

Determination of the *in vitro* antidiabetic potential of a polyherbal commercial tea

by

Veronica Paddy

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Supervisor: Prof. V. Steenkamp

Co-supervisor: Dr. J.J. van Tonder

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Declaration

University of Pretoria

Faculty of Health Sciences

Department of Pharmacology

I, Veronica Paddy,

Student number: 27058175

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Publications and conferences

To be submitted

1. V. Paddy, J.J. van Tonder, V. Steenkamp. The *in vitro* antioxidant activity and polyphenolic content of a commercial tea mixture and its constituents.
2. V. Paddy, J.J. van Tonder, V. Steenkamp. The *in vitro* hypoglycaemic activity of a commercial tea mixture and its constituents.

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“Nothing you have not given away will ever really be yours.”

— C.S. Lewis

Abstract

Type 2 diabetes mellitus (T2DM) is an increasing global health concern, currently affecting an estimated 382 million individuals. There is no cure for T2DM and the search for new and improved treatments is ongoing. Presently, various pharmacological regimens are available to treat T2DM, but with varied success. Thousands of traditional herbs are also used to treat T2DM, but mainly without scientific validation. The aim of this study was to assess the polyphenolic content, antioxidant capacity, as well as *in vitro* toxicity and hypoglycaemic activity of a commercial 'antidiabetic' tea mixture (Diabetea) and its individual constituents: *Achillea millefolium* L. (Yarrow), *Agathosma betulina* Bartl. & Wendl. (Buchu), *Salvia officinalis* L. (Sage), *Taraxacum officinalis* L. (Dandelion), *Thymus vulgaris* L. (Thyme), *Trigonella foenum-graecum* L. (Fenugreek) and *Urtica urens* L. (Nettle).

All herbs were tested as crude extracts, prepared using hot water (HW) and dichloromethane (DCM). The total polyphenolic content of each extract was determined using the Folin-Ciocalteu and aluminium trichloride methods. The non-cellular antioxidant activity was assessed using 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) methods. The cell-based antioxidant activity was measured against *p*-chloranil-induced generation of reactive oxygen species (ROS) in Ea.hy926 cells, using the fluorescent dye, 2',7'-dichlorofluorescein-diacetate (DCFH-DA). The effect of each extract on the viability of C2C12 myotubes, Ea.hy926 endothelial cells and human lymphocytes (HL) was determined using sulforhodamine B (SRB). The *in vitro* hypoglycaemic activity was assessed against α -amylase and α -glucosidase activity using 3,5-dinitrosalicylic acid (DNSA) and *p*-nitrophenyl- α -D-glucopyranoside (*p*-NPG), respectively. The type of

inhibition exerted on these enzymes was determined using the Michaelis-Menten enzyme kinetics model, expressed as mixed, competitive, non-competitive and uncompetitive. Glucose uptake activity was measured using the 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-*D*-glucose (2-NBDG) fluorescent analogue.

T. vulgaris and *S. officinalis* had the highest amount of polyphenols of all extracts tested. The HW extracts of *T. vulgaris* and *S. officinalis* showed significant ($p < 0.05$) cell-free antioxidant activity and cell-based radical scavenging activity. In addition, *U. urens* (HW) also limited cell-based ROS generation ($p < 0.05$). The Diabetea extracts presented with poor antioxidant activities, of which some had a pro-oxidant effect on Ea.hy926 cells. The positive linear relationship between antioxidant activity and polyphenolic content was shown to be dependent on the solvent type used. All of the DCM extracts had low antioxidant activity and polyphenolic content.

None of the extracts produced $< 50\%$ cell density at the concentrations tested (1.3 - 20 $\mu\text{g}/\text{m}\ell$). In general, the DCM extracts showed a greater decrease in cell density than the HW extracts. The Ea.hy926 cells were the least affected by the extracts in terms of decreased cell density.

