THE EFFECTS OF *RHOICISSUS TRIDENTATE* DICHLOROMETHANE EXTRACT AND ITS BIOACTIVE COMPOUNDS ON UTEROTONIC AND GLUCOSE LOWERING ACTIVITY *IN-VITRO*

by

Zinhle Mvelase

211532767

Supervisor: Prof M.V. Mabandla

Discipline of Human Physiology

School of Laboratory Medicine and Medical Sciences

College of Health Sciences

2019

Submitted as the dissertation component in partial fulfilment for the degree of Master of Medical Science in the School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal

Preface

Pregnancy related complications continue to be a major clinical burden, especial in developing countries due inequities in access to health services. Intriguingly, recent retrospective studies have reported co-existence of poor myometrium contractility and gestational diabetes in some pregnancies.Poor myometrium contractility is one of the factors causing difficult labour. Conventional drugs that are used to manage pregnancy related complications are costy, inaccessible and elicit severe undesirable effects. Currently, there is a shift toward exploring other alternatives. Traditionally, the medicinal plant, *Rhoicissues tridentate* (RT) have been used to promote labour induction, and to attenuate hyperglycaemia. However, very few studies have been done to ascertain these claims. Accordingly, in this study we sought to investigate RT and its bioactive ingredients, beta-sitesterol (BS) and arjunolic acid (AA) on rate and force of uterine muscle contractility. Since poor myometrium contractility parallels with gestational diabetes, we further sought to explore the glucose lowering effects of RT and its bioactive compounds on liver and muscle cell lines *in-vitro.*

Declaration

I, **Zinhle Mvelase** student number: **211532767**, hereby declare that the dissertation entitled, **The Effects of *Rhoicissus Tridentate* Dichloromethane Extract and it's Bioactive Compounds on Uterotonic and Glucose Lowering Activity *In-vitro*** is the result of my own investigation and research and that it has not been submitted in part or full for any other degree or to any other University or Tertiary Institution. Where use was made of others work, it has been duly acknowledged. This study was carried out under the supervision of Prof M.V. Mabandla.

_____

Student: Zinhle Mvelase (211532767)

_____

Supervisor: Prof M.V. Mabandla

DATE: _____

PLAGIARISM DECLARATION

School of Laboratory Medicine and Medical Sciences, College of Health Sciences

MASTER'S DEGREE IN MEDICAL SCIENCES

1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's own.
2. Each contribution to, and quotation in, this thesis from the works of other people has been attributed and has been cited and referenced.
3. This thesis is my own work.
4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

Signature_____

Acknowledgements

- First, praises and thanks to the God, the Almighty, for His blessings throughout my research work and for giving me strength to complete the research successfully.

- I would like to express my deep and sincere gratitude to my research supervisor, Professor M.V. Mabandla for giving me the opportunity to do research and invaluable guidance throughout this research. His dynamism, vision, sincerity and motivation deeply inspired deeply me. He has taught me the methodology to carry out the research and to present the research work as clearly as possible. It was a great privilege and honour to work and study under his guidance. I am extremely grateful.

- I would like to pay my great thanks to my mentor, Dr. Ntethelelo Sibiya for her advice, motivation and helpful comments throughout the course of this research project. There was a time where things were not going well, Dr. Sibiya was always there to support, motive and encourage.

- I would like to pay my great thanks to my colleagues Dr. Andile Khathi, Mlindeli Gamede, Mluleki Luvuno and Cleopatra Rakgantsho and for all the support. I wish to acknowledge the technical assistance rendered by the staff at traditional medicine. Thank you so much Dr. Mlungisi Ngcobo.

- Special thanks are due to Siboniso Nkosi for his continuous support. I thank you for everything. You are my strongest support structure. Thank you for your valuable support.

- I am extremely grateful to my dearest parents, Dumisani Mvelase and Lindiwe Mvelase for their love, prayers, caring and sacrifices for educating and preparing me for my future. I am very much thankful. This dissertation stands as a testament to your unconditional love and encouragement.
- I would like to thank the National Research Foundation (NRF) and the University of KwaZulu Natal for granting me the funds to pursue my studies.

TABLE OF CONTENTS

TABLE OF FIGURES

LIST OF TABLES

List of abbreviations

A. africanus	Agapanthus africanus
AA	Arjunolic acid
Ach	Acetylcholine
ACTH	Adenocorticotrophin hormone
(ANOVA)	One-way analysis of variance
(DM)	Diabetes mellitus
DAG).	Diacylglycerol (DAG).
ALT	Alanine amino transferase
AST	Aspartate amino transferase
Arj	Arjunolic acid
ATR	Atropine
ANOVA	One-way analysis of variance
ATP	Adenosine triphosphate
BS	Beta- Sitosterol
Ca2+	Calcium ion
cAMP	Cyclic adenosine monophosphate
Con	Control
Cl-	Chloride
CRH	Corticotrophin releasing hormone
DCM	Dichloromethane
KCl	Potassium chloride

MLCK	Myosin light chain kinase
Na+	Sodium
NaCl	Sodium chloride
NO	Nitric oxide
P. prunelloides	Pentanasia prunelloides
PEPCK	phosphoenolpyruvate carboxykinase
PGE2	Prostaglandin E2
PGF2α	Prostaglandins F2α
PLCβ	Phospholipase-C β
(PIP_2)	Phosphatidylinositol bisphosphate
PPH	Postpartum haemorrhage
PR	Progesterone receptors
ROC	Receptor operated channels
R. tridentata	Rhoicissus tridentate
RT	Rhoicissus tridentate
TGF-β1	Transforming growth factor-β1
VGC	Voltage gated channels

STUDY OUTLINE

This dissertation is presented in manuscript format. It consists of two studies and five chapters. The background chapter has been included to bring the reader up to speed in understanding the problem statement and study. Chapter 1 is the literature review which general discusses the topics covered in the study and how the current study sought to fill the gaps in literature. Chapter 2 contains the first research study. This work will be submitted for publication in **The Canadian Journal of Physiology and Pharmacology,** Authors: Zinhle Mvelase, Ntethelelo Sibiya and Musa Mabandla. Chapter 3 is the second research study, which will be submitted for publication in **The Journal of Ethno-Pharmacology,** Authors: Zinhle Mvelase, Ntethelelo Sibiya and Musa Mabandla. Chapter 4 is the synthesis chapter that links the research chapters to the aims of the project. Chapter 5 consists of the references list.

ABSTRACT

Poor myometrial contractility during labour is associated with an extended partuition process that can affect the neonate's health status. Chronic use of conventional treatments for diabetes mellitus is sometimes associated with undesirable effects. In an effort to overcome these challenges, we explored the ability of a crude medicinal plant extract of *Rhoicissus tridentate* (RT) and its bioactive compounds, beta-sitesterol (BS) and arjunolic acid (AA) to act as a multi-target agent to manage both poor myometrium contractility and diabetes mellitus. We thus investigated the uterotonic effects of RT and its bioactive ingredients BS and AA using uterine strips *in-vitro*. Further we studied the glucose lowering effects and possible mechanisms of action of RT and its bioactive compounds using muscle (C2C12) and liver (Chang) cell lines.For uterotonic studies, diethylstilboestrol-treated female Sprague-Dawley rats were sacrificed by decapitation and 2-3 cm of the uterine muscle was isolated and suspended in an organ bath containing aerated de Jalon's buffer maintained at 32 ºC. Each muscle strip was treated with graded concentrations of RT (0.24-62.08 mg/mL) or BS (0.09-57.10 µmol/mL) or AA (0.37-22.80 µmol/mL). Rhythmic contractions in myometrial tissue incubated in the absence of plant extract or oxytocin served as the absolute control. To determine the synergistic effect of RT and its bioactive ingredients, the uterine strips were pre-treated with oxytocin (1.11 nmol/mL) before being treated with various concentrations of RT, BS or AA. Afterwards, the force and rate of contractions were recorded. Thereafter, the uterine strips were harvested for prostaglandin F2 alpha (PGF2α) and oxytocin-receptor concentration measurements. In experiment 2, we assessed the effects of RT/BS/AA on glucose metabolism. Liver (Chang) and muscle (C2C12) cell lines were cultured in Eagle's Minimal Essential Medium and Dulbecco's Modified Eagle's Medium, respectively. To initiate glucose utilization experiments, separate preparations of muscle and liver cell lines were incubated at 37 $^{\circ}$C in a humidified incubator with 5% CO_2 with the following concentrations of each compound, 12.5, 25 and 50 µg/ml. Both the liver and muscle cell lines were challenged with 19 mmol and 29 mmol of glucose respectively. Cells incubated in DMSO (0.1%), insulin (40 µg/mL) or metformin (16 µg/ml) served as untreated and treated positive controls. Media glucose concentration and cell viability were measured at 0, 12, 24 and 48 hour post incubation. Thereafter, cells were assessed for glycogen, GLUT 4 and glycogen synthase while the media harvested was assessed for Alanine

amino transferase (ALT) and aspartate amino transferase (AST) activity. Our results showed an increase in the force and rate of uterine muscle contraction following treatment with RT, BS and AA ($p<0.05$). While co-treatment of RT/BS with oxytocin had a synergistic effect on force and rate of myometrium contractility, RT treatment resulted in decreased PGF2α and oxytocin receptor concentration. Treatment with BS/AA resulted in an increase in PGF2α receptor concentration, ($p<0.05$) but a decrease in oxytocin receptors. RT/BS and oxytocin resulted in increased PGF2α and oxytocin receptor concentration. The administration of RT/BS/AA did not result in cell toxicity in both cell lines. The individual treatments of RT and BS resulted in decreased ``media glucose concentration with concomitant increases in glycogen concentration after 48 hours in both cell lines. The increased GLUT4 concentration in muscle cell lines following treatment RT or BS supported this. Furthermore, RT administration resulted in increased glycogen synthase concentration in both cell lines. The observations suggest that RT promotes uterine muscle contractility and glucose disposal in the liver and skeletal muscle cell lines. This may suggest that RT and its associated bioactive compounds may be of benefit in alleviating poor myometrium contractility and diabetes-associated hyperglycaemia with BS playing a more active role than AA.

CHAPTER 1

INTRODUCTION/LITERATURE REVIEW

1. Background

Pregnancy is associated with significant fetal and maternal morbidity and mortality. These outcomes are instigated by various complications associated with pregnancies including gestational diabetes and poor myometrium complications amongst others[29]. Although, fore-mentioned complications can occur independently of one another, scientific reports have indicated that diabetic patients experience a higher rate of failed labour induction than non-diabetic patients do[62]. Oxytocin remains the mainstay drug for labour-induction, however, it exhibit sides effects. Oxytocin have been reported to cause premature ventricular contractions, sinus tachycardia and hyperstimulation of the myometrium leading to complications such as asphyxia[162]. The administration of various conventional anti-hyperglycaemic drugs have been shown to relieve glycaemia, however, undesirable effects have been reported[77]. The oral antidiabetic agent metformin and glyburide are associated with long-term undesirable effects. Despite the beneficial effects of insulin administration, scientific reports have pointed out the risk of cardiovascular diseases due to high insulin concentration administered. These reasons have prompted researchers to search for alternative modalities with promising outcomes. Medicinal plants have drawn increasing attention as a potential viable source of treating pregnancy related complications [78]. Studies have documented that experimental diabetes facilitates gestation-induced denervation and increases myometrial sensitivity to oxytocin in late pregnancy. This could contribute to premature uterine contractions in diabetic pregnancies. Studies have however, reported that medicinal plants are on the threshold of becoming a practical alternative in obstetrics and gynecology as they have been found to exert various oxytocic and diabetic effects. These reports indicate an increase in scientific evaluation on plants with oxytocic properties, especially in diabetic patients. Medicinal plants such as the *Agapanthus africanus (Alliaceae), Clivia miniata (Amaryllidaceae), Combretum erythrophyllum (Combretaceae), Gunnera perpensa (Gunneraceae) and Pentanisia prunelloides (Rubiaceae)* have been used by South African women to promote favorable course of pregnancy and facilitate quick uncomplicated labour. During pregnancy, these plants are used for different reasons, firstly, to prevent miscarriage, abdominal pain and to lower blood glucose levels.

Rhoicissus tridentate (RT) is amongst the plants used by pregnant women to attenuate pregnancy related complications. *Rhoicissus tridentate* (RT) has been shown to contain phytochemicals such as arjunolic acid (AA) and beta-sitosterol (AA). The paucity of scientific information on the effects and mechanism(s) of action of this plant extract and bioactive compounds remains a concern, hence, it's vital that we scientifically evaluate and validate effects on pregnancy related complications. Despite the traditional use of this plant to relieve these complications, there has been sparse scientific research aiming to scientifically validate therapeutic value of RT in pregnancy-associated disorders. For these reason, the study sought to elucidate the uterotonic effect and mechanisms employed by RT using isolated uterine strips and shed light on the mechanisms in which RT relieves gestational diabetes , hence, we investigated the effect of RT on glucose handling in isolated system, using liver and muscle cells *in-vitro*

2. Normal response to pregnancy: glucose metabolism

The state of pregnancy requires an increased fuel to sustain increasing fetal demand. The production of pregnancy-related hormones alter the metabolism of nutrients, specifically glucose, to shift the priority of metabolic products toward the growing fetus[1]. Maternal glucose homeostasis is sustained by the delicate interplay of maternal hormones designed to increase fat storage, decrease energy expenditure, and delay glucose clearance. Hormones such as cortisol contribute to the rising demand for glucose and provides the strongest diabetogenic property[1]. In the context of glucose metabolism, these adaptations occur to ensure adequate shunting of glucose to promote fetal development while maintaining adequate maternal nutrition[2, 3]. This balance in glucose regulation is of paramount importance to maternal-fetal health during gestation. During this period of increased glucose utilization by the fetal-placental unit, maternal insulin sensitivity decreases[4]. Other contributing factors may include altered pancreatic β-cell–mediated insulin secretion and hepatic gluconeogenesis[5].

Insulin resistance during pregnancy stems from a variety of factors, including alterations in growth hormone and cortisol secretion, human placental lactogen secretion and insulinase secretion[6, 7]. In addition, estrogen and progesterone also contribute to a disruption of the glucose insulin balance[8, 9]. Increased maternal adipose deposition, decreased exercise, and increased caloric intake also contribute to this state of relative glucose intolerance. When the maternal endocrine pancreatic function is impaired, and she is unable to overcome the insulin resistance associated

with pregnancy then gestational diabetes develops[10]. Insulin stimulates the uptake of glucose from the blood by body cells, particularly the muscle and adipose tissue [11]. Furthermore, insulin also stimulates conversion of excess glucose to glycogen synthesis in the liver. In the absence of insulin, hyperglycaemia is established and may lead to glucotoxity in those tissues which are insulin independent. The liver and a muscle play a critical role in glucose homeostasis and diabetes mellitus (DM). In this study, we investigated the effect of RT and associated bioactive compounds on glucose handling in liver and muscle cells.

3. Role of liver in glucose homoeostasis

The liver plays a central role in metabolic homeostasis and is a major site for synthesis, metabolism, storage and redistribution of carbohydrates, proteins and lipids. About 90% of all circulating glucose is not derived directly from the diet[12] .However, most of this glucose comes from the liver. The liver contains significant amounts of stored glycogen available for rapid release into circulation and is capable of synthesizing large quantities of glucose from substrates such as lactate, amino acids and glycerol released by other tissues[13]. In addition to controlling plasma glucose, the liver is responsible for synthesis and release of the lipoproteins[14]. Insulin's metabolic effects result to a decrease glucose output through inhibition of gluconeogenesis and glycogenolysis[5]. In the liver, insulin stimulates glycogen synthesis and inhibits glycogen breakdown[5]. Insulin also stimulates glycolysis and inhibits gluconeogenesis[15]. The rate of gluconeogenesis is controlled principally by the activities of unidirectional enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase and glucose-6-phosphate (G6Pase)[16]. PEPCK catalyses one of the rate limiting steps of gluconeogenesis, the conversion of oxaloacetate to phosphoenolpyruvate , while G6Pase catalyses the final step of gluconeogenesis, the production of free glucose from glucose-6-phosphate (G6Pase) [17]. Insulin inhibits gluconeogenesis by suppressing the expression of PEPCK and G6Pase. Medicinal plants extract such as *Vernonia amygdalina, Hypoxis hemerocallidea and Leonotis leonurus* have been reported to inhibit glycogen breakdown by inhibiting glucose-6-phosphatase and phosphorylase[18, 19]. Interestingly, these medicinal plants extracts have been shown to improve hexokinase and pyruvate kinase. In DM, glycogen synthesis is inhibited meanwhile release of glucose synthesized from gluconeogenesis is enhanced leading to high blood glucose. The liver cells cannot respond to insulin properly in pregnancy, hence the development of gestational

diabetes mellitus (GDM). In the study we investigated, the effects of a pure medicinal plant extract of *Rhoicissus tridentate* (RT) and its bioactive compounds (beta-sitosterol and Arjunolic acid) on glucose metabolism using liver (Chang) cell line. The liver also metabolize and degrade exogenous substance, however administration of some compounds have been shown to damage the liver. The biochemical markers of hepatic damage include enzymes such as alanine amino transferase (ALT), aspartate amino transferase, alkaline phosphatase, gamma glutamyl transferase and aspartate transaminase (AST). In the presence study we were also interested on the toxicological effect RT may have on liver, hence ALT and AST activity was assessed.

4. Role of skeletal muscle in glucose homoeostasis

The skeletal muscle cannot release glucose into the circulation, however, their ability to rapidly increase glucose uptake is critical for dealing with sudden increases in plasma glucose[20]. The Skeletal muscle has an additional role in maintaining plasma glucose levels by releasing free amino acids into circulation to serve as substrates for gluconeogenesis in the liver[21]. The skeletal muscle can use glucose, fatty acids, and ketone bodies. In skeletal muscle, insulin stimulates amino acid uptake, protein synthesis, glucose uptake and incorporation into glycogen[22].The muscle expresses significant amounts of the GLUT4 transporters which upon insulin stimulation are translocated to the plasma membrane allowing glucose uptake[23, 24]. Gestational diabetes mellitus (GDM) is associated with reduced skeletal muscle oxidative phosphorylation and disordered calcium homeostasis. These relationships deserve further attention as they may represent novel risk factors for development of GDM and may have implications on treatment interventions for GDM[25]. Traditional medicinal plants derived products have been found to improve glucose homeostasis by promoting the activation of GLUT4 and hence promoting glucose uptake in skeletal muscle[26]. Diminished translocation of GLUT4 to the plasma membrane due to defective intracellular signaling may account for insulin resistance mainly in the skeletal muscle. In DM, the absence of insulin effects results in inadequate uptake of glucose by muscle tissue leading hyperglycaemia[27]. Glucose handling impairment have been shown to affect myometrium function[28]. Previous studies have documented that the changes in contractility in human myometrium from diabetic patients, and it reveals that the spontaneous contractions are significantly lower in diabetic patients compared with non-diabetic patients[29]. Furthermore, studies have reported that uncontrolled glycaemia may lead to labour

complications[30]. Despite tremendous advances in medicine during the past century, it is of paramount importance to investigate alternative methods to ameliorate pregnancy related complications. In the current study, RT and associated bioactive compounds were investigated on glucose handling as a well as possible mechanism by assessing the expression of GLUT4 transporter and glycogen synthase**.**

5. Myometrium contractions

The onset of labour is facilitated by phasic myometrial contractions that are driven or initiated by development of action potential across the plasma membrane. The myometrium contraction is the calcium dependent muscle contraction where calcium from sarcoplasmic reticulum and extracellular fluid enter the smooth muscle cell through voltage gated calcium channels. Inside the cell, calcium binds to calmodulin forming the calmodulin-calcium complex which activates the myosin light chain kinase. The activated myosin light chain kinase phosphorylates the myosin light chain, leading to structural changes on myosin head. Consequently, the actin activation by myosin ATPase causes myometrium contraction [31, 32]. G-protein and G-protein coupled receptors can result in stimulatory or inhibitory effects on myometrial contraction [33]. The receptor coupled to $G\alpha_q$ can stimulate contractility by activating the phospholipase C pathway whereas the receptors coupled to $G\alpha_s$ relax the uterus by stimulating adenylyl cyclase and increase myometrial cAMP concentration [34]. Receptor coupled to $G\alpha_i$ potentiates contractility by inhibiting cAMP production. When G-protein subunits are activated, they trigger effectors that regulate the calcium ion channel activity by direct or indirect inhibition or stimulation of phosphorylation pathway, leading to the initiation of cascades that lead to elevation of cytosolic calcium ions [35, 36].The rise of intracellular Ca^{2+} leads to cycling of actomyosin cross bridges, the hydrolysis of ATP and contraction**.** Oxytocin induces the muscle contraction by elevating the intracellular calcium ion concentration through different pathways[37, 38]. Binding of oxytocin to the receptors that are the member of the G-protein coupled receptors family, stimulates the muscle and induces the myometrial muscle contraction[39]. Oxytocin, have been shown to be the most potent of the endogenous oxytocics and acts on oxytocin receptors to induce contractions by elevating intracellular calcium concentration [Ca2+], first by PLC-mediated d-myoinositol 1, 4,5-triphosphate (IP3) induced release of Ca2+ from internal stores and by calcium influx of extracellular via voltage- and receptor-operated calcium channels[39]. Moreover, oxytocin also stimulates an increase in cytoplasmic phospholipase A2 activity and induces cyclooxygenase-2

thus increasing contractions[40].The increase in the circulating oxytocin increases the uterine production of prostaglandins. The uterine prostaglandins production induces luteolysis and hence ovarian progesterone production ceases[41]. The fall of progesterone preludes the onset of parturition. Prostaglandins (PGs) are 20-carbon chain fatty acids that are the member of eicosanoid family that are produced by the uterus, fetal membrane and placenta during the late stages of gestation[41]. Prostaglandins bind to their specific receptors and activate contractile intracellular pathway (EP1, EP3, FP and thromboxane) or relaxant receptor isoforms (EP2, EP4, EP)[42]. Prostaglandins E2 and F2α play an important role in uterine muscle contractility and initiation of parturition in human[43]. PG-E2 and F2α stimulate calcium release from the myomentrial sarcoplasmic reticulum. The binding of the prostaglandin to the receptors stimulate the G-protein activation of phospholipase C and A2, which stimulate production of IP3 and intracellular calcium release[44]. IP3 mobilizes the intracellular calcium release from sarcoplasm reticulum, which then increases uterine contractility[45]. In the current study, the focus was directed at evaluating the effects of medicinal plants on uterotonic activity *in-vitro* and to further evaluate the mechanism of action of the plant extract (RT) and its bioactive compounds on rate and force of uterine muscle contractility. To further elucidate this mechanism, expression of biochemical markers such as oxytocin and PGF2 alpha-receptors were evaluated using isolated uteri. Scientific report indicates that poor myometrium contraction may cause poor myometrium contractility

Gestational diabetes: effects on myometrium contractility

Worldwide, diabetes in pregnancy is associated with significant fetal and maternal morbidity and mortality[46]. Gestational diabetes has been reported to affect a significant proportion of pregnant women [48]. Diabetic pregnancies are associated with prolonged labour and failed induction of labour[47]. Hyperglycaemia observed in gestational diabetes is mainly attributed to placental hormones :progesterone and chorionic somatomamotrophin[49, 50]. Despite the availability of conventional pharmacological agents, the treatment of diabetes with medicinal plants is often successful. Herbal medicines and plant components with insignificant toxicity and side effects are notable therapeutic options for the treatment of this disease worldwide [51].

