L	Milli mol per litre
MP	Malaysian propolis
NADPH	Nicotinamide adenine phosphate
NF-κβ	Nuclear factor-kappaβ
Nrf2	Nuclear factor erythroid 2
PAI-1	Plasminogen activator inhibitor-1
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PHK	Phosphorylase kinase
PI3K	Phosphoinositide 3-kinase

PKA	Protein kinase A
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor type gamma
PTP	Protein tyrosine phosphatase
PTP1β	Protein tyrosine phosphatase 1β
PYK	Pyruvate kinase
RIN	Rat insulinoma
RLU	Relative lights units
ROS	Reactive oxygen species
RPMI	Rosal Park Memorial Institute
RT-qPCR	Real time-quantitative polymerase chain reaction
SA	South Africa
SOD	Superoxide dismutase
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
STZ	Streptozotocin
SUR	Sulphonylurea Receptor
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBARS	Thiobarbituric acid reactive substances
TG	Triglycerides
TGF	Transforming growth factor
TGF-β1	Transforming growth factor-beta 1

THP	Traditional health practitioner
TZDs	Thiozolidinediones
UKZN	University of Kwa Zulu - Natal
USA	United States of America
v: v	Volume:volume
VEGF	Vascular endothelial growth factor
α	Alpha
β	Beta
μ	Micro

ABSTRACT

Background: By the year 2025, prevalence of diabetes mellitus is estimated to reach 300 million globally; with type 2 diabetes mellitus comprising more than 90% of these cases. Due to the side effects which are sometimes adverse and gradual loss of efficacy with time, presented by conventional regimens; a number of diabetic patients have been reported to be using both traditional medicines and conventional regimens concurrently. Experimental and clinical experiments have yielded positive results on studies performed on conventional treatments in combination with traditional medicines and medicinal plant extracts. Most these studies have been performed using a single medical plant, whilst African traditional medicine (ATM) products constitute of a variety of medical plants.

Aim: This study aimed to investigate the concurrent treatment of *in vitro* cell lines with *Uthuli Lwezichwe*™, an African traditional medicine used in the management of diabetes mellitus in KwaZulu-Natal, in combination with conventional treatments and their effects on glucose uptake and insulin secretion.

Methods: Cell viability was used to establish the IC_{50} doses of *Uthuli Lwezichwe*™ for HepG2 liver, C2C12 muscle and RIN-5 pancreatic beta cell lines. The IC_{50} doses were used in combination with known effective doses of metformin, insulin and tolbutamide to treat liver, skeletal muscle and beta cells, respectively. Glucose uptake was monitored at 0, 6, 12 and 24 h time intervals. Changes in glycogen and glutathione (GSH) levels in treated liver cells were evaluated using a glycogen assay kit (MAK0160) and GSH Glo glutathione kits, respectively. Insulin secretion in treated pancreatic cells was assessed using an ultra-sensitive rat insulin ELISA kit.

Results: In comparison to the untreated control, treatment with *Uthuli Lwezichwe*™ in combination with conventional drugs significantly increased ($p<0.05$) glucose uptake after 24 h in liver cells, which was further evidenced by an increase in glycogen synthesis in liver. Concurrent treatment of liver cells with *Uthuli Lwezichwe*™ in combination with metformin was shown to significantly ($p<0.05$) decrease GSH expression when compared to the untreated control over 24 h. Post 24 h treatment of beta cells indicated that co-administration of *Uthuli*

Lwezichwe™ and tolbutamide significantly ($p<0.05$) increased insulin secretion in comparison to all treatment groups.

Conclusion: Interaction of anti-diabetic agents studied, resulted in ameliorated glucose metabolism, both via glucose uptake and insulin secretion. This could be beneficial both in modulating diabetes mellitus and its comorbidities.

CHAPTER 1

INTRODUCTION

Diabetes mellitus (DM) refers to a collective group of endocrinological disorders resulting from sustained hyperglycaemia. DM may be due to a disturbed insulin secretion, target tissues being resistant to the action of insulin or sometimes both (Njølstad et al., 2003). This collective group of endocrinological disorders comprise of type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) and gestational diabetes mellitus (GDM). T1DM arises due to an attenuated insulin secretion by the beta (β) cells of the pancreas (~ 90% destruction of β cells) (Holt, 2004). T2DM is due to inadequate insulin secretion by the pancreatic β cells or highly reduced sensitivity of the insulin sensitive tissues to the action of insulin (Holt, 2004). Gestational diabetes mellitus (GDM) is referred to as insulin resistance that leads to elevated blood glucose levels during the period of pregnancy (Setji, Brown, & Feinglos, 2005). Sustained hyperglycaemia is due to increased levels of hormones such as cortisol, prolactin, human placental lactogen, and leptin as these hormones are antagonists of insulin (Gunton, Hitchman, & McElduff, 2001; Metzger et al., 2007). Sustained hyperglycaemia which results in DM leads to impaired metabolism of carbohydrates, proteins, fats, water and electrolytes; resulting in structural and functional changes of vital body organs (Cho et al., 2018). Depending on the severity of DM together with complications that may arise due to DM, combination therapy to manage the disease is often recommended as some hypoglycaemic agents only lower glucose but do not modulate co-morbidities that result from diabetes mellitus (Qaseem, Humphrey, Sweet, Starkey, & Shekelle, 2012).

Biguanides, insulin secretagogues, insulin injections and thiazolidinediones (TZDs) are amongst the widely used therapies in the management of DM (Bastaki, 2005). Insulin secretagogues and insulin injections are widely used to manage T1DM; as they stimulate insulin secretion (Bastaki, 2005). Biguanides and TZDs are utilised in the management of T2D as they facilitate skeletal muscle and the liver in glucose uptake (Kirpichnikov, McFarlane, & Sowers, 2002; Singh, Loke, & Furberg, 2007). Weight gain, hypoglycaemia, oedema and loss of efficacy over time are side effects that have been reported in the therapies; whether administered as mono or combination therapy (Diabetes Control Complications Trial Research Group, 1994).

According to conventional medicine, DM cannot be cured but is managed through mono or combination therapy. However, since DM also results in co-morbidities such as sustained high blood pressure, oxidative stress of various body tissues, gangrene and neuropathy; it is essential that therapies do not only target lowering blood glucose but also alleviate the complications that arise due to DM (Diabetes Control Complications Trial Research Group, 1994; Jousilahti et al., 2011; Lind et al., 2014; Livingstone et al., 2015; Rehman, Choi, Choe, & Yoo, 2015; Reichard, Nilsson, & Rosenqvist, 1993; UK Prospective Diabetes Study Group, 1998a). Hence, research has advanced towards the utilisation of medicinal plants, which are the basis of traditional medicine. Findings reported on medicinal plants have yielded positive results as modulation of various diseases via various mechanisms of action has been documented together with less side effects and toxicity (Gupta et al., 2017a; Mangoyi, Ngcobo, & Gomo, 2017).

It is reported that by the year 2025, the number of diabetic patients would have risen to 300 million globally (Zimmet, 2003). With this predicted increase in the number of diabetic cases in developing countries such as South Africa and globally, this calls for research to explore spheres such as medicinal plants which have long been utilised by African traditional health practitioners (THPs) in treating and curing diseases. Moreover, with the current economic status of African communities, it is of high importance to develop therapies that are easily accessible, affordable, more potent and present less side effects in comparison to conventional therapies. Hence, this study aimed to investigate the *in vitro* effects of an African traditional medicine product, *Uthuli Lwezichwe*™, in combination with conventional treatment on glucose utilisation and insulin secretion.

CHAPTER 2

LITERATURE REVIEW

DIABETES MELLITUS

DM refers to a collective group of endocrinological disorders resulting from sustained hyperglycaemia, which may be due to impaired insulin secretion, target tissues being resistant to the action of insulin or sometimes both (Dey et al., 2015; Matzinger, Fischhuber, & Heiss, 2018; Njølstad et al., 2003). Impaired insulin secretion observed in diabetic individuals results in disturbed carbohydrates, lipid and protein metabolism, which is evidenced by elevated blood glucose (Ghai, 2020). DM comprise of type 1 (T1DM), type 2 (T2DM) and gestational diabetes mellitus (GDM) (Egan & Dinneen, 2019; Holt, 2004; Ighodaro, 2018; Pociot & Lernmark, 2016). T1DM, sometimes referred to as juvenile onset diabetes mellitus; is reported to be responsible for approximately 10% of all cases of DM which translate to about 20 million people globally (Dey et al., 2015; Mokdad et al., 2001). T1DM arises due to an attenuated insulin secretion by the beta (β) cells of the pancreas. The impaired secretion of insulin observed in T1DM is due to an auto-immune attack (~ 90% destruction of β cells) in response to proteins presented by islet cells of the pancreas (Egan & Dinneen, 2019; Holt, 2004; Ighodaro, 2018; Pociot & Lernmark, 2016). T2DM is due to inadequate insulin secretion by the pancreatic β cells and/or highly reduced sensitivity of the insulin sensitive tissues to the action of insulin (Chaudhury et al., 2017; Holt, 2004; Malvandi, Loretelli, Nasr, Zuccotti, & Fiorina, 2019). Hence, glucose cannot be utilised by insulin sensitive tissues, thus accumulates in the blood stream (Egan & Dinneen, 2019; Holt, 2004; Hupfeld, Courtney, & Olefsky, 2013; Ighodaro, 2018; Rhodes, 2005; Turner et al., 2008). T2DM is responsible for most of the cases of diabetes mellitus, as it account for over 90% of DM cases reported globally (González, Johansson, Wallander, & Rodríguez, 2009; Malvandi et al., 2019). The prevalence of T2DM is predicted to rise by 70% in developing countries as these countries are gradually adopting the Western lifestyles whereby high energy diets are employed without much physically activity of the body (Shaw, Sicree, & Zimmet, 2010). Over the years DM has become the third leading cause of global mortality whereby Asian, African and Mediterranean countries are largely affected (Asrani, Devarbhavi, Eaton, & Kamath, 2019; Assah & Mbanya, 2017). A significant marked increase is predicted to be in developing countries which includes Africa as a whole, due to urbanisation and a shift in diet leading to the late on-set DM which is

T2DM (Green, Christian Hirsch, & Krøger Pramming, 2003). South Africa being a country that is still developing; scientific data reports the prevalence of T2DM to rise in developing areas whereby a rural-urban gradient in cases of DM was reported (Norman, Bradshaw, Schneider, Pieterse, & Groenewald, 2006). Higher prevalence of T2DM was reported in the developing urban areas than in the rural areas (Norman et al., 2006). Studies indicate that the highest prevalence is in the Indian population, followed by the Coloured population then African populations (Bradshaw, Norman, Pieterse, & Levitt, 2007). In the overall South African population, it is reported that persons aged thirty years old and upwards are at risk of developing T2DM which is the third leading cause of death globally and in South Africa (Hall, Thomsen, Henriksen, & Lohse, 2011). DM is associated with cataracts, which may lead to blindness, coronary heart disease, end stage renal disease which requires dialysis or transplantation, hypertension and gangrene which may result in amputation (Rhodes, 2005). South Africa being a country that is still developing, the health care system has limited resources to help manage DM and its related comorbidities that eventually lead to death. Hence, there is a need for efficient use of all the available resources to respond to the high prevalence of DM in South Africa. As a contribution to this fight and widening the available range of anti-diabetic medications, the aim of this study was to investigate effects of concurrent treatment on *in vitro* cell lines with an ATM product with conventional drugs on glucose uptake and insulin secretion.

ETIOLOGY OF TYPE 2 DIABETES MELLITUS AND ASSOCIATED COMORBIDITIES

T2DM is generally developed by morphological then functional damage of pancreatic β-cells and unresponsive insulin receptors (Holt, Cockram, Flyvbjerg, & Goldstein, 2017). In diabetic patients, insulin resistance is mainly characterised by increased glucose generation coupled with decreased insulin secretion. Increased levels of free fatty acids and lipolysis are also responsible for sustained hyperglycaemia, highly reduced β-cell function, and glucose utilisation. In addition, ROS alter cell function and induce lipid peroxidation (LPO), inflammation, and apoptosis in the pancreas, kidneys, liver, nerves, and vasculature (Tiwari, Pandey, Abidi, & Rizvi, 2013). T2DM is characterised by insulin resistance and a gradual decline in the functioning of pancreatic β cells or both, resulting in sustained hyperglycaemia (Kahn, Cooper, & Del Prato, 2014). Insulin resistance attenuates the action of insulin signaling on insulin sensitive tissues (Petersen & Shulman, 2018).

Insulin resistance observed in T2DM is reported to be as a result of an imbalance between the insulin receptor (IR) and protein tyrosine phosphatase (PTP) on insulin sensitive tissues. PTP enzyme modulates the insulin signalling pathway in skeletal muscles, liver, adipose tissues via negative feedback (Combs, 2010). PTP dephosphorylates insulin receptors which blocks the signalling cascade of insulin. This phenomenon results in insulin resistance and eventually DM, which presents comorbidities such as nephropathy, retinopathy, neuropathy, atherosclerosis, myocardial infarction and stroke (Chawla, Chawla, & Jaggi, 2016; Jeevathayaparan, Tennekoon, & Karunanayake, 1995). In the pancreas, oxidative stress results in morphological changes of the insulin secreting β cells, followed by a decline of insulin biosynthesis and secretion and β cell apoptosis (Hasnain, Prins, & McGuckin, 2016) . Particularly, DM has been reported to concomitantly attenuate superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR) activities, while accentuating hepatic lipid peroxides, hydrogen peroxides, protein carbonyl and nitric oxide, up-regulate pro-inflammatory mediators like nuclear factor kappa B (NF-kB), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6 in hepatic tissues (Haligur, Topsakal, & Ozmen, 2012; Ingaramo et al., 2011; Palsamy, Sivakumar, & Subramanian, 2010). Other studies have also reported an up-regulation of caspase-3 and increased apoptotic index in hepatic tissues of diabetic experimental rats (Elattar, Estaphan, Mohamed, Elzainy, & Naguib, 2017; Tunçdemir, Ertürküner, & Özçelik, 2017). In skeletal muscles, ROS overproduction as a results of advanced glycation end-products (AGEs) exhibit its insulin resistance effects by downregulating the insulin receptor substrate 1 (IRS-1) tyrosine phosphorylation and eventually PI3K which result in the inactivation of the glucose transporter 4 (GLUT4) translocation to the plasma membrane (Geer, Islam, & Buettner, 2014; Gerber & Rutter, 2017; Styskal, Van Remmen, Richardson, & Salmon, 2012). As DM develops and progresses due to oxidative stress, there's also a parallel development of diabetic comorbidities (Ahmed, 2005). Progression of DM together with its comorbidities can be explained by the following mechanisms: formation of AGEs, polyol, protein kinase C and hexosamine pathways (Aragno & Mastrocola, 2017; Sada et al., 2016; Singh, Bali, & Jaggi, 2014).