The DCM extract of *U. urens* inhibited α -amylase activity in a mixed manner, which was comparable to the percentage inhibition exerted by the commercial drug, acarbose. Both the HW and DCM extracts of *U. urens* caused a significant ($p < 0.05$) increase in glucose uptake into C2C12 myotubes. The HW extract of *T. vulgaris* had a significant ($p < 0.05$) inhibitory activity against α -glucosidase (mixed). It also caused the uptake of glucose into C2C12 myotubes, which was significantly ($p <$

0.05) more active than insulin. *S. officinalis* (DCM extract) also inhibited α -glucosidase activity ($p < 0.05$) in a mixed manner. Its HW extract displayed potent hypoglycaemic potential by causing glucose uptake into C2C12 myotubes, which was more significant ($p < 0.05$) than the activity of the positive control, insulin. The DCM extract of *A. betulina* was active against α -glucosidase (non-competitive), which was comparable to the activity of acarbose. Its HW extract also showed a significant ($p < 0.05$) glucose uptake activity. Furthermore, the DCM extracts of *T. officinalis*, *A. millefolium*, Diabetea and HW extracts of *T. foenum-graecum* and *T. officinalis* also caused a significant ($p < 0.05$) increase in glucose uptake into C2C12 myotubes.

This study provides evidence for the antidiabetic potential of *T. vulgaris* and *S. officinalis*, in terms of antioxidant capacity and potential to prevent of post-prandial hyperglycaemia and alleviate hyperglycaemia by mimicking the action of insulin. In addition, the organic preparation of *U. urens* is also a potent α -amylase inhibitor. All herbs tested in this study exerted some form of *in vitro* antidiabetic activity. The Diabetea mixture, as a traditional preparation, did not have a significant antidiabetic capacity. *In vitro* observations from this study do not support the use of Diabetea as an antidiabetic preparation and reveal that some of the individual extracts prove more efficacious than the herb mixture.

Key words: antioxidant activity, cytotoxicity, Diabetea, glucose uptake, herbs, polyphenolic content, reactive oxygen species, type 2 diabetes mellitus, α -amylase, α -glucosidase

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LIST OF ABBREVIATIONS

µg	Microgram
µM	Micromolar
2-NBDG	2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-d-glucose
ABTS ^{•+}	2,2-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid free radical
AcOH	Acetic acid
AGEs	Advanced glycated end products
AlCl ₃	Alluminium trichloride
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ANSA	3-amino-5-nitrosalicylic acid
ATCC	American type culture collection
B.C.	Before Christ
BMI	Body mass index
BSA	Bovine serum albumin
C2C12	Mouse myoblasts (CRL-1772)
CO ₂	Carbon dioxide
DCF	2',7'-dichlorodihydrofluorescein

DCFH-DA	2',7'-Dichlorodihydrofluorescein diacetate
DCM	Dichloromethane
DF	Dilution factor (volume reagent solution/volume of solvent)
dH ₂ O	Distilled water
DI	Deionized
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DMEM-	DMEM without 1% penicillin G/ 1% streptomycin
DMEM+	DMEM with 1% penicillin G/ 1% streptomycin
DMSO	Dimethyl sulfoxide
DNSA	3,5-Dinitrosalicylic acid
DPP-4	Dipeptidyl aminopeptidase-4
DPPH [•]	1,1-Diphenyl-2-picrylhydrazyl free radical
Ea.hy926	Human umbilical vein endothelial cell line (CRL-2922)
EDTA	Ethylenediaminetetra-acetic acid
EtOH	Ethanol
FCR	Folin-Ciocalteu reagent
FCS	Foetal calf serum
FPG	Fasting plasma glucose

FXR	Farnesoid X factor
g	Gram
<i>g</i>	Gravitational force
GA	Gallic acid
GAE	Gallic acid equivalence
GDM	Gestational diabetes mellitus
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporters
h	Hour
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
HBSS	Hank's balanced salt solution
SEM	Standard error of the mean
HClO	Hypochlorous acid
HL	Human lymphocytes
HW	Hot water
IC ₅₀	Half maximal inhibitory concentration
IDDM	Insulin-dependent diabetes mellitus

IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
<i>In vacuo</i>	Under vacuum
$K_2C_4H_4O_6$	Sodium potassium tartrate
$K_2S_2O_8$	Potassium persulfate
K_{ATP}	Adenosine triphosphate-sensitive potassium channels
K_M	Michaelis constant
MeOH	Methanol
min	Minute
mℓ	Milliliter
MRC	Medical Research Council
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NCDs	Non-communicable diseases
NH_2Cl	Chloramine