Further evidence to implicate poor myometrial contractility is that post-partum haemorrhage is six times more common in diabetic women[29] .Post-partum haemorrhage may be attributed to altered oxytocin responsiveness in diabetic patients. Poor myometrial contractility has been suggested as an important factor in diabetic pregnancies, thus the reduction in force and rate of contraction in diabetic patients. This impaired contractility, if translated *in vivo* would lead to poor labour contractions. Furthermore, studies have reported that calcium transient amplitude and duration are less in diabetic compared with non-diabetic samples. Therefore, a defect in channel activation, function or expression may occur because of the diabetic environment. Scientific studies have demonstrated the benefits of medicinal plants containing hypoglycemic properties in diabetes management. Uterine oxytocic properties of some folk medicine play a major role in averting uterotonic defects/ poor uterine contractility associated with gestational diabetes. Although researchers have shown interest in isolating, the active ingredients in plants to further elucidate their mechanisms of action. Some herbal remedies have been shown to have beneficial effects in the stimulation of uterine contractions [52]. Medicinal plants such as *A. africanus* and *C. miniata* activate the uterus through different mechanisms[52, 54]. *A. africanus* stimulates myometrial contractility by binding to muscarinic receptors and by promoting prostaglandin synthesis. Similarly, *C. miniat*a stimulates myometrial contraction through the activation of cholinergic receptors and prostaglandin synthesis[55].Previous studies in our laboratory have shown that *Syzygium aromaticum*-derived oleanolic acid (OA) and maslinic acid (MA) use various mechanisms to lower blood glucose concentrations in experimental diabetes[56, 57]. Bioactive compounds are classified as flavonoids, tannins, phenolic, and alkaloid. Tannin have been shown to improve the function of pancreatic Beta-cells and increases insulin secretion[53]. In this study, we also sought to investigate the potential anti-diabetic effects of RT and active ingredients contained by our plant of choice RT on glucose metabolism and on uterotonic activity *in-vitro.*

6. Diabetes-induced pregnancy complications

7.1 Preterm labor

Preterm labor is defined as a regular uterine contractions and progressive cervical change after the gestational age of viability and before 37 completed weeks of pregnancy[58]. Premature births have major neonatal implications and are the single most common cause of perinatal death with an overall neonatal mortality rate of 41/1000 live births[59, 60]. Consequently, long-term morbidity including cerebral palsy, delayed neurological development and chronic lung disease result[61]. Preterm labor is precipitated by a number of factors which include multiple pregnancies, polyhydramnios, intra-uterine growth restriction, intra-uterine death, congenital anomalies and congenital infection, cervical incompetence, preterm rupture of the membranes, systemic disease, uterine anomalies and placenta previa[62]. There is some evidence for a premature activation of oxytocin secretion in preterm birth, suggesting a pathogenic role for it in preterm labour. Numerous pharmacological agents have been utilised to inhibit preterm labour, but none has proven to be ideal[63, 64]. Currently, there is a shift towards exploring therapeutic value of natural or traditional medicine due to the high prescription costs and high incidence of adverse effects and resistance associated with these western medicines. As it is, traditional medicines are more readily available and have less adverse effects on the patient. This indicates a need for further evaluation of traditional medicine that may perhaps be freely available to everyone regardless of socio- economic issues

7.2 Placental abruption

Placental abruption also called abruption placentae is defined as the premature separation of the placenta from the uterus. Patients with placental abruption, typically present with bleeding, uterine contractions and fetal distress[65]. Placental abruption is a serious obstetric complication that occurs in about 1–2 per 100 pregnancies and the precise etiology of abruption is unknown[66, 67]. However, abruption may result from a variety of different pathways such as from the direct abdominal trauma and cocaine use, which causes vasospasm that may result to placental separation[68].Placental abruption is evoked by haemorrhage behind the placenta in the decidua basalis [69]. A decidual haematoma leads to separation and compression of the adjacent parts. The

retroplacental haematoma is most probably caused by a rupture of a decidual spiral artery [70].Maternal hyperglycemia and gestational diabetes mellitus (GDM) have been associated with an adverse gyneacological outcomes such as placental abruption and maternal hyperglycemia[71]. These effects of hyperglycemia are usually attributed to medical comorbid conditions co-occurring in GDM patients, rather than to hyperglycemia per se.

7.3 Post-term labour

Post-term pregnancy refers to a pregnancy that has extended to or beyond 42 weeks (294 days) of gestation. Perinatal mortality rate after 42 weeks of gestation is twice that at term (4 to 7 versus 2 to 3 deaths per 1000 deliveries) and is increased fourfold at 43 weeks and five- to sevenfold at 44 weeks compared with 40 weeks [72]. The post term pregnancy is the result of the post term labour which is said to be caused by progesterone by preventing the onset of labour during gestation. The progesterone prevents the onset of labour, which alters the myometrium activation, including excitation-contraction uncoupling, inhibition of oxytocin and prostaglandin receptors, stimulation of nitric oxide synthase, inhibition of gap junctions, and the suppression of oxytocin and prostaglandin secretion. Exogenous oxytocin may be administered intravenously and results in uterine contractions. Oxytocin induction of labour may have a role to play in high-risk patients whose foetuses may be at increased risk for intolerance of labour but further research into this area is required. Plants extracts such as *Calotropis procera*, *Commelina africana*, *Duranta repens*, *Hyptis suaveolens*, *Ocimum gratissimum*, *Saba comorensis*, *Sclerocarya birrea*, *Sida corymbosa* and *Vernonia amygdalina* have been shown to induce significant sustained increases in human myometrial muscle cell contractility[73]. These plants have been shown to facilitate the birth process and, to reduce the time and associated pain of labor and manage postpartum complications. The mechanism of action of these plants on uterotonic activity is not fully understood. Hence, in the current study, we evaluated the uterotonic effects of RT and its bioactive compounds *in-vitro*. The force and rate of uterine muscle contractility was recorded following treatment with the plant extract (RT) and its bioactive compound RT.

7.4 Postpartum hemorrhage

Postpartum hemorrhage is the most common cause of maternal mortality and accounts for one quarter of all maternal deaths worldwide [74].The postpartum hemorrhage is the excess loss of blood after giving birth. Inversion of the maternal spiral arterioles which create the a wide-diameter, the non-contractile vessels and ensuring the high volume of blood flow to the placenta. This happens during pregnancy, then during after the delivery the placenta separate with myometrium and those no-contractile vessel allow bleeding to continue after delivery.

7. Conventional management of hyperglycaemia

There are various strategies used to manage hyperglyceamia in GDM[75, 76]. There are five classes of hypoglycaemic agents; these include insulin and its analogues, sulphonylureas derivatives, biguanides, α_glucosidase inhibitors and thiazolidinediones (TZDs) [77-79]. These treatments presents with various limitations, for example, insulin is associated with multiple painful injections. Furthermore, prolonged higher doses of insulin cause insulin resistance. Metformin exhibits its effect by suppressing hepatic glucose production, improves peripheral insulin resistance and inhibits glucose absorption by small intestines [77]. Metformin also reduces plasma triglyceride and LDL-cholesterol levels [77].However, metformin later induces undesirable side effects such as diarrhoea, nausea and lactic acidosis. Another group known as sulphonylureas, examples include drugs such tolbutamide, glibenclamide, glimepiride and glipizide. These treatment have been regarded as the first-line drug treatment in type 2 diabetes patients who are not very obese. The adverse side effects of sulphonylureas include weight gain, hyperinsulinaemia and hypoglycaemia. There is another category known as alpha-glucosidase inhibitors. This drug category decreases glucose concentration by delaying gastrointestinal absorption of glucose. Furthermore, alpha-glucosidase inhibitors decrease hyperinsulinaemia and improve insulin sensitivity[77]. However, these drugs also have mild side effects such as flatulence and diarrhoea due to delayed degradation of complex carbohydrates. Lastly, there is a group known as thiazolidinediones (TZDs), this group is represented by troglitazone, rosiglitazone and pioglitazone. These expensive oral agents manage glycaemia by improving insulin sensitivity in muscle and, to a much lesser extent, in the liver [78]. They decrease plasma triglyceride levels; however, such decrease may be associated with obesity and an increase in

low-density lipoprotein-cholesterol levels . TZDs are associated with liver toxicity. The cost burden of these therapies together with the inability of these agents to treat DM efficiently, drive researchers to explore other alternatives, not only to broaden treatment availability but also to ensure efficient treatment of DM [80]. These observations suggest and highlight a need to further evaluate the effects of medicinal plants on GDM

8. Conventional oxytocic drugs

Conventional treatment used to enhance uterine muscle contractility and to prevent postpartum bleeding include uterotonic agents such as oxytocin, prostaglandins (PGF2α, PGE2) and ergometrine[81-83] .The use of these drugs has been associated with serious undesirable side effects such as premature ventricular contractions, sinus tachycardia and hyperstimulation of the myometrium leading to complications such as asphyxia[84, 85]. Due to the high prescription costs and high incidence of adverse effects associated with the use of conventional pharmaceuticals, there is currently a shift towards exploring the use of natural or traditional medicinal products [86, 87] .Moreover, previous studies have documented that about 70-80% of women worldwide rely on traditional medicines during pregnancy [88-90]. Prostaglandins are used to induce labour and to prevent post-partum haemorrhage [162]. Abnormal uterine action is one of the factors causing dystocia(difficult labor) in which uterine forces are insufficiently strong or inappropriately coordinated to efface and dilate the cervix .Traditionally used herbal medicines and their active ingredients, such as cyclotides found in flowering plants, are ideal starting point for alleviating uncoordinated uterine muscle contractility[91, 92]. Plants extracts such as *Clivia miniaia*, *Crinum bulbispermum and Cyrtanthus obliquus* are used by South African women to alleviate pregnancy related complications. However, there is little documented scientific evidence on the exact mechanism(s) of action of these plants on post term labor and related complications. Hence, the current study evaluated the effects of RT on the force and rate of contraction; furthermore, we looked at the effects of RT on oxytocin receptors.

9. Traditional medicinal plants

Current conventional treatments for pregnancy related complications have some limitations. As a result, alternative methods for treating gynecological complications are therefore needed. The World Health Organization (WHO) estimated that 80 % of the population of developing countries relies on traditional medicines, mostly plant based drugs, for their primary health care needs. Scientific investigations of the plant extracts that possess uterotonic effects not only assists in validating indigenous knowledge systems, but may also provide a more affordable alternative source of pregnancy complications. In developing countries such as South Africa decoctions of the plants *Agapanthus africanus(Alliaceae),Clivia miniata(Amaryllidaceae),Combretum erythrophyllum (Combretaceae), Gunnera perpensa (Gunneraceae), and Pentanisia prunelloides (Rubiaceae)* have been used extensively by women to promote child birth[93, 94].Currently, several exotic, endemic and indigenous plants are sold as decoctions in several markets to treat gyneacological complications globally. However, very few of these plants have been validated scientifically for their therapeutic efficacy. Some of the compounds found in these extracts are not naturally toxic; nonetheless, because they are given in excessively high amounts they become toxic. This has led to the identification and isolation of the bioactive compounds responsible for the noted desirable effects. In the field of obstreatics and gynecology, labour complications such preterm labour, postparturm bleeding and post term labour are of greater concern. For amelioration of pregnancy related complications, synthetic drugs are utilized. The oxytocic treatments are costly and have so many undesirable side effects. Hence, in this study we are interested in evaluating the effects of traditional plant extract as an alternative source of managing pregnancy related complication

9.1 *Rhoicissus tridentate*

Rhoicissus tridentate is one of the most commonly selected plant species for South African traditional medicines used during pregnancy and childbirth. Decoctions and infusions of *Rhoicissus tridentate* roots is widely used in South African traditional herbal remedies during pregnancy[95]. Scientific reports have indicated that *Rhoicissus tridentate* promotes childbirth by triggering hyperstimulation of the uterus. *Rhoicissus tridentate* contains several bioactive compounds with beneficial effects which have been shown to be non-toxic at the doses used by

traditional healers[96] .These findings were investigated *in vitro* using human fibroblast and monkey cell lines[97]. Biological and pharmacological studies of various extracts and isolated compounds from the plant confirmed antibacterial activities antifungal activity antimicrobial activity, antinociceptive and anti-inflammatory antioxidant activity [98]. Interestingly, previous studies have shown that RT extract inhibits muscle atrophy and promotes muscle hypertrophy thus promoting glucose uptake by the muscle [99]. RT extract also promotes downstream mRNAs that promote glucose utilization by muscle [100]. The data suggest that RT could be used as a potential treatment for management or treatment of hyperglyceamia associated with GDM. In the current study, we sought to evaluate the effect of this plant together with its bioactive compounds on uterotonic activity, furthermore, we evaluated the effect of the RT on glucose metabolism *in-vitro.*

10. Bioactive compounds

A large number of bioactive components have been isolated from crude plant extracts. There is a growing public interest for traditional medicine because of the potential health benefits linked to phytochemical compounds present in these plants. RT plant extract have been shown to contain the bioactive compounds such as triterpene saponins, flavonoids, isoflavonoids and chalcones, beta sitosterol (sterols) and arjunolic acid (triterpene saponins). Although literature evidence suggests that RT possess uterotonic activity, it's still not clear which bioactive is responsible for this activity. For this reason, in this study we also investigated the effects of the major bioactive compounds found in RT in isolated uterine tissue.

10.1 Beta-sitosterol

Beta-sitosterol is a known a "plant sterol ester." It is found in fruits, vegetables, nuts, and seeds [101-103]. Beta-sitosterol have been used to alleviate conditions such as heart disease and high cholesterol. It is also used to boost the immune system, preventing colon cancer, tuberculosis, psoriasis, allergies, cervical cancer, fibromyalgia and systemic lupus erythematosus (SLE)[103, 104]. Literature has reported more than 100 different types of phytosterols[105]. Beta sitosterol ,3 stigmasterol, and campesterol are the most abundant in plants such as wild ginger *(Costus speciosus (Koen) Smith, Costaceae)* rhizome extract have been shown to contain sterols such as beta sitosterol[106]. β-sitosterol has important anti-inflammatory properties, it inhibits proinflammatory mediators in mouse model. This anti-inflammatory effect appears to be mediated

by the calcium uptake in activated neutrophils in a time-dependent and dose-dependent manner through L-type voltage dependent calcium channels, intracellular calcium, PI3K activity, and microtubule modulation[107]. Studies by have shown that Pomegranate derived beta-sitosterol increases the rate and force of myometrium contraction *in-vitro*[108]. β-sitosterol has been show to promote downstream mRNAs that promote glucose utilization by muscle. Literature has documented that BS increases energy expenditure, leading to reduced obesity, improved glucose tolerance and decreased hepatic steatosis.

10.2 Arjunolic acid

Arjunolic acid is amongst other bioactive compounds contained in RT medicinal plant extract it is classified as triterpenoid saponins. Triterpenoid saponins are globally recognized for their heath beneficial properties such as cardio-protection properties. Arjunolic acid is used in folk medicine to treat several disorders, including pain, infections, prevention of myocardial necrosis, platelet aggregation and coagulation and amelioration of diabetic renal dysfunctions[109]. Triterpenoid saponins such as cyclaminorin, deglucocyclamin, cyclacoumin and mirabilin were isolated from the tubes of the crude plant extract *Cyclamen mirabile.* The bioactive compound stimulated uterine contractility when tested on rats[110]. The mechanism of cytoprotection conferred by arjunolic acid can be explained by its property to reduce the oxidative stress by enhancing the antioxidant levels[111]. Though the beneficial role of this triterpenoid has been assessed from various angles, there are no comprehensive studies regarding its effects on uterotonic activity. The current study evaluated the effect of arjunolic acid on uterotonic activity.

Justification for the study

The factors that influence smooth muscle contractility were considered in an effort to establish the therapeutic efficacy of plant–derived products. The rationale being that these plant derived products can be used to manage gestational diabetes mellitus and pregnancy associated complications of uterine smooth muscle contractility. This is specifically in relation to RT plant extract, which have been used by the traditional healers in KwaZulu Natal, South Africa. The plant extract RT together with other plants extracts are used as a decoction known as *isihlambezo. Isihlambezo* has been proven to potentiate uterine muscle contractions in pregnant women. Again,

Literature evidence suggest that these medicinal plant extracts exhibit uterotonic activity on uterine smooth muscle and might potentiate antidiabetic properties. This study sought to elucidate the effects and possible mechanisms of RT on glucose metabolism *in-vitro* and to elucidate the effects of RT and its bioactive compounds on uterine muscle contraction

11. Aims

The aim of the study was to evaluate the effects of ethnomedicinal plant extract and its bioactive ingredients on uterotonic activity and pregnancy related metabolic changes *in-vitro*

12. Objectives

The objectives of the study were to determine:

a) Effects of RT on force and rate of uterine muscle contractility
b) Synergistic effects of RT and oxytocin on force and rate of uterine muscle contractility
c) Effects of bioactive compounds (AA/BS) on force and rate of uterine muscle contractility
d) Synergistic effects of bioactive compounds (AA/BS) and oxytocin on force and rate of uterine muscle contractility
e) Expression of oxytocin receptor and PGF2 alpha receptor
f) Cell viability studies
g) Effects of RT on glucose concentrations
h) Glycogen analysis
i) Expression of GLUT4 and Glycogen synthase
j) Expression of ALT and AST

13. References

1. Cowett, R.M., *Principles of perinatal-neonatal metabolism*. 2012: Springer Science & Business Media.
2. Angueira, A.R., et al., *New insights into gestational glucose metabolism: lessons learned from 21st century approaches.* Diabetes, 2015. **64**(2): p. 327-334.
3. Baschat, D.A.A., *Fetal responses to placental insufficiency: an update.* BJOG: An International Journal of Obstetrics & Gynaecology, 2004. **111**(10): p. 1031-1041.
4. Girard, J.R., et al., *Fetal metabolic response to maternal fasting in the rat.* American Journal of Physiology-Endocrinology and Metabolism, 1977. **232**(5): p. E456.
5. Saltiel, A.R. and C.R. Kahn, *Insulin signalling and the regulation of glucose and lipid metabolism.* Nature, 2001. **414**(6865): p. 799.
6. Catalano, P. and T. Buchanan, *Metabolic changes during normal and diabetic pregnancies.* Diabetes in women: adolescence, pregnancy and menopause. Lippincott Williams & Wilkins, Philadelphia, Pa, 2004: p. 129-145.
7. Ural, S.H. and J.T. Repke, *Gestational diabetes mellitus.* Reviews in Obstetrics and Gynecology, 2008. **1**(3): p. 129.
8. Livingstone, C. and M. Collison, *Sex steroids and insulin resistance.* Clinical Science, 2002. **102**(2): p. 151-166.
9. Casals-Casas, C. and B. Desvergne, *Endocrine disruptors: from endocrine to metabolic disruption.* Annual review of physiology, 2011. **73**: p. 135-162.
10. Boinpally, T. and L. Jovanovič, *Management of type 2 diabetes and gestational diabetes in pregnancy.* Mount Sinai Journal of Medicine: A Journal of Translational and Personalized Medicine, 2009. **76**(3): p. 269-280.
11. Anabela, P., Rolo, Carlos, M., Palmeira, *Diabetes and mitochondrial function: Role of hyperglycemia and oxidative stress.* Toxicology and Applied Pharmacology, 2006. **212**: p. 167-178.
12. Bechmann, L.P., et al., *The interaction of hepatic lipid and glucose metabolism in liver diseases.* Journal of hepatology, 2012. **56**(4): p. 952-964.
13. Ruderman, N.B., *Muscle amino acid metabolism and gluconeogenesis.* Annual review of medicine, 1975. **26**(1): p. 245-258.

14. Durrington, P.N., et al., *Effects of insulin and glucose on very low density lipoprotein triglyceride secretion by cultured rat hepatocytes.* The Journal of clinical investigation, 1982. **70**(1): p. 63-73.
15. James, C. and K. Hussain, *Hypoglycemia*, in *Textbook of Clinical Pediatrics*. 2012, Springer. p. 3803-3817.
16. Williamson, J.R., R. Kreisberg, and P. Felts, *Mechanism for the stimulation of gluconeogenesis by fatty acids in perfused rat liver.* Proceedings of the National Academy of Sciences, 1966. **56**(1): p. 247-254.
17. Postic, C., R. Dentin, and J. Girard, *Role of the liver in the control of carbohydrate and lipid homeostasis.* Diabetes & metabolism, 2004. **30**(5): p. 398-408.
18. Kibiti, C.M. and A.J. Afolayan, *Herbal therapy: A review of emerging pharmacological tools in the management of diabetes mellitus in Africa.* Pharmacognosy magazine, 2015. **11**(Suppl 2): p. S258.
19. Kibiti, C.M., *Evaluation of the medicinal potentials of Bulbine abyssinica A. Rich in the management of diabetes mellitus in the Eastern Cape, South Africa*. 2016, University of Fort Hare.
20. Sinacore, D.R. and E.A. Gulve, *The role of skeletal muscle in glucose transport, glucose homeostasis, and insulin resistance: implications for physical therapy.* physical therapy, 1993. **73**(12): p. 878-891.
21. Wollheim, C.B. and G. Sharp, *Regulation of insulin release by calcium.* Physiological reviews, 1981. **61**(4): p. 914-973.
22. Koves, T.R., et al., *Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance.* Cell metabolism, 2008. **7**(1): p. 45-56.
23. Lund, S., et al., *Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin.* Proceedings of the National Academy of Sciences, 1995. **92**(13): p. 5817-5821.
24. Michael, L.F., et al., *Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1.* Proceedings of the National Academy of Sciences, 2001. **98**(7): p. 3820-3825.

25. Boyle, K.E., et al., *Gestational diabetes is characterized by reduced mitochondrial protein expression and altered calcium signaling proteins in skeletal muscle.* PloS one, 2014. **9**(9): p. e106872.
26. Babu, P.V.A., D. Liu, and E.R. Gilbert, *Recent advances in understanding the anti-diabetic actions of dietary flavonoids.* The Journal of nutritional biochemistry, 2013. **24**(11): p. 1777-1789.
27. Zhang, C.-Y., et al., *Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, β cell dysfunction, and type 2 diabetes.* Cell, 2001. **105**(6): p. 745-755.
28. Prossnitz, E.R. and M. Barton, *Signaling, physiological functions and clinical relevance of the G protein-coupled estrogen receptor GPER.* Prostaglandins & other lipid mediators, 2009. **89**(3-4): p. 89-97.
29. Al-Qahtani, S., et al., *Diabetes is associated with impairment of uterine contractility and high Caesarean section rate.* Diabetologia, 2012. **55**(2): p. 489-498.
30. White, P., *Diabetes complicating pregnancy.* American Journal of Obstetrics and Gynecology, 1937. **33**(3): p. 380-385.
31. Fu, X., et al., *Prostaglandin F2α-induced Ca++ oscillations in human myometrial cells and the role of RU 486.* American Journal of Obstetrics and Gynecology, 2000. **182**(3): p. 582-588.
32. Gemzell-Danielsson, K., M. Bygdeman, and A. Aronsson, *Studies on uterine contractility following mifepristone and various routes of misoprostol.* Contraception, 2006. **74**(1): p. 31-35.
33. Ulloa-Aguirre, A., et al., *Structure-Activity Relationships of G Protein-Coupled Receptors.* Archives of Medical Research, 1999. **30**(6): p. 420-435.
34. Manning, M., et al., *Peptide and non-peptide agonists and antagonists for the vasopressin and oxytocin V1a, V1b, V2 and OT receptors: research tools and potential therapeutic agents*, in *Progress in Brain Research*, D.N. Inga and L. Rainer, Editors. 2008, Elsevier. p. 473-512.
35. Du, J., et al., *Ligustilide inhibits spontaneous and agonists- or K+ depolarization-induced contraction of rat uterus.* Journal of Ethnopharmacology, 2006. **108**(1): p. 54-58.

36. Loch-Caruso, R., *Uterine muscle as a potential target of polychlorinated biphenyls during pregnancy.* International Journal of Hygiene and Environmental Health, 2002. **205**(1–2): p. 121-130.
37. Altura, B., et al., *Mg2+–Ca2+ interaction in contractility of vascular smooth muscle: Mg2+ versus organic calcium channel blockers on myogenic tone and agonist-induced responsiveness of blood vessels.* Canadian journal of physiology and pharmacology, 1987. **65**(4): p. 729-745.
38. Sanborn, B.M., *Hormones and calcium: mechanisms controlling uterine smooth muscle contractile activity.* Experimental physiology, 2001. **86**(2): p. 223-237.
39. Gimpl, G. and F. Fahrenholz, *The oxytocin receptor system: structure, function, and regulation.* Physiological reviews, 2001. **81**(2): p. 629-683.
40. Katori, M. and M. Majima, *Cyclooxygenase-2: its rich diversity of roles and possible application of its selective inhibitors.* Inflammation Research, 2000. **49**(8): p. 367-392.
41. Baldwin, D.M. and G.H. Stabenfeldt, *Endocrine changes in the pig during late pregnancy, parturition and lactation.* Biology of reproduction, 1975. **12**(4): p. 508-515.
42. Hindson, J., B.M. SCHOFIELD, and W. Ward, *The effect of progesterone on recorded parturition and on oxytocin sensitivity in the sheep.* Journal of Endocrinology, 1969. **43**(2): p. 207-215.
43. Karim, S., *Appearance of prostaglandin F2α in human blood during labour.* Br Med J, 1968. **4**(5631): p. 618-621.
44. Parkington, H.C., et al., *Hyperpolarization and slowing of the rate of contraction in human uterus in pregnancy by prostaglandins E2 and F2α: involvement of the Na+ pump.* The Journal of physiology, 1999. **514**(1): p. 229-243.
45. Fischer, D.P., *The influence of the hormonal milieu on functional prostaglandin and oxytocin receptors and their downstream signal pathways in isolated human myometrium*. 2010, University of Bradford.
46. Bener, A., N.M. Saleh, and A. Al-Hamaq, *Prevalence of gestational diabetes and associated maternal and neonatal complications in a fast-developing community: global comparisons.* International journal of women's health, 2011. **3**: p. 367.