EPIDEMIOLOGY OF TYPE 2 DIABETES MELLITUS

DM has gradually become the third leading cause of worldwide mortality (Assah & Mbanya, 2017). The increasing incidents of T2DM globally and in Africa are due to several factors which include ageing, economic status which relates to food one can afford, as most of the African communities consume high carbohydrate diets (Steyn, Kazenellenbogen, Lombard, & Bourne, 1997). Urbanisation also presents a shift in nutritional diets as we see a major increase in the consumption of fast foods which constitute a high fat content that may result in obesity which is a major cause for insulin resistance leading to T2DM (Steyn et al., 1997). The number of people with DM was estimated to be 451 million in 2017 and this figure is expected to reach 693 million by 2045 (Cho et al., 2018). The international diabetes federation reports that there are approximately half a billion people who suffer from DM, globally, and 80% of these cases are from low-income and middle-income countries like South Africa. In Africa T2DM and its comorbidities is reported to result in 77% of deaths annually (International Diabetes Federation, 2017). Reports have also indicated that in South Africa, 87% of DM cases are related to excess body weight which gives rise to T2DM; this figure translates to 3.15million people living with T2DM. (Joubert et al., 2007). Such statistics furthermore emphasise how much of a burden T2DM is to Africa, and the whole world. Hence, development of treatments that will facilitate, improve the potency and efficacy of current treatments is very much needed.

CURRENT TREATMENTS FOR TYPE 2 DIABETES MELLITUS

Prescription of DM treatments mostly depends on the number of years the patient has been diagnosed with the disease and also the severity of the disease (American Diabetes Association, 2016; Nathan et al., 2009). This is due to how effective the treatment is, with less intensive intervention on patients who have recently been diagnosed with DM, while more rapid glucose lowering interventions are effective on cases of chronic exposure to DM (American Diabetes Association, 2016; Nathan et al., 2009). This is employed as a strategy to prevent the risk of hypoglycaemia in patients with on-set DM and also to prevent sustained hyperglycaemia even when receiving treatment (Peters & Davidson, 1996). Clinical trials performed over the years have reported that decreasing hyperglycaemia and/or maintaining it as closely as possible to euglycaemic levels is effective in reducing long term microvascular complications that are due to

DM (retinopathy, neuropathy and nephropathy) (Reichard et al., 1993; UK Prospective Diabetes Study Group, 1998a, 1998b).

Biguanides

Biguanides include metformin, phenformin and buformin, with metformin being the widely used drug in the management of T2DM. Metformin is the only safe drug in the biguanides family that is recommended as first-line therapy for T2DM (Zhou, Xu, Du, Zhao, & Wang, 2018). Biguanides present a class of drugs which exert anti-hyperglycaemic effects by suppressing gluconeogenesis but some reports have documented that metformin promote glucose uptake in the skeletal muscles by enhancing insulin sensitivity (Cheng & Fantus, 2005; Gomez-Peralta et al., 2017; Kirpichnikov et al., 2002). Metformin is reported to modulate hyperglycaemia by lowering hepatic glucose output and fasting plasma glucose by sensitizing the liver to actions of insulin to promote glycogenesis (Cheng & Fantus, 2005; DeFronzo & Goodman, 1995). Mechanism of action of metformin is documented to be via AMP-activated protein kinase (AMPK) pathway in hepatocytes, as AMPK is a cellular regulator of lipid and glucose metabolism (Holmes, Kurth-Kraczek, & Winder, 1999; Qiang et al., 2016). In skeletal muscles modulation of hexokinase and glucose transporters subtype-4 (GLUT 4) have been reported on as some of the other mechanisms of action for metformin (Holmes et al., 1999). Side effects such as diarrhea, anorexia, bloating and abdominal discomfort have been reported on patients receiving metformin as a therapy for the management of T2DM (Chaudhury et al., 2017; Stein, Lamos, & Davis, 2013). Metformin has also been reported to disrupt vitamin B_{12} absorption as a side effect which may give rise to anemia. It is also rendered unsafe when used by patients with impaired renal, hepatic and cardiac function, which on most cases are diabetes mellitus related comorbidities observed in diabetic patients (Bailey & Turner, 1996). This is said to be due to lactic acidosis which is often reported in T2DM patients receiving metformin (Salpeter, Greyber, Pasternak, & Salpeter, 2010). Decreased glucose absorption has also been reported as a side effect presented by metformin. T2DM patients in inadequate long-term blood glucose control with metformin monotherapy require additional treatment (Goldstein, Pans, & Rubin, 2003).

Insulin secretagogues

This class of anti-diabetic drugs facilitates or promotes the secretion of insulin by the β cells of the pancreas; and are namely sulphonylureas and non sulphonylureas (Cheng & Fantus, 2005; Groop, 1992). Sulphonylureas modulate hyperglycaemia by stimulating insulin secretion by binding on the sulphonylurea receptor (SUR) on the surface of the pancreatic β cell (Cheng & Fantus, 2005; Groop, 1992). The SUR is a subunit of potassium adenosine phosphate-dependent (K^+-ATP) channels located in the β-cell membrane. This leads to the blockage the potassium (K^+) channels which results in the influx of calcium (Ca^{2+}) into the β cells (Nenquin & Henquin, 2016). The entry of Ca^{2+} into the β cells leads to cell depolarization and subsequently insulin exocytosis occurs (Kalra et al., 2018). Furthermore, owing to their non-glucose concentration dependent action, sulphonylureas are more effective in the on-set stages of T2DM whilst most of the β cell function is better (Kalra et al., 2018). Documented side effects presented by sulphonylureas include hypoglycaemia that may arise to over-secretion of the hormone insulin whereby more β cells are recruited and stimulated to secrete insulin since sulphonylureas do not rely on elevated blood glucose levels to exert their effects (Andre J Scheen, 2016). The hypoglycaemia may result in a coma or episodes of seizures, weight gain (~2 – 5 kg) and nausea (Kahn et al., 2006; Khunti, Chatterjee, Gerstein, Zoungas, & Davies, 2018; Zimmerman, 1997). Non-sulphonylureas present a relative new class of drugs represented by nateglinide and repaglinide. Repaglinide is derived from benzoic acid while nateglinide is derived from phenylalanine (Hatorp, 2002; McLeod, 2004). This new class of drugs mimic the mechanism of sulphonylureas the way they exert their effects but they bind on a different side of the SUR to promote insulin secretion (McLeod, 2004). Risks of hypoglycaemia have been reported on this particular insulin secretagogue but the side effects are less deleterious in comparison to sulphonylureas (Nattrass & Lauritzen, 2000). Weight gain also presents as a side effect but less pronounced in comparison to sulphonylureas (Inzucchi, 2002).

Insulin

The hormone insulin is known as one of the most potent glucose lowering agents. Insulin decreases hepatic glucose production while promoting glucose uptake on extra-hepatic insulin sensitive tissues (Tripathi & Srivastava, 2006). Experimental evidence suggests that the beneficial effects of insulin include alleviation of glucotoxicity and eventually oxidative stress, in a dose dependent

manner; since insulin presents hypoglycaemia when administered at supra-physiological doses (Bastaki, 2005; Ohkubo et al., 1995; Popovic-Djordjevic, Jevtic, & Stanojkovic, 2018). Insulin therapy is associated with weight gain of approximately 2 – 4 kg which is reported to be as a result of ameliorated glycosuria, hence an increase in glucose reabsorption by the kidneys (Ohkubo et al., 1995). In some cases, insulin therapy has been reported to result in hypoglycaemia whereby patients suffered a coma (Ohkubo et al., 1995). One to four injections daily, weight gain, hypoglycemia, expensive insulin analogues are short falls of receiving insulin as therapy to manage DM. It is also reported that diabetic patients receiving insulin injections as a form of therapy, have a poor glycaemic control when compared to other groups receiving oral hypoglycaemic agents (Bastaki, 2005). A global major predicament involving insulin is its high cost price; and it is predicted that within the next 5 – 10 years most developing countries will not afford insulin (Basu et al., 2019).

Thiazolidinediones

Thiazolidinediones (TZDs) are peroxisome proliferator activated receptor gamma (PPAR-γ) antagonists as they inhibit PPAR γ, thereby sensitise the insulin sensitive tissues to the action of insulin to facilitate glucose uptake in the state of insulin resistance (Yki, 2004). TZDs are associated with an increased risk of fluid retention which presents a life-threatening risk of congestive heart failure as a result of oedema (Davidson, Mattison, Azoulay, & Krewski, 2018; Home et al., 2007; Singh et al., 2007). Weight gain is also one of the adverse side effects presented by TZDs on diabetic patients which is a risk factor for T2DM and related complications (Davidson et al., 2018; Home et al., 2007; Singh et al., 2007). Troglitazone was the first TZDs approved in the United States for management of T2DM in 1997, three years later troglitazone was withdrawn due to increased frequency of liver injury that resulted in acute liver failure following administration (Tan et al., 2019).

Sodium-glucose cotransporters type 2 inhibitors

Sodium-glucose cotransporters type 2 (SGLT2) inhibitors are glucose modulating agents for T2DM patients (Tahrani, Barnett, & Bailey, 2013). The insulin-independent mode of action for SGLT2 exerted on the kidneys, inhibits the reabsorption of filtered glucose by 30-50%. This results in increased urinary glucose excretion, which lowers blood glucose from hyperglycaemic state (A. Scheen & Paquot, 2014). Regarding Adverse events that have been reported on SGLT2 inhibitors

were as a result of their mode of action, whereby urinary glucose excretion resulted in urinary, genital infections and diabetic ketoacidosis (Peters et al., 2015; Andre J Scheen, 2015; André J Scheen, 2020).

Alpha-glucosidase inhibitors

Alpha-glucosidase inhibitors (AGIs) are oral antihyperglycemic drugs that exert their effects via the inhibition of intestinal alpha-glucosidase, which is an enzyme that breaks down polysaccharide carbohydrates into glucose (Bischoff, 1995; Van De Laar et al., 2005). Therefore, by inhibiting alpha-glucosidase, the absorption of glucose is delayed, therefore slowing down surges in postprandial blood glucose and improved glycaemic control (Van De Laar et al., 2005). The most common documented side effects are diarrhea, abdominal pain and rare cases of hepatic lesion (Chiasson et al., 2003; Zhu, Tong, Wu, Li, & Tong, 2013).

Dipeptidyl peptidase-4 inhibitors

Dipeptidyl peptidase-4 (DPP-4) is an enzyme that breaks down incretin hormones. Incretin hormones (glucagon like peptide (GLP)-1 and glucose-dependent insulinotropic polypeptide (GIP)) play a crucial role in the maintenance of glucose homeostasis. GLP-1 and GIP are secreted from L- and K-cells of the intestinal mucosa, during a meal. This results in indirect plasma glucose regulations (Theodorakis et al., 2006). In humans, GLP-1 increases cells β responsiveness to the stimulus of glucose, inhibits gastric emptying and reduces appetite (Deacon, Nauck, Meier, Hücking, & Holst, 2000; Kjems, Holst, Vølund, & Madsbad, 2003). Inhibition of DPP-4 activity allows the activity of endogenous incretins, GLP-1 and GIP (Patel & Ghate, 2014). Scientific evidence indicates that DPP-4 inhibitors are moderately safe and tolerable, with a low incidences of hypoglycaemia reported (Niswender, 2010).