NH ₄ Cl	Ammonium chloride
NIDDM	Non-insulin dependent diabetes mellitus
nm	Nanometer
nM	Nanomolar
NPH	Neutral protamine Hagedorn
O ₂ ⁻	Superoxide
O ₂ [*]	Singlet oxygen
OH ⁻	Hydroxyl radical
PBS	Phosphate buffered saline
PG	Plasma glucose
pH	Negative logarithm of the hydrogen ion concentration
PHA	Phytohaemagglutinin
PI	Polarity index
p-NPG	<i>p</i> -Nitrophenyl- α - <i>D</i> -glucopyranoside
PP	Polypropylene
PPA	Porcine pancreatic α -amylase
PPAR- γ	Peroxisome proliferator-activated receptor - γ
PS	Polystyrene
RE	Rutin equivalence

RFI	Relative fluorescence intensity
ROS	Reactive oxygen species
RPG	Random plasma glucose
RPMI	Roswell Park Memorial Institute
RPMI-	RPMI without 1% penicillin G/ 1% streptomycin
RPMI+	RPMI with 1% penicillin G/ 1% streptomycin
RSC	Radical scavenging
SEM	Standard error of the mean
SRB	Sulforhodamine B
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA	Trichloroacetic acid
TE	Trolox equivalents
TFC	Total flavonoid content
TNF- α	Tumour necrosis factor alpha
TPC	Total phenolic content
TRIS	Trisaminomethane
UCPs	Uncoupling proteins
V_0	Initial reaction velocity

V_{\max} Maximal reaction velocity

WHO World Health Organization

LIST OF SYMBOLS AND NOTATIONS

% Percentage

%v/v Milliliters per 100 milliliter in solution

%w/v Grams of solid per 100 milliliter of solvent

λ_{em} Emission wavelength

λ_{ex} Excitation wavelength

ΔP Delta P: difference between end and initial product concentration ($\mu\text{g}/\text{m}\ell$)

Δt Delta t: difference between end and initial time (seconds)

[S] Substrate concentration

$^{\circ}\text{C}$ Degrees Celsius

G_0 Resting phase of cell cycle

ℓ Liter

p Probability

t Test statistic

α Alpha

β	Beta
λ_{re}	Reference wavelength
ψ	Psi

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Diabetes mellitus (DM) is a chronic endocrine disorder associated with impaired blood glucose control as a consequence of defective insulin production and/or action¹. The result is dysfunctional carbohydrate, fat and protein metabolism^{1,2}. Currently, it is estimated that 382 million individuals suffer from DM, which may escalate to 592 million by 2035³. DM is associated with life threatening secondary complications that, in 2013, claimed the lives of 5.1 million individuals worldwide³. The exact aetiology of DM remains elusive, but, its onset has been coupled to obesity, gestation, sedentary lifestyle and/or a hereditary predisposition⁴.

The word 'diabetes' originated in the 16th century⁵. It is originally a Greek word for 'siphon' from '*diabetein*', which means to 'go through'. *Mellitus* on the other hand is a latin word for 'sweet'⁵ and was first used in context of diabetes in the 18th century⁶. Diabetes mellitus, therefore, literally means the 'going through of something sweet'. This describes the classical sign of DM namely, glucosuria¹. The very first record of DM, dates back to 1550 B.C., written on papyrus by an Egyptian physician named Hesy-Ra, which noted the 'passing of too much urine'⁶. Although DM has been identified ages ago, there is still no cure for it or complete satisfactory conventional treatment⁷. Therefore, there is a continual focus on improving and developing safer and more effective treatments/regimens⁷.

DM has been treated with herbs for centuries and there are many herbs that have been reported to exert antidiabetic activity⁸⁻¹¹. Historically, traditional herbal treatments have been shown to possess successful pharmacological activity, such as in the case with metformin, isolated from *Galega officinalis*¹².

Within the last decade the global prevalence of DM climbed from 5.1 to 8.3%, indicating the unrestrained surge in DM cases^{3,13}. At present, 80% of DM sufferers live in low- to middle-income countries, and the greatest increase in DM diagnoses is expected to occur in developing countries³. An ageing population groups, urbanization, unhealthy diets and sedentary lifestyles all contribute to this increase¹⁴.

1.2 Prevalence of diabetes mellitus

In 1995, 135 million individuals, globally, were directly affected by DM, which were estimated to double to 270 million by 2010³. However, around 2010, 366 million individuals were already living with DM³. In 2003, there were an estimated 197 million cases of DM and it was further projected to reach 333 million by 2025. However, this estimate was surpassed only a decade later with up to ~382 million DM sufferers in 2013³. There has been an increase, globally, in the annual mortality rate over the last six years from 4 to 5.1 million³. The global expenditure on diabetes in 2013 was R5 500 billion³, which equates to a mean expenditure per person per year of ~R13 000³.