47. Arrowsmith, S., S. Wray, and S. Quenby, *Maternal obesity and labour complications following induction of labour in prolonged pregnancy.* BJOG: An International Journal of Obstetrics & Gynaecology, 2011. **118**(5): p. 578-588.
48. Ferrara, A., *Increasing prevalence of gestational diabetes mellitus.* Diabetes care, 2007. **30**(Supplement 2): p. S141-S146.
49. Di Cianni, G., et al., *Intermediate metabolism in normal pregnancy and in gestational diabetes.* Diabetes/metabolism research and reviews, 2003. **19**(4): p. 259-270.
50. Hu, L., et al., *Detection of placental growth hormone variant and chorionic somatomammotropin-L RNA expression in normal and diabetic pregnancy by reverse transcriptase-polymerase chain reaction.* Molecular and cellular endocrinology, 1999. **157**(1-2): p. 131-142.
51. Gupta, P.D. and A. De, *Diabetes mellitus and its herbal treatment.* International Journal of Research in Pharmaceutical and Biomedical Sciences, 2012. **3**(2): p. 706-721.
52. Meier, S. and P. Wright, *The induction of parturition in the bitch using sodium cloprostenol.* Theriogenology, 2000. **54**(3): p. 457-465.
53. Abdollahi, M., O. Tabatabaei-Malazy, and B. Larijani, *A systematic review of in vitro studies conducted on effect of herbal products on secretion of insulin from Langerhans islets.* Journal of Pharmacy & Pharmaceutical Sciences, 2012. **15**(3): p. 447-466.
54. Veale, D., et al., *Discrimination between the functional and biochemical effects of two herbal oxytocics on the rat myometrium.* Journal of Pharmacy and Pharmacology, 2001. **53**(8): p. 1145-1151.
55. Gruber, C.W. and M. O'Brien, *Uterotonic plants and their bioactive constituents.* Planta medica, 2011. **77**(03): p. 207-220.
56. Khathi, A., et al., *Effects of Syzygium aromaticum-derived triterpenes on postprandial blood glucose in streptozotocin-induced diabetic rats following carbohydrate challenge.* PLoS One, 2013. **8**(11): p. e81632.
57. Madlala, H.P., et al., *Changes in renal function and oxidative status associated with the hypotensive effects of oleanolic acid and related synthetic derivatives in experimental animals.* PloS one, 2015. **10**(6): p. e0128192.
58. Offenbacher, S., et al., *Potential pathogenic mechanisms of periodontitis-associated pregnancy complications.* Annals of periodontology, 1998. **3**(1): p. 233-250.

59. Gonik, B. and R.K. Creasy, *Preterm labor: its diagnosis and management.* American Journal of Obstetrics & Gynecology, 1986. **154**(1): p. 3-8.
60. Marchant, T., et al., *Neonatal mortality risk associated with preterm birth in East Africa, adjusted by weight for gestational age: individual participant level meta-analysis.* PLoS medicine, 2012. **9**(8): p. e1001292.
61. Atiah, F.A., *Contribution of Very Low Birth Weight to Preterm Neonatal Mortality at 37 Military Hospital, Accra*. 2016, University of Ghana.
62. Clifford, A., et al., *Obstetrics, Gynaecology and Women's Health on the Move*. 2012: CRC Press.
63. Caritis, S.N., *Treatment of preterm labour.* Drugs, 1983. **26**(3): p. 243-261.
64. Goldenberg, R.L., *The management of preterm labor.* Obstetrics & Gynecology, 2002. **100**(5): p. 1020-1037.
65. Baskett, T.F., *Essential management of obstetric emergencies*. 2002: Orient Blackswan.
66. Ananth, C.V., et al., *Placental abruption and adverse perinatal outcomes.* Jama, 1999. **282**(17): p. 1646-1651.
67. Oyelese, Y. and C.V. Ananth, *Placental abruption.* Obstetrics & Gynecology, 2006. **108**(4): p. 1005-1016.
68. Yeo, L., C. Ananth, and A. Vintzileos, *Placenta abruption.* Gynecology and Obstetrics. Lippincott, Williams & Wilkins, 2003.
69. Paz, N.C.d., et al., *Risk of placental abruption in relation to maternal depressive, anxiety and stress symptoms.* Journal of Affective Disorders 2011. **130**: p. 280–284.
70. Eskes, T.K.A.B., *Clotting disorders and placental abruption: homocysteine- a new risk factor.* European Journal of Obstetrics & Gynecology and Reproductive Biology 2001. **95**: p. 206-212.
71. Group, H.S.C.R., *Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) Study: associations with maternal body mass index.* BJOG: An International Journal of Obstetrics & Gynaecology, 2010. **117**(5): p. 575-584.
72. Hurd, W., et al., *Shortening increases spontaneous contractility in myometrium from pregnant women at term.* American Journal of Obstetrics and Gynecology (2005) **192**: p. 295–303.

73. Attah, A.F., et al., *Uterine contractility of plants used to facilitate childbirth in Nigerian ethnomedicine.* Journal of ethnopharmacology, 2012. **143**(1): p. 377-382.

74. Tourne, G.r., et al., European Journal of Obstetrics & Gynecology and

Reproductive Biology 2003. **110** p. 29–34.

75. Turner, R.C., C.A. Cull, and V. Frighi, *Glycaemic control with diet, sulphonylurea, metformin or insulin in patients with type 2 diabetes, a progressive repuirement for multiple therapies.* JAMA, 1999. **281**: p. 2005-212.

76. Forbes, J.M., M.E. Cooper, and V. Thallas, *Reduction of the accumulation of advanced glycation end products by ACE inhibition in experimental diabetic nephropathy.* Diabetes, 2002. **51**: p. 3274-3282.

77. Ohiraa, M., et al., *Effect of metformin on serum lipoprotein lipase mass levels and LDL particle size in type 2 diabetes mellitus patients.* Diabetes Research and Clinical Practice 2007. **78**: p. 34-41.

78. Edgerton, D.S., K.M. Johnson, and A.D. Cherrington, *Current strategies for the inhibition of hepatic glucose production in type 2 diabetes.* Front Biosci (Landmark Ed), 2009. **14**: p. 1169-1181.

79. Correia, S., et al., *Metformin protects the brain against the oxidative imbalance promoted by type 2 diabetes.* Med Chem, 2008. **4**: p. 358-64.

80. Storr, T., et al., *Vanadyl-thiazolidinedione combination agents for diabetes therapy.* Bioconjugate chemistry, 2003. **1**(14): p. 212-221.

81. Kelly, A.J., et al., *Vaginal prostaglandin (PGE2 and PGF2a) for induction of labour at term.* The cochrane library, 2009.

82. Karim, S. and S. Sharma, *Therapeutic abortion and induction of labour by the intravaginal administration of prostaglandins E2 and F2α.* BJOG: An International Journal of Obstetrics & Gynaecology, 1971. **78**(4): p. 294-300.

83. Smith, R., *Parturition.* New England Journal of Medicine, 2007. **356**(3): p. 271-283.

84. Ronsmans, C., W.J. Graham, and L.M.S.S.s. group, *Maternal mortality: who, when, where, and why.* The Lancet, 2006. **368**(9542): p. 1189-1200.

85. Haddad, S., et al., *Cardiorespiratory crisis at the end of pregnancy: a case of pheochromocytoma.* Middle East J Anesthesiol, 2013. **22**(2): p. 195-202.

86. Gutierrez, R.M.P., *Orchids: A review of uses in traditional medicine, its phytochemistry and pharmacology.* Journal of Medicinal Plants Research, 2010. **4**(8): p. 592-638.
87. Organization, W.H., *WHO traditional medicine strategy 2002-2005.* 2002.
88. Kamatenesi Mugisha, M. and R. Bukenya Ziraba, *Ethnobotanical survey methods to monitor and assess the sustainable harvesting of medicinal plants in Uganda.* Plant conservation in the tropics: perspectives and practice, 2002: p. 467-482.
89. Kamatenesi-Mugisha, M., R. Bukenya-Ziraba, and R. Hoeft, *Ethnomedical use of Rytigynia (nyakibasi) in Bwindi Impenetrable National Park, SW Uganda.* Lidia (Norway), 2000.
90. Kamatenesi-Mugisha, M. and H. Oryem-Origa, *Traditional herbal remedies used in the management of sexual impotence and erectile dysfunction in western Uganda.* African Health Sciences, 2005. **5**(1): p. 40-49.
91. Gruber, C.W. and M. O'Brien, *Uterotonic plants and their bioactive constituents.* Planta medica, 2011. **77**(3): p. 207.
92. Hu, E., et al., *Novel cyclotides from Hedyotis diffusa induce apoptosis and inhibit proliferation and migration of prostate cancer cells.* International journal of clinical and experimental medicine, 2015. **8**(3): p. 4059.
93. Kilpatrick, S.J. and R.K. Laros Jr, *Characteristics of normal labor.* Obstetrics & Gynecology, 1989. **74**(1): p. 85-87.
94. Kunene, N., R. Wilson, and N. Myeni, *The use of trees, shrubs and herbs in livestock production by communal farmers in northern KwaZulu-Natal, South Africa.* African Journal of Range and Forage Science, 2003. **20**(3): p. 271-274.
95. Katsoulis, L.C., *The pharmacological actitvity of Rhoicissus tridentata subsp cuneifolia in relation to parturition*. 2014.
96. Brookes, K. and L. Katsoulis, *Bioactive components of Rhoicissus tridentate: a pregnancy-related traditional medicine.* South African journal of science, 2006. **102**(5-6): p. 267-272.
97. Naidoo, V., et al., *Antioxidant compounds in Rhoicissus tridentata extracts may explain their antibabesial activity: research in action.* South African journal of science, 2006. **102**(5-6): p. 198-200.

98. Maroyi, A., *From traditional usage to pharmacological evidence: Systematic review of Gunnera perpensa L.* Evidence-Based Complementary and Alternative Medicine, 2016. **2016**.
99. Gruden, G., G. Setti, and A. Hayward, *Mechanical stretch induces monocyte chemoattractant activity via an NF-kappaB-dependent monocyte chemoattractant protein-1-mediated pathway in human mesangial cells: inhibition by rosiglitazone.* .J Am Soc Nephrol, 2005. **16**: p. 688-696.
100. Kota, S.K., et al., *Aberrant angiogenesis: The gateway to diabetic complications.* Indian journal of endocrinology and metabolism, 2012. **16**(6): p. 918-930.
101. Awad, A., et al., *beta-Sitosterol inhibits HT-29 human colon cancer cell growth and alters membrane lipids.* Anticancer research, 1995. **16**(5A): p. 2797-2804.
102. Sugano, M., H. Morioka, and I. Ikeda, *A comparison of hypocholesterolemic activity of beta-sitosterol and beta-sitostanol in rats.* The Journal of nutrition, 1977. **107**(11): p. 2011-2019.
103. Farquhar, J.W., R.E. Smith, and M.E. Dempsey, *The effect of beta sitosterol on the serum lipids of young men with arteriosclerotic heart disease.* Circulation, 1956. **14**(1): p. 77-82.
104. Awad, A., et al., *beta-Sitosterol inhibits growth of HT-29 human colon cancer cells by activating the sphingomyelin cycle.* Anticancer research, 1998. **18**(1A): p. 471-473.
105. Moreau, R.A., B.D. Whitaker, and K.B. Hicks, *Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses.* Progress in lipid research, 2002. **41**(6): p. 457-500.
106. Lijuan, W., et al., *The effects of wild ginger (Costus speciosus (Koen) Smith) rhizome extract and diosgenin on rat uterine contractions.* Reproductive Sciences, 2011. **18**(6): p. 516-524.
107. Liz, R., et al., *Acute effect of β-sitosterol on calcium uptake mediates anti-inflammatory effect in murine activated neutrophils.* Journal of Pharmacy and Pharmacology, 2013. **65**(1): p. 115-122.
108. Malini, T. and G. Vanithakumari, *Effect of beta-sitosterol on uterine biochemistry: a comparative study with estradiol and progesterone.* Biochemistry and molecular biology international, 1993. **31**(4): p. 659-668.

109. Paarakh, P.M., *Terminalia arjuna (Roxb.) Wt. and Arn.: a review.* Int j pharmacol, 2010. **6**(5): p. 515-534.
110. Calis, I. and O. Sticher, *Triterpene saponins from plants of the flora of Turkey*, in *Saponins Used in Traditional and Modern Medicine*. 1996, Springer. p. 485-500.
111. Hemalatha, T., et al., *Arjunolic acid: a novel phytomedicine with multifunctional therapeutic applications.* 2010.

CHAPTER 2

PROLOGUE

Manuscript 1

Abnormal uterine muscle contractility might underlie common and important disorders such as infertility, spontaneous miscarriage or preterm birth. In this study, we evaluated the effects of a medicinal plant extract and its bioactive compounds on uterine muscle contractility

“Evaluation of an ethnomedicinal plant extract on uterine muscle contraction”

EVALUATION OF AN ETHNOMEDICINAL PLANT EXTRACT ON UTERINE MUSCLE CONTRACTION

Zinhle Mvelase[*], Ntethelelo Sibiya*, Musa V. Mabandla[*#]

[*]Discipline of Human Physiology, School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa

[#]Author for Correspondence: Prof. Musa Mabandla; Tel: (+27) 31 2604603; Email: mabandlam@ukzn.ac.za

Abstract

Rhoicissus tridentate (RT) is a plant that is used by many expectant mothers to alleviate pregnancy related complications. However, its effects on myometrial contractions are not well known. In the present study, we investigated the uterotonic activity of RT and that of two of its bioactive compounds, beta-sitosterol (BS) and arjunolic acid (AA)**.** Non-pregnant female rats were injected with diethylstilbestrol and the uteri (2-3 cm) were removed for *in-vitro* contraction studies 24 hours later. The uteri were treated with graded concentrations of RT (0.24-62.08 mg/mL) or BS (0.09-57.10 µmol/mL) or AA (0.37-22.80 µmol/mL). A subset of uteri was co-treated with RT/BS/AA and oxytocin. Oxytocin treated uteri served as the positive control while untreated uteri served as an absolute control. The force and rate of uterine muscle contractions were recorded. After the experimental period, the uteri were analysed for oxytocin and PGF2α receptor expression. RT, BS and AA treatment produced a dose-dependent increase in the force and rate of uterine muscle contraction. Co-treatment with RT/BS and oxytocin had a synergistic effect on force and rate of myometrium contractility. RT treatment resulted in downregulation while AA/BS resulted in PGF2α receptor concentration increase but a decrease in oxytocin receptors. Similarly, RT treatment decreased oxytocin receptor concentration. AA and oxytocin co-treatment decreased PGF2α and oxytocin receptor concentration. While RT/BS and oxytocin co-treatment resulted in increased PGF2α and oxytocin receptor concentration. RT/AA/BS exhibited oxytocin-like effects on uterine contraction *in vitro*. These observations suggest that RT may be beneficial in inducing labour and alleviating uterine atony

Keywords: *Rhoicissus tridentate,* β-sitosterol, arjunolic acid, oxytocin, uterine contractions, PGF2α

1. Introduction

It has been suggested that globally, about 600 000 women die from pregnancy-related complications each year[1]. Among delivery pathologies such as pre-term labour, post-term labour, postpartum haemorrhage and placental abruption, uterine atony remains the principal cause of maternal mortality[2]. Postpartum haemorrhage is a life-threatening obstetric emergency caused mainly by uterine atony[3, 4]. Uterine atony occurs after childbirth when the uterus fails to contract[5]. Under normal conditions, uterine muscle contractility during labour compresses the blood vessels and reduces flow, thereby increasing the likelihood of coagulation and preventing haemorrhage[6]. Thus, better contraction of the uterus allows for good parturition and the avoidance of post-partum bleeding. Conventional drugs used to enhance uterine muscle contractility and to prevent postpartum bleeding include uterotonic agents such as oxytocin, prostaglandins (PGF2α, PGE2) and ergometrine [7-9]. The use of ergometrine or drugs containing oxytocin and prostaglandins has been associated with side effects such as premature ventricular contractions, sinus tachycardia and hyperstimulation of the myometrium leading to complications such as birth asphyxia [10, 11]. Due to the high prescription costs and high incidence of adverse effects associated with the use of conventional pharmaceuticals, there has been a trend to explore the use of natural or traditional medicinal products [12, 13].Previous studies have documented that about 70-80% of women worldwide rely on traditional medicines during pregnancy [14-16]. In KwaZulu Natal, the crude extracts of *Agapanthus africanus* (Alliaceae), *Clivia miniata* (Amaryllidaceae), *Combretum erythrophyllum* (Combretaceae), *Gunnera perpensa* (Gunneraceae) and *Pentanisia prunelloides* (Rubiaceae) have been used in folk medicine in a decoction known as *Isihlambezo*[17]. *Isihlambezo* is used to induce labour, achieve relatively painless delivery, terminate unwanted pregnancy, extrude retained placenta and alleviate uterine atony [18] [19]. Currently there is little documented scientific evidence concerning the uterotonic effects of the plants found in *Isihlambezo*. Approximately 60% of the herbal remedy known as *Isihlambezo* is made up *R.tridentate (RT)* crude extract[20]. *R.tridentate* contains bioactive compounds that include β- sitosterol and arjunolic acid [21-24].The uterotonic effects and mechanism(s) of action of this plant and its associated bio-active compounds remains unclear. Accordingly, we investigated uterotonic effect of *R.tridentate (RT)* and its bioactive ingredients, beta-sitesterol (BS) and arjunolic acid (AA) using uterine strips *in-vitro*. Additionally, the effects exhibited RT /BS/AA on oxytocin and PGF2 alpha concentration.

2. Materials and methods

2.1 Chemicals and drugs

The following chemicals of analytical grade quality were purchased from Sigma Chemicals (St Louis, MO, USA); Diethylstilbestrol, acetylcholine hydrochloride, oxytocin (α-eypophamine), β-sitosterol and arjunolic acid. Sodium chloride (NaCl), potassium chloride (KCl), magnesium sulphate (MgSO4), calcium chloride (CaCl), sodium bicarbonate (NaHCO4) and glucose were purchased from Merck, Johannesburg, South Africa.

2.2 Plant extract

R. tridentate was collected from Silverglen Nursery, Durban and identified by a botanist, Miss Christina Potgieter at the Bews Herbarium at the University of KwaZulu Natal, Pietermaritzburg campus. (Voucher specimen number - 086931, C.E. Moss Herbarium). Leaves were harvested in summer.

2.3 Plant preparation

Dried leaves (143.4 g) of *R. tridentate* were ground into powder and extracted with dichloromethane (DCM) for 48 hours. The resulting extract was filtered and concentrated to dryness yielding 13 g DCM extract. For uterotonic studies, *R. tridentata* (RT), beta-sitosterol (BS) and arjunolic acid (AA) were freshly prepared in De-Jalon's solution.

2.4 Animals

Adult female Sprague-Dawley rats bred in the Biomedical Resource Unit of the University of KwaZulu-Natal's Westville Campus were used in the study. The animals were kept in a room with a temperature of 22± ℃, carbon dioxide content of <5000 p.p.m, relative humidity of 55± 5% and a 12-hour light/dark cycle (with lights on at 07h00). The animals were fed standard rat chow (Meadows, Pietermaritzburg, South Africa) and had free access to drinking water. The University

of KwaZulu-Natal's Animal Research Ethics Committee approved all experimental procedures (Ref: AREC/084/015M).

2.5 Oestrous induction

Female Sprague-Dawley rats (250-300 g) were treated with diethylstilboestrol (12 mg/kg i.p.), 24 hours prior to experimentation to ensure regular uterine muscle contractility. Diethylstilboestrol is a synthetic form of oestrogen used to ensure regular uterine muscle contractility. The animals were sacrificed by decapitation and uterine muscle 2-3 cm long was removed as described in detail by Simelane *et al.*[20] . Briefly, the inferior lateral part abdomen was cut open and the two uterine horns of approximately 2-3 cm were exposed without stretching the uterine smooth muscle. The uterine strips were removed and were maintained under physiological conditions for the duration of the experiments. The two uterine horn segments were separately suspended in 25ml organ bath containing de Jalon's physiological solution.

2.6 Determination of *in-vitro* contractions

The excised uterine horns were suspended in an organ bath containing aerated De-Jalons buffer (mmol/L: NaCl, 154; KCl, 5.6, CaCl2.2H2O, 0.4; NaHCO3, 5.8 and glucose; 2.8) maintained at 32 ºC. The De-Jalon's solution was aerated continuously with 5% of carbogen. Each uterine strip was subjected to an applied resting tension of 1.0g and was allowed to equilibrate for 45 minutes. After the equilibration period, the muscle strips were separately challenged with RT (0.24-62.08 mg/mL), beta-sitesterol (BS, 0.09-57.10 µmol/mL) and arjunolic acid (AA, 0.37-22.80 µmol/mL). Following the assessment of the above mentioned concentrations on uterine smooth muscle function, to determine the synergistic effect of RT and its bioactive ingredients, the uterine strips were incubated in oxytocin (1.11 nmol/mL) before being treated with various concentrations of RT, BS or AA. Uterine strips treated with graded concentration of oxytocin (0.01-109.00 nmol/mL) served as the positive controls, while contractions generated in a treatment-free buffer served as the absolute control. Following treatment, the force and rate of contraction were recorded using a method adapted from Datte *et al [25]*. Briefly, force and rate of contraction were recorded electronically using the Powerlab system (AD Instruments, Bella Vista, Australia). The rate of contractions is the number of rhythmic contractions, during 5-minute period of contraction after adding each concentration of RT/AA/BS. After experimentation, the uterine strips were harvested for biochemical analysis.

2.7 Biochemical analysis

The concentration of oxytocin and prostaglandin (PG) F2 alpha-receptors in the uterine horns was measured using separate specific sandwich ELISA kits (Ela Science and Biotechnology, Wuhan, China) according to the manufacturer's instructions. Briefly, the kits included micro ELISA plates which were coated with antibody specific to oxytocin and PGF2 receptors. Samples were pipetted into the appropriate wells of the micro ELISA plates and incubated for 90 minutes and 30 minutes for PGF2 alpha and oxytocin receptors respectively. All samples were pipetted in triplicate. This was followed by the addition of relevant biotinylated detection antibody (100 µL). After incubating for 30 minutes, Avidin-Horseradish Peroxidase (HRP) conjugate (90µL) was added to each micro-plate well. After incubating for 30 minutes, the unbound components were washed away with a wash buffer solution. Substrate solution (100 µL) was added into each micro-plate well. After incubating for a further 15 minutes, stop solution (50 µL) was added. Optical density (OD) was measured at 450 nm within 10 minutes using a Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Wurttemberg, Germany). The concentration of the samples was extrapolated from the respective standard curves.

2.8 Data analysis

Data obtained from treating uterine muscle strips with the crude extract, bioactive compounds, oxytocin and combined treatment oxytocin with RT, BS OR AA was calculated as a percentage of contraction with reference to the non-treated controls. The values were expressed as mean ± SEM. Statistical analysis of differences between the means of the control and treated groups were performed on Graph Pad InStat Software Version 5.00 (Graph Pad Software, Inc., San Diego, California, USA), using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. A value of $p < 0.05$ was considered significant.