MEDICINAL PLANTS UTILISED IN THE MANAGEMENT OF DIABETES MELLITUS

The world-wide utilisation of complementary and alternative medicine (CAM) in the treatment of ailments and diseases such as DM has rapidly increased over the last decade. It is reported that approximately 73% of diabetic patients globally utilise herbal medicines, dietary supplements and CAM therapies (Chang, Wallis, & Tiralongo, 2007). ATM is reported to be the most easily accessible and economically available system of health care for most African communities, due

to its potency and efficacy in curing ailments and some diseases (Kasilo et al., 2010). The World Health Organization (WHO) estimates that over 80% of the population in developing countries utilise traditional herbal medicines (World Health Organization, 2003). Generally, herbs are used for nourishing, strengthening and co-ordinating the function of the body. However, without scientific evidence herbal medicines cannot be commercialised as either as antidiabetic supplements or as anti-diabetic drugs (Singh, Nayak, & Mishra, 2020). In the recent years leading to the present, extracts and bioactive compounds from traditional medicinal plants have been studied extensively and documented to possess beneficial properties in the management of DM (Khan et al., 2012). Documented active constituents derived from medicinal plants and their extracts include polyphenols alkaloids, flavonoids, saponins, steroidal saponins, terpenoids, steroids, tannins and anthocyanidin; to name a few (Modak, Dixit, Londhe, Ghaskadbi, & Devasagayam, 2007; Prabhakar, Kumar, & Doble, 2014; Sales, Souza, Simeoni, Magalhães, & Silveira, 2012). In order to broaden the portfolio of anti-diabetic drugs available, research studies investigating the anti-diabetic potential of medicinal plants and their extracts have continuously grown worldwide (Wiedemann et al., 2015). According to the world ethnobotanical inspection, over 800 plants are utilised medicinally for the prevention, management and treatment of DM (Ekar & Kreft, 2019; Modak et al., 2007; Prabhakar et al., 2014). Studies aimed at the modulation of DM have rapidly grown towards the utilisation of plant extracts. This is reported to be due to these extracts possessing bio-active compounds that modulate DM together with its related complications, while presenting less side effects (Zaman, 1989). Antidiabetic effects of these plant species extracts have been confirmed using human and animal models of DM (Modak et al., 2007). One such plant that has been extensively studied is *Momordica charantia* (Linn/Family: Cucurbaceae). Communities in the eastern region of Africa are known for utilising the fruit (Karela/corilla or bitterground) of this plant for health benefits (Akhtar, 1982). This is further evidenced by experimental findings whereby circulating levels of plasma glucose were significantly reduced following treatment with *M. Charantia* (freshy prepared juice or dried fruits)(Sarkar, Pranava, & Marita, 1996). *Allium cepa* (onion) is reported to modulate circulating levels of glucose by acting on the enzymes; hexokinase and glucose-6-phosphatase in alloxan induced diabetic rats when administered at a dose of 200mg/kg for 45 days. In rabbit models the very same extract was reported to be both antidiabetic and anti-oxidative (El-Soud & Khalil, 2010; Kumari, Mathew, & Augusti, 1995). Diabetic patients that were treated with *Coccinia indica* (*C.*

indica) (500 mg/kg body weight) and studied over a six week period were reported to have restored activities and functioning of lipoprotein lipase enzyme (LPL) which was markedly suppressed in the diabetic untreated group; glucose 6-phosphatase and lactate dehydrogenase which were elevated in the untreated groups (Kamble, Kamlakar, Vaidya, & Bambole, 1998). Documented mechanisms of actions of medicinal plant extracts and herbal medicines, including ATM products exert anti-diabetic effects and/or alleviate diabetic complications via:

i. Reducing the expression of gluconeogenic enzymes (Shibib, Khan, & Rahman, 1993);
ii. Sensitise insulin sensitive tissues via translocation of glucose transporters, promote glucose uptake (Shibib et al., 1993);
iii. Preserving the structure and function of β cells, results in increased insulin secretion (Jeevathayaparan et al., 1995);
iv. Alleviating oxidative stress via modulating the expression of glutathione (GSH), therefore maintaining structural and functional of vital organs (Karasu, Dewhurst, Stevens, & Tomlinson, 1995).

Although most studies on traditional remedies have focused on the use of a single medicinal plant extract, it is known that traditional health practitioners (THPs) use a mixture of plants when preparing medicines. Therefore, the efficacy of ATMs is due to the mixed herbs/medical plants possessing a wide range of therapeutic benefits that are brought by the medicinal plants having multiple mechanisms of action to modulate glucose uptake and related complications (Baldé et al., 2006).

DUAL CONVENTIONAL THERAPY FOR DIABETES MELLITUS

A vast majority of patients diagnosed with DM as reported receive more than one class of diabetic medication (Qaseem et al., 2012). In most cases 14% of patients are both on insulin and an oral anti-hyperglycaemic agent; while 58% of patients are on a combination of oral hypoglycaemic agents (Qaseem et al., 2012; Qaseem, Snow, Owens, & Shekelle, 2010). Research findings indicate that most people who utilise CAM therapies do so concurrently with conventional medicines, rather than in place of, conventional medicines (Manya, Champion, & Dunning, 2012). Synergism may help improvise potency of medication, but it has been reported that conventional medication used for the management of DM in combination with ATM may present an adverse effect of

hypoglycaemia. This is what this study aims to investigate. Maintaining euglycaemia either pharmacologically (through mono/dual therapy) or by adopting a healthy lifestyle has been reported to have positive effects in halting the progression of co-morbidities that arise due to glucotoxicity in diabetics (Bennett, Maruthur, et al., 2011). The potent effects reported on combination therapy when compared to monotherapy, maybe due to synergism resulting from more than one mechanism of lowering blood glucose; which often presents cases of hypoglycaemia when in combination with insulin secretagogues (Nattrass & Lauritzen, 2000). Malabsorption of glucose has also been reported in combination therapy that includes metformin as one of the hypoglycaemic agents (Bennett, Wilson, et al., 2011). Hence, this study aimed to establish the effects of combination therapy, of an ATM product in combination with conventional anti-diabetic agent on glucose utilisation and insulin secretion.

HERB-DRUG INTERACTIONS ON THE MODULATION OF DIABETES MELLITUS

Extensive work still needs to be done in drug herb interactions in the modulation of T2DM, but some reports indicate synergistic and antagonistic interactions of the herbs and drug molecules may achieve the desired pharmacological effects (Gupta et al., 2017b). The possibility of synergism would allow effective doses of conventional anti-diabetic drugs to be decreased, while the supplementation with herbal medicines would increase the efficacy of the reduced effective dose via synergism with conventional anti-diabetic drugs (Gupta et al., 2017b). The herbal extracts may reduce toxicity or side effects of the conventional anti-diabetic drugs while widening the pharmaco-dynamics by acting on receptors which are not ligands of conventional regimen (Yang et al., 2014). In diabetic experiments, blood glucose lowering effects of anti-diabetic drugs have been shown to be increased by medicinal plants. *Panax ginseng* and *Panax quiquefolium* when administered in combination with metformin have been shown to be more potent in comparison to metformin or the plant extracts monotherapy. This was further evidenced by circulating levels of glucose and insulin in homeostasis model assessment - insulin resistance (HOMA-IR) and haematoxylin and eosin-stained liver tissues (Vuksan et al., 2008; Yoon, Han, Sung, & Chung, 2007). Another study documented that a combination of *Momordica charantia* and metformin has been studied extensively in experimental and clinical studies. In one study, administration of 400mg chloroform/benzene extract of the plant in combination with *Momordica charantia* plant

extracts yielded desired glycaemic control; whereby combination therapy proved to be more potent when compared with doses of metformin alone (Tongia, Tongia, & Dave, 2004). Opuntia (*Opuntia aciculata*) which is widely utilised by the Mexican community is reported to be more efficient when administered in combination with metformin. In streptozotocin (STZ) induced diabetic rats, this combination treatment was reported to significantly increase muscle and liver glycogen storage, while reducing plasma glucose levels (Sobieraj & Freyer, 2010).

CONCLUSION

Drug research and development employs various methods in the production of new drugs. One such method is molecular synergism, whereby fusion herbs and synthetic drugs is performed to enhance and modify different pharmacological bio-activities of conventional treatments (Viegas-Junior, Danuello, da Silva Bolzani, Barreiro, & Fraga, 2007). Exploration of herb drug treatment seems to yield desirable pharmacological effects but most of these studies have been performed using only single extracts. Since THPs use various medicinal plants when preparing medicines and this study aims to investigate the *in vitro* effects of *Uthuli Lwezichwe*^{TM}, ATM product administered in combination with conventional treatment on glucose utilisation and insulin secretion.

RESEARCH QUESTION

What are the effects of concurrent treatment of *in vitro* cell lines with *Uthuli Lwezichwe*™ and conventional drugs on glucose uptake and insulin secretion?

HYPOTHESIS

Concurrent treatment of *in vitro* cell lines with an *Uthuli Lwezichwe*™ and conventional drugs increases glucose uptake and insulin secretion.

OBJECTIVES

i. To establish the cytotoxic effects of co-administration of *Uthuli Lwezichwe*™ and conventional treatments on *in vitro* cell lines;

ii. To evaluate the effects of co-administration of an *Uthuli Lwezichwe*™ and conventional treatments on glucose uptake, glycogen synthesis, insulin secretion and GSH activity;

iii. Assess the possible mechanisms involved in the anti-diabetic effects observed of the combination treatment.

CHAPTER 3

MATERIALS AND METHODS

ETHICAL CLEARANCE

The present study received ethical clearance from Biomedical Research Ethic Committee of University of KwaZulu-Natal (BREC reference number: BE329/19). A copy of current BREC approval certificate in Appendix I.

MATERIALS

Chemicals and drugs were sourced as follows:

Dimethyl sulphoxide (DMSO), phosphate buffered saline (PBS), metformin, insulin, tolbutamide, glycogen assay kit (MAK016), ultra-sensitive rat insulin ELISA kit (Sigma-Aldrich, St Louis, Missouri, USA); Dulbecco's Modified Essential Medium (DMEM), Eagle's Minimum Essential Medium (EMEM), Roswell Park Memorial Institute Medium (RPMI) 1640, L-glutamine, penicillin/streptomycin (Pen/Strep), foetal calf serum (FCS) and trypsin (Highveld Biological, Johannesburg, South Africa); metformin, tolbutamide (Merck chemicals, Johannesburg, South Africa); insulin (NovoRapid Pen Refill, Novo Nordisk Pty Ltd, Sandton, South Africa); horse radish peroxidase (HRP) (Abcam, Cambridge, United Kingdom) GSH-Glo™ glutathione assay kit and Cell Titer-Glo™ Luminescent cell viability assay kit (Promega, Madison, Wisconsin, USA). Accu-Check Active glucose test strips (Roche Diabetes Care South Africa (Pty) Ltd. Midrand, South Africa). All chemicals used were of analytical grade.

METHODS

Study design

This was a lab-based experimental study that employed liver (Hep G2), skeletal muscle (C2C12) and pancreatic β (RIN-5) cell lines to evaluate the antidiabetic effects of *Uthuli Lwezichwe*™ in combination with conventional anti-diabetic drugs (tolbutamide, metformin and insulin) and their effects on glucose uptake and insulin secretion *in vitro*.

Data collection

Botanical identification

Botanical identification was performed by a botanist. The names of the medicinal plants were withheld for protection of intellectual property of the THP and protection of indigenous knowledge. The THP was assisted by the botanist to preserve specimens of the medicinal plants used in the preparation of the ATM product (*Uthuli Lwezichwe*™). Scientific names of the plants only will be revealed when the intellectual property rights have been addressed and the complete bio-medical data collected. Phytochemical analyses of the *Uthuli Lwezichwe*™ using LC-MS is in Appendix II.

Preparation of *Uthuli Lwezichwe*™

The freshly prepared *Uthuli Lwezichwe*™ was collected from the THP in the pristine form the THP prescribes it to his/her patients which is in an aqueous form. *Uthuli Lwezichwe*™ was filtered by high speed, centrifuged then freeze-dried to a powder. The powdered extract obtained was stored at -20 °C until time of use. Before the experiments were performed, *Uthuli Lwezichwe*™ powder was dissolved in phosphate buffered saline (PBS) and filter sterilized. To prepare doses required for each experiment, *Uthuli Lwezichwe*™ was prepared in PBS and subsequently diluted in fully supplemented respective cell culture media.

Experimental design

The study was divided into 3 sections. The first of experiments investigated the effects of *Uthuli Lwezichwe*™ on viability of liver, muscle and pancreatic β cell lines. The second series investigated the effects of *Uthuli Lwezichwe*™ plant extracts on glucose uptake and glycogen synthesis in treated muscle and liver cells. Lastly, third series investigated the effects of *Uthuli Lwezichwe*™ plant extracts on insulin secretion and GSH activity in treated pancreatic and liver cells, respectively. An outline summarizing the experimental protocols is shown in figure 1 below.