The top 10 countries with regards to the highest number of DM cases from highest to lowest are: China, India, USA, Brazil, Russian Federation, Mexico, Indonesia, Germany, Egypt and Japan³. The top 10 countries with the greatest prevalence of

DM, from highest to lowest, are: Tokelau Federated States of Micronesia, Marshall Islands, Kiribati, Cook Islands, Vanuatu, Saudi Arabia, Kuwait, Qatar and Nauru, ³. Taken together, this makes the Western Pacific, Middle East and North Africa the regions with the greatest incidence of DM, hosting approximately 60% of all diabetes sufferers worldwide³.

Currently, there are approximately 20 million people with DM on the African continent³. This figure is estimated to double by 2035³. South Africa has the second highest number of DM affected individuals in Africa (2.6 million), second to Nigeria (3.9 million)³. Within the last decade the prevalence of DM in South Africa has more than doubled, from 3.4 to 8.3%, indicating a growing problem of epidemic proportion³. DM is the seventh leading cause of death in South Africa¹⁵. In 2013, there were approximately 83 000 diabetes-related deaths in South Africa, which is four times higher than in 2007¹⁶. Obesity-related DM was the second greatest cause of an increase in the number of deaths between 2004 and 2005 in South Africa¹⁶. In South Africa the cost per person per year is approximately R9 500³.

A global estimate of 175 million persons, affected by DM, remains undiagnosed³. DM education and healthcare is not a priority in some developing countries and therefore many diabetes sufferers remain undiagnosed³. Long-term undiagnosed diabetes is associated with severe secondary complications such as heart failure, retinopathy, neuropathy and kidney disease^{14,17}. Global healthcare costs related to complications associated with undiagnosed diabetes, amounts to approximately R180 billion per year³.

On the African continent, 76.4% of diabetes-related deaths have been found to occur in patients younger than 60 years of age³, disqualifying the notion that diabetes is an elderly disease¹⁸. The majority of diabetes patients in the world are between the age of 40 and 59 years, a productive and adept time of life³. In South Africa, one-fifth of DM patients are between the ages of 20 and 39 years³.

1.3 Classification of diabetes mellitus

There are three main types of DM (1, 2 and gestational) and one 'other' type, which are classified according to clinical representation or pathological aetiology^{18,19}.

Type 1 DM (T1DM) is an insulin-dependent DM (IDDM), which is the result of a destructive autoimmune response, instigating damage to pancreatic β -cells, causing an insulin deficiency²⁰⁻²². The exact trigger of this immune response is unknown and can affect individuals at any age, however, it is predominantly present in children and young adults^{3,22}. The onset of T1DM is abrupt and associated with polydipsia, polyuria, polyphagia, fatigue, weight loss, reoccurring infections, impeded wound healing and blurred vision¹. T1DM can be managed successfully by means of daily insulin supplementation, a controlled diet and regular physical activity^{3,22}. Globally, T1DM affects 7.9 million individuals below the age of 15 years and is growing by 3% annually³. The prevalence of T1DM in South Africa is ~3%³.

Type 2 DM (T2DM), is the consequence of insulin defect(s)²⁰⁻²². T2DM is the most common type of DM affecting between 85 – 95% of all DM patients^{3,22}. This type of DM is the focus of this study and will be discussed at length in section 1.4.

Gestational DM (GDM) is found in women who develop insulin resistance and hyperglycemia around the 24th week of gestation, which is said to be as a result of an interaction between placental hormones and insulin^{3,22}. The likelihood of incipient GDM occurring during successive pregnancies is high, as is the chance of developing T2DM during later stages of life³. Unmanaged GDM can pose a risk to both the mother and infant by increasing the risk of preeclampsia, fetal macrosomia and infant hypoglycaemia³. An estimated 21 million women were affected by GDM in 2013³.

The 'other' type of DM is caused by genetic defects affecting β -cell function, type A insulin resistance, pancreatitis, acromegaly, abnormal drug interaction, infection-induced or as a result of other genetic syndromes such as Down syndrome^{1,19,22}. This type of DM is rare¹⁹.