3. Results

3.1 Contractility: Force and rate

3.1.1 Oxytocin effect on the myometrium

Figure 1 shows the uterine muscle response curves following exposure to oxytocin. There was a concentration dependent increase on the force and rate of contraction of the myometrium in response to increase in oxytocin concentration *(control vs oxytocin, $p<0.05$, Figures 1 A and B).

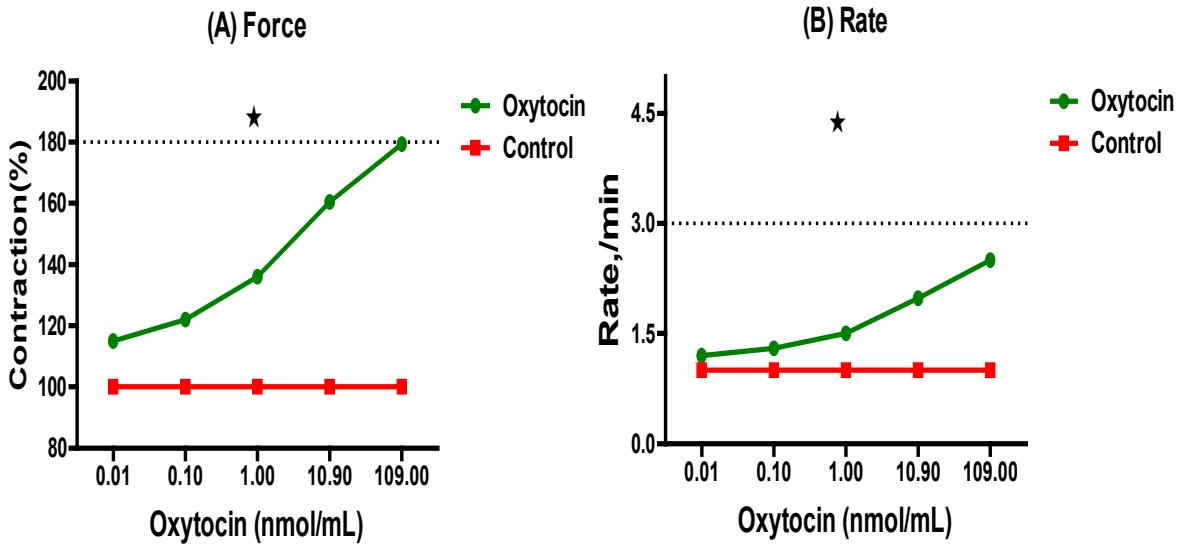

Figure 1: Effects of oxytocin on the Force **(A)** and Rate **(B)** of uterine muscle contraction. Values are presented as means ± SEM, n=6 for each concentration, *$p < 0.05$ when compared to the control group

3.1.2 *R.tridantate*, β-sitosterol and arjunolic acid effect on the myometrium

Figure 2 shows the effect of *R.tridantate* (RT), β- sitosterol (BS) and arjunolic acid (AA) treatment on the force and rate of uterine muscle contraction. The administration of graded concentrations of crude RT extract induced an increase in the force and rate of myometrium contraction *(control vs RT, $p< 0.05$, Figures 2 A and B). A similar effect was observed following treatment with BS and AA on both force and rate *(control vs AA and control vs BS, $p< 0.05$, Figures 2 A and B). Treatment with RT crude extract resulted in a greater effect on force and rate contraction $^{\alpha}$ (RT vs BS, $p< 0.05$ figure 2A and B) and $^{\beta}$ (RT vs AA, $p< 0.05$ figure 2A and B)

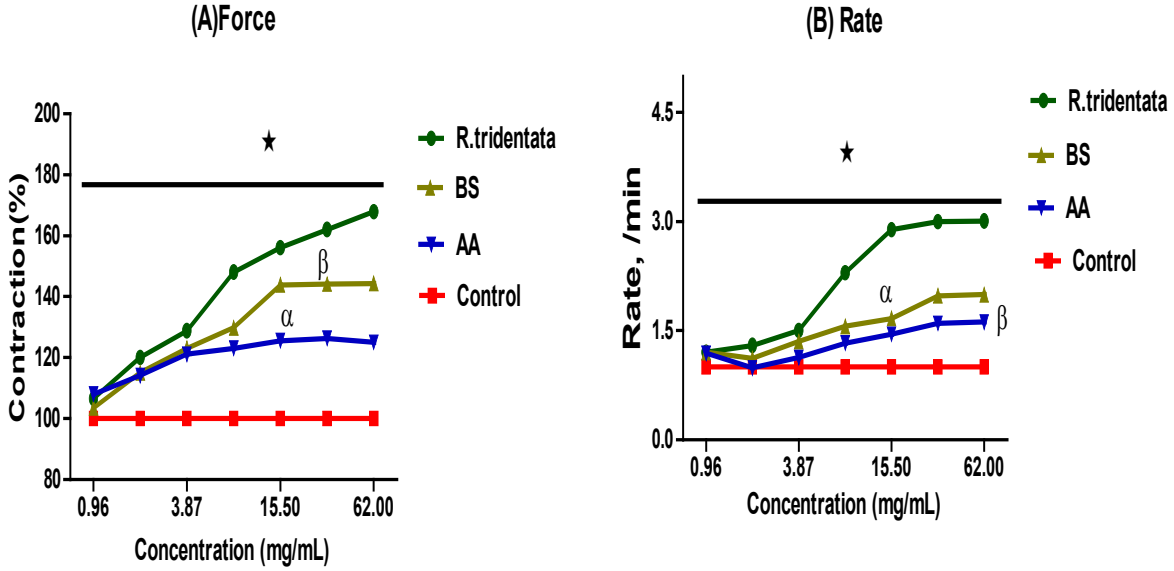

Figure 2: Effects of RT/BS/AA on the Force **(A)** and Rate **(B)** of uterine muscle contraction. Values are presented as means ± SEM, n=6 for each concentration, * control vs RT/BS/AA, $p< 0.05$; α (RT vs BS, $p< 0.05$) and β (RT vs AA, $p< 0.05$)

3.2 Effects of co-treatment on myometrium contraction

3.2.1 Oxytocin and *R. Tridentate*

Figure 3 shows the effect of combined oxytocin and *R. tridentate* (RT) treatment on the force and rate of contraction on uterine strips. Co-treatment with RT and oxytocin had a synergistic effect on force and rate of myometrium contraction, ∗ (RT vs RT+Oxy, $p<0.05$, Figure 3 A and B) and α (Oxy vs RT+Oxy, $p<0.05$, Figures 3 A and B).

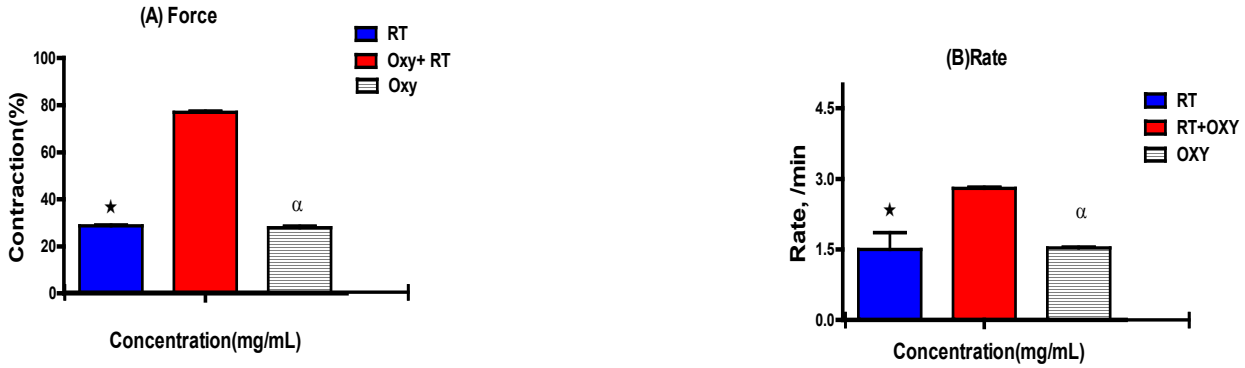

Figure 3: Effect of co-administration of oxytocin and *R.tridantate* extract on the Force **(A)** and Rate **(B)** of uterine muscle contraction. Values are presented as means ± SEM (n=6 for each concentration), * (RT vs RT+Oxy, $p<0.05$) and $^{\alpha}$ (Oxy vs RT+Oxy, $p<0.05$)

3.2.2 Oxytocin and beta-sitosterol

Figure 4 below shows the results of the combined treatment of oxytocin and BS on the force and rate of contraction on uterine strips. Co-treatment had a synergistic effect on the force of contraction ∗ (BS vs BS+Oxy, $p<0.05$, Figure 4 A and B) and $^{\alpha}$ (Oxy vs BS+Oxy, $p<0.05$, Figures 4 A and B).

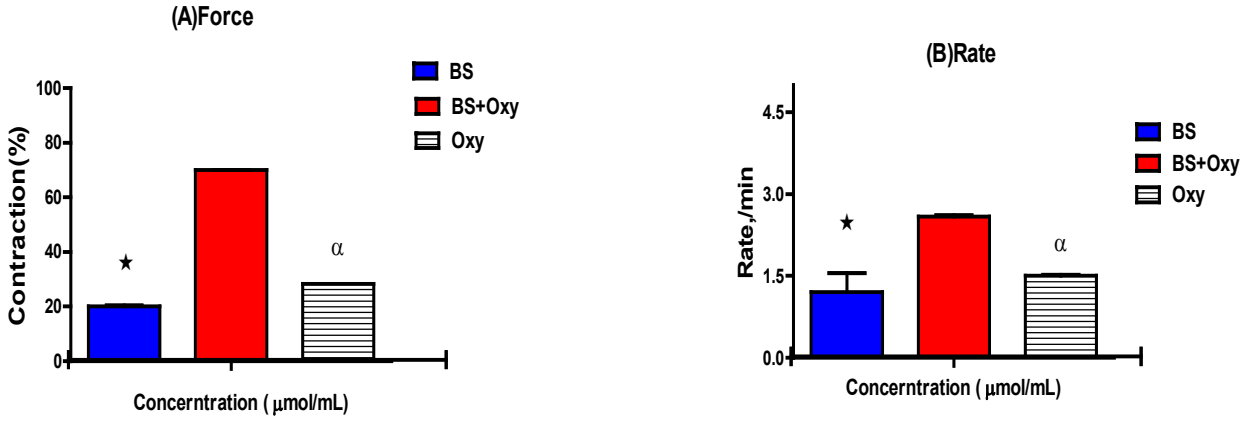

Figure 4: Effects of combined oxytocin and β- sitosterol on the Force **(A)** and Rate **(B)** of uterine muscle contraction. Values are presented as means ± SEM (n=6 for each concentration) *(BS vs BS+Oxy, $p<0.05$) and $^{\alpha}$ (Oxy vs BS+Oxy, $p<0.05$).

3.2.3 Oxytocin and arjunolic acid

Figure 5 below shows the results from the combined treatment with oxytocin and arjunolic acid on uterotonic activity.

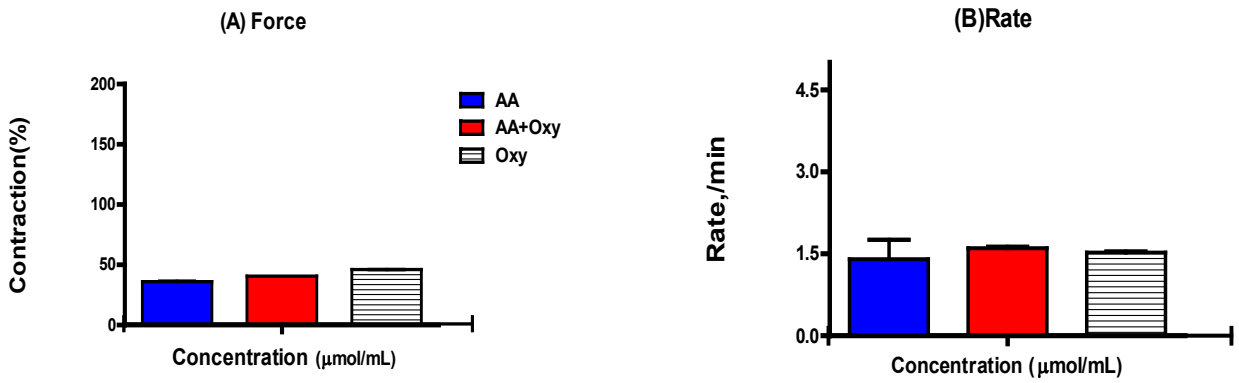

Figure 5: Effects of simultaneous administration of oxytocin and arjunolic acid on the Force **(A)** and Rate **(B)** of uterine muscle contraction. Values are presented as means ± SEM (n=6 for each concentration)

3.3 Biochemical analysis

3.3.1 PGF2α receptor concentration

In Table 1, PGF2α receptor concentration was measured in uterine muscle tissue following treatment with RT, BS, or AA as well as co-treatment with oxytocin (Oxy +RT / BS / AA). There was a decreased PGF2α receptor concentration following treatment with RT *(control vs RT, $p<0.05$). Treatment with BS and AA resulted in upregulation of the PGF2 alpha receptor $^{\alpha}$(control vs BS and control vs AA, $p<0.05$). There was a decrease in PGF2 α receptor following co-treatment with oxytocin and AA $^{\#}$ (AA vs AA+Oxy, $p<0.05$).

Table 1. PGF2α receptor concentration in the uterine muscle tissues treated with RT /AA/BS followed by individual combination of BS/AA/RT with oxytocin

Experimental groups	**PGF2α receptor concentration (µg/mL)**
Control	1.2±0.0
RT	0.8±0.0*
Oxytocin (Oxy)	1.3±0.1
Arjunolic acid (AA)	4.8±0.0 $^{\alpha}$
β-sitosterol (BS)	4.9±0.0 $^{\alpha}$
RT +Oxytocin	1.9±0.3
Arjunolic acid +Oxytocin	0.2±0.4 $^{\#}$
β-sitosterol+oxytocin	3.6±0.1

Data are expressed as mean ± SEM, n=6 in each group *=$p<0.05$ when comparing bioactive compounds or the plant extract and untreated control, #=$p<0.05$ when comparing RT/BS/AA with co-treatment.

3.3.2 Oxytocin receptor concentration

Table 2: Oxytocin receptor concentration was measured in uterine muscle tissue following treatment with AA, BS or RT and co-treatment with oxytocin. Treatment with RT or AA resulted in decrease in the oxytocin receptor concentration $*$ (control vs RT and control vs AA, $p<0.05$). Co-treatment with oxytocin and RT/BS/AA resulted in upregulation of the oxytocin receptor $^{\#}$ (RT vs RT + Oxy, BS vs BS+oxytocin and AA vs AA+Oxy, $p<0.05$).

Table 2. Oxytocin receptor concentration in the uterine muscle tissue treated with RT /AA/BS followed by co-treatment with oxytocin and RT/BS/AA

Experimental groups	**Oxytocin receptor concentration (µg/mL)**
Control	0.33±.0.1
Oxytocin (Oxy)	1.1±0.3
RT	0.12 ±0.0*
Arjunolic acid (AA)	0.1±0.2*
β-sitosterol (BS)	0.44±0.1
RT +Oxytocin	1.41 ±0.4#
Arjunolic acid +Oxytocin	0.99±0.3#
β-sitosterol+oxytocin	0.79±0.1#

Data is expressed as mean ± SEM, n=6 in each group *=$p<0.05$ when comparing bioactive compounds or the plant extract and untreated control, #=$p<0.05$ when comparing RT/BS/AA with a co-treatment

Discussion

In this study we sought to scientifically validate the uterotonic effects and elucidate possible mechanisms of action of the crude extract of *Rhoicissus tridentate* (RT) and two of its bioactive compounds, beta sitosterol (BS) and arjunolic acid (AA) on force and rate of uterine muscle contraction. The force of uterine muscle contraction increases with increasing rate of stimulation, i.e. a positive force-rate relationship. The force-rate relationship is often used to describe the contractile state and can be altered by inotropic intervention[26]. Myometrial dysfunction has been shown to result in amongst others, uncoordinated contractions leading to a miscarriage, pre-term delivery or stronger than necessary contractions which usually cause asphyxia which leads to foetal distress, hypoxia, and even death of the foetus[27]. The results of the current study show that the administration of oxytocin on the uteri horns induced an increase in the force and rate of contractions which occurred in a concentration dependent manner. Oxytocin has been shown to be the most potent endogenous uterotonic and acts on oxytocin receptors leading to increased intracellular calcium concentration which induce contractions[28, 29]. Briefly, depolarization of the plasma membrane opens L-type Ca^{2+} channels resulting in Ca^{2+} influx into the cell[30]. Calcium then complexes with calmodulin protein and activates myosin light chain kinase which phosphorylates the myosin light chain[31]. Phosphorylated myosin binds to actin and initiate cross bridge cycling leading to uterine contraction[32]. Therefore, one may expect to observe marked uterotonic effects elicited by oxytocin on uterine strips as we have shown. Similarly, RT administration showed comparable effects to oxytocin suggesting uterotonic activity. The increase in the force and rate of uterine muscle contraction following RT administration may suggest that RT also utilises voltage-dependent calcium channels, allowing an influx of extracellular calcium and enhancing contraction. Moreover, it has been documented that myometrial gap junctions improve coordination and increase the force of uterine contraction during parturition[33]. This process involves smooth muscle cells of the myometrium communicating via gap junctions which synchronize myometrial function via conduction of electrophysiological stimuli during labour[34]. We therefore speculate that RT may mediate its effects by stimulating gap-junction function within the myometrium, thus causing the observed uterotonic effects. However, this remains to be verified. Furthermore, the extract may inhibit voltage-gated potassium channels, which have been proposed as a major contributing factor to basal myometrial contractility[35, 36]. During gestation, potassium channels maintain the uterus in a state of quiescence by contributing to the resting

membrane potential and counteracting contractile stimuli[37]. Inhibition of the myometrium K^+ channels expression can translate into an inadequate repolarization leading to aberrant uterine activity[38]. A decreased expression of K^+ channels diminishes myometrial smooth muscle cells repolarizing current resulting in induction of uterine contractions[38, 39]. Thus, K^+ channel alterations may contribute to the increased force and rate of uterine muscle contractility observed in this study. BS and AA have been found to be the main components found in the RT extract[20]. Indeed, our results revealed that BS and AA increase the rate and force of contraction in a concentration-dependent manner. The ability of these compounds to increase both the force and rate of contraction is crucial for adequate parturition. As previously stated, an increase in force of contraction only, may lead to distress and even death of the foetus[27, 40]. It has been shown that pomegranate seed-derived β-sitosterol exhibits uterotonic effects suggesting that this effect may be due to Ca^{2+} influx and through activation of myosin light kinase [41]. This suggests that BS in the current study may have used the same mechanism. On the other hand, the voltage gated and receptor operated Ca^{2+} channels are responsible for the influx of Ca^{2+} from the extracellular fluid into the cytosol thus triggering contractions[42, 43]. Studies have shown that some plant-derived triterpenes (resembling similar characteristics as AA) exerts uterotonic effects through increased extracellular Ca^{2+} influx and release of intracellular Ca^{2+} from stores[44]. Hence, we speculate that AA effects may be mediated through the opening of these ion channels.

Synergism between oxytocin and other uterotonic drugs is not well studied. In this study we investigated the effect of RT (and its bioactive compounds), co-treatment with oxytocin on uterine muscle contractility. The co-treatment of oxytocin with RT or BS exhibited additive effects on the force and rate of contractility. This effect is also supported by the effect of co-treatment RT and BS with oxytocin on PGF2α and oxytocin receptors where we have shown that receptor concentration increases thus suggesting more binding surface and hence an increase in the force and rate of contraction. During the third trimester of pregnancy, the elevation in the concentration of oestrogen increases the expression of both oxytocin and PGF2α receptors[45]. These receptors have been shown to play an important role in myometrial contraction[46].There was a decreased PGF2α and oxytocin receptors following treatment with RT. The uterotonic effect seen following RT treatment may suggest its affinity for PGF2α and oxytocin receptors thus causing RT- induced desensitization or internalization of these receptors. Conversely, we also noted an increase in PGF2α and a decrease in oxytocin receptor concentration following treatment with BS, suggesting

that BS facilitated contractions influence the increase in PGF2α receptor concentration. AA and oxytocin co-treatment resulted in a muted increase in PGF2A and oxytocin receptor concentration. The observations in this study complement those of Katsoulis *et al*[47] and Simelane *et al* [46] who showed that co-treatment with oxytocin and resveratrol glycoside exhibit synergistic uterotonic effects in rats[46, 47]. Studies have shown that G protein coupled receptors such as muscarinic (M_1, M_3) receptors can activate both cAMP and Ca^{2+} signalling pathways[48, 49]. Muscarinic (M1 and M2) receptors cause the activation of phospholipase C, generating two secondary messengers (IP3 and DAG) eventually leading to an intracellular increase in calcium concentration which results in an increase in uterine muscle contractility[50, 51]. We therefore speculate that AA in the presence of oxytocin may be mediating its functions by binding to G protein coupled receptors (GPCRs), thus promoting the activation of different intracellular signalling cascades causing the observed effects. Apart from AA and BS, there are other bioactive compounds found in RT. These bioactive compounds include compound 285, resveratrol glycoside and morin rhamnoside[20]. These bioactive compounds are classified as either phenols, flavonoids, alkaloids, saponins, terpenoids, steroids and tannins. Due to differences in the structural configuration of AA and BS compounds, one may expect the differences in the observed effects elicited by BS/AA.

RT, AA and BS increase the rate and force of uterine muscle contraction. These finding have shed some light on the mechanism by which RT may exert its uterotonic effects. Moreover, the observations further encourage the use of medicinal plant concoction in alleviating pregnancy complications especially in the economy depressed regions. The effect elicited by RT and its bioactive compounds on myometrial contraction is enhanced by co-treatment with oxytocin suggesting that this co-treatment has potential as an effective oxytocic during labour

Declaration

All the authors declare that the results presented in this paper have not been published previously in whole or part.

Funding source

The study was funded by the National Research Foundation South Africa and University of KwaZulu Natal, collage of health Sciences

Conflict of interests: None.

References

1. Khan, K.S., et al., *WHO analysis of causes of maternal death: a systematic review.* The lancet, 2006. **367**(9516): p. 1066-1074.
2. Kota, S.K., et al., *Fetal endocrinology.* Indian journal of endocrinology and metabolism, 2013. **17**(4): p. 568.
3. Pelage, J.-P., et al., *Life-threatening primary postpartum hemorrhage: treatment with emergency selective arterial embolization.* Radiology, 1998. **208**(2): p. 359-362.
4. Sheldon, I.M., *The postpartum uterus.* Veterinary Clinics: Food Animal Practice, 2004. **20**(3): p. 569-591.
5. Hayashi, R.H., M.S. Castillo, and M.L. Noah, *Management of Severe Postpartum Hemorrhage due to Uterine Atony Using an Analogue of Prostaglandin F2 [alpha]*. Obstetrics & Gynecology, 1981. **58**(4): p. 426-429.
6. Garfield, R.E. and C. Yallampalli, *Control of myometrial contractility and labor*, in *Basic mechanisms controlling term and preterm birth*. 1994, Springer. p. 1-28.
7. Kelly, A.J., et al., *Vaginal prostaglandin (PGE2 and PGF2a) for induction of labour at term.* The cochrane library, 2009.
8. Karim, S. and S. Sharma, *Therapeutic abortion and induction of labour by the intravaginal administration of prostaglandins E2 and F2α.* BJOG: An International Journal of Obstetrics & Gynaecology, 1971. **78**(4): p. 294-300.
9. Smith, R., *Parturition.* New England Journal of Medicine, 2007. **356**(3): p. 271-283.
10. Ronsmans, C., W.J. Graham, and L.M.S.S.s. group, *Maternal mortality: who, when, where, and why.* The Lancet, 2006. **368**(9542): p. 1189-1200.
11. Haddad, S., et al., *Cardiorespiratory crisis at the end of pregnancy: a case of pheochromocytoma.* Middle East J Anesthesiol, 2013. **22**(2): p. 195-202.
12. Gutierrez, R.M.P., *Orchids: A review of uses in traditional medicine, its phytochemistry and pharmacology.* Journal of Medicinal Plants Research, 2010. **4**(8): p. 592-638.
13. Organization, W.H., *WHO traditional medicine strategy 2002-2005.* 2002.