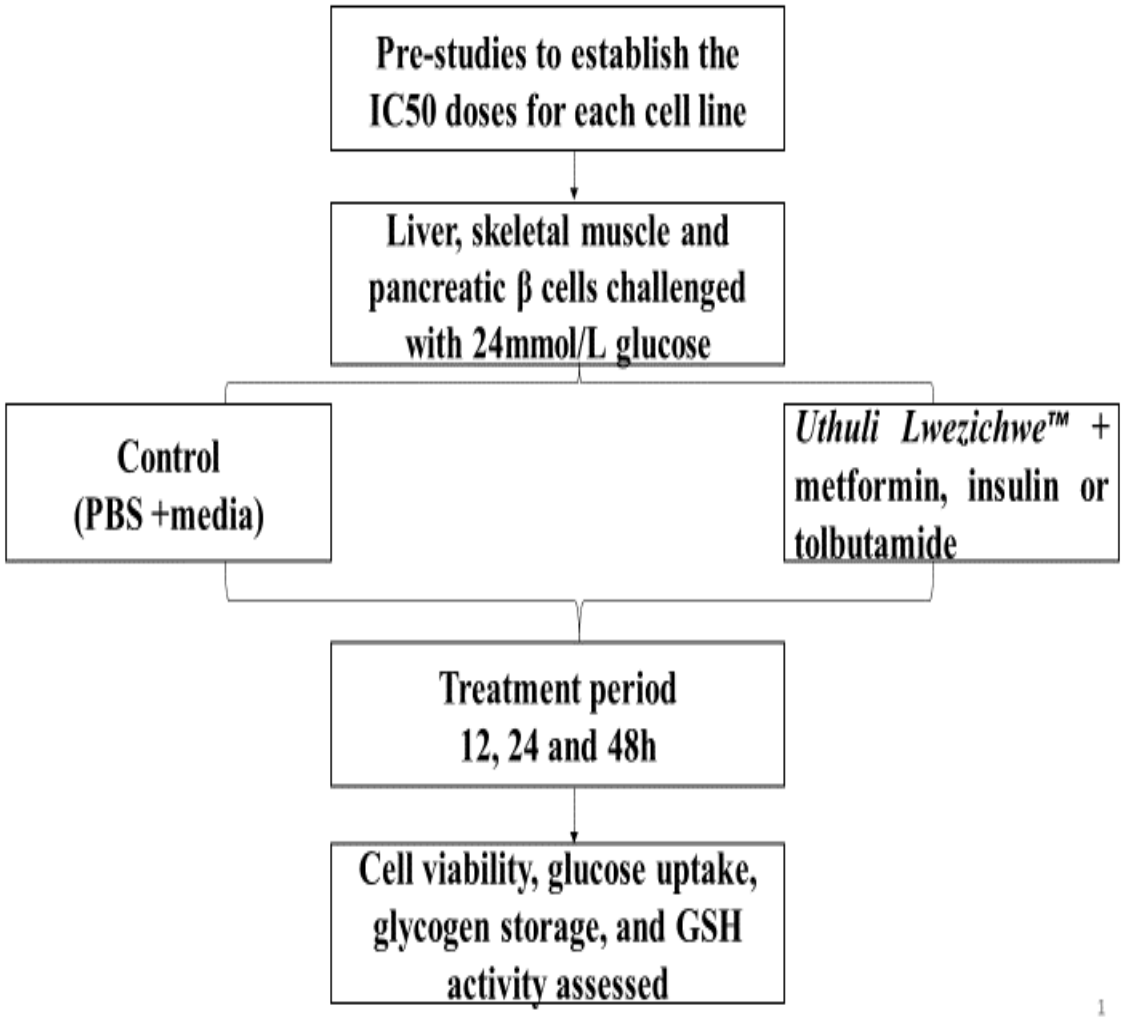

Figure 1: Summarized outline of protocols used in the study. The effects of *Uthuli Lwezichwe*™ plant extracts on cell viability were investigated in liver, skeletal muscle and pancreatic β cell lines. Subsequently, the effects of *Uthuli Lwezichwe*™ in combination with conventional treatments were evaluated on glucose utilisation and glycogen storage in liver and muscle cell lines, followed by GSH activity analysis. The effects of *Uthuli Lwezichwe*™ plant extracts on insulin secretion were investigated in pancreatic cell line.

Cell culture protocol

The pancreatic β cell line used in the study were kindly donated by Ms. Cabangile Mbili from the Discipline of Physiology at the University of KwaZulu - Natal (UKZN), Durban, South Africa while the muscle and liver cell lines were purchased from Cellonex, Johannesburg, South Africa. Cell culture studies were conducted according to a protocol established by (Czifra et al., 2006). Briefly, RPMI, EMEM, and DMEM for culturing muscle (C2C12), liver (Hep G2) and pancreatic (RIN-5) cell lines, respectively, were supplemented with foetal calf serum (FCS) (10%) and penicillin/streptomycin **(**1%). Frozen pancreatic (RIN-5), liver (Hep G2), skeletal muscle (C2C12) cell lines were reconstituted in respective media then transferred into 25cm^3 flasks which were incubated at 37 ºC in a CO_2 (5%) incubator with humidity of (95%) (Shel Lab, Cornelius, Oregon, USA). The cells were allowed to attach, grow, and reach confluency. Once confluency was reached, cells were trypsinized via the action of trypsin (0.25%) following washing three times with PBS. The cells were then sub-cultured into 75cm^3 flasks and incubated. Again, the cells were allowed to attach, grow, and reach confluency. Once confluency was reached, cells were trypsinized via the action of trypsin (0.25%) following washing three times with phosphate buffered saline (PBS). The cells were then sub-cultured into new flasks and incubated. Thereafter, the cells were plated in 24 and 96 well plates for assays.

Cell viability studies

Cell viability studies were conducted in pancreatic β (RIN-5), liver (Hep G2) and skeletal muscle (C2C12) cells to assess the cytotoxic effects of *Uthuli Lwezichwe*™ and the combination therapy treatment with conventional anti-diabetic drugs (metformin, insulin and tolbutamide). Cell viability was assessed using the Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, Wisconsin, USA). The assay reagent produces luminescence in the presence of ATP from viable cells. The luminescence produced is proportional to the quantity of ATP present. Hence, the quantity of ATP is proportional to the number of viable cells. The cell viability assay was conducted as follows; cells (10, 000 cells/mL) were seeded in 96-well plates and incubated in their respective media (200 µL) overnight. Thereafter the cells were treated with IC_{50} doses of *Uthuli Lwezichwe*™ (1070 µg/mL for Hep G2 cells, 2612 µg/mL for C2C12 cells and 2117 µg/mL for RIN-5 cells) and/or in combination with metformin (160 µg/mL), insulin (4 µg/mL) and

tolbutamide (200 µM). Cells incubated with DMSO (0.1%), metformin (160µg/mL), insulin (4µg/mL) or tolbutamide (200 µM) served as vehicle and treated positive controls, respectively. Each treatment was conducted in 6 separate wells (n=6). After each incubation period, plates were equilibrated at room temperature for 30 minutes. The assay reagent (100 µL) was added to each well, followed by shaking the plate for 2 minutes to induce cell lysis. After shaking, plates were incubated at room temperature for 10 minutes to stabilise the luminescence signal. Thereafter, the luminescence was read using the GloMax microplate luminometer (Promega, Madison, Wisconsin, USA). For background correction, wells with media only were used as blank. Data were expressed as relative light units (RLU) read by the luminometer.

Glucose utilisation studies

The glucose utilisation experiments were conducted as previously described by (van de Venter et al., 2008) with slight modifications. Briefly, 90% confluent liver and muscle cells (1.5×10^5) plated in 24 well plates were incubated at 37ºC with EMEM and DMEM (1 mL) containing 24 mmol/L of glucose, in the presence of IC_{50} doses of *Uthuli Lwezichwe*™ (1070 µg/mL for Hep G2 cells and 2612 µg/mL for C2C12 cells) and/or in combination with metformin (160µg/mL) and insulin (4µg/mL). Cells incubated with DMSO (0.1%), metformin (160µg/mL) and insulin (4µg/mL) served as vehicle and treated positive controls, respectively. Each treatment was conducted in 6 separate wells (n=6). Media glucose concentrations were measured at 0, 6 12 and 24 h using an Accu-Chek® Active glucometer (Roche Diabetes Care GmbH, Mannheim, Germany). After each treatment period, liver cells were trypsinised and harvested to quantify glycogen synthesis and GSH activity.

Glycogen synthesis assay

Glycogen synthesis experiments were conducted according to the manufacturer's instructions. Briefly, 90% confluent liver cells (1.5×10^5) plated in 24 well plates were incubated at 37ºC with EMEM (1 mL) containing 24 mmol/L of glucose, in the presence of IC_{50} dose of *Uthuli Lwezichwe*™ (1070 µg/mL) and/or in combination with metformin (160µg/mL). Cells incubated with DMSO (0.1%) and metformin (160µg/mL) served as vehicle and treated positive controls, respectively. Each treatment was conducted in 6 separate wells (n=6).

Glycogen synthesis assay was performed using a glycogen assay kit MAK016 (Sigma-Aldrich, Missouri, USA). The kit is equipped with hydrolysis buffer, development buffer, fluorescent peroxidase substrate, hydrolysis enzyme mix, development enzyme mix and glycogen standard (2 mg/mL). Briefly, the harvested liver cells (1 x 10^6) were washed with cold PBS and homogenised in 100 mL of water on ice. Thereafter, the samples were boiled at 100ºC for 5 minutes to inactivate enzymes. Homogenates were centrifuged at 13,000 rpm at 4^o C for 10 min, and supernatants were recovered for glycogen analysis. Standards (0.4, 0.8, 1.2, 1.6, and 2.0 µg/well) were brought to a final volume of 50 µL by adding the hydrolysis buffer. Hydrolysis enzyme mix (2 µL) was added to the samples, followed by incubation for 30 min at room temperature, protected from light. Master reaction mix (50 µL) was then added to samples followed by shaking the plate while incubating for 30 min protected from light. After cooling, the absorbance was read using the EZ Read 400 spectrophotometer (Biochrom, Oslo, Norway) at 570 nm. The glycogen concentrations were calculated from the glycogen standard curve.

Insulin secretion studies

Separate 90% confluent pancreatic cells (1.5 x10^5) in 24 well plates were incubated at 37ºC with RPMI (1 mL) containing 24 mmol/L of glucose, in the presence of an IC_{50} dose of *Uthuli Lwezichwe*™ (2117 µg/mL for RIN-5 cells) and/or in combination with tolbutamide (200 µM). Cells incubated with DMSO (0.1%), tolbutamide (200 µM) served as vehicle and treated positive controls, respectively. Glucose concentrations were monitored at 0, 6, 12 and 24 h. The media samples were harvested at 6, 12 and 24 h for insulin secretion measurements.

Media samples collected at the different incubation periods were assayed for insulin secretion. Insulin secretion was quantified using an ultra-sensitive rat insulin ELISA kit (Sigma-Aldrich, Missouri, USA). The kit was equipped with a 96 well plate coated with mouse monoclonal anti-insulin, calibrator, enzyme conjugate, enzyme conjugate buffer, wash buffer, substrate 3,3',5,5'-tetramethylbenzidine (TMB) and a stop solution. The principle of the assay is a solid phase two-site enzyme immunoassay based on the direct sandwich technique in which two monoclonal antibodies are directed towards separate antigenic determinants on the insulin molecule. During the incubation period insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microtitration well. The washing steps removed

unbound enzyme labelled antibody, leaving the bound conjugate which reacts with TMB. This reaction was stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically. Each determination was conducted in duplicate for both calibrators and samples. The insulin assay was performed as follows; calibrators (0, 3.40, 8.50, 25.50, 68.10 and 170.27 pmol/L) or samples (10 µL) were added in each well of the plate coated with anti-insulin antibody. Thereafter, an enzyme conjugate solution (100 µL) was added in each well, followed by incubation of the plate at room temperature on an orbital shaker for 2 hours. After incubation, the reaction volume was discarded followed by washing (5 times) using a wash buffer solution (350 µL) per well. After washing, a substrate TMB (200 µL) was added in each well and incubated for 15 min at room temperature. Thereafter, the stop solution (50 µL) was added in each well, followed by shaking for 5 seconds. The absorbance was read using an EZ Read 400 spectrophotometer (Biochrom, Oslo, Norway) at 450 nm. Sample insulin concentrations were extrapolated from the insulin standard curve.

Glutathione content

Glutathione level quantification was conducted on liver (Hep G2) cell lines to assess the antioxidant effects of *Uthuli Lwezichwe*™ and the combination of treatment with conventional anti-diabetic drugs (metformin). GSH-Glo^{TM} Glutathione Assay (Promega, Madison, Wisconsin, USA). The GSH-Glo™ Glutathione Assay is a luminescence-based assay for detecting and quantifying GSH. The assay is based on the conversion of a luciferin derivative into luciferin in the presence of glutathione, catalysed by glutathione S-transferase. The signal generated in a coupled reaction with firefly luciferase is proportional to the amount of glutathione present in the sample. The GSH-Glo™ Glutathione assay was conducted as follows; cells (10, 000 cells/mL) were seeded in 96-well plates and incubated in their respective media (200 µL) overnight. Thereafter the cells were treated with IC_{50} dose of *Uthuli Lwezichwe*™ 1070 µg/mL for Hep G2 cells in combination with metformin (160 µg/mL). Cells incubated with DMSO (0.1%) and metformin (160 µg/mL) served as vehicle and treated positive controls, respectively. Each treatment was conducted in 6 separate wells (n=6). GSH-Glo^{TM} reagent (100 µL) was added to each well, followed by incubation at room temperature for 30 min while mixing on a plate shaker; protected from light. Thereafter, Luciferin detection reagent (100 µL) was added to each well and

allowed to mix on a plate shaker while incubating at room temperature for 15 min; protected from light. Thereafter the luminescence was read using the Promega microplate luminometer (Promega, Madison, Wisconsin, USA). For background correction, wells with medium only were used as blank.

Statistical analysis

All data was expressed as means ± standard error of means (SEM). Statistical analysis was performed using GraphPad Prism Instat Software (version 5.00, GraphPad Software, San Diego, California, USA). One-way analysis of variance (ANOVA) was followed by Bonferroni test was used for analysis of differences between control and experimental groups.

CHAPTER 4

RESULTS

Cell viability

The effects of *Uthuli Lwezichwe*™ monotherapy on liver, skeletal muscle and β cells to establish IC50 doses

Figures 2, 3 and 4, depict the effects of *Uthuli Lwezichwe*™ various doses [(untreated control) 0 – 2500 µg/mL)] on the viability of liver, skeletal muscle and pancreatic β cells, over 24 h incubation.

Figure 2 shows the viability of liver cells, following administration of *Uthuli Lwezichwe*™ doses ranging from 0 – 2000 µg/mL, whereby an IC_{50} dose of 1070 µg/mL was established for liver cells.

Figure 3 shows the viability of skeletal muscle cells, following administration of *Uthuli Lwezichwe*™ at doses ranging from 0 – 4000 µg/mL, whereby an IC_{50} dose of 2612 µg/mL was established.

Figure 4 shows the viability of pancreatic β cells, following administration of *Uthuli Lwezichwe*™ ranging from 0 – 2500 µg/mL, whereby an IC_{50} dose of 2117 µg/mL was established.

The IC50 was calculated using the formula Y = a * X + b; whereby Y (y-axis) represented cell viability, X (x-axis) represented *Uthuli Lwezichwe*™ doses, a represented a gradient of the straight line ((plotted against cell viability vs dose response)) whilst b represented the starting point of the straight line (plotted against cell viability vs dose response).