1.4 Type 2 diabetes mellitus

1.4.1 Overview

T2DM is associated with insulin insufficiency, insulin resistance or both, resulting in defective carbohydrate metabolism in adipocytes, myocytes and hepatocytes³. This leads to chronic hyperglycaemia, which is the hallmark of T2DM³. Hyperglycaemia is an abnormally high and persistent plasma glucose concentration characterised by a chronic fasting plasma glucose (FPG) level ≥ 7.0 mmol/l and a two hour (or at random) plasma glucose (PG) level ≥ 11.1 mmol/l. It is associated with symptoms such as dizziness, confusion and seizures^{19,23}.

Hyperglycaemia mainly affects the cardiovascular, optic, renal and neural systems, producing severe secondary pathologies and comorbidities. These include, amongst others, gastro-paresis, gangrene, amputations, myocardial infarction, nephropathy, retinopathy, strokes, atherosclerosis and neuropathy^{2,7,24}. Cardiovascular complications are the primary cause of T2DM-related debility and death³ and microvascular damage is the leading cause of blindness, end-stage renal failure and nerve damage^{20,25}.

T2DM is generally asymptomatic and is therefore diagnosed at later stages when chronic symptoms such as polydipsia, polyuria, polyphagia, syncope, emesis and insomnia are present¹. Individuals that are asymptomatic are at risk of developing T2DM if they have a body mass index (BMI) of ≥ 25 kg/m² combined with one persistent risk indicator such as; physical inactivity, hypertension, hereditary diabetes, dyslipidaemia, polycystic ovarian syndrome, certain ethnicities, cardiovascular disease, occurrence of GDM, birth of infant over 4 kg, past impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) and other illnesses resulting in insulin resistance¹⁹. The diagnosis of T2DM, in asymptomatic individuals, is made in association with a fasting plasma glucose (FPG) of ≥ 7.0 mmol/l, a random plasma glucose (RPG) of ≥ 11.1 mmol/l and HbA_{1c} of $\geq 6.5\%$ ¹⁹. HbA_{1c} is a form of glycated haemoglobin, that is representative of the mean plasma glucose concentration over the 2-3 months period prior to testing²⁶.

1.4.2 Pathophysiology of T2DM

1.4.2.1 Carbohydrate metabolism

Carbohydrate digestion starts in the mouth with salivary α -amylase (ptyalin)². Ptyalin hydrolyzes polysaccharides through dextrinization, whereby the α 1-4 linkages in carbohydrates are digested, resulting in smaller molecules known as dextrins² (Figure 1). Dextrins are then further hydrolyzed into maltose (87%) and glucose (13%)². Due to the short duration of mastication, only 3-5% of polysaccharides are converted to disaccharides in the mouth². Ptyalin remains active until it reaches the stomach, where it is inactivated by a low pH².

The majority of carbohydrate digestion and absorption occurs within/in the small intestine, mediated through digestive enzymes such maltase, sucrose, lactase, α -glucosidase and pancreatic α -amylase². α -Amylase and α -glucosidase have been studied as potential drug targets to treat diabetes for half a century already^{271,272}. Pancreatic α -amylase is produced by the β -cells of the pancreas (*Islets of Langerhans*) and released into the intestinal lumen in response to an increase in carbohydrates². It mediates the hydrolysis of the internal 1,4- α -glycosidic linkages of saccharides containing three or more 1,4- α -D-glucose units into di- and tri-saccharides, which is further digested by α -glucosidase² (Figure 1).