14. Kamatenesi Mugisha, M. and R. Bukenya Ziraba, *Ethnobotanical survey methods to monitor and assess the sustainable harvesting of medicinal plants in Uganda.* Plant conservation in the tropics: perspectives and practice, 2002: p. 467-482.
15. Kamatenesi-Mugisha, M., R. Bukenya-Ziraba, and R. Hoeft, *Ethnomedical use of Rytigynia (nyakibasi) in Bwindi Impenetrable National Park, SW Uganda.* Lidia (Norway), 2000.
16. Kamatenesi-Mugisha, M. and H. Oryem-Origa, *Traditional herbal remedies used in the management of sexual impotence and erectile dysfunction in western Uganda.* African Health Sciences, 2005. **5**(1): p. 40-49.
17. Abdillahi, H.S. and J. Van Staden, *Application of medicinal plants in maternal healthcare and infertility: a South African perspective.* Planta medica, 2013. **79**(07): p. 591-599.
18. McFarlin, B.L., et al., *A national survey of herbal preparation use by nurse-midwives for labor stimulation.* Journal of nurse-midwifery, 1999. **44**(3): p. 205-216.
19. Morris, D.G. and M.B. Mdlalose, *The use of isihlambezo in the upper Tugela Region.* South African Family Practice, 2012. **12**(5).
20. Brookes, K. and L. Katsoulis, *Bioactive components of Rhoicissus tridentate: a pregnancy-related traditional medicine.* South African journal of science, 2006. **102**(5-6): p. 267-272.
21. Attah, A.F., et al., *Uterine contractility of plants used to facilitate childbirth in Nigerian ethnomedicine.* Journal of ethnopharmacology, 2012. **143**(1): p. 377-382.
22. Bryant, A.T., *Zulu medicine and medicine-men*. 1966: C. Struik.
23. Edeoga, H., D. Okwu, and B. Mbaebie, *Phytochemical constituents of some Nigerian medicinal plants.* African journal of biotechnology, 2005. **4**(7): p. 685-688.
24. Ogbe, F.M.D., O.L. Eruogun, and M. Uwagboe, *Plants used for female reproductive health care in Oredo local government area, Nigeria.* Scientific Research and Essays, 2009. **4**(3): p. 120-130.
25. Datte, J., A. Offoumou, and O. Manda, *Uterotonic effects of hydromethanolic extract of Parquetina nigrescens (Periplocaceae) on spontaneous contractile activity in the isolated myometrium of pregnant rats.* Journal of ethnopharmacology, 1996. **53**(1): p. 15-20.
26. Blumhardt, L. and G. Barrett, *Differential effects of demyelination on the components of the pattern visual evoked response (PVER) in man.* COMMUNICATIONS, 1991. **295**.
27. Tiran, D., *Baillière's midwives' dictionary*. 2008: Elsevier Health Sciences.

28. Arrowsmith, S. and S. Wray, *Oxytocin: its mechanism of action and receptor signalling in the myometrium.* Journal of neuroendocrinology, 2014. **26**(6): p. 356-369.
29. Arias, F., *Pharmacology of oxytocin and prostaglandins.* Clinical Obstetrics and Gynecology, 2000. **43**(3): p. 455-468.
30. Meldolesi, J. and T. Pozzan, *Pathways of Ca2+ influx at the plasma membrane: voltage-, receptor-, and second messenger-operated channels.* Experimental cell research, 1987. **171**(2): p. 271-283.
31. Blumenthal, D.K. and J.T. Stull, *Activation of skeletal muscle myosin light chain kinase by calcium (2+) and calmodulin.* Biochemistry, 1980. **19**(24): p. 5608-5614.
32. Kamm, K.E. and J.T. Stull, *The function of myosin and myosin light chain kinase phosphorylation in smooth muscle.* Annual review of pharmacology and toxicology, 1985. **25**(1): p. 593-620.
33. Garfield, R., et al., *Possible role of gap junctions in activation of myometrium during parturition.* American Journal of Physiology-Cell Physiology, 1978. **235**(5): p. C168-C179.
34. Sims, S., E.E. Daniel, and R. Garfield, *Improved electrical coupling in uterine smooth muscle is associated with increased numbers of gap junctions at parturition.* The Journal of general physiology, 1982. **80**(3): p. 353-375.
35. Bafor, E. and U. Sanni, *Uterine contractile effects of the aqueous and ethanol leaf extracts of newbouldia laevis (Bignoniaceae) in vitro.* Indian journal of pharmaceutical sciences, 2009. **71**(2): p. 124.
36. Zingg, H.H. and S.A. Laporte, *The oxytocin receptor.* Trends in Endocrinology & Metabolism, 2003. **14**(5): p. 222-227.
37. Buxton, I.L., C.A. Singer, and J.N. Tichenor, *Expression of stretch-activated two-pore potassium channels in human myometrium in pregnancy and labor.* PLoS One, 2010. **5**(8): p. e12372.
38. Brainard, A.M., V.P. Korovkina, and S.K. England. *Potassium channels and uterine function*. in *Seminars in cell & developmental biology*. 2007. Elsevier.
39. Knock, G.A., S.V. Smirnov, and P.I. Aaronson, *Voltage-gated K+ currents in freshly isolated myocytes of the pregnant human myometrium.* The Journal of physiology, 1999. **518**(3): p. 769-781.

40. Vallbo, A., *Human muscle spindle discharge during isometric voluntary contractions. Amplitude relations between spindle frequency and torque.* Acta Physiologica, 1974. **90**(2): p. 319-336.
41. Yadav, P., et al., *Madhuca longifolia (Sapotaceae), a review of its traditional uses, phytochemistry and pharmacology.* International Journal of Biomedical Research, 2012. **3**(7): p. 291-305.
42. Khalil, R.A. *Regulation of vascular smooth muscle function*. in *Colloquium Series on Integrated Systems Physiology: From Molecule to Function*. 2010. Morgan & Claypool Life Sciences.
43. Aqel, M. and R. Shaheen, *Effects of the volatile oil of Nigella sativa seeds on the uterine smooth muscle of rat and guinea pig.* Journal of ethnopharmacology, 1996. **52**(1): p. 23-26.
44. Guo, L., et al., *Active pharmaceutical ingredients and mechanisms underlying phasic myometrial contractions stimulated with the saponin extract from Paris polyphylla Sm. var. yunnanensis used for abnormal uterine bleeding.* Human Reproduction, 2008. **23**(4): p. 964-971.
45. Wathes, D., et al., *Oxytocin receptor expression in human term and preterm gestational tissues prior to and following the onset of labour.* Journal of endocrinology, 1999. **161**(1): p. 143-151.
46. Simelane, M., et al., *In vitro antioxidant and cytotoxic activity of Gunnera perpensa L.(Gunneraceae) from South Africa.* Journal of Medicinal Plants Research, 2010. **4**(21): p. 2181-2188.
47. Katsoulis, L., D. Veale, and I. Havlik, *The pharmacological action of Rhoicissus tridentata on isolated rat uterus and ileum.* Phytotherapy Research, 2000. **14**(6): p. 460-462.
48. Felder, C.C., *Muscarinic acetylcholine receptors: signal transduction through multiple effectors.* The FASEB Journal, 1995. **9**(8): p. 619-625.
49. Berridge, M.J., *Inositol trisphosphate and calcium signalling.* Nature, 1993. **361**(6410): p. 315.
50. Felder, C.C., et al., *Therapeutic opportunities for muscarinic receptors in the central nervous system.* Journal of medicinal chemistry, 2000. **43**(23): p. 4333-4353.

51. Waller, D.G. and T. Sampson, *Medical pharmacology and therapeutics E-Book*. 2017: Elsevier Health Sciences.

CHAPTER 3

PROLOGUE

Manuscript 2

Hyperglycaemia is a serious health threat, especially in a pregnant woman. Therefore, adequate glycaemic control is critical for preventing the onset and progression of all possible disturbances which may delay labour onset. In this study, we evaluated the effects and possible mechanism (s) of action of *Rhoicissus tridentate* and associated bioactive compounds, arjunolic acid (AA) and beta sitosterol (BS) on glucose metabolism.

"*Rhoicissus tridentate* and associated bioactive compounds effects on glucose metabolism in liver and muscle cell lines"

RHOICISSUS TRIDENTATE AND ASSOCIATED BIOACTIVE COMPOUNDS EFFECTS ON GLUCOSE METABOLISM IN LIVER AND MUSCLE CELL LINES

Zinhle Mvelase[*], Ntethelelo Sibiya*, Musa V. Mabandla[*#]

[*]Discipline of Human Physiology, School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa

[#]Author for Correspondence: Prof. Musa V. Mabandla; Tel: (+27) 31 2604603; Email: mabandlam@ukzn.ac.za

Abstract

Rhoicissus tridentate (RT) extract is used to alleviate hyperglycaemia and associated diabetes mellitus complications. In this study we sought to validate the effects of RT and its bioactive compounds arjunolic acid (AA) and beta sitosterol (BS) on glucose metabolism and the possible mechanism(s) involved using liver and muscle cell lines. Three concentrations (12.5, 25 and 50 µg/ml) of each compound (RT/AA/BS) were administered to liver and muscle cell lines challenged with 19 mmol and 29 mmol of glucose respectively. Cells incubated in DMSO (0.1%), insulin (40 µg/mL) or metformin (16 µg/ml) served as untreated and treated positive controls. Media glucose concentration was measured at 0, 12, 24 and 48 hours post incubation. Thereafter, media glucose or cells were harvested for ALT, AST, glycogen, GLUT 4 and glycogen synthase analysis. AA (50 µg/mL) negatively affected cell viability in both cell lines after 48h and resulted in increased ALT concentration in liver cells. Treatment with BS (50 µg/ml) decreased cell viability (muscle) after 48h. RT/BS treatment decreased media glucose with concomitant increase in glycogen concentrations after 48 hours in both cell lines. Interestingly, both RT/BS stimulated an increase in GLUT4 concentrations in muscle cells while RT administration resulted in increased glycogen synthase concentration in both cell lines.These observations suggest that RT/BS promote glucose utilisation via GLUT 4 and glycogen synthase stimulation, thus promoting anti-diabetic activity in diabetes mellitus.

Keywords: *Rhoicissus tridentate,* β- sitosterol, arjunolic acid, glucose utilization, cell viability

1. Introduction

Diabetes mellitus (DM) is a major health concern affecting communities in developed and developing countries[7]. Worldwide approximately 150-300 million people suffer from this debilitating disease[42, 57]. The hallmark of diabetes is hyperglycaemia resulting from poor glucose uptake by insulin dependent tissues such as the liver and skeletal muscle[59]. Therefore, the primary intervention for alleviating diabetes associated complications is through stimulating glucose disposal in insulin sensitive tissues[44]. Despite solid progress made towards the management of the disease, much controversy surrounds the management of diabetes mellitus[23]. To date, insulin injections and a vast number of oral anti-hyperglycaemic drugs are used to manage hyperglycaemia and DM associated complications[48]. These drugs are relatively expensive while they also possess life threatening effects including hypoglycaemia as well as cardiovascular hazards[52]. Furthermore, the unavailability together with the inability of these agents to treat DM efficiently has driven researchers to explore other alternatives not only to broaden treatment availability but also to ensure the availability of efficient treatment. Several medicinal plants such as *Allium cepa, Anacardium occidentale, Andrographic paniculata, Momordica charantia, Azadirachtha indica, Brassica oleraccia, Cinnamomum tamala and Withania sominifera* have been used to control diabetes in many cultures worldwide[4, 8, 25, 46]. Moreover, the bioactive compounds isolated from these plants have been shown to possess antidiabetic activity with more efficacy than oral conventional hypoglycaemic agents used in clinical therapy[35]. Some studies have suggested that traditional medicinal plant extracts may be at the threshold of becoming practical alternatives to conventional hypoglycaemic agents as they have been found to exert various insulino-mimetic effects including glucose disposal stimulation[2, 36]. The medicinal plant *Rhoicissus tridentate (RT*) commonly known as Bushman's grape is used by many pregnant women to alleviate pregnancy related complications[11]. *Rhoicissus tridentate (RT)* contains bioactive compounds such as β- sitosterol and arjunolic acid[3, 5, 12, 33]. These bioactive compounds have been shown to possess antidiabetic and antioxidant potential[18]. Despite these proclaimed properties, very few studies have been conducted to provide scientific insight regarding their effect on glucose homeostasis. Therefore, in this study, we aimed to investigate the effects of the plant extract *R.tridentate (RT*) and its bioactive compounds beta-sitosterol (BS) and Arjunolic acid (AA) on glucose metabolism in liver (Chang) and muscle (C2C12) cell lines *in vitro.*

2. Methods and Materials

2.1 Plant collection and identification

R. tridentate (RT) was collected from Silverglen Nursery, Durban and identified by a botanist, Miss Christina Potgieter at the Bews herbarium of the University of KwaZulu Natal, Pietermaritzburg campus. (Voucher specimen number - 086931, C.E. Moss Herbarium). Leaves of the plant were harvested in summer.

2.2 Plant preparation

Dried leaves (143.4 g) of *R. tridentate* (RT) were ground into powder and extracted with dichloromethane (DCM) for 48 hours. The yielded extract was filtered and concentrated to dryness yielding 13 g of extract. In our laboratory, we extracted 6 pure compounds from crude *R. tridentate* (RT) extract viz; arjunolic acid, β-sitosterol **,** compound 285, oleanolic acid, resveratrol glycoside and morin rhamnoside (Table 1)[11]. For this study, we only focused on two compounds viz; arjunolic acid (AA) and β-sitosterol (BS)

Table 1: Summary showing the list of bioactive compounds obtained from *R. tridentate (RT)* leaves extracted with DCM and acetone solvents for 48 hours.

Acetone solvent	**DCM solvent**
• Compound 285 • Resveratrol glycoside • **Arjunolic acid (AA)** • Morin rhamnoside	• **β-sitosterol (BS)** • Oleanolic acid

2.3 Cell culture

2.3.1 Cell lines

The muscle (C2C12) and liver (Chang) cell lines were used for the study. The C2C12 muscle cell line is a subclone from a myoblast line established from normal mouse muscle[21]. The C2C12 cell line differentiates rapidly, forming contractile myotubes and producing characteristic muscle proteins [10]. Chang liver cells are immortalized non tumour cells derived from normal liver tissue of a mouse [43]. Chang cells are adherent cells and grow as undifferentiated hepatoblasts in growth medium [43]. Both cell lines, can divide an unlimited number of times in a laboratory cell culture plate as long as fundamental cell survival conditions are met [43].

2.3.2 Cell culture protocol

Experiments were conducted using a well-established cell culture protocol[22]. Briefly, the cells were grown in a humidified atmosphere containing 5% CO_2 at 37ºC (Shel Lab, Cornelius, Oregon, USA).The cells were routinely maintained in growth medium which consisted of Dulbecco's Modified Eagle Medium (DMEM) and Eagle's Minimal Essential Medium for the muscle and the liver respectively. Culture media was supplemented with 10% heat-inactivated foetal calf serum (FCS), 1 % L-glutamate and 1% penicillin/streptomycin/fungizone. The reconstituted muscle and liver cell lines were plated in 25 cm^3 flasks followed by the addition of 10 mL fully supplemented DMEM and EMEM media, respectively. Thereafter, the flasks were incubated at 37 $^{\circ}$C in a humidified incubator (Shel Lab, Cornelius, Oregon, USA) with 5% CO_2. The cells were allowed to grow and become confluent. Growth media was replaced every 2-3 days. For routine maintenance, the confluent cells were trypsinised with trypsin (1mL) after washing three times with PBS. The trypsinised cells were sub-cultured into new flasks and some were stored in a nalgene cooler (Sigma-Aldrich, St Louis, Missouri, USA) at -80ºC for future studies.

2.3.3 Cell seeding

For the glucose utilization experiments, both muscle (C2C12) and liver (Chang) cells were seeded at a density of (1.5 x 10^5 cells/mL). The cell lines were allowed to grow and reach an 80% confluence monolayer.

2.4 Cell viability assay

Due to the variability of cell numbers in different wells, the viability of the cells in each well in each experiment was determined so that any significant difference between the cells exposed to the treatments compared to the control wells could be determined. The Cell Titer-Glo viability assay kit (Promega, USA) was used in accordance with the instruction manual protocol. This assay uses an indicator dye to measure the metabolic capacity of cells, thereby indicating cell viability. In this protocol, the amount of ATP is deemed to be proportional to the number of viable cells. Both muscle and liver 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 12, 25 and 50 µg/mL of *Rhoicissus tridentate (RT*), Arjunolic acid (AA) and beta sitesterol (BS). The control well remained untreated. After 12, 24 and 48 hours, the plates were equilibrated at room temperature for 30 minutes. The assay reagent (100 µL) was added to each well, followed by shaking the plates for 2 minutes to induce cell lysis. After shaking, the plates were incubated at room temperature for 10 minutes to stabilize the luminescence signal. Thereafter, the luminescence was read on the Promega Microplate Luminometer (Promega, Madison, Wisconsin, USA). All plates had control wells containing media without cells for background correction. For comparative purposes, we used a dose of 25mg/mL for all biochemical analysis.

2.5 Alanine amino transferase (ALT) and aspartate amino transferase (AST) measurements

We confirmed the toxicity at high doses by looking at the effect treatment has on the liver enzymes alanine amino transferase (ALT) and aspartate amino transferase (AST). The elevated concentration of these enzymes may suggest the presence of hepatic inflammation, which is known to impair the insulin signalling.ALT and AST concentrations were analysed using separate specific assays (elabscience Biotechnology, Wuhan) following the manufacturer's instructions. Briefly, the kits included micro plates specific for ALT or AST. The substrate solution (20µL) was added to both sample and control wells. This was followed by the addition of sample (5 µL) into the sample wells. After full agitation, the microplate was incubated at 37°C for 30 minutes. Following incubation, the relevant DNPH (20 µL) solution was added into the appropriate wells. The microplate was then incubated at 37°C for 30 minutes followed by the addition of 0.4 mol/L NaOH (200 µL) which was followed by a further incubation at room temperature for 15 minutes. Optical density (OD) was measured at 510 nm using a Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Wurttemberg, Germany). ALT and AST activity of the samples was extrapolated from the respective standard curves

2.6 Glucose utilization

Glucose utilization experiments were performed as previously described by Van de Venter *et al*., with slight modifications[53]. Liver and muscle cells were seeded in 24 well plates in their respective media. Thereafter, the plates were incubated at 37 $^{\circ}$C for 48 hours in a humidified incubator (Shel Lab, Cornelius, Oregon, USA) with 5% CO_2. The cells were allowed to attach and become confluent. To initiate the glucose utilization experiment, the confluent cell line monolayers were challenged with 19 mmol (liver) and 29 mmol (muscle) glucose. To examine the glucose utilisation effect of the crude RT extract, arjunolic acid (AA) and beta- sitostestol (BS), concentrations of 12.5, 25 and 50 µg/ml were administered separately to the cells. The cells were then incubated in DMSO (0.1%) and insulin (40 µg/mL) or metformin (16 µg/mL) which served as untreated (DMSO) or treated (insulin or metformin) controls. Doses of insulin (40 µg/mL) or metformin (16

μg/mL) were selected on the basis of toxicity studies that were previously conducted in our laboratory for the dose-response trial.

Each treatment was conducted in 6 separate wells. Media glucose concentration was measured at 0, 12, 24 and 48 hours with the OneTouch select glucometer (Lifescan, Mosta, Malta, and United Kingdom). After the 48 hours period, cells were harvested for measurements of glycogen, GLUT4 and glycogen synthase concentration. Media from the liver cells was also harvested for the analysis of alanine amino transferase (ALT) and aspartate amino transferase (AST) concentrations. To examine the effects of RT/AA/BS on ALT and AST concentration, a concentration of 25.5 μg/mL of RT/BS/AA was used.

2.7 Biochemical analysis

2.7.1 Glycogen concentration

Glycogen assay was performed using a well-established laboratory protocol [29, 32]. The harvested muscle and liver cells were heated with KOH (30%, 2 mL) at 100ºC for 30 minutes. Thereafter, $NaSO_4$ (10%, 0.194 mL) was added to stop the reaction and the cells were allowed to cool. For glycogen precipitation, the cooled mixture (200 μL) was aspirated and mixed with ethanol (95%, 200 μL). The precipitated glycogen was pelleted, washed and resolubilized in H_2O (1 mL). Thereafter, anthrone (0.5g dissolved in 250ml of sulphuric acid, 4 mL) was added and boiled for 10 minutes. After cooling, the absorbance was read using the Spectrostar Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Württemberg, Germany) at 620 nm. The glycogen concentration was calculated from the glycogen standard curve. The standards ranged from 200 to 1000 mg/L.

2.7.2 GLUT4 and glycogen synthase concentration

GLUT4 and glycogen synthase concentrations were analyzed using separate specific sandwich ELISA kits (Elabscience Biotechnology, Wuhan, USA) following the manufacturer's instructions. Briefly, the kits included micro ELISA plates which were pre-coated with antibody specific to GLUT 4 or glycogen synthase. Standards and samples were added into the appropriate wells of the micro ELISA plate and incubated for 90 minutes at 37 °C .This was followed by the addition of the relevant biotinylated detection antibody (100 µL). After incubating for 1 hour, avidin-horseradish peroxidase conjugate (100µL) was added to each micro-plate well. After incubating for 30 minutes at 37 °C the unbound components were washed away. Briefly, each wells were washed 3 times with diluted HRP wash Buffer, 300 µL per well per wash, this was followed by decant and tap after each wash to remove residual buffer. The substrate solution (90 µL) was added to each micro-plate well. This was followed by a further 15 minute incubation (37 °C), after which the stop solution (50 µL) was added. Optical density (OD) was measured at 450 nm within 10 minutes using a Nano spectrophotometer (BMG Labtech, Ortenburg, Baden-Wurttemberg, Germany). The concentration of the samples was extrapolated from the respective standard curves.

2.8 Statistical analysis

Data is expressed as means ± standard error of the mean (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) followed by the Tukey-Kramer post hoc test was used. Values of $p<0.05$ indicate statistical significance.

3. Results

3.1 Cell viability

Figure1(a-f) show percentage cell viability in muscle and liver cell lines treated with *R. tridentate* (RT) /arjunolic acid (AA)/ beta sitosterol (BS) at concentrations of 12.5, 25 and 50 µg/ml over a 48 hour period. Treatment with the highest concentration (50 µg/ml) of BS decreased cell viability in muscle cells after 48 hours of treatment * (BS vs control, $p<0.05$, Figure 1c). A similar effect was present in liver cells following treatment with AA (50 µg/ml) in both liver and muscle cell lines*(AA vs control, $p<0.05$, Figure 1e and f).