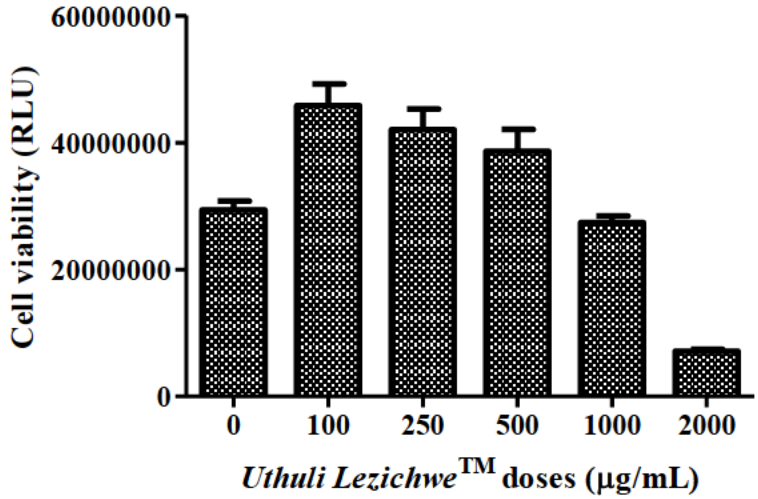

Figure 2: The effects of *Uthuli Lwezichwe*™ on cell viability of HepG2 liver cells after 24 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group).

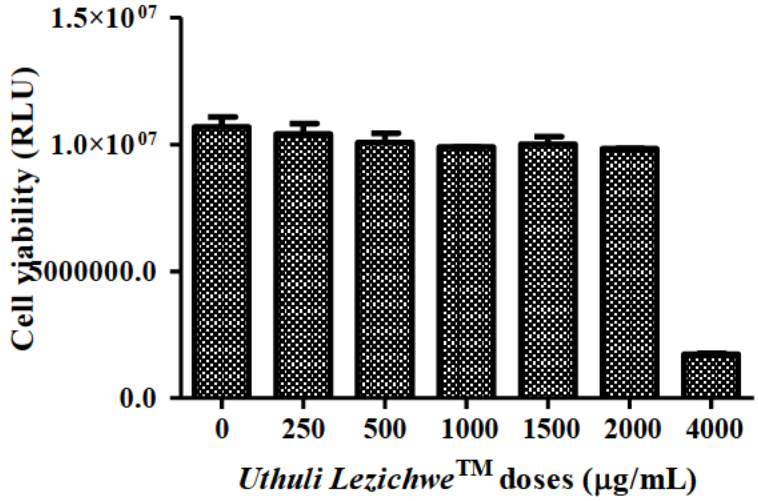

Figure 3: The effects of *Uthuli Lwezichwe*™ on cell viability of C2C12 skeletal muscle cells after 24 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group).

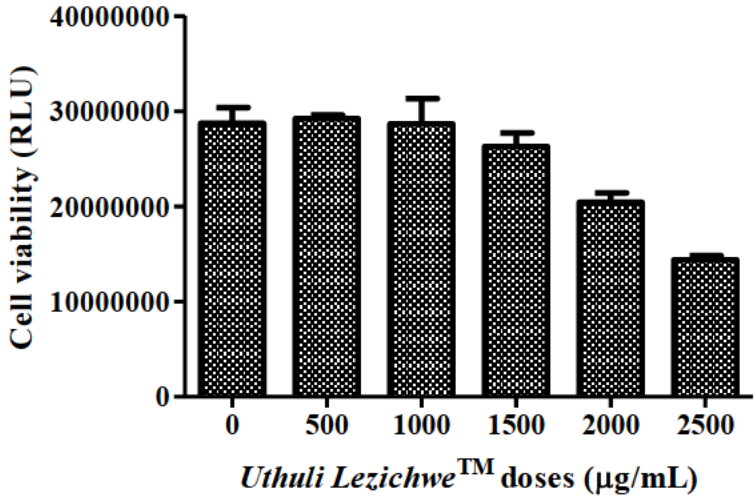

Figure 4: The effects of *Uthuli Lwezichwe*™ on cell viability of RIN-5 pancreatic β cells after 24 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group).

The effects of *Uthuli Lwezichwe*™ and a combination with metformin, insulin or tolbutamide

Figures 5, 6 and 7, depict the effects of *Uthuli Lwezichwe*™ IC_{50} doses as monotherapy and/or as combination therapy with metformin (160 µg/mL), insulin (4 µg/mL) or tolbutamide (200 µM) on the viability of liver, skeletal muscle and pancreatic β cells, after 6, 12 and 24 h incubation periods.

Figure 5 shows the viability of liver cells, following administration of *Uthuli Lwezichwe*™ at an IC_{50} dose (1070 µg/mL) and/or in combination therapy with metformin (160µg/mL). *Uthuli Lwezichwe*™ monotherapy treated liver cells showed significant ($p<0.05$) increase of cell viability in comparison to the untreated control and combined treated cells at 6 and 12 h incubation periods. However, at the 24 h incubation period, *Uthuli Lwezichwe*™ monotherapy treated liver cells had a significant ($p<0.05$) decrease in viability that was approximately 50% in comparison to the untreated control cells and below 50% in relation to the combined therapy treated liver cells, which had a time dependent (6 – 24 h incubation period) gradual increase in viability that was significant ($p<0.05$) at the 24 h incubation period.

Figure 6 shows the viability of skeletal muscle cells, following administration of *Uthuli Lwezichwe*™ at an IC_{50} dose of 2612 µg/mL and/or in combination with insulin (4µg/mL). *Uthuli Lwezichwe*™ monotherapy and combination therapy treated cells had a significant ($p<0.05$) decrease on the viability of skeletal muscle cells in comparison to the untreated and positive control treated cells from 6 -24 h incubation period.

Figure 7 shows the viability of pancreatic β cells, following administration of *Uthuli Lwezichwe*™ at an IC_{50} dose of 2117 µg/mL and/or in combination with tolbutamide (200 µM). *Uthuli Lwezichwe*™ monotherapy had a non-significant increase in cell viability in comparison to untreated pancreatic β cells; and a significant ($p<0.05$) increase in comparison to combination therapy treated cells at 6 h incubation period. This was followed by a significant ($p<0.05$) decrease in viability *Uthuli Lwezichwe*™ monotherapy and combination therapy treated pancreatic β cells at 12 – 24 h incubation periods.

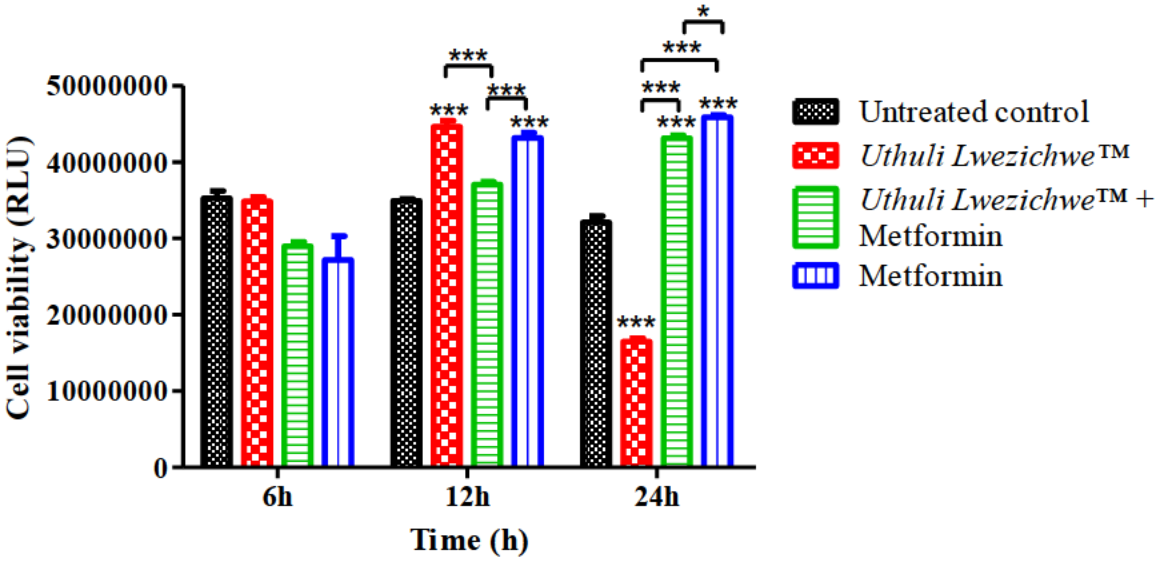

Figure 5: The effects of *Uthuli Lwezichwe*™ and a combination with metformin on cell viability of liver cells after 6, 12 and 24h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). Differences were statistically significant when $p < 0.05$.

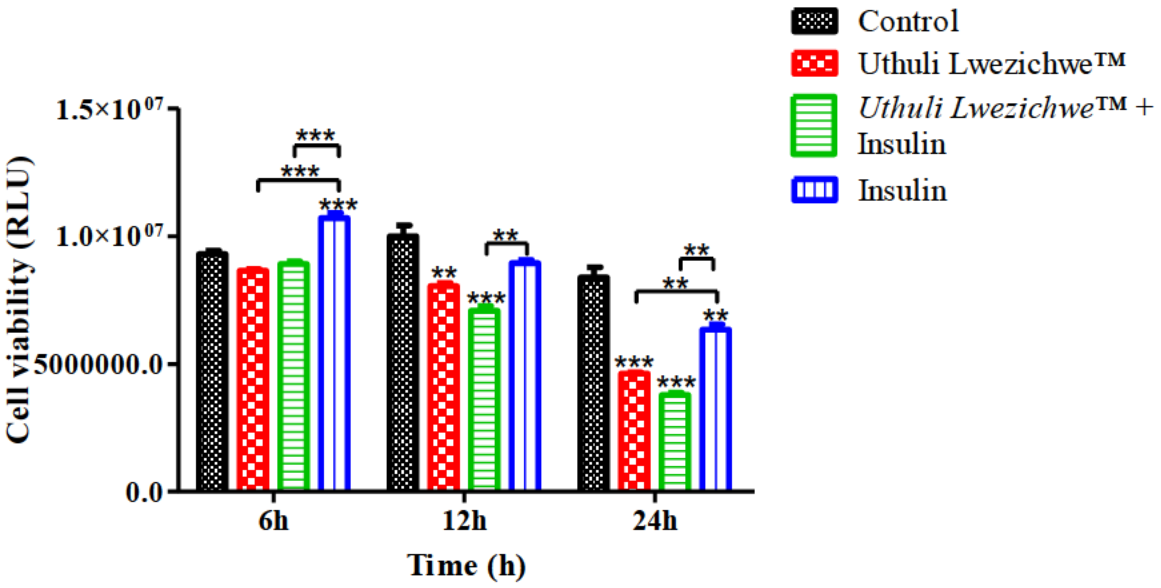

Figure 6: The effects of *Uthuli Lwezichwe*™ and a combination with insulin on cell viability of skeletal muscle cells after 6, 12 and 24 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). Differences were statistically significant when $p < 0.05$.

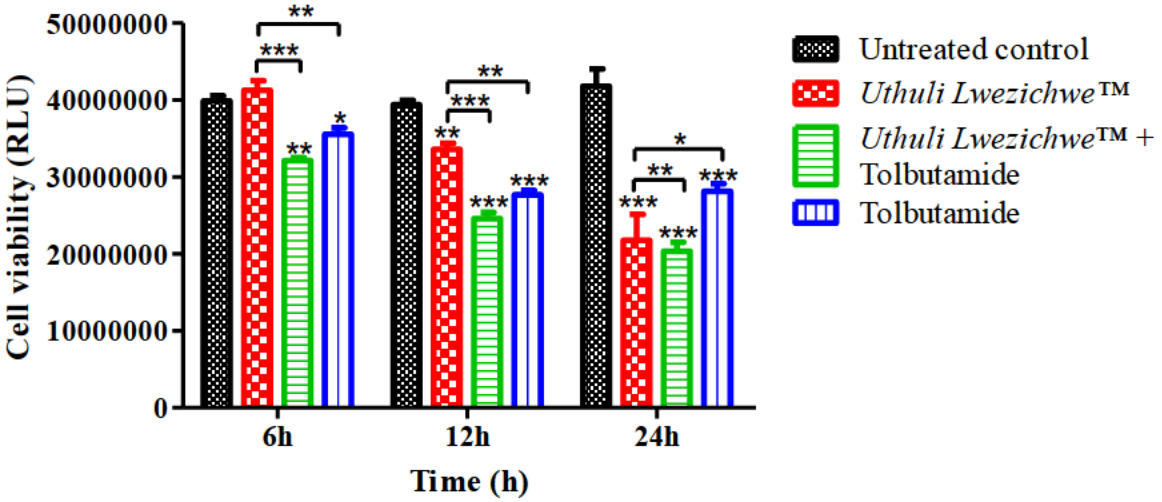

Figure 7: The effects of *Uthuli Lwezichwe*™ and a combination with tolbutamide on cell viability of pancreatic β cells after 6, 12 and 24 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). Differences were statistically significant when $p < 0.05$.

Glucose utilisation

The effects of *Uthuli Lwezichwe*™ and a combination with either metformin or insulin

Figures 8 and 9 show the effects *Uthuli Lwezichwe*™ IC_{50} doses as monotherapy and/or as combination therapy with either, metformin (160µg/mL) or insulin (4µg/mL) on glucose uptake in liver and skeletal muscle cells; after 0, 6, 12 and 24h incubation periods. The untreated control groups for both muscle and liver cells showed a steady decline in glucose concentrations over 24 h experimental period. *Uthuli Lwezichwe*™ mono and combination therapy treated liver and muscle cells in comparison to the respective controls significantly ($p<0.05$) decreased media glucose concentrations in both liver and muscle cells after 6, 12 and 24 h incubation periods. *Uthuli Lwezichwe*™ in combination with either metformin or insulin showed to be the most significant ($p<0.05$) in reducing media glucose concentrations in both liver and muscle cells at 24 h.