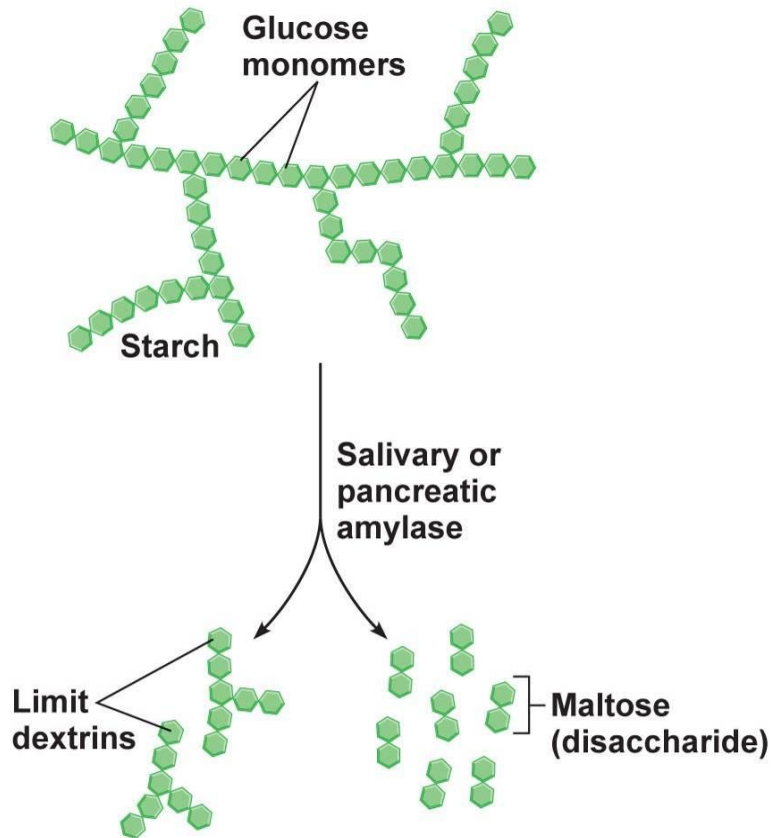


Figure 1: Salivary and pancreatic α -amylase activity²⁷.

α -Glucosidase is one of the most important enzymes for the digestion of dietary carbohydrates and is found in the brushborder of the enterocytes of the small intestinal wall² (Figure 2). This enzyme mediates the hydrolysis of external 1,4- α -D-glucose linked residues from the non-reducing ends². This releases β -glucose and further hydrolyses 1,6- α -glucosidic bonds into monosaccharides such as glucose, galactose and fructose². Therefore, the inhibition of α -glucosidase, as by the drug acarbose (competitive inhibitor), is clinically associated with a decrease in post-prandial hyperglycaemia² (Figure 2), which is therapeutically important in the treatment of T2DM.

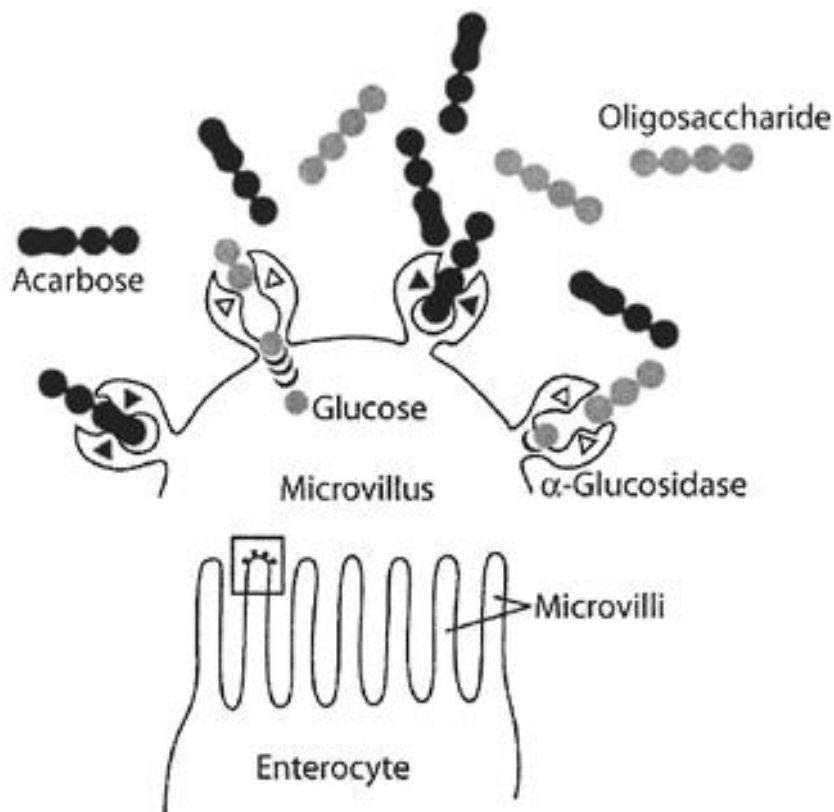


Figure 2: α -Glucosidase activity and the action of acarbose²⁸.