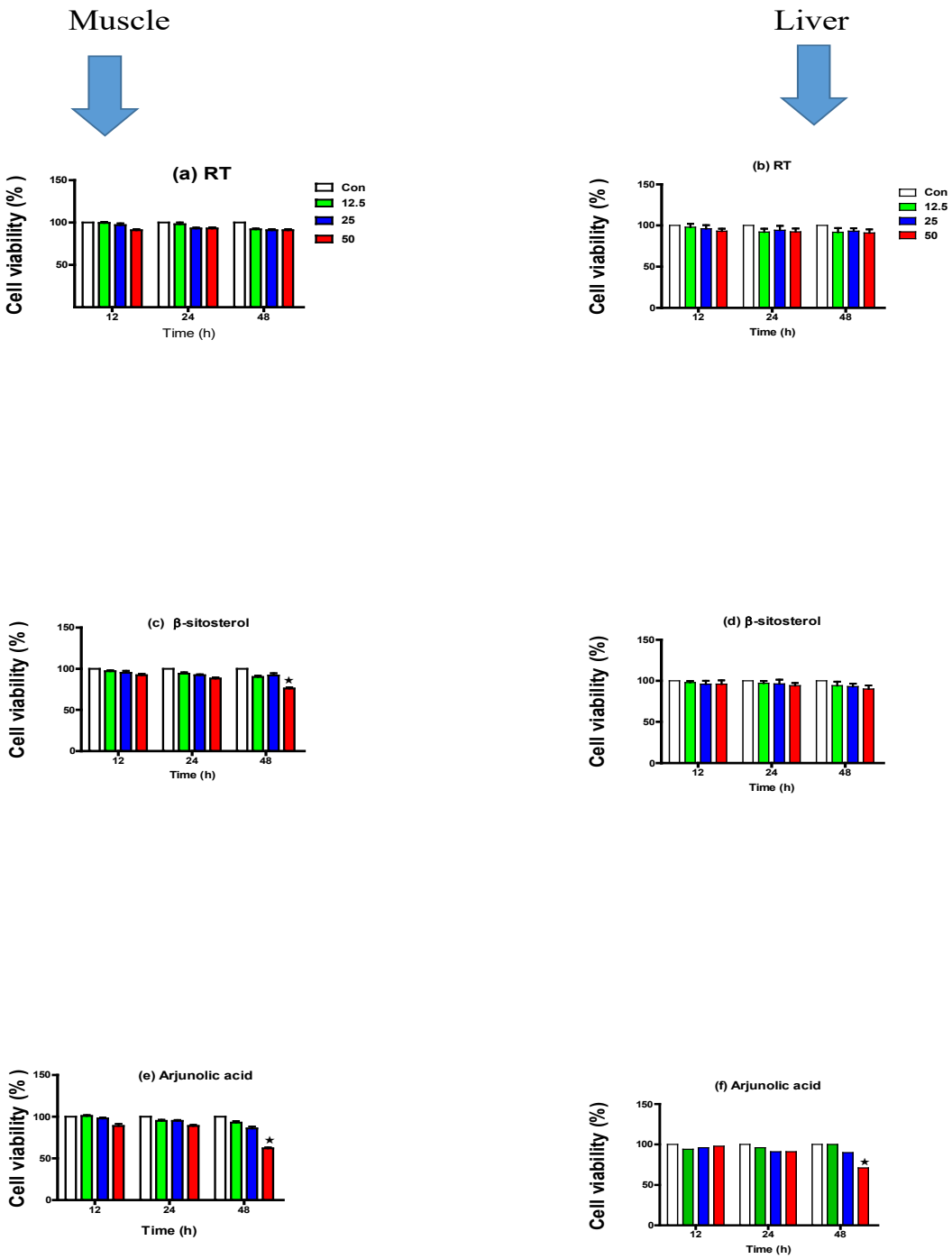

Figure 1: The effects of *R. tridentata* (RT), beta sitosterol (BS) and arjunolic acid (AA) following treatment with 12.5, 25 and 50 µg/ml concentration on cell viability (%)on muscle and liver cell lines following a 12, 24 and 48 hour incubation period. Values are presented as means ± SEM (n=6/group). * =p<0.05 when comparing treatment group to the control.

3.2 Alanine amino transferase (ALT) and aspartate amino transferase (AST)

In Table 2 We showed the effects of the administration of *R. tridentate* (RT) /arjunolic acid (AA)/ beta sitosterol (BS) (25µg/ml) as well as insulin (40 µg/m) and metformin (16 µg/mL) on liver alanine amino transferase (ALT) and aspartate amino transferase (AST) concentration. There was a treatment effect on ALT concentration following AA administration *(control vs AA, $p < 0.05$, table 2).

Table 2: The effects of *R. tridentata* (RT) /arjunolic acid (AA)/ beta sitosterol (BS) as well as insulin (40 µg/m) and metformin (16 µg/mL) on liver alanine amino transferase (ALT) and aspartate amino transferase (AST) concentrations after a 48 hour treatment period.

Experimental groups	Liver ALT (Karman unit)	Liver AST (Karman unit)
Control	1.11±0.03	2.10±0.06
RT (25µg/ml)	1.00±0.02	1.99±0.01
AA (25µg/ml)	3.02±0.00*	2.19±0.03
BS (25µg/ml)	1.21±0.10	1.89±0.05
Insulin (40 µg/m)	1.20±0.01	2.00±0.02
Metformin (16 µg/mL)	1.23±0.10	1.80±0.01

Data expressed as mean ± SEM (n=6) in each group. *=$p<0.05$ when comparing with the untreated control

3.3 Glucose utilisation

Figure 2 (a-f) show the effects of RT (25 µg/ml) / insulin (40 µg/ml) /metformin (16 µg/ml) treatment as well as arjunolic acid and beta-sitosterol at concentrations 12.5 25 and 50 µg/ml on glucose concentration in muscle and liver cell lines over a 48 hour incubation period. There was a time dependant increase in glucose utilisation following treatment with insulin / metformin in both liver and muscle cells *(insulin vs control and metformin vs control, $p<0.05$, Figure 2a and b). RT treatment also decreased media glucose concentration in both liver and muscle cells at all time points *(RT vs control, $p<0.05$, Figure 3a and b). Treatment with BS exhibited a decrease in media glucose concentration (in a dose dependent manner) at all time intervals *(BS vs control, $p<0.05$, figure 3c and d).

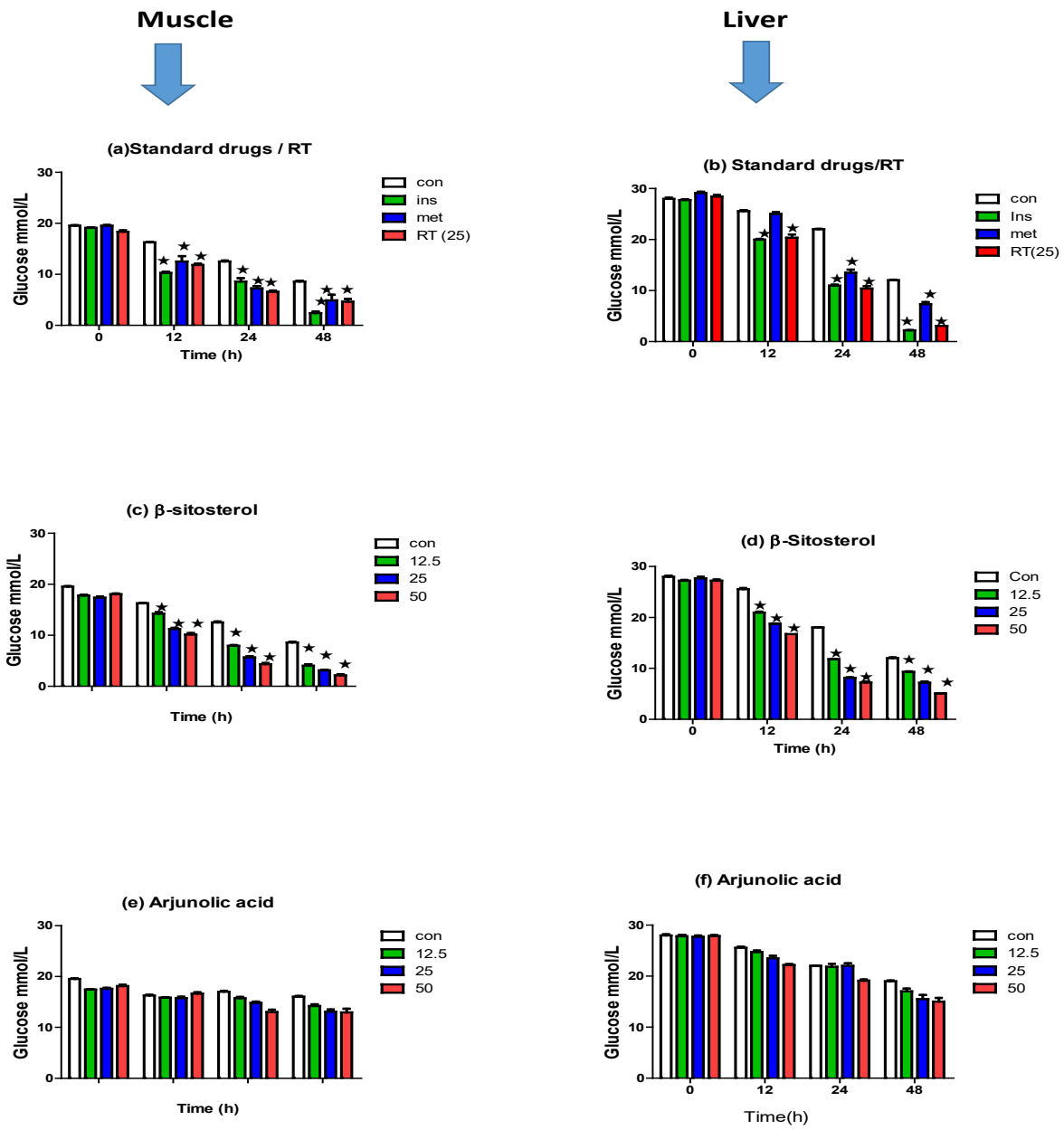

Figure 2: The effects of insulin (40 µg/ml)/metformin (16 µg/ml) /RT (25 µg/ml) and the bioactive compounds arjunolic acid (AA)/ beta sitosterol (BS) at concentrations 12.5, 25 and 50 µg/ml on glucose utilization in muscle and liver cells following 0, 12, 24 and 48 hour incubation period. * =p< 0.05 when comparing insulin/metformin/RT/AA/BS with control group at the corresponding time interval

3.4 Glycogen concentration

Table 3 shows the effects of *R. tridentata* (RT) /arjunolic acid (AA)/ beta sitosterol (BS) at a concentration of (25 mL µg/) on glycogen concentration in both liver and muscle cells after a 48 hour incubation period. The administration of metformin and insulin resulted in increased liver and muscle glycogen concentration *(metformin vs control and insulin vs control, $p<0.05$). The administration of RT/BS increased glycogen concentration in both muscle and liver cell lines *(RT vs control and BS vs control, $p<0.05$).

Table 3: Glycogen concentration in muscle and liver cell lines following treatment with RT/AA/BS (25 µg/mL) after a 48 hour treatment period. Insulin (concentration) and metformin (concentration) were used as positive controls

Groups	Muscle cells		Liver cells	
	Media Glucose (mmol/L)	Glycogen (mmol/cells)	Media Glucose (mmol/L)	Glycogen (mmol/cells)
Control	8.2±0.16	0.51±0.05	12±0.16	0.62±0.02
RT(25µg/ml)	3.9±0.90*	1.32±0.03*	2.1±0.72*	1.75±0.01*
AA (25µg/ml)	8.1±0.18	0.54±0.01	11.1±0.24	0.60±0.00
BS (25µg/ml)	2.3±0.01a*	1.68±0.00*	5.1±0.31*	1.21±0.01*
Insulin(40µg/ml)	2.4±0.13*	1.62±0.01*	2.5±0.12*	1.51±0.05*
Metformin (16µg/ml)	4.9±0.16*	1.12±0.03*	7.3±0.1*	0.99±0.03*

Data expressed as mean ± SEM (n=6) in each group. *=$p<0.05$ when comparing with the control

3.5 GLUT4 concentration

Figure 3 shows the effects of *R. tridentata* (RT) /arjunolic acid (AA)/ beta sitosterol (BS) (25 µg/ mL) on GLUT4 concentration in muscle cell lines following a 48 hour incubation period. The administration of RT and BS increased GLUT4 concentration *(RT vs control and BS vs control, $p<0.05$, Figure 3), similar to the increase seen following treatment with insulin and metformin.

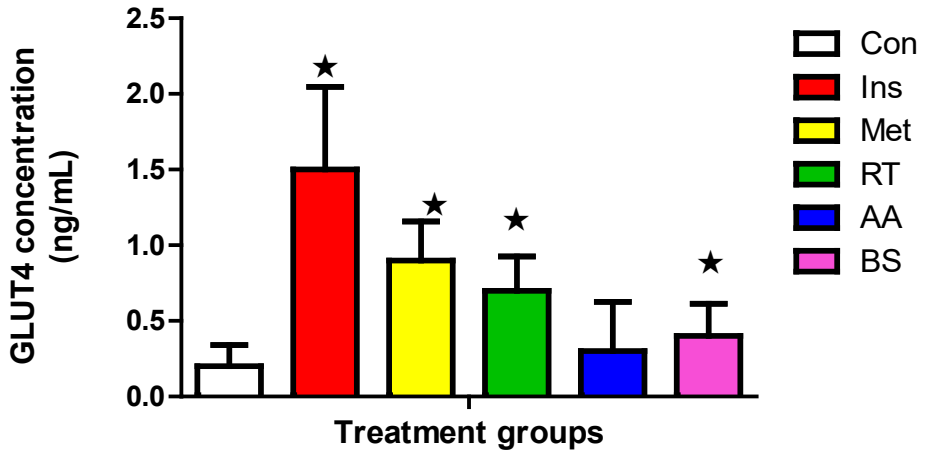

Figure 3: Effects of *R. tridentate (RT)* /arjunolic acid (AA)/ beta sitosterol (BS) at (25 µg/ mL) as well as insulin (40 µg/mL) and metformin (16 µg/mL) on GLUT 4 concentration in muscle cell line following a 48 hour treatment period. Values are presented as means ± SEM (n=6 in each group). * =$p< 0.05$ in comparison to control at 48 hours.

3.6 Glycogen synthase concentration

Figure 4 shows the effects of *R. tridentate* (RT) /arjunolic acid (AA)/ beta sitosterol (BS) (25µg/ml) as well as insulin (40 µg/ml) and metformin (16 µg/ml) on glycogen synthase concentration in muscle and liver cells after a 48 hour incubation period. There was an RT effect on glycogen synthase concentration in both the liver and muscle cells *(control vs RT, $p<0.05$, Figure 4). These effects are similar to the increase seen following insulin and metformin exposure *(control vs insulin and control vs metformin, $p<0.05$)

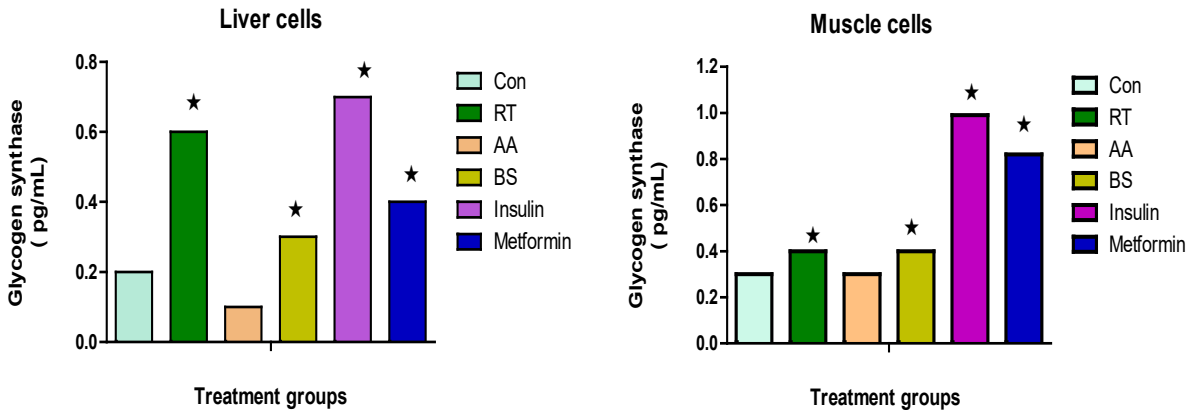

Figure 4: Effects of RT/AA/BS (25 µg/ml), insulin (40 µg/mL) and metformin (16 µg/mL) on glycogen synthase (GS) concentration in liver and muscle cells after a 48 hour incubation period. Values are presented as mean ±SEM (n=6 in each group). * =$p< 0.05$ in comparison to the control group.

4. Discussion

In this study we investigated the efficacy of *Rhoicissus tridentate* (RT) and two of its bioactive compounds beta-sitosterol (BS) and arjunolic acid (AA) on glucose metabolism using liver and muscle cell lines. The cell viability studies are crucial for determining the concentration tolerated by the cells. In this study, the concentrations used for the crude extract (RT) did not affect cell viability suggesting negligible toxicity to the liver and muscle cells. The highest concentration of AA (50 mg/mL) used resulted in decreased % cell viability suggesting toxicity in liver and muscle cell lines after a 48 hour incubation period. This decrease in the number of viable cells at this concentration may be due to an increase in oxidative stress. Studies have shown that the administration of bioactive compounds at high doses induces oxidative stress which may damage crucial cellular elements including membranes, DNA and proteins[16, 31].

Since the liver is an important site for insulin clearance and production of inflammatory cytokines[55], we confirmed the toxicity at high doses by looking at the effect treatment has on the liver enzymes alanine amino transferase (ALT) and aspartate amino transferase (AST). Studies have shown that the elevated concentration of these enzymes may also suggest the presence of inflammation which is known to impair insulin signalling [15, 55, 19, 37, 51]. Inflammation in the liver has been reported in diabetes and inflammatory markers such as ALT and AST have been shown to predict the risk of hepatic damage [51]. Hepatic inflammation causes an increase in mitochondrial oxidative stress which results in DNA mutation damage and triggers a series of deleterious effects in the mitochondrial respiratory chain by producing reactive oxygen species [58]. This vicious cycle escalates exponentially overcoming normal physiological negative feedback control thus leading to hepatocellular injury and insulin resistance[41]. In the present study, ALT was elevated following treatment with AA (25 mg/mL). This may confirm the decrease in cell viability following treatment with AA thus suggesting that AA may be toxic when administered in isolation at high concentrations.

Muscle and liver tissue play a significant role in glucose homeostasis[27]. Glucose metabolism in muscle and liver is mainly modulated by insulin[39]. In skeletal muscle, glucose uptake is mediated through the translocation of the glucose transporter GLUT4 from the interior to the cell surface, an effect stimulated by the activation of insulin receptors on the cell surface[6]. In our study, the

decrease in media glucose concentration indicated an increase in glucose uptake by the cells. In the liver control cells, the steady decline in media glucose may be attributed to the presence of a bidirectional GLUT2 which facilitates glucose uptake into the liver cells[28, 56]. Indeed, insulin administration in both liver and muscle cells increased glucose uptake as evidenced by a sharp decline in media glucose concentrations. Insulin facilitates glucose uptake in muscle and adipose tissue through GLUT4 translocation and enhanced glycogen synthesis[20]. Studies suggest that certain plant-derived products exert insulin mimetic effects[34]. We found that administration of RT increased glucose uptake in both liver and muscle cells suggesting insulin-like effects. This insulin mimetic effect was also present following treatment with BS. We attribute this effect to BS's ability to increase the expression of GLUT4 in muscle [45].The decrease in media glucose concentration in the liver may be attributed to BS's ability to inhibit mitochondrial glycerophosphate dehydrogenase (mGPD) therefore decreasing the conversion of lactate and pyruvate to glucose which results in decreased gluconeogenesis[14]. It has been reported that skeletal muscle expression of GLUT4 in type 2 diabetes patients is significantly reduced, as evidenced by the patients' decreased capability to process glucose[24]. In this study, we have shown that treatment with RT or BS increased GLUT4 concentration which is critical for glucose uptake and this effect may be of benefit in counteracting insulin resistance seen in type 2 and gestational diabetes. This may be attributed to the activation of the AMPK signalling pathway which has been shown to increase glucose uptake through increased GLUT4 translocation, hexokinase concentration and enhanced glycogen synthesis via the Akt pathway[38, 47]. Apart from the AMPK signalling pathway, anti-hyperglycaemic drugs such as thiozolidinediones interact with peroxisome proliferator-activated receptor-gamma (PPARγ) to promote glucose disposal in muscle and adipose tissue[49]. The improvement in glucose homeostasis is essential and is regarded as the primary intervention in the management of diabetes in order to delay the onset or progression of diabetes complications[49, 54]. In addition to insulin, conventional anti-hyperglycaemic drugs such as metformin and rosiglitazone achieve glucose lowering effects through enhancing glucose disposal in liver and skeletal muscle[26, 30]. Although metformin aims to enhance insulin sensitivity, it also promotes glucose uptake in the liver and skeletal muscle through the AMPK pathway[50]. This explains the increase in glucose utilisation observed in metformin treated cells in the present study.

After observing improved glucose disposal following RT and BS administration, we further investigated the effect of RT/AA/BS on glycogen synthesis. In the absence of insulin, glycogen synthesis in the liver and skeletal muscle has been shown to be compromised[9]. Indeed, untreated muscle and control liver cells showed poor glycogen synthesis. Furthermore, these observations correlated with reduced glycogen synthase concentration in control liver cells. The administration of insulin increased glycogen and glycogen synthase concentration. We also found an increase in glycogen synthesis following RT or BS administration in liver and muscle cells. The increase in glycogen synthesis may be partly mediated via glycogen synthase expression which was also found to be elevated in liver cells. Crude extracts of *Sclerocarya birrea and C. edulis* (khat) have been shown to stimulate glycogen synthesis both *in vivo* and *in vitro*[1, 13 17]. These plants together with their bioactive compounds may also directly enhance the enzyme activity of glycogen synthase[40]. Previous studies have shown a correlation between glycogen synthesis and glycogen synthase expression *in vitro* which we also observed in this study. Since the causal link of diabetes mellitus and associated complications is hyperglycaemia, the ability of an agent to efficiently lower blood glucose concentration or facilitate glucose disposal is of importance in diabetes therapy. The current study showed that RT and its bioactive compound BS exhibit insulin mimetic effects as evidenced by the increase in glucose utilisation. The ability of RT and BS to increase glucose utilization may be ascribed to an increase in glycogen synthesis and GLUT 4 expression. Taken together these observations suggest that RT and its bioactive compound BS have the ability to promote glucose uptake and activate glycogenesis in the liver and muscle cells in a manner comparable to insulin. This suggests that RT and BS may be effective in improving glycaemic control in diabetes. As both RT and BS were shown to be non-toxic to the cells, further development on RT and BS as alternative therapy for diabetes mellitus may be warranted.

Declaration

All the authors declare that the results presented in this paper have not been published previously in whole or part.

Funding source

The study was funded by the National Research Foundation South Africa and University of KwaZulu Natal, collage of health Sciences

Conflict of interests: None.

References

1 Afolayan AJ, Sunmonu TO. In vivo studies on antidiabetic plants used in South African herbal medicine. Journal of clinical biochemistry and nutrition 2010; 47: 98-106.

2 Andrade-Cetto A, Heinrich M. Mexican plants with hypoglycaemic effect used in the treatment of diabetes. Journal of ethnopharmacology 2005; 99: 325-348.

3 Attah AF, O'Brien M, Koehbach J, Sonibare MA, Moody JO, Smith TJ *et al*. Uterine contractility of plants used to facilitate childbirth in Nigerian ethnomedicine. Journal of ethnopharmacology 2012; 143: 377-382.

4 Bailey CJ, Day C. Traditional plant medicines as treatments for diabetes. Diabetes care 1989; 12: 553-564.

5 Bryant AT. *Zulu medicine and medicine-men*. C. Struik, 1966.

6 Bryant NJ, Govers R, James DE. Regulated transport of the glucose transporter GLUT4. Nature reviews Molecular cell biology 2002; 3: 267.

7 Carson JL, Scholz PM, Chen AY, Peterson ED, Gold J, Schneider SH. Diabetes mellitus increases short-term mortality and morbidity in patients undergoing coronary artery bypass graft surgery. Journal of the American College of Cardiology 2002; 40: 418-423.

8 Chauhan A, Sharma P, Srivastava P, Kumar N, Dudhe R. Plants having potential antidiabetic activity: a review. Der Pharmacia Lettre 2010; 2: 369-387.

9 Cline GW, Petersen KF, Krssak M, Shen J, Hundal RS, Trajanoski Z *et al*. Impaired glucose transport as a cause of decreased insulin-stimulated muscle glycogen synthesis in type 2 diabetes. New England Journal of Medicine 1999; 341: 240-246.

10 Diel P, Baadners D, Schlupmann K, Velders M, Schwarz JP. C2C12 myoblastoma cell differentiation and proliferation is stimulated by androgens and associated with a modulation of myostatin and Pax7 expression. Journal for molecular endocrinology 2008; 40

231–241: 231–241.

11 Dube SC. Contractile Effects of Gunnera Perpensa and Rhoicissus Tridentata Bioactive Extracts in Isolated Rat Uterine Muscles, University of KwaZulu-Natal, Durban, 2014.

12 Edeoga H, Okwu D, Mbaebie B. Phytochemical constituents of some Nigerian medicinal plants. African journal of biotechnology 2005; 4: 685-688.

13 Erasto P, Adebola P, Grierson D, Afolayan A. An ethnobotanical study of plants used for the treatment of diabetes in the Eastern Cape Province, South Africa. African Journal of Biotechnology 2005; 4.