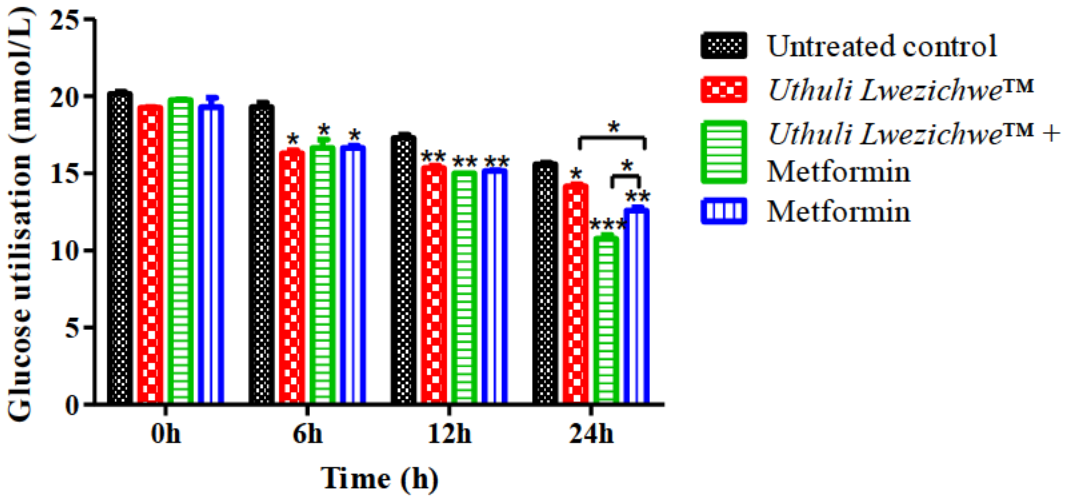

Figure 8: The effects of *Uthuli Lwezichwe*™ and a combination with metformin on glucose utilisation in liver cells after 0, 12, 24 and 24 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). Differences were statistically significant when $p < 0.05$.

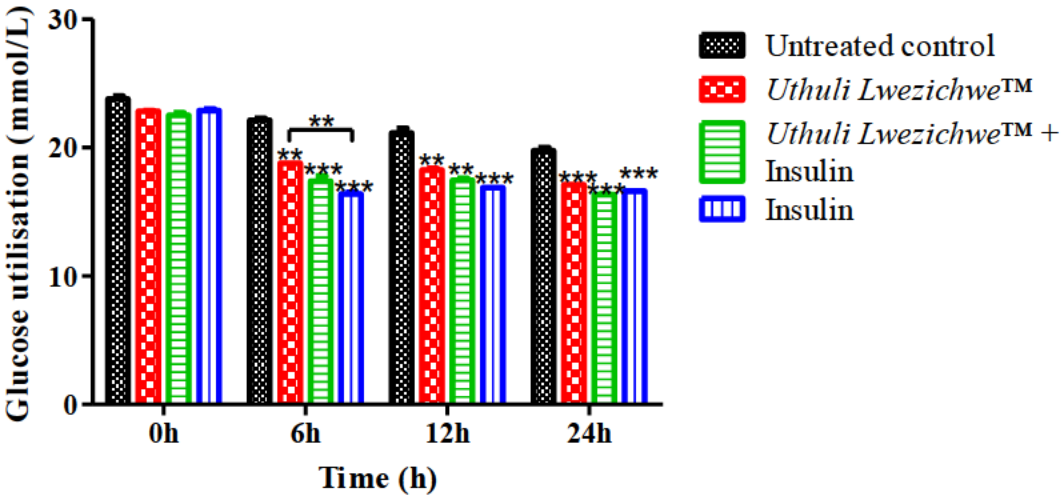

Figure 9: The effects of *Uthuli Lwezichwe*™ and a combination with insulin on glucose utilisation in skeletal muscle cells after 0, 12, 24 and 24 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). Differences were statistically significant when $p < 0.05$.

Glycogen storage

Figure 10 show the effects *Uthuli Lwezichwe*™ IC_{50} dose as monotherapy and/or as combination therapy with metformin (160µg/mL) on glycogen storage potential of liver cells, after 6, 12 and 24h. Administration of *Uthuli Lwezichwe*™ either as monotherapy or combination therapy with metformin significantly increased ($p<0.05$) glycogen concentrations when compared to the untreated and positive control (160µg/mL) liver cells. A non-significant difference was observed with untreated control and metformin treated liver cells. Interestingly, the effects of the combination therapy were the most potent and statistically significant.

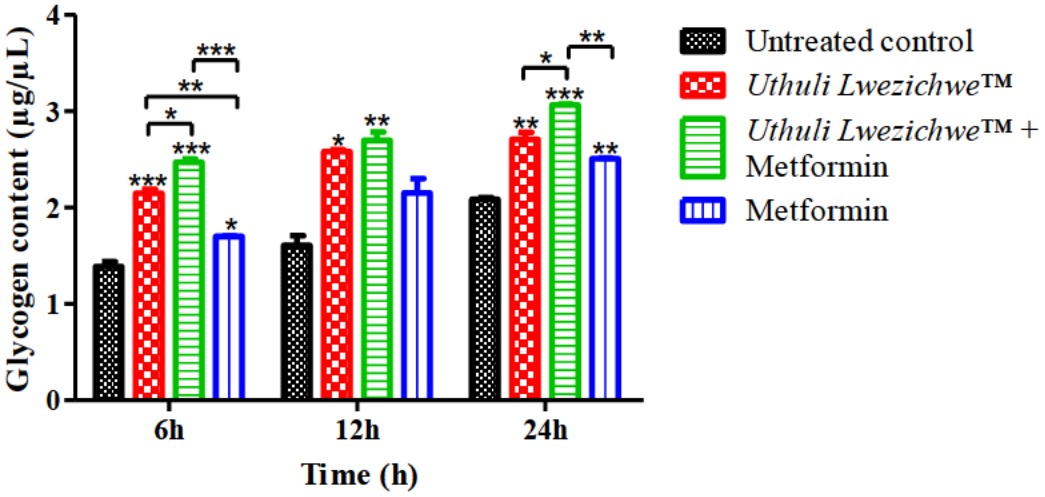

Figure 10: The effects of *Uthuli Lwezichwe*™ and a combination with metformin on glycogen storage in liver cells after 24 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). Differences were statistically significant when $p< 0.05$.

Insulin secretion

Figure 11 shows the effects of *Uthuli Lwezichwe*™ IC_{50} dose as monotherapy and/or as combination therapy tolbutamide (200 µM) on insulin secretion. Administration of *Uthuli Lwezichwe*™ as monotherapy or combination therapy significantly decreased ($p < 0.05$) media glucose concentrations followed by a significant ($p<0.05$) increase of insulin secretion; except for *Uthuli Lwezichwe*™ monotherapy at 6 h whereby by a significant decrease in media glucose was accompanied by a non-significant increase of insulin secretion.

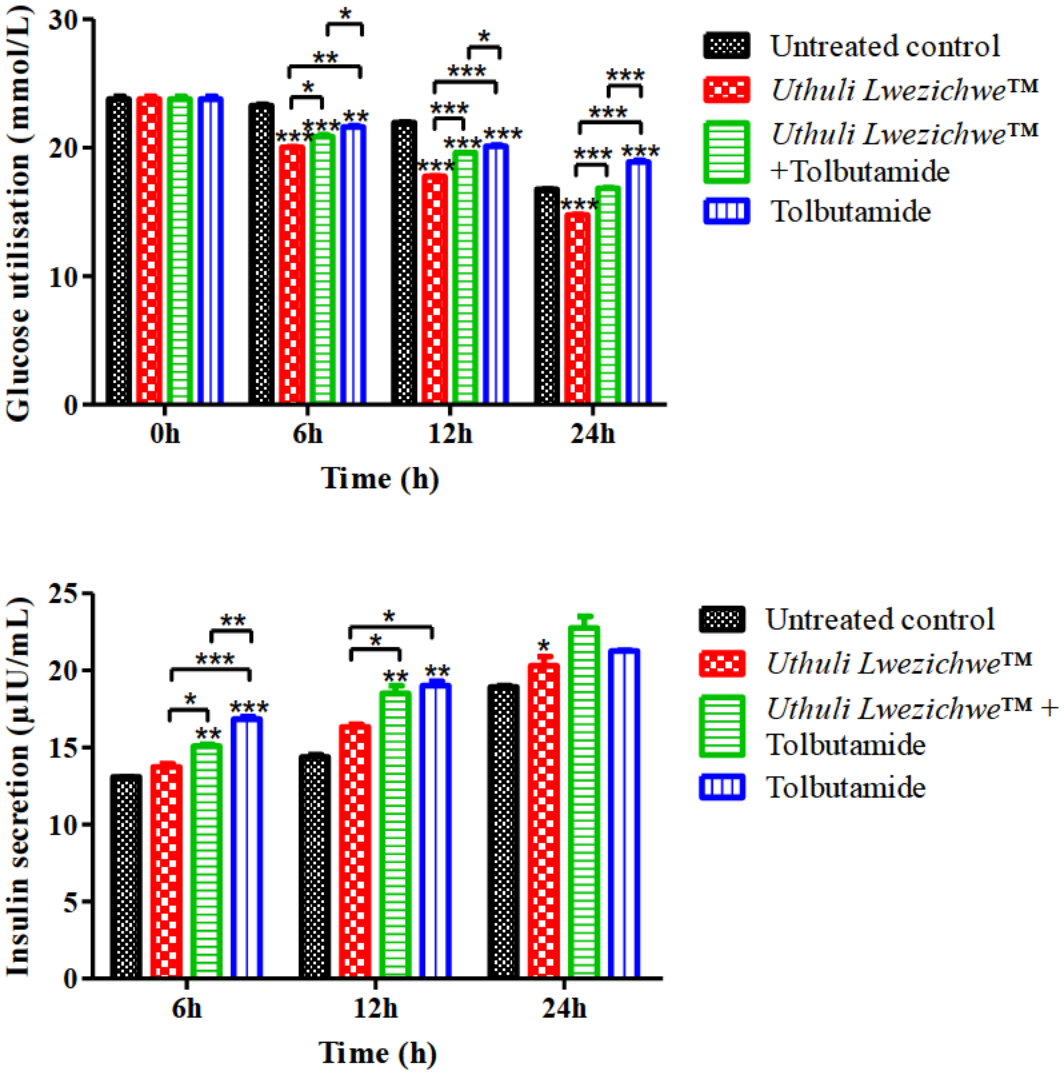

Figure 11: The effects of *Uthuli Lwezichwe*™ and a combination with tolbutamide on insulin of β cells after 6, 12 and 24 h, in comparison to glucose utilisation. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). Differences were statistically significant when $p < 0.05$.

GSH content

Figure 12 shows the effects *Uthuli Lwezichwe*™ IC_{50} dose as monotherapy and/or as combination therapy with metformin (160 µg/mL) on the activity of GSH in liver cells, after 6, 12 and 24h incubation periods.

The administration of *Uthuli Lwezichwe*™ either as monotherapy or combination therapy with metformin significantly decreased ($p < 0.05$) GSH content when compared to the untreated control liver cells at the 6 h incubation period. A significant ($p < 0.05$) difference of a similar trend was observed between the 12 -24 h incubation periods.

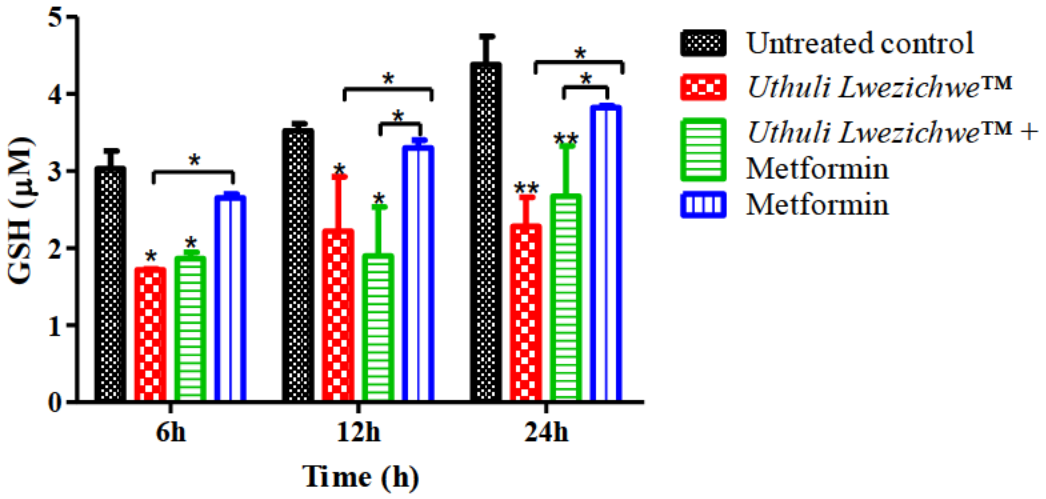

Figure 12: The effects of *Uthuli Lwezichwe*™ and a combination with metformin on GSH content of liver cells after 6, 12 and 24 h. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). Differences were statistically significant when $p < 0.05$.