14 Ferrannini E. The target of metformin in type 2 diabetes. New England Journal of Medicine 2014; 371: 1547-1548.

15 Freeman DJ, Norrie J, Caslake MJ, Gaw A, Ford I, Lowe GD *et al*. C-reactive protein is an independent predictor of risk for the development of diabetes in the West of Scotland Coronary Prevention Study. Diabetes 2002; 51: 1596-1600.

16 Gaetke LM, Chow CK. Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology 2003; 189: 147-163.

17 Goldwaser I, Gefel D, Gershonov E, Fridkin M, Shechter Y. Insulin-like effects of vanadium: basic and clinical implications. Journal of Inorganic Biochemistry 2000; 80: 21-25.

18 Gupta R, Sharma AK, Dobhal M, Sharma M, Gupta R. Antidiabetic and antioxidant potential of β-sitosterol in streptozotocin-induced experimental hyperglycemia. Journal of diabetes 2011; 3: 29-37.

19 Jan M, Virtue AT, Pansuria M, Liu J, Xiong X, Fang P *et al*. The role of immunogenicity in cardiovascular disease. World heart journal 2011; 3: 1.

20 Khan A, Pessin J. Insulin regulation of glucose uptake: a complex interplay of intracellular signalling pathways. Diabetologia 2002; 45: 1475-1483.

21 Kubo Y. Comparison of initial stages of muscle differentiation in rat and mouse myoblastic and mouse mesodermal stem cell lines. The Journal of physiology 1991; 442: 743-759.

22 Kühn C, Arapogianni NE, Halabalaki M, Hempel J, Hunger N, Wober J *et al*. Constituents from cistus salvifolius (cistaceae) activate peroxisome proliferator-activated receptor-γ but Not-δ and stimulate glucose uptake by adipocytes. Planta medica 2011; 77: 346-353.

23 Langer O. Management of gestational diabetes. Clinical obstetrics and gynecology 2000; 43: 106-115.

24 Maier V, Gould G. Long-term insulin treatment of 3T3-L1 adipocytes results in mis-targeting of GLUT4: implications for insulin-stimulated glucose transport. Diabetologia 2000; 43: 1273-1281.

25 Malviya N, Jain S, Malviya S. Antidiabetic potential of medicinal plants. Acta Pol Pharm 2010; 67: 113-118.

26 Mayerson AB, Hundal RS, Dufour S, Lebon V, Befroy D, Cline GW *et al*. The effects of rosiglitazone on insulin sensitivity, lipolysis, and hepatic and skeletal muscle triglyceride content in patients with type 2 diabetes. Diabetes 2002; 51: 797-802.

27 Meyer C, Dostou JM, Welle SL, Gerich JE. Role of human liver, kidney, and skeletal muscle in postprandial glucose homeostasis. American Journal of Physiology-Endocrinology And Metabolism 2002; 282: E419-E427.

28 Mokuno T, Uchimura K, Hayashi R, Hayakawa N, Makino M, Nagata M *et al*. Glucose transporter 2 concentrations in hyper-and hypothyroid rat livers. Journal of Endocrinology 1999; 160: 285-289.

29 Musabayane CT, Mahlalela N, Shode FO, Ojewole JAO. Effects of Syzygium cordatum (Hochst.) [Myrtaceae] leaf extract on plasma glucose and hepatic glycogen in streptozotocin-induced diabetic rats. Journal of Ethnopharmacology 2005; 97: 485-490.

30 Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O *et al*. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. Diabetes 2002; 51: 2074-2081.

31 Nel A, Xia T, Mädler L, Li N. Toxic potential of materials at the nanolevel. science 2006; 311: 622-627.

32 Ngubane PS, Musabayane CT, Masola B. The effects of *Syzygium aromaticum*-derived oleanolic acid on glycogenic enzymes in streptozotocin-induced diabetic rats. Ren Fail 2011; 33: 434-439.

33 Ogbe FMD, Eruogun OL, Uwagboe M. Plants used for female reproductive health care in Oredo local government area, Nigeria. Scientific Research and Essays 2009; 4: 120-130.

34 Patel D, Kumar R, Laloo D, Hemalatha S. Natural medicines from plant source used for therapy of diabetes mellitus: An overview of its pharmacological aspects. Asian Pacific Journal of Tropical Disease 2012; 2: 239-250.

35 Patel D, Prasad S, Kumar R, Hemalatha S. An overview on antidiabetic medicinal plants having insulin mimetic property. Asian Pacific journal of tropical biomedicine 2012; 2: 320-330.

36 Rahmatullah M, Azam NK, Khatun Z, Seraj S, Islam F, Rahman A *et al*. Medicinal plants used for treatment of diabetes by the Marakh sect of the Garo tribe living in Mymensingh district, Bangladesh. African Journal of Traditional, Complementary and Alternative Medicines 2012; 9: 380-385.

37 Ridker PM, Nader R, Rifai N, Rose L. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. J Engl Med 2002; 347: 1157-1165.

38 Sakamoto K, Holman GD. Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. American journal of physiology-endocrinology and metabolism 2008; 295: E29-E37.

39 Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 2001; 414: 799.

40 Shechter Y, Goldwaser I, Mironchik M, Fridkin M, Gefel D. Historic perspective and recent developments on the insulin-like actions of vanadium; toward developing vanadium-based drugs for diabetes. Coordination chemistry reviews 2003; 237: 3-11.

41 Sodhi C, Rana S, Mehta S, Vaiphei K, Attari S, Mehta S. Study of oxidative-stress in isoniazid-rifampicin induced hepatic injury in young rats. Drug and chemical toxicology 1997; 20: 255-269.

42 Soriguer F, Goday A, Bosch-Comas A, Bordiú E, Calle-Pascual A, Carmena R *et al*. Prevalence of diabetes mellitus and impaired glucose regulation in Spain: the Di@ bet. es Study. Diabetologia 2012; 55: 88-93.

43 Strick-Marchand H, Weiss MC. Embryonic liver cells and permanent lines as models for hepatocyte and bile duct cell differentiation. Mechanisms of development 2003; 120: 89-98.

44 Stumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: principles of pathogenesis and therapy. The Lancet 2005; 365: 1333-1346.

45 Sujatha S, Anand S, Sangeetha K, Shilpa K, Lakshmi J, Balakrishnan A *et al*. Biological evaluation of (3β)-STIGMAST-5-EN-3-OL as potent anti-diabetic agent in regulating

glucose transport using in vitro model. International Journal of Diabetes Mellitus 2010; 2: 101-109.

46 Tahraoui A, El-Hilaly J, Israili Z, Lyoussi B. Ethnopharmacological survey of plants used in the traditional treatment of hypertension and diabetes in south-eastern Morocco (Errachidia province). Journal of ethnopharmacology 2007; 110: 105-117.

47 Taylor EB, An D, Kramer HF, Yu H, Fujii NL, Roeckl KS *et al*. Discovery of TBC1D1 as an insulin-, AICAR-, and contraction-stimulated signaling nexus in mouse skeletal muscle. Journal of Biological Chemistry 2008; 283: 9787-9796.

48 Tieu J, Coat S, Hague W, Middleton P. Oral anti-diabetic agents for women with pre-existing diabetes mellitus/impaired glucose tolerance or previous gestational diabetes mellitus. Cochrane Database of Systematic Reviews 2009.

49 Tomić-Naglić D, Mitrović M, Novaković-Paro J, Pejin R, Popović ĐS, Pejaković S *et al*. Impact of currently used oral antihyperglycemic drugs on dysfunctional adipose tissue. Srp Arh Celok Lek 2017; 145: 643-645.

50 Towler MC, GrahameHardie D. AMP-activated protein kinase in metabolic control and insulin signaling. Circulation research 2007.

51 Tsuruda T, Jougasaki M, Boerrigter G. Cardiotrophin-1 stimulation of cardiac fibroblast growth: roles for glycoprotein 130/leukemia inhibitory factor receptor and the endothelin type A receptor. Circ Res 2002; 90: 128–134.

52 Tzoulaki I, Molokhia M, Curcin V, Little MP, Millett CJ, Ng A *et al*. Risk of cardiovascular disease and all cause mortality among patients with type 2 diabetes prescribed oral

antidiabetes drugs: retrospective cohort study using UK general practice research database. Bmj 2009; 339: b4731.

53 van de Venter M, Roux S, Bungu LC, Louw J, Crouch NR, Grace OM *et al*. Antidiabetic screening and scoring of 11 plants traditionally used in South Africa. Journal of ethnopharmacology 2008; 119: 81-86.

54 Viollet B, Lantier L, Devin-Leclerc J, Hébrard S, Amouyal C, Mounier R *et al*. Targeting the AMPK pathway for the treatment of Type 2 diabetes. Frontiers in bioscience (Landmark edition) 2009; 14: 3380.

55 Wannamethee SG, Shaper AG, Lennon L, Whincup PH. Hepatic enzymes, the metabolic syndrome, and the risk of type 2 diabetes in older men. Diabetes care 2005; 28: 2913-2918.

56 Weinstein SP, O'Boyle E, Fisher M, Haber RS. Regulation of GLUT2 glucose transporter expression in liver by thyroid hormone: evidence for hormonal regulation of the hepatic glucose transport system. Endocrinology 1994; 135: 649-654.

57 Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes care 2004; 27: 1047-1053.

58 Yakes FM, Van Houten B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proceedings of the National Academy of Sciences 1997; 94: 514-519.

59 Yki-Järvinen H, Helve E, Koivisto VA. Hyperglycemia decreases glucose uptake in type I diabetes. Diabetes 1987; 36: 892-896.

CHAPTER 4

4.1 Synthesis

Poor myometrium contractility is associated with significant foetal as well as maternal morbidity and mortality. Scientific reports indicate that most of the pregnancy-related complications such as pre-term and post-term labour as well as post-partum haemorrhage mainly result from poor uterine muscle contractility, implying that uterine contractions play a vital role in childbirth. Currently, exogenous oxytocin is used to aid myometrium contractions to relieve post-term labour. Although oxytocin has been shown to yield positive outcomes, some limitations still exist. Additionally, poor myometrium contractility has been shown to co-exist with gestational diabetes in some pregnancies. Traditionally, medicinal plants such as *Rhoicissus tridentate* (RT) have been used to promote labour induction and to attenuate hyperglycaemia. However, less has been done scientifically to ascertain the veracity of these claims. Accordingly, in this study we sought to investigate uterotonic effects of RT and its bioactive ingredients beta-sitesterol (BS) and arjunolic acid (AA) using uterine strips *in-vitro*. We also looked at the possible mechanism(s) of action by which RT and its bioactive compounds achieved the uterotonic effects observed. Since it has been shown in some studies that poor myometrium contractility is accompanied by hyperglycaemia, we further explored the glucose lowering effects of RT and its bioactive compounds using muscle (C2C12) and liver (Chang) cell lines *in-vitro*. We envisage that the outcomes of this study may assist in expanding the current literature regarding the beneficial therapeutic properties of RT and associated bioactive compounds in pregnancy associated complications.

In the current study we evaluated the effects of *Rhoicissus tridentate* (RT) and its bioactive compounds (BS and AA) on force and rate of uterine muscle contraction. The positive force-rate relationship is crucial to promote labour and partuition. The positive force-rate relationship observed following treatment with RT or BS is of paramount importance in regulating contractility. The myometrium contraction is mediated via oxytocin and PGF2α receptors. We therefore investigated the mechanisms of action RT and its bioactive compounds (AA and BS) by

studying the expression of oxytocin and PGF2α receptor concentration. We showed that the RT crude plant extract resulted in decreased oxytocin receptor concentration, indicating that RT may in part be exerting its uterotonic effect through oxytocin receptor activation. Taken together, the observations from this study suggest that RT and its associated bioactive compounds promote uterine muscle contraction. Moreover, studies have shown that conventional drugs can be used in combination with other uterotonic drugs to promote efficacy. Oxytocin has been shown to be more effective when combined with other uterotonic drugs in ameliorating pregnancy complications. Indeed, the combination treatment with oxytocin and RT or oxytocin and BS resulted in synergism of the force and rate of contraction.

Since diabetic patients often present with complications during labour resulting from poor uterine muscle contractility, we investigated the effects of RT, BS and AA on glucose utilization in liver and muscle cell lines. Hyperglycaemia is a serious health threat, especially during pregnancy. Therefore, adequate glycaemic control is critical for preventing the onset and progression of all possible disturbances that may delay labour and child birth. The observations in this study show that RT and BS have beneficial effects on glucose handling by promoting glucose utilisation in cell lines. In the present study, we were also interested in whether RT and its bioactive compounds exhibited toxicity on the cell lines. Therefore, in addition to the cell viability test, we also measured ALT and AST concentration in the liver cell line. We showed that AA possesses toxic effects on the liver cells, as evidenced by increased ALT concentrations following treatment with AA.

In skeletal muscle, glucose uptake is mediated by GLUT 4. A decrease in GLUT 4 expression has been reported in type 2 diabetes mellitus. In the present study, treatment with RT or BS increased GLUT 4 expression and decreased media glucose concentration. This process may be of benefit in counteracting insulin resistance observed in type 2 and in gestational diabetes thus preventing the onset of diabetes associated complications. We also investigated the effects of RT/BS/AA treatment on glycogen synthesis. Physiologically, glycogen synthase expression is modulated by insulin or AMPK through PI3-K activation which further activates GS3-K to enhance GS 57 expression for glycogen synthesis. As in previous studies on glucose metabolism, we were able to show that there is a correlation between glycogen synthesis and glycogen synthase expression.

The overall observations of the study suggest that treatment with RT/BS/AA is associated with uterotonic and anti-hyperglycaemic properties. These observations taken together suggest that RT and BS may be beneficial in managing pregnancy related complications as evidenced by its ability to promote myometrium contraction and glucose disposal in the liver and skeletal muscle cells.

4.2 Conclusions

Our results suggest that the RT crude extract and its associated bioactive compounds BS and AA promote uterine muscle contraction and glucose disposal in the liver and skeletal muscle cells. Taken together, further developments and evaluation may be necessary on RT as a potential remedy for gestational diabetes and labour-related complications

4.3 Recommendations

Further studies are required to elucidate the mechanisms of action of RT extracts and associated bioactive compounds on glucose metabolism and possible uterotonic activity. Moreover, detailed studies on RT/BS/AA using different doses on animal models are needed before reaching a clear-cut conclusion. Further research developments to refine the extraction of bioactive compounds from medicinal plants could lead to improved pharmaceutical products.

CHAPTER 5

References

1. Cowett, R.M., *Principles of perinatal-neonatal metabolism*. 2012: Springer Science & Business Media.
2. Angueira, A.R., et al., *New insights into gestational glucose metabolism: lessons learned from 21st century approaches.* Diabetes, 2015. **64**(2): p. 327-334.
3. Baschat, D.A.A., *Fetal responses to placental insufficiency: an update.* BJOG: An International Journal of Obstetrics & Gynaecology, 2004. **111**(10): p. 1031-1041.
4. Girard, J.R., et al., *Fetal metabolic response to maternal fasting in the rat.* American Journal of Physiology-Endocrinology and Metabolism, 1977. **232**(5): p. E456.
5. Saltiel, A.R. and C.R. Kahn, *Insulin signalling and the regulation of glucose and lipid metabolism.* Nature, 2001. **414**(6865): p. 799.
6. Catalano, P. and T. Buchanan, *Metabolic changes during normal and diabetic pregnancies.* Diabetes in women: adolescence, pregnancy and menopause. Lippincott Williams & Wilkins, Philadelphia, Pa, 2004: p. 129-145.
7. Ural, S.H. and J.T. Repke, *Gestational diabetes mellitus.* Reviews in Obstetrics and Gynecology, 2008. **1**(3): p. 129.
8. Livingstone, C. and M. Collison, *Sex steroids and insulin resistance.* Clinical Science, 2002. **102**(2): p. 151-166.
9. Casals-Casas, C. and B. Desvergne, *Endocrine disruptors: from endocrine to metabolic disruption.* Annual review of physiology, 2011. **73**: p. 135-162.

10. Boinpally, T. and L. Jovanovič, *Management of type 2 diabetes and gestational diabetes in pregnancy.* Mount Sinai Journal of Medicine: A Journal of Translational and Personalized Medicine, 2009. **76**(3): p. 269-280.
11. Anabela, P., Rolo, Carlos, M., Palmeira, *Diabetes and mitochondrial function: Role of hyperglycemia and oxidative stress.* Toxicology and Applied Pharmacology, 2006. **212**: p. 167-178.
12. Bechmann, L.P., et al., *The interaction of hepatic lipid and glucose metabolism in liver diseases.* Journal of hepatology, 2012. **56**(4): p. 952-964.
13. Ruderman, N.B., *Muscle amino acid metabolism and gluconeogenesis.* Annual review of medicine, 1975. **26**(1): p. 245-258.
14. Durrington, P.N., et al., *Effects of insulin and glucose on very low density lipoprotein triglyceride secretion by cultured rat hepatocytes.* The Journal of clinical investigation, 1982. **70**(1): p. 63-73.
15. James, C. and K. Hussain, *Hypoglycemia*, in *Textbook of Clinical Pediatrics*. 2012, Springer. p. 3803-3817.
16. Williamson, J.R., R. Kreisberg, and P. Felts, *Mechanism for the stimulation of gluconeogenesis by fatty acids in perfused rat liver.* Proceedings of the National Academy of Sciences, 1966. **56**(1): p. 247-254.
17. Postic, C., R. Dentin, and J. Girard, *Role of the liver in the control of carbohydrate and lipid homeostasis.* Diabetes & metabolism, 2004. **30**(5): p. 398-408.
18. Kibiti, C.M. and A.J. Afolayan, *Herbal therapy: A review of emerging pharmacological tools in the management of diabetes mellitus in Africa.* Pharmacognosy magazine, 2015. **11**(Suppl 2): p. S258.
19. Kibiti, C.M., *Evaluation of the medicinal potentials of Bulbine abyssinica A. Rich in the management of diabetes mellitus in the Eastern Cape, South Africa*. 2016, University of Fort Hare.
20. Sinacore, D.R. and E.A. Gulve, *The role of skeletal muscle in glucose transport, glucose homeostasis, and insulin resistance: implications for physical therapy.* physical therapy, 1993. **73**(12): p. 878-891.
21. Wollheim, C.B. and G. Sharp, *Regulation of insulin release by calcium.* Physiological reviews, 1981. **61**(4): p. 914-973.

22. Koves, T.R., et al., *Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance.* Cell metabolism, 2008. **7**(1): p. 45-56.
23. Lund, S., et al., *Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin.* Proceedings of the National Academy of Sciences, 1995. **92**(13): p. 5817-5821.
24. Michael, L.F., et al., *Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1.* Proceedings of the National Academy of Sciences, 2001. **98**(7): p. 3820-3825.
25. Boyle, K.E., et al., *Gestational diabetes is characterized by reduced mitochondrial protein expression and altered calcium signaling proteins in skeletal muscle.* PloS one, 2014. **9**(9): p. e106872.
26. Babu, P.V.A., D. Liu, and E.R. Gilbert, *Recent advances in understanding the anti-diabetic actions of dietary flavonoids.* The Journal of nutritional biochemistry, 2013. **24**(11): p. 1777-1789.
27. Zhang, C.-Y., et al., *Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, β cell dysfunction, and type 2 diabetes.* Cell, 2001. **105**(6): p. 745-755.
28. Prossnitz, E.R. and M. Barton, *Signaling, physiological functions and clinical relevance of the G protein-coupled estrogen receptor GPER.* Prostaglandins & other lipid mediators, 2009. **89**(3-4): p. 89-97.
29. Al-Qahtani, S., et al., *Diabetes is associated with impairment of uterine contractility and high Caesarean section rate.* Diabetologia, 2012. **55**(2): p. 489-498.
30. White, P., *Diabetes complicating pregnancy.* American Journal of Obstetrics and Gynecology, 1937. **33**(3): p. 380-385.
31. Fu, X., et al., *Prostaglandin F2α-induced Ca++ oscillations in human myometrial cells and the role of RU 486.* American Journal of Obstetrics and Gynecology, 2000. **182**(3): p. 582-588.
32. Gemzell-Danielsson, K., M. Bygdeman, and A. Aronsson, *Studies on uterine contractility following mifepristone and various routes of misoprostol.* Contraception, 2006. **74**(1): p. 31-35.

33. Ulloa-Aguirre, A., et al., *Structure-Activity Relationships of G Protein-Coupled Receptors.* Archives of Medical Research, 1999. **30**(6): p. 420-435.
34. Manning, M., et al., *Peptide and non-peptide agonists and antagonists for the vasopressin and oxytocin V1a, V1b, V2 and OT receptors: research tools and potential therapeutic agents*, in *Progress in Brain Research*, D.N. Inga and L. Rainer, Editors. 2008, Elsevier. p. 473-512.
35. Du, J., et al., *Ligustilide inhibits spontaneous and agonists- or K+ depolarization-induced contraction of rat uterus.* Journal of Ethnopharmacology, 2006. **108**(1): p. 54-58.
36. Loch-Caruso, R., *Uterine muscle as a potential target of polychlorinated biphenyls during pregnancy.* International Journal of Hygiene and Environmental Health, 2002. **205**(1–2): p. 121-130.
37. Altura, B., et al., *Mg2+–Ca2+ interaction in contractility of vascular smooth muscle: Mg2+ versus organic calcium channel blockers on myogenic tone and agonist-induced responsiveness of blood vessels.* Canadian journal of physiology and pharmacology, 1987. **65**(4): p. 729-745.
38. Sanborn, B.M., *Hormones and calcium: mechanisms controlling uterine smooth muscle contractile activity.* Experimental physiology, 2001. **86**(2): p. 223-237.
39. Gimpl, G. and F. Fahrenholz, *The oxytocin receptor system: structure, function, and regulation.* Physiological reviews, 2001. **81**(2): p. 629-683.
40. Katori, M. and M. Majima, *Cyclooxygenase-2: its rich diversity of roles and possible application of its selective inhibitors.* Inflammation Research, 2000. **49**(8): p. 367-392.
41. Baldwin, D.M. and G.H. Stabenfeldt, *Endocrine changes in the pig during late pregnancy, parturition and lactation.* Biology of reproduction, 1975. **12**(4): p. 508-515.
42. Hindson, J., B.M. SCHOFIELD, and W. Ward, *The effect of progesterone on recorded parturition and on oxytocin sensitivity in the sheep.* Journal of Endocrinology, 1969. **43**(2): p. 207-215.
43. Karim, S., *Appearance of prostaglandin F2α in human blood during labour.* Br Med J, 1968. **4**(5631): p. 618-621.
44. Parkington, H.C., et al., *Hyperpolarization and slowing of the rate of contraction in human uterus in pregnancy by prostaglandins E2 and F2α: involvement of the Na+ pump.* The Journal of physiology, 1999. **514**(1): p. 229-243.

45. Fischer, D.P., *The influence of the hormonal milieu on functional prostaglandin and oxytocin receptors and their downstream signal pathways in isolated human myometrium*. 2010, University of Bradford.
46. Bener, A., N.M. Saleh, and A. Al-Hamaq, *Prevalence of gestational diabetes and associated maternal and neonatal complications in a fast-developing community: global comparisons.* International journal of women's health, 2011. **3**: p. 367.
47. Arrowsmith, S., S. Wray, and S. Quenby, *Maternal obesity and labour complications following induction of labour in prolonged pregnancy.* BJOG: An International Journal of Obstetrics & Gynaecology, 2011. **118**(5): p. 578-588.
48. Ferrara, A., *Increasing prevalence of gestational diabetes mellitus.* Diabetes care, 2007. **30**(Supplement 2): p. S141-S146.
49. Di Cianni, G., et al., *Intermediate metabolism in normal pregnancy and in gestational diabetes.* Diabetes/metabolism research and reviews, 2003. **19**(4): p. 259-270.
50. Hu, L., et al., *Detection of placental growth hormone variant and chorionic somatomammotropin-L RNA expression in normal and diabetic pregnancy by reverse transcriptase-polymerase chain reaction.* Molecular and cellular endocrinology, 1999. **157**(1-2): p. 131-142.
51. Gupta, P.D. and A. De, *Diabetes mellitus and its herbal treatment.* International Journal of Research in Pharmaceutical and Biomedical Sciences, 2012. **3**(2): p. 706-721.
52. Meier, S. and P. Wright, *The induction of parturition in the bitch using sodium cloprostenol.* Theriogenology, 2000. **54**(3): p. 457-465.
53. Abdollahi, M., O. Tabatabaei-Malazy, and B. Larijani, *A systematic review of in vitro studies conducted on effect of herbal products on secretion of insulin from Langerhans islets.* Journal of Pharmacy & Pharmaceutical Sciences, 2012. **15**(3): p. 447-466.
54. Veale, D., et al., *Discrimination between the functional and biochemical effects of two herbal oxytocics on the rat myometrium.* Journal of Pharmacy and Pharmacology, 2001. **53**(8): p. 1145-1151.
55. Gruber, C.W. and M. O'Brien, *Uterotonic plants and their bioactive constituents.* Planta medica, 2011. **77**(03): p. 207-220.