CHAPTER 5

DISCUSSION

The WHO expert committee recommended that medicinal herbs be further scientifically investigated for their effects on diseases such as DM, as herbal medicines are constantly reported to present less side effects than conventional therapies (World Health Organization, 2016). This study investigated the concurrent treatment effects of *Uthuli Lwezichwe*™, an ATM used for treatment and /or management of DM in KwaZulu-Natal, South Africa with conventional treatments on glucose uptake and insulin secretion using cultured cells. *Uthuli Lwezichwe*™ is a polyherbal mixture, that is formulated using six medicinal plants. Identities of the constituent medicinal plants are withheld for protection of trade secrets and intellectual property rights of the THP, who is indigenous knowledge holder who owns *Uthuli Lwezichwe*™. Previous studies by (Mangoyi, Ngcobo, Gqaleni, & Gomo, 2019) reported the anti-hyperglycaemic, antioxidant and phytochemistry of *Uthuli Lwezichwe*™ *in vitro* and in type-2 diabetes rat models. Phytochemical analysis indicated that *Uthuli Lwezichwe*™ contains alkaloids, flavonoids, coumarins, saponins, tannins and terpenoids as secondary metabolites (Mangoyi et al., 2019). Previous studies have indicated that herbal medicines and ATM products would not have enough efficacy to achieve desirable glycaemic control, but the utilisation of various herbal extracts at concentrations below effective doses concurrently with conventional drugs has been scientifically proven to elicit a synergistic effect while presenting less side effects (Low Wang, Hess, Hiatt, & Goldfine, 2016; Tandon, Ali, & Narayan, 2012). This phenomenon signifies how herbal medicines and ATM formulas such as tonics may be effective without presenting side effects. This is due to multiple bioactive compounds acting synergistically at low concentrations, whereby each medicinal plant extract's bioactive compound(s) exert pharmacological effects that may be potent when combined (Qiao et al., 2016). Hence, the current study investigated the effects of an ATM (*Uthuli Lwezichwe*™) in combination with conventional anti-diabetic drugs to evaluate glucose utilisation and insulin secretion *in vitro*.

The cell cytotoxicity effects of *Uthuli Lwezichwe*™ either as monotherapy or in combination with metformin, insulin and tolbutamide were investigated on liver (Hep G2), skeletal muscle (C2C12) and pancreatic β cells (RIN – 5 cells); respectively. These cell lines were employed as models to study the effects of *Uthuli Lwezichwe*™ and conventional anti-hyperglycaemic drugs on glucose

utilisation and insulin secretion. A gradual time dependent cytotoxicity in the *Uthuli Lwezichwe*™ monotherapy treated cells was recorded across all cell lines as expected following treatment with an IC_{50} dose. According to another *in vitro* study, liver and skeletal muscle cells treated with sub IC_{50} doses of *Uthuli Lwezichwe*™ monotherapy, exhibited over 80% viability when compared to the untreated cells, post 24 h treatment (Mangoyi et al., 2019). The results indicated that *Uthuli Lwezichwe*™ is safe at sub IC_{50} doses. This is in line with the present study findings whereby liver cells were more susceptible to the toxic effects of *Uthuli Lwezichwe*™ with an IC_{50} dose of 1070 µg/mL while the IC_{50} dose of skeletal muscle cells was 2612 µg/mL. A similar trend *in vitro* following administration of *Uthuli Lwezichwe*™ documented that liver cells were more susceptible when compared to liver cells (Mangoyi et al., 2019). A beneficial therapeutic effect was observed in the combined therapy treated liver cells whereby cell proliferation almost comparable to that of metformin therapy treated liver cells was observed. The viability of combined therapy treated liver cells may be attributed to the synergistic hepatoprotective effects of both *Uthuli Lwezichwe*™ and metformin. *Uthuli Lwezichwe*™ is reported to be constituted of secondary metabolites such as alkaloids, flavonoids, coumarins, saponins, tannins and terpenoids which are all reported to elicit anti-oxidant effects on various body tissues whilst metformin is a well-known antioxidant promoter (An et al., 2016; Hasanpour Dehkordi, Abbaszadeh, Mir, & Hasanvand, 2019; Hou et al., 2010; Mangoyi et al., 2017). In β cells, a time dependant decline in the viability of treated β cells across all treatment groups was observed. The gradual decline in the viability of β cells treated with *Uthuli Lwezichwe*™ monotherapy is due to the cytotoxicity of the IC_{50} dose used to treat β cells. By definition, an IC50 dose is the concentration of a particular treatment which will result in 50% inhibition in cell viability time dependently at 24 h post treatment. Herbal extracts including *Rhizoma coptidis* and kinsenoside from *Anoectochilus roxburghii* are reported to be pancreo-protective by increasing pancreatic β-cell regeneration at sub IC_{50} doses (Oh, 2015). Therefore, we can conclude that such findings were not documented in the present because we used IC_{50} doses to treat cells. *In vitro* studies conducted on tolbutamide reported a dose and time dependent decline on viability of β cells due to apoptosis following administration of tolbutamide (Sarnobat, Moffett, Flatt, & Tarasov, 2021). Taken together with the cytotoxic effect induced by the IC_{50} dose of *Uthuli Lwezichwe*™, we speculate that the marked time dependant decline in the viability of *Uthuli Lwezichwe*™ and tolbutamide combined therapy treated β cells, which was not

observed in the untreated control cells may have resulted from the cytotoxicity of the IC_{50} dose of *Uthuli Lwezichwe*™ and tolbutamide.

DM is a multi-organ disease whereby disturbed insulin secretion and sensitivity by insulin sensitive tissues, or sometimes both; results in unregulated carbohydrates, lipids and proteins metabolism which manifest as sustained hyperglycaemia (Arora, Sarup, Tomar, Singh, & Kumar, 2019). Sustained hyperglycaemia then gives rise to ROS which results in tissue damage of both insulin and non-insulin sensitive tissues (Lu et al., 2016). Scientific evidence indicates that the manifestation of inflammation in the pathogenesis of DM is related to insulin resistance in insulin sensitive tissues (mostly liver, adipose and skeletal muscles) of diabetic patients (Khodabandehloo, Gorgani-Firuzjaee, Panahi, & Meshkani, 2016). Inflammation refers to an immune response whereby white blood cells protect body tissues against infections and injuries (due to ROS in DM); often presented as swelling, redness and pain (Reaven, 2005). The process of inflammation depends on metabolic support and energy redistribution (Reaven, 2005). Hence, during an inflammatory response, the body is continuously on a catabolic state, which highly inhibits the insulin signalling pathway (Martinez‐Herrera et al., 2018). Therefore, modulation of ROS production is pivotal in treatment or management DM as the physiological oxidant/antioxidant homeostasis can ameliorate DM comorbidities progression, associated with tissue lesion due to hyperglycaemia (Panigrahy, Kumar, & Bhatt, 2020). We evaluated the GSH content of liver cells as a measure of the antioxidant potential of concurrent treatment of these cells with metformin and *Uthuli Lwezichwe*™. Our findings were consistent with previous reports whereby, following treatment; a time dependent increase of GSH content was recorded across all treatment groups. *Uthuli Lwezichwe*™ monotherapy treated liver cells expressed the least GSH content, followed by combination therapy and metformin therapy treated liver cells at the end of the experimental period. Alkaloids, flavonoids, coumarins, saponins, tannins and terpenoids which are constituents of *Uthuli Lwezichwe*™ are well studied promoters of GSH in diabetic experiments (Mangoyi et al., 2019). However, administration of the IC_{50} dose to liver cells may have resulted in a time dependent suppression of GSH activity, resulting in a time dependent gradual decline in the activity of GSH in *Uthuli Lwezichwe*™ monotherapy treated liver cells. The antioxidant activities of medicinal plants has been attributed to their properties of free radicals scavenging, hydrogen (H^+) donation, breaking of radical chain reactions, and reduction of a singlet oxygen (Duthie,

Duthie, & Kyle, 2000). A previous study conducted on Malaysian propolis (MP) from stingless bees, *Heterotrigona itama*, indicated that MP as monotherapy significantly promoted antioxidant activity of hepatocytes (Nna, Bakar, Lazin, & Mohamed, 2018). It was reported that the high flavonoid content of MP may directly scavenge ROS in the liver, and/or may have indirectly regulated hepatic oxidant/antioxidant homeostasis via the promotion of glucose uptake (Kolluru, Bir, & Kevil, 2012; Nna, Bakar, & Mohamed, 2018). The combined therapy treated liver cells GSH content may be attributed to the synergistic hepatoprotective effects of both *Uthuli Lwezichwe*™ and metformin. Since *Uthuli Lwezichwe*™ constitutes of secondary metabolites such as alkaloids, flavonoids, coumarins, saponins, tannins and terpenoids; which are all reported by diabetic experiments to elicit anti-oxidant effects on various body tissues whilst metformin is a well-known antioxidant promoter (An et al., 2016; Hasanpour Dehkordi et al., 2019; Hou et al., 2010; Mangoyi et al., 2017). Scientific findings indicate that metformin suppress intracellular ROS levels by enhancing the expression of the antioxidant proteins such as GSH via the downregulation of NADPH oxidase through the AMPK-FOXO-3 pathway (An et al., 2016; Hasanpour Dehkordi et al., 2019; Hou et al., 2010).

Our study also documented that *Uthuli Lwezichwe*™ as mono or combination therapy promotes glucose uptake *in vitro,* whereby a time dependent glucose uptake was recorded across all treatment groups in both liver and skeletal muscle cell lines, with the combination therapy being the most potent at the end of each experimental period. Previous glucose uptake studies indicated that *Uthuli Lwezichwe*™ monotherapy promoted glucose uptake in liver and skeletal muscle cells by up to 10% in comparison to untreated control cells, over 24 h incubation period, which is in accordance with our present findings (Mangoyi et al., 2019). Another similar study suggested that the range by which three different medicinal plant extracts of the plant species *Oryza sativa* L. extracts promoted glucose uptake was between 9% and 39.5% (Krishnan et al., 2021). The results acquired in the present study are in accordance with existing previous findings, reported by similar studies. The previous studies documented a time dependent glucose uptake on both the mono and combination therapy treated liver and skeletal muscle cells, with combination therapy being the most potent. The findings of previous studies were validated by glycogen synthesis results of treated liver and skeletal muscle cells (Nna, Bakar, Lazin, et al., 2018; Nna, Bakar, & Mohamed, 2018). Metformin and herbal extract combinations have been reported to exert synergistic antidiabetic effects via increasing AMPK and PPAR-γ gene expression *in vitro* resulting in a more

amplified glucose uptake and glycogen synthesis (Kim et al., 2014). Therefore, we speculate that the potent glucose utilisation coupled with glycogen storage, recorded at the 24 h incubation period on the combination therapy treated liver cells was evident of the synergistic effects between *Uthuli Lwezichwe*™ and metformin since biguanides exert their anti-diabetic effects by activating AMPK, inhibiting hepatic glucose production.

Following treatment with *Uthuli Lwezichwe*™ and insulin as either monotherapy or polytherapy, skeletal muscle cells had a significant change in media glucose uptake when compared to the control skeletal muscle cells throughout the experimental period. Concurrent therapy treated skeletal muscle cells were comparable to those of the monotherapy treated liver cells, though shown the most potent glucose uptake which was not statistically significant when compared to the monotherapy treatment with either *Uthuli Lwezichwe*™ or insulin. The anti-diabetic mechanism(s) of *Uthuli Lwezichwe*™ monotherapy can be explained via the following similar studies of medicinal plants that possess similar secondary metabolites. Administration of nigelladine A, B and C (alkaloids) inhibited (PTP1B) expression in L6 myotubes via the PI3K/Akt signalling pathway. The net effect was enhanced glucose uptake (Tang, Chen, Xin, & Aisa, 2017). (Turner et al., 2008) also reported that berberine (an alkaloid), reduced the respiratory chain complex I in the mitochondria of muscle cells in a dose-dependent manner, as reported in the conventional anti-diabetic drugs, metformin and rosiglitazone. This signifies that *Uthuli Lwezichwe*™ and insulin may be competing for the same receptors to exert their glucose uptake effects. Hence, the comparable glucose uptake observed between the monotherapy and combination therapy treated skeletal muscle cells.

Insulin is a hormone that maintains glucose homeostasis by promoting glucose utilisation, absorption, and breakdown by exerting its effects on insulin sensitive tissues (skeletal muscle, liver and adipose). Insulin is secreted by the β cells of the pancreas which was represented by RIN – 5 cell line in this study. Glucose stimulated insulin secretion (GSIS) is the principle mechanism that β cells undergo to secrete insulin, in response to hyperglycaemic blood levels (Corkey, 2008). Glucose enters β cells via facilitated diffusion through (GLUT-2 in rodents, GLUT-1 and GLUT-3 in humans), which results in glucose being phosphorylated in a reaction catalysed by GK (Corkey, 2008). The following step in this cascade is glycolysis, which occurs in the cytoplasm. Pyruvate is then generated by glycolysis in the cytoplasm then metabolised by pyruvate carboxylase and pyruvate dehydrogenase in the mitochondria, which disturbs the ATP/ADP ratio

via the generation of ATP (Gheibi & Ghasemi, 2020). Disturbance of the ATP/ADP ratio results in the closure of K^+-ATP channels which results in depolarization of the β cell membrane followed by the activation of voltage dependent Ca^{2+} channels. Opening of the voltage dependent Ca^{2+} channels allows the influx of the intracellular Ca^{2+} which results in the fusion of insulin containing secretory granules with the plasma membrane; and insulin is secreted via exocytosis (Rorsman & Ashcroft, 2018). This cascade is referred to as the first phase insulin secretion, and is followed by a more sustained second phase of insulin secretion (Rorsman & Ashcroft, 2018).

In the present study we compared *Uthuli Lwezichwe*™ monotherapy, combination therapy with tolbutamide and tolbutamide monotherapy to investigate the herb-drug induced anti-diabetic effects, thereby identifying a therapeutic alternative. The combination therapy treated β cells were comparable to tolbutamide treated β cells at 6 and 12 h while the combination therapy was more potent at 24 h. This may be due to the activation of both K^+-ATP channel dependent and independent pathways due to the synergism of *Uthuli Lwezichwe*™ and tolbutamide. We also speculate that *Uthuli Lwezichwe*™ can further stimulate cAMP dependent and PKA pathway insulin secreting pathways independent of the K^+-ATP channel as reported on coumarins, alkaloids, saponins, tannins terpenoids and flavonoids extracts (Chueh & Lin, 2011; Wang et al., 2008). A monotherapy study conducted on a water-soluble herbal extract derived from *Triticum aestivum* sprout was evaluated for insulin secretion properties *in vitro* using the RIN-5F pancreatic β-cell line and rat pancreatic islets. It was documented that the extract induced GSIS from the pancreatic RIN-5F cells in a dose dependent manner when challenged with 16.7 mM glucose concentration. These results of the study suggested that herbal extracts induce GSIS via modulation of the K^+-ATP channels in hyperglycaemic states (Wiedemann et al., 2015). A separate study conducted on flavonoids (a similar secondary metabolite as the one identified by phytochemistry of *Uthuli Lwezichwe*™) were reported by (Aherne & O'Brien, 2002; Chemler, Lock, Koffas, & Tzanakakis, 2007; Hameed et al., 2019; Pinent et al., 2008) to possess insulin secreting properties in previous studies whereby tambulin of the plant species *Zanthoxylem armatum* exhibited *in vitro*:

i. no effect on insulin secretion in basal glucose (< 3mM) conditions which is an indication that traditional medicines present less side effects associated with hypoglycaemia as GSIS was documented at elevated glucose (<15mM) concentrations;
ii. significant insulinotropic effect through the PKA signalling pathway;

iii. flavonoids, namely genistein and eriodictyol enhance insulin secretion in pancreatic β-cells through cAMP dependent PKA pathway;
iv. an additive effect in tolbutamide-induced insulin secretion, which is not the case when synergism of tolbutamide is studied with other conventional anti-diabetic drugs.

Taken together, these findings suggest that the K^+ current is integral to GSIS; however, is bypassed by medicinal plant extracts (Aherne & O'Brien, 2002; Chemler et al., 2007; Hameed, Ashraf, Khan, Hafizur, & Ul-Haq, 2018; Hameed, Hafizur, et al., 2018; Hameed et al., 2019; Liu et al., 2006; Pinent et al., 2008; Rorsman & Ashcroft, 2018). Insulin secretagogues such as tolbutamide stimulate insulin secretion via depolarisation of the plasma membrane and activation of voltage gated Ca^{2+} influx in β cells (Stumvoll, Goldstein, & van Haeften, 2005). Therefore, tolbutamide only facilitates insulin secretion without promoting insulin synthesis (Del Prato & Pulizzi, 2006). Based on the results obtained in this study, the recorded significant increase at 24 h on the combination therapy treated β cells may be due to:

i. the effective properties of facilitating insulin production and secretion, reported on coumarins, alkaloids, saponins, tannins terpenoids and flavonoids which are constituents of *Uthuli Lwezichwe*™;
ii. closure of K^+-ATP channels and the influx of Ca^{2+} via voltage-dependent Ca^{2+} channels by *Uthuli Lwezichwe*™ and tolbutamide.

Since in diabetic patients, pancreatic islets secrete inadequate insulin or the insulin sensitive tissues do not respond to the stimulus of insulin, ameliorating insulin secretion via pancreatic β regeneration or activation of the insulin receptors has been one of the most explored approach to treat DM (Shou, Chen, & Xiao, 2020). Most importantly, with the number of diabetic patients growing every day, there is an urgent need for alternative therapies or modifications of how the conventional treatments are administered.

CHAPTER 6

CONCLUSIONS

The findings of the study revealed that concurrent administration of *Uthuli Lwezichwe*™ with antidiabetic drugs enhanced glucose uptake in both liver and muscle cell lines *in vitro*. Furthermore, *Uthuli Lwezichwe*™ as mono or combination therapy promotes insulin secretion in pancreatic β cells *in vitro*. The antidiabetic ability of *Uthuli Lwezichwe*™ may be beneficial in the management of both type 1 and type 2 diabetes mellitus, as well also delaying the onset of diabetes mellitus or its related co-morbidities since the combination therapy increased GSH content on hepatocytes. The overall observations warrant further clinical investigations to determine whether concurrent administration of *Uthuli Lwezichwe*™ with antidiabetic drugs may exert a tight glycaemic control *in vivo*.

Limitations of the study

In the current study we did not investigate the blood glucose lowering effects and insulin secreting of *Uthuli Lwezichwe*™ in combination with antidiabetic drugs *in vivo*. In addition, doses of *Uthuli Lwezichwe*™ below the IC_{50} established in this study may work better with less cytotoxicity to cells. IC_{50} doses were utilized only in this study in order allow for easy of comparison between monotherapy and concurrent treatment with both the ATM and conventional drugs.

Recommendations for future studies

Further studies to investigate the effects of *Uthuli Lwezichwe*™ whether alone or in combination on other parts of the pancreas (alpha and delta cells), adipose and kidneys both in *vitro* and *in vivo* are necessary to provide full data of the effects of this ATM product.

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APPENDICES

Appendix I: BREC ethical approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

14 May 2021

Mr S Hlatshwayo (210505551)
School of Nursing and Public Health
College of Health Sciences
210505551@stu.ukzn.ac.za

Dear Mr Hlatshwayo

Protocol: Effects of concurrent treatment of in vitro celllines with an African traditional medicine in and drugs on glucose uptake and insulin secretion
Degree: MMedSc
BREC Ref No: BE329/19

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	**29 May 2021**
Expiration of Ethical Approval:	**28 May 2022**

I wish to advise you that your application for recertification received on 03 May 2021 for the above study has been **noted and approved** by a subcommittee of the Biomedical Research Ethics Committee (BREC).The start and end dates of this period are indicated above.

Note to PI: In future please supply some quantitative information if possible for table 2.3

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 08 June 2021.

Yours sincerely

[redacted]

.....
Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Appendix II: Phytoconstituents in *Uthuli Lwezichwe*™ as revealed by Liquid Chromatography – Quadrupole Time of Flight – Mass Spectrometer

Phytoconstituents in *Uthuli Lwezichwe*™ as revealed by Liquid Chromatography – Quadrupole Time of Flight – Mass Spectrometer (LC-QTOF-MS) (Mangoyi et al., 2019)

Class	**Phytoconstituent**
Alkaloids	Lamprolobine, 1,2-Dihydrovomilenine, lochinerine, coronaridine, gabunine, sarpagine, 11 - methyoxy-vinorine, erysopine, isorhynchophylline, diferuloylputrescine, coronalidine, glaucine, cinnamoylcocaine, sinactine, senaetnine, corydaline, melostanin A, licorice glycoside A, skimmianine,
Flavonoids	6'-Hydroxy-O-desmethylangolensin, phloridizin, 4'-O-methyl-(-)-epicatechin-7-O-beta-glucuronide, desmethylxanthohumol, aromadendrin 4'-methyl ether 7-rhamnoside, spinosin A, (2R,3R)-3,3',4',7-Tetrahydroxyflavanone 7-O-alpha-L-Rhamnopyranoside, 1,2-Dimethoxy-13-methyl-[1,3]benzodioxolo[5,6-c]phenanthridine, 7-Hydroxy-2',4',5'-trimethoxyisoflavan, 7,8-Dihydro-3b,6a-dihydroxy-alpha-ionol 9-glucoside, gallocatechin-(4alpha->8)-catechin-(4alpha->8)-catechin
Quinones & quinoline	Cularine, pulmatin, dihydrocoriandrin, geranylbenzoquinone,
Terpenoids	Dimethylaminoethyl reserpilinate, tokoronin, gossyrubilone, esculentoside E, hiyodorilactone A, 11,12-dimethylrosmanol, (2S,4R,6S)-2-[2-(4-hydroxy-3-methoxyphenyl)ethyl]tetrahydro-6-(4-hydroxy-3,5-dimethoxyphenyl)-2H-pyran-4-ol, picrasin F, arabsin, simalikilactone D, trilobolide, picrasin C, 6,10,14-trimethyl-5,9,13-pentadecatrien-2-one, cucurbitacin P, all trans decaprenyl diphosphate
Saponins	Glucoconvallasaponin B
Coumarins	Nodakenin, methyl (2r*,3s*)-2,3-dihydro-3-hydroxy-2-isopropenyl-5-benzofurancarboxylate

Glycoside	Orientaloside, dide-o-methyl-4-o-alpha-d-glucopyranosylsimmondsin, glucocaffeic acid, coroloside, (1alpha,3beta,20S,22R,24S,25S)-Pubescenin, 2'-oxoaloesol 7-glucoside, (1r,2r,4s)-p-menthane-1,2,8-triol 8-glucoside, PE(18:4(6Z,9Z,12Z,15Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), licorice glycoside, 1alpha,3beta,22R-Trihydroxyergosta-5,24E-dien-26-oic acid 3-O-b-D-glucoside 26-O-[b-D-glucosyl-(1->2)-b-D-glucosyl] ester, 1-Octen-3-yl glucoside, alpha – antiarin, corchorusoside A, canesceol,
Other	LysoPE(0:0/20:5(5Z,8Z,11Z,14Z,17Z)), aflatoxin exb2, AFN911, citreovirenone, (2E,8E)-Piperamide-C9:2, genipic acid, coriandrone E, sapidolide A, 1-benzyloxy-1-(2-methoxyethoxy)ethane, khelloside, dukunolide C, eupatoriochromene, 1-(beta-d-glucopyranosyloxy)-3-octanone, dihydro daidzin, 7-hydroxy-2,5-dimethyl-4h-1-benzopyran-4-one, gibberellin a105, pterosin E, homodihydrocapsaicin, lactariamide b, 5-Hexyl-1,4-dioxan-2-one, 10,20-dihydroxyeicosanoic acid, N-hexadecanoylpyrrolidine, osmundalactone

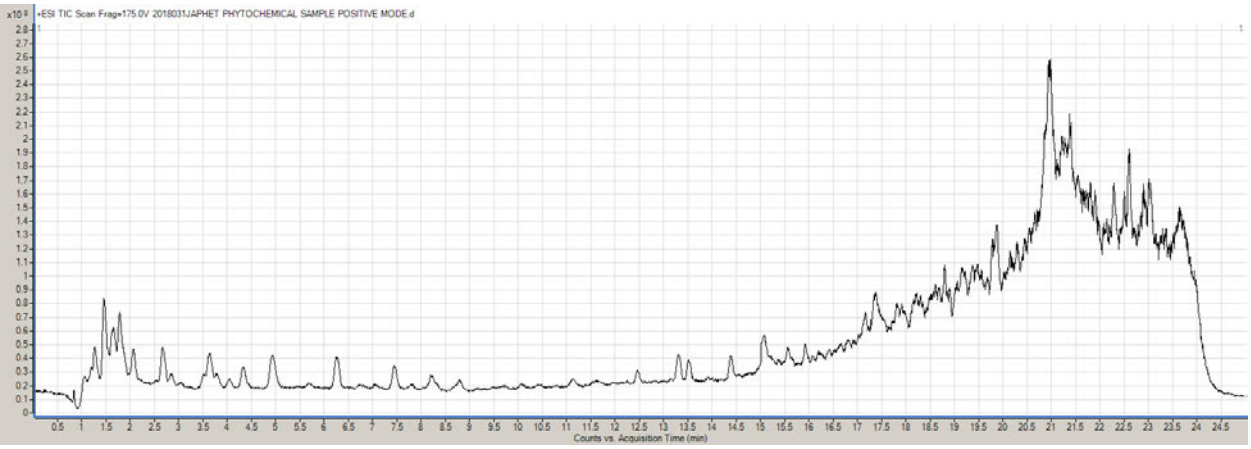

Figure 16: Chromatogram run in positive mode (Mangoyi et al., 2019).

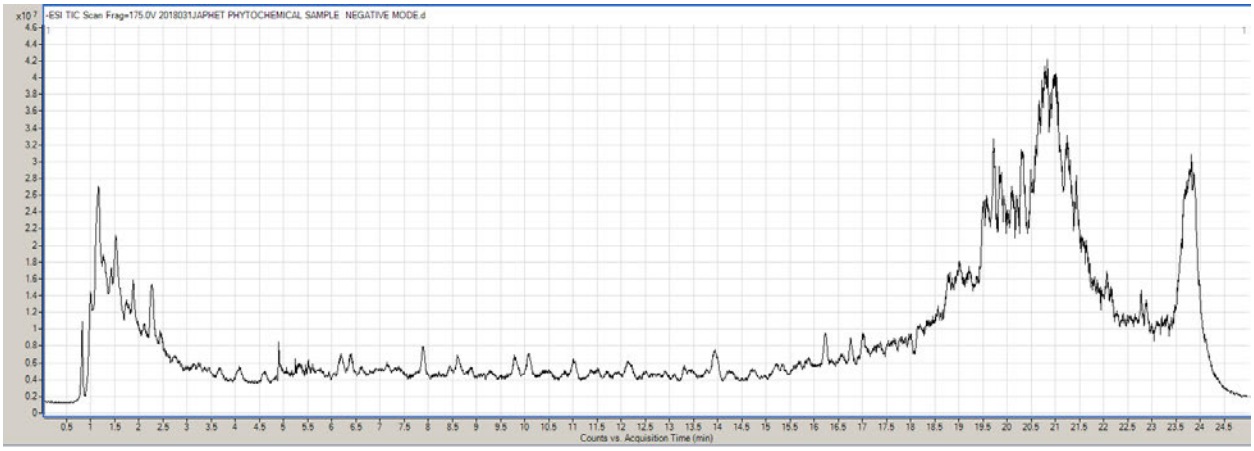

Figure 17: Chromatogram run in negative mode (Mangoyi et al., 2019).

Appendix III: Turnitin report

S. Hlatshwayo MMed Thesis

ORIGINALITY REPORT

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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

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