56. Khathi, A., et al., *Effects of Syzygium aromaticum-derived triterpenes on postprandial blood glucose in streptozotocin-induced diabetic rats following carbohydrate challenge.* PLoS One, 2013. **8**(11): p. e81632.
57. Madlala, H.P., et al., *Changes in renal function and oxidative status associated with the hypotensive effects of oleanolic acid and related synthetic derivatives in experimental animals.* PloS one, 2015. **10**(6): p. e0128192.
58. Offenbacher, S., et al., *Potential pathogenic mechanisms of periodontitis-associated pregnancy complications.* Annals of periodontology, 1998. **3**(1): p. 233-250.
59. Gonik, B. and R.K. Creasy, *Preterm labor: its diagnosis and management.* American Journal of Obstetrics & Gynecology, 1986. **154**(1): p. 3-8.
60. Marchant, T., et al., *Neonatal mortality risk associated with preterm birth in East Africa, adjusted by weight for gestational age: individual participant level meta-analysis.* PLoS medicine, 2012. **9**(8): p. e1001292.
61. Atiah, F.A., *Contribution of Very Low Birth Weight to Preterm Neonatal Mortality at 37 Military Hospital, Accra*. 2016, University of Ghana.
62. Clifford, A., et al., *Obstetrics, Gynaecology and Women's Health on the Move*. 2012: CRC Press.
63. Caritis, S.N., *Treatment of preterm labour.* Drugs, 1983. **26**(3): p. 243-261.
64. Goldenberg, R.L., *The management of preterm labor.* Obstetrics & Gynecology, 2002. **100**(5): p. 1020-1037.
65. Baskett, T.F., *Essential management of obstetric emergencies*. 2002: Orient Blackswan.
66. Ananth, C.V., et al., *Placental abruption and adverse perinatal outcomes.* Jama, 1999. **282**(17): p. 1646-1651.
67. Oyelese, Y. and C.V. Ananth, *Placental abruption.* Obstetrics & Gynecology, 2006. **108**(4): p. 1005-1016.
68. Yeo, L., C. Ananth, and A. Vintzileos, *Placenta abruption.* Gynecology and Obstetrics. Lippincott, Williams & Wilkins, 2003.
69. Paz, N.C.d., et al., *Risk of placental abruption in relation to maternal depressive, anxiety and stress symptoms.* Journal of Affective Disorders 2011. **130**: p. 280–284.

70. Eskes, T.K.A.B., *Clotting disorders and placental abruption: homocysteine- a new risk factor.* European Journal of Obstetrics & Gynecology and Reproductive Biology 2001. **95**: p. 206-212.
71. Group, H.S.C.R., *Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) Study: associations with maternal body mass index.* BJOG: An International Journal of Obstetrics & Gynaecology, 2010. **117**(5): p. 575-584.
72. Hurd, W., et al., *Shortening increases spontaneous contractility in myometrium from pregnant women at term.* American Journal of Obstetrics and Gynecology (2005) **192**: p. 295–303.
73. Attah, A.F., et al., *Uterine contractility of plants used to facilitate childbirth in Nigerian ethnomedicine.* Journal of ethnopharmacology, 2012. **143**(1): p. 377-382.
74. Tourne, G.r., et al., European Journal of Obstetrics & Gynecology and

Reproductive Biology 2003. **110** p. 29–34.
75. Turner, R.C., C.A. Cull, and V. Frighi, *Glycaemic control with diet, sulphonylurea, metformin or insulin in patients with type 2 diabetes, a progressive repuirement for multiple therapies.* JAMA, 1999. **281**: p. 2005-212.
76. Forbes, J.M., M.E. Cooper, and V. Thallas, *Reduction of the accumulation of advanced glycation end products by ACE inhibition in experimental diabetic nephropathy.* Diabetes, 2002. **51**: p. 3274-3282.
77. Ohiraa, M., et al., *Effect of metformin on serum lipoprotein lipase mass levels and LDL particle size in type 2 diabetes mellitus patients.* Diabetes Research and Clinical Practice 2007. **78**: p. 34-41.
78. Edgerton, D.S., K.M. Johnson, and A.D. Cherrington, *Current strategies for the inhibition of hepatic glucose production in type 2 diabetes.* Front Biosci (Landmark Ed), 2009. **14**: p. 1169-1181.
79. Correia, S., et al., *Metformin protects the brain against the oxidative imbalance promoted by type 2 diabetes.* Med Chem, 2008. **4**: p. 358-64.
80. Storr, T., et al., *Vanadyl-thiazolidinedione combination agents for diabetes therapy.* Bioconjugate chemistry, 2003. **1**(14): p. 212-221.
81. Kelly, A.J., et al., *Vaginal prostaglandin (PGE2 and PGF2a) for induction of labour at term.* The cochrane library, 2009.

82. Karim, S. and S. Sharma, *Therapeutic abortion and induction of labour by the intravaginal administration of prostaglandins E2 and F2α.* BJOG: An International Journal of Obstetrics & Gynaecology, 1971. **78**(4): p. 294-300.
83. Smith, R., *Parturition.* New England Journal of Medicine, 2007. **356**(3): p. 271-283.
84. Ronsmans, C., W.J. Graham, and L.M.S.S.s. group, *Maternal mortality: who, when, where, and why.* The Lancet, 2006. **368**(9542): p. 1189-1200.
85. Haddad, S., et al., *Cardiorespiratory crisis at the end of pregnancy: a case of pheochromocytoma.* Middle East J Anesthesiol, 2013. **22**(2): p. 195-202.
86. Gutierrez, R.M.P., *Orchids: A review of uses in traditional medicine, its phytochemistry and pharmacology.* Journal of Medicinal Plants Research, 2010. **4**(8): p. 592-638.
87. Organization, W.H., *WHO traditional medicine strategy 2002-2005.* 2002.
88. Kamatenesi Mugisha, M. and R. Bukenya Ziraba, *Ethnobotanical survey methods to monitor and assess the sustainable harvesting of medicinal plants in Uganda.* Plant conservation in the tropics: perspectives and practice, 2002: p. 467-482.
89. Kamatenesi-Mugisha, M., R. Bukenya-Ziraba, and R. Hoeft, *Ethnomedical use of Rytigynia (nyakibasi) in Bwindi Impenetrable National Park, SW Uganda.* Lidia (Norway), 2000.
90. Kamatenesi-Mugisha, M. and H. Oryem-Origa, *Traditional herbal remedies used in the management of sexual impotence and erectile dysfunction in western Uganda.* African Health Sciences, 2005. **5**(1): p. 40-49.
91. Gruber, C.W. and M. O'Brien, *Uterotonic plants and their bioactive constituents.* Planta medica, 2011. **77**(3): p. 207.
92. Hu, E., et al., *Novel cyclotides from Hedyotis diffusa induce apoptosis and inhibit proliferation and migration of prostate cancer cells.* International journal of clinical and experimental medicine, 2015. **8**(3): p. 4059.
93. Kilpatrick, S.J. and R.K. Laros Jr, *Characteristics of normal labor.* Obstetrics & Gynecology, 1989. **74**(1): p. 85-87.
94. Kunene, N., R. Wilson, and N. Myeni, *The use of trees, shrubs and herbs in livestock production by communal farmers in northern KwaZulu-Natal, South Africa.* African Journal of Range and Forage Science, 2003. **20**(3): p. 271-274.

95. Katsoulis, L.C., *The pharmacological actitvity of Rhoicissus tridentata subsp cuneifolia in relation to parturition*. 2014.
96. Brookes, K. and L. Katsoulis, *Bioactive components of Rhoicissus tridentate: a pregnancy-related traditional medicine.* South African journal of science, 2006. **102**(5-6): p. 267-272.
97. Naidoo, V., et al., *Antioxidant compounds in Rhoicissus tridentata extracts may explain their antibabesial activity: research in action.* South African journal of science, 2006. **102**(5-6): p. 198-200.
98. Maroyi, A., *From traditional usage to pharmacological evidence: Systematic review of Gunnera perpensa L.* Evidence-Based Complementary and Alternative Medicine, 2016. **2016**.
99. Gruden, G., G. Setti, and A. Hayward, *Mechanical stretch induces monocyte chemoattractant activity via an NF-kappaB-dependent monocyte chemoattractant protein-1-mediated pathway in human mesangial cells: inhibition by rosiglitazone.* .J Am Soc Nephrol, 2005. **16**: p. 688-696.
100. Kota, S.K., et al., *Aberrant angiogenesis: The gateway to diabetic complications.* Indian journal of endocrinology and metabolism, 2012. **16**(6): p. 918-930.
101. Awad, A., et al., *beta-Sitosterol inhibits HT-29 human colon cancer cell growth and alters membrane lipids.* Anticancer research, 1995. **16**(5A): p. 2797-2804.
102. Sugano, M., H. Morioka, and I. Ikeda, *A comparison of hypocholesterolemic activity of beta-sitosterol and beta-sitostanol in rats.* The Journal of nutrition, 1977. **107**(11): p. 2011-2019.
103. Farquhar, J.W., R.E. Smith, and M.E. Dempsey, *The effect of beta sitosterol on the serum lipids of young men with arteriosclerotic heart disease.* Circulation, 1956. **14**(1): p. 77-82.
104. Awad, A., et al., *beta-Sitosterol inhibits growth of HT-29 human colon cancer cells by activating the sphingomyelin cycle.* Anticancer research, 1998. **18**(1A): p. 471-473.
105. Moreau, R.A., B.D. Whitaker, and K.B. Hicks, *Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses.* Progress in lipid research, 2002. **41**(6): p. 457-500.

106. Lijuan, W., et al., *The effects of wild ginger (Costus speciosus (Koen) Smith) rhizome extract and diosgenin on rat uterine contractions.* Reproductive Sciences, 2011. **18**(6): p. 516-524.
107. Liz, R., et al., *Acute effect of β-sitosterol on calcium uptake mediates anti-inflammatory effect in murine activated neutrophils.* Journal of Pharmacy and Pharmacology, 2013. **65**(1): p. 115-122.
108. Malini, T. and G. Vanithakumari, *Effect of beta-sitosterol on uterine biochemistry: a comparative study with estradiol and progesterone.* Biochemistry and molecular biology international, 1993. **31**(4): p. 659-668.
109. Paarakh, P.M., *Terminalia arjuna (Roxb.) Wt. and Arn.: a review.* Int j pharmacol, 2010. **6**(5): p. 515-534.
110. Calis, I. and O. Sticher, *Triterpene saponins from plants of the flora of Turkey*, in *Saponins Used in Traditional and Modern Medicine*. 1996, Springer. p. 485-500.
111. Hemalatha, T., et al., *Arjunolic acid: a novel phytomedicine with multifunctional therapeutic applications.* 2010.
112. Carson, J.L., et al., *Diabetes mellitus increases short-term mortality and morbidity in patients undergoing coronary artery bypass graft surgery.* Journal of the American College of Cardiology, 2002. **40**(3): p. 418-423.
113. Wild, S., et al., *Global prevalence of diabetes: estimates for the year 2000 and projections for 2030.* Diabetes care, 2004. **27**(5): p. 1047-1053.
114. Soriguer, F., et al., *Prevalence of diabetes mellitus and impaired glucose regulation in Spain: the Di@ bet. es Study.* Diabetologia, 2012. **55**(1): p. 88-93.
115. Yki-Järvinen, H., E. Helve, and V.A. Koivisto, *Hyperglycemia decreases glucose uptake in type I diabetes.* Diabetes, 1987. **36**(8): p. 892-896.
116. Stumvoll, M., B.J. Goldstein, and T.W. van Haeften, *Type 2 diabetes: principles of pathogenesis and therapy.* The Lancet, 2005. **365**(9467): p. 1333-1346.
117. Langer, O., *Management of gestational diabetes.* Clinical obstetrics and gynecology, 2000. **43**(1): p. 106-115.
118. Tieu, J., et al., *Oral anti-diabetic agents for women with pre-existing diabetes mellitus/impaired glucose tolerance or previous gestational diabetes mellitus.* Cochrane Database of Systematic Reviews, 2009(2).

119. Tzoulaki, I., et al., *Risk of cardiovascular disease and all cause mortality among patients with type 2 diabetes prescribed oral antidiabetes drugs: retrospective cohort study using UK general practice research database.* Bmj, 2009. **339**: p. b4731.
120. Bailey, C.J. and C. Day, *Traditional plant medicines as treatments for diabetes.* Diabetes care, 1989. **12**(8): p. 553-564.
121. Tahraoui, A., et al., *Ethnopharmacological survey of plants used in the traditional treatment of hypertension and diabetes in south-eastern Morocco (Errachidia province).* Journal of ethnopharmacology, 2007. **110**(1): p. 105-117.
122. Malviya, N., S. Jain, and S. Malviya, *Antidiabetic potential of medicinal plants.* Acta Pol Pharm, 2010. **67**(2): p. 113-118.
123. Chauhan, A., et al., *Plants having potential antidiabetic activity: a review.* Der Pharmacia Lettre, 2010. **2**(3): p. 369-387.
124. Patel, D., et al., *An overview on antidiabetic medicinal plants having insulin mimetic property.* Asian Pacific journal of tropical biomedicine, 2012. **2**(4): p. 320-330.
125. Rahmatullah, M., et al., *Medicinal plants used for treatment of diabetes by the Marakh sect of the Garo tribe living in Mymensingh district, Bangladesh.* African Journal of Traditional, Complementary and Alternative Medicines, 2012. **9**(3): p. 380-385.
126. Andrade-Cetto, A. and M. Heinrich, *Mexican plants with hypoglycaemic effect used in the treatment of diabetes.* Journal of ethnopharmacology, 2005. **99**(3): p. 325-348.
127. Dube, S.C., *Contractile Effects of Gunnera Perpensa and Rhoicissus Tridentata Bioactive Extracts in Isolated Rat Uterine Muscles*. 2014, University of KwaZulu-Natal, Durban.
128. Bryant, A.T., *Zulu medicine and medicine-men*. 1966: C. Struik.
129. Edeoga, H., D. Okwu, and B. Mbaebie, *Phytochemical constituents of some Nigerian medicinal plants.* African journal of biotechnology, 2005. **4**(7): p. 685-688.
130. Ogbe, F.M.D., O.L. Eruogun, and M. Uwagboe, *Plants used for female reproductive health care in Oredo local government area, Nigeria.* Scientific Research and Essays, 2009. **4**(3): p. 120-130.
131. Gupta, R., et al., *Antidiabetic and antioxidant potential of β-sitosterol in streptozotocin-induced experimental hyperglycemia.* Journal of diabetes, 2011. **3**(1): p. 29-37.

132. Kubo, Y., *Comparison of initial stages of muscle differentiation in rat and mouse myoblastic and mouse mesodermal stem cell lines.* The Journal of physiology, 1991. **442**(1): p. 743-759.

133. Diel, P., et al., *C2C12 myoblastoma cell differentiation and proliferation is stimulated by androgens and associated with a modulation of myostatin and Pax7 expression.* Journal for molecular endocrinology, 2008. **40**

231–241: p. 231–241.

134. Strick-Marchand, H. and M.C. Weiss, *Embryonic liver cells and permanent lines as models for hepatocyte and bile duct cell differentiation.* Mechanisms of development, 2003. **120**(1): p. 89-98.

135. Kühn, C., et al., *Constituents from cistus salvifolius (cistaceae) activate peroxisome proliferator-activated receptor-γ but Not-δ and stimulate glucose uptake by adipocytes.* Planta medica, 2011. **77**(04): p. 346-353.

136. van de Venter, M., et al., *Antidiabetic screening and scoring of 11 plants traditionally used in South Africa.* Journal of ethnopharmacology, 2008. **119**(1): p. 81-86.

137. Musabayane, C.T., et al., *Effects of Syzygium cordatum (Hochst.) [Myrtaceae] leaf extract on plasma glucose and hepatic glycogen in streptozotocin-induced diabetic rats.* Journal of Ethnopharmacology, 2005. **97**(3): p. 485-490.

138. Ngubane, P.S., C.T. Musabayane, and B. Masola, *The effects of Syzygium aromaticum-derived oleanolic acid on glycogenic enzymes in streptozotocin-induced diabetic rats.* Ren Fail, 2011. **33**: p. 434-439.

139. Nel, A., et al., *Toxic potential of materials at the nanolevel.* science, 2006. **311**(5761): p. 622-627.

140. Gaetke, L.M. and C.K. Chow, *Copper toxicity, oxidative stress, and antioxidant nutrients.* Toxicology, 2003. **189**(1-2): p. 147-163.

141. Wannamethee, S.G., et al., *Hepatic enzymes, the metabolic syndrome, and the risk of type 2 diabetes in older men.* Diabetes care, 2005. **28**(12): p. 2913-2918.

142. Freeman, D.J., et al., *C-reactive protein is an independent predictor of risk for the development of diabetes in the West of Scotland Coronary Prevention Study.* Diabetes, 2002. **51**(5): p. 1596-1600.

143. Tsuruda, T., M. Jougasaki, and G. Boerrigter, *Cardiotrophin-1 stimulation of cardiac fibroblast growth: roles for glycoprotein 130/leukemia inhibitory factor receptor and the endothelin type A receptor.* Circ Res, 2002. **90**: p. 128–134.
144. Jan, M., et al., *The role of immunogenicity in cardiovascular disease.* World heart journal, 2011. **3**(1): p. 1.
145. Ridker, P.M., et al., *Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events.* J Engl Med, 2002. **347**: p. 1157-1165.
146. Yakes, F.M. and B. Van Houten, *Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress.* Proceedings of the National Academy of Sciences, 1997. **94**(2): p. 514-519.
147. Sodhi, C., et al., *Study of oxidative-stress in isoniazid-rifampicin induced hepatic injury in young rats.* Drug and chemical toxicology, 1997. **20**(3): p. 255-269.
148. Meyer, C., et al., *Role of human liver, kidney, and skeletal muscle in postprandial glucose homeostasis.* American Journal of Physiology-Endocrinology And Metabolism, 2002. **282**(2): p. E419-E427.
149. Bryant, N.J., R. Govers, and D.E. James, *Regulated transport of the glucose transporter GLUT4.* Nature reviews Molecular cell biology, 2002. **3**(4): p. 267.
150. Mokuno, T., et al., *Glucose transporter 2 concentrations in hyper-and hypothyroid rat livers.* Journal of Endocrinology, 1999. **160**(2): p. 285-289.
151. Weinstein, S.P., et al., *Regulation of GLUT2 glucose transporter expression in liver by thyroid hormone: evidence for hormonal regulation of the hepatic glucose transport system.* Endocrinology, 1994. **135**(2): p. 649-654.
152. Khan, A. and J. Pessin, *Insulin regulation of glucose uptake: a complex interplay of intracellular signalling pathways.* Diabetologia, 2002. **45**(11): p. 1475-1483.
153. Patel, D., et al., *Natural medicines from plant source used for therapy of diabetes mellitus: An overview of its pharmacological aspects.* Asian Pacific Journal of Tropical Disease, 2012. **2**(3): p. 239-250.
154. Sujatha, S., et al., *Biological evaluation of (3β)-STIGMAST-5-EN-3-OL as potent anti-diabetic agent in regulating glucose transport using in vitro model.* International Journal of Diabetes Mellitus, 2010. **2**(2): p. 101-109.

155. Ferrannini, E., *The target of metformin in type 2 diabetes.* New England Journal of Medicine, 2014. **371**(16): p. 1547-1548.
156. Maier, V. and G. Gould, *Long-term insulin treatment of 3T3-L1 adipocytes results in mis-targeting of GLUT4: implications for insulin-stimulated glucose transport.* Diabetologia, 2000. **43**(10): p. 1273-1281.
157. Sakamoto, K. and G.D. Holman, *Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic.* American journal of physiology-endocrinology and metabolism, 2008. **295**(1): p. E29-E37.
158. Taylor, E.B., et al., *Discovery of TBC1D1 as an insulin-, AICAR-, and contraction-stimulated signaling nexus in mouse skeletal muscle.* Journal of Biological Chemistry, 2008. **283**(15): p. 9787-9796.
159. Tomić-Naglić, D., et al., *Impact of currently used oral antihyperglycemic drugs on dysfunctional adipose tissue.* Srp Arh Celok Lek, 2017. **145**(11-12): p. 643-645.
160. Viollet, B., et al., *Targeting the AMPK pathway for the treatment of Type 2 diabetes.* Frontiers in bioscience (Landmark edition), 2009. **14**: p. 3380.
161. Mayerson, A.B., et al., *The effects of rosiglitazone on insulin sensitivity, lipolysis, and hepatic and skeletal muscle triglyceride content in patients with type 2 diabetes.* Diabetes, 2002. **51**(3): p. 797-802.
162. Musi, N., et al., *Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes.* Diabetes, 2002. **51**(7): p. 2074-2081.
163. Towler, M.C. and D. GrahameHardie, *AMP-activated protein kinase in metabolic control and insulin signaling.* Circulation research, 2007.
164. Cline, G.W., et al., *Impaired glucose transport as a cause of decreased insulin-stimulated muscle glycogen synthesis in type 2 diabetes.* New England Journal of Medicine, 1999. **341**(4): p. 240-246.
165. Erasto, P., et al., *An ethnobotanical study of plants used for the treatment of diabetes in the Eastern Cape Province, South Africa.* African Journal of Biotechnology, 2005. **4**(12).
166. Afolayan, A.J. and T.O. Sunmonu, *In vivo studies on antidiabetic plants used in South African herbal medicine.* Journal of clinical biochemistry and nutrition, 2010. **47**(2): p. 98-106.

167. Goldwaser, I., et al., *Insulin-like effects of vanadium: basic and clinical implications.* Journal of Inorganic Biochemistry, 2000. **80**: p. 21-25.
168. Shechter, Y., et al., *Historic perspective and recent developments on the insulin-like actions of vanadium; toward developing vanadium-based drugs for diabetes.* Coordination chemistry reviews, 2003. **237**(1-2): p. 3-11.

APPENDICES

Appendix 1 - AREC Ethics Approval Letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

18 August 2015

Ms Zinhle Mvelase (211532767)
Department of Physiology
School of Laboratory Medicine and Medical Sciences
Westville Campus

Dear Ms Mvelase,

Protocol reference number: AREC/084/015M
Project title: Evaluation of ethnomedicinal plants extracts on uterine contraction

Full Approval – Research Application

With regards to your revised application received on 11 August 2015. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

CONDITION:

1. **A total of 90 animals to be used for all groups.**

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 18 August 2016.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....

Dr Shahidul Islam
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Dr MV Mabandla
Cc Dean / Head of School: Professor W Daniels
Cc Acting Registrar: Mr Baatile Poo
Cc NSPCA: Ms Lebo Sentle
Cc BRU – Dr S Singh

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

Appendix 2 – ALT ELISA protocol

Elabscience®

8th Edition, revised in February, 2018

（FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSIS !）

Alanine Aminotransferase (ALT/GPT) Assay Kit

Catalog No: E-BC-K235
Method: Colorimetric method
Specification: 96T

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help.

Phone: 240-252-7368(USA) Fax: 240-252-7376(USA)
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

Appendix 3 – AST ELISA protocol

Elabscience®

7th Edition, revised in April, 2017

(FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSTICS !)

Rat AST(Aspartate Aminotransferase) ELISA Kit

Synonyms: SGOT, serum glutamic oxaloacetic transaminase, GOT1, AspAT, ASAT, AAT

Catalog No : E-EL-R0076
96T

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help (info in the header of each page).

Phone: 240-252-7368(USA) 240-252-7376(USA)
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please refer to specific expiry date from label on the side of box.

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service.