Following digestion, glucose and galactose are actively transported into the interstitium, while fructose is absorbed via passive diffusion². Glucose is transported into the interstitium by means of glucose transporters (GLUT)² (Figure 3). Following absorption into the systematic circulation, glucose is then transported into insulin-dependent cells (myocytes and adipocytes) by means of GLUT-4 receptors that are instigated by the binding of insulin to an insulin-receptor complex² (Figure 3). The insulin receptor is a glycoprotein with two subunits; α and β , which are linked by disulphide bonds². Upon insulin binding, α units activate β units that, in turn, activate tyrosine kinase, leading to a kinase cascade². This cascade causes the phosphorylation of insulin, activating it, with serine and tyrosine residues functioning as phosphorylation and dephosphorylation triggers². The activation of insulin leads

to an increase of GLUT and clathrin-covered complexes on the cell membrane, mediating endocytosis of glucose². This is followed by the activation of an enzymatic cascade leading to the dislodgement of insulin from its receptor and cell membrane². The free insulin attaches to other cells or undergoes digestion after 7-10 hours². This makes insulin the primary gluco-regulatory hormone in carbohydrate metabolism².

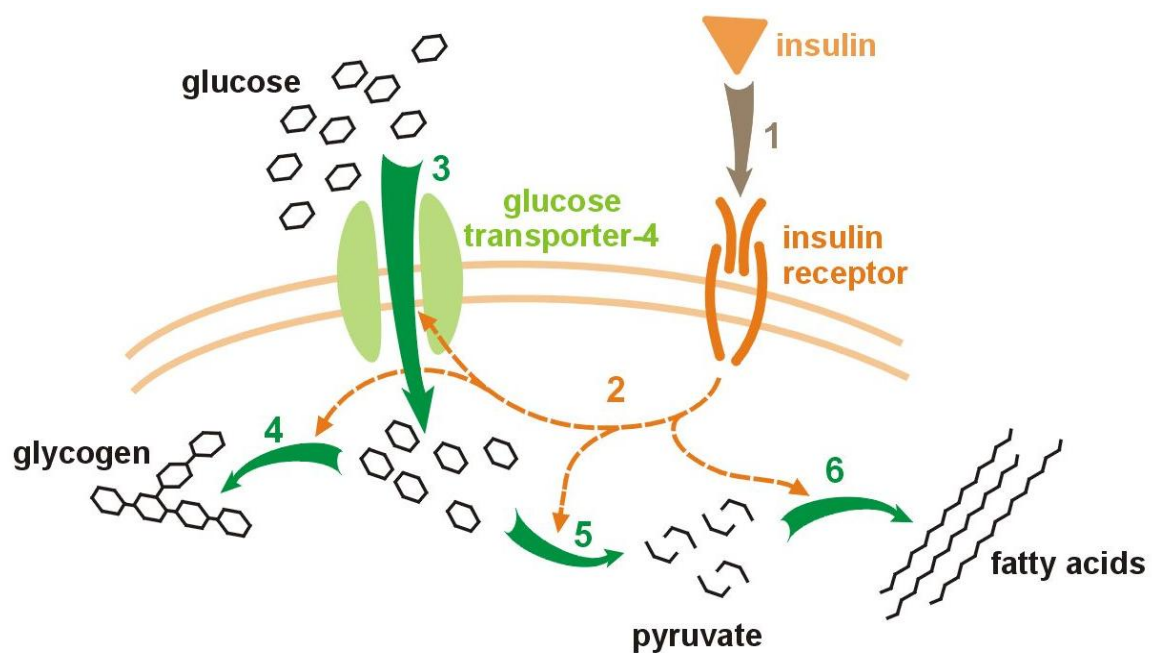


Figure 3: Glucose uptake and metabolism mediated through insulin. (1) Insulin binds to its receptor, (2) the activation of protein cascades, (3) which include: translocation of GLUT-4 transporter to the plasma membrane and influx of glucose, (4) glycogen synthesis, (5) glycolysis and (6) fatty acid synthesis. This image was obtained from an open source public domain and information validated to be in accordance with Meyer *et al.*².

During carbohydrate metabolism, two important basal carbohydrate pathways; glycogenolysis and gluconeogenesis, are also regulated by insulin². Glycogenolysis is a hydrolytic process initiated by adrenaline and glucagon, whereby glucagon is

converted into glucose-1-phosphate. This process is inhibited by insulin^{2,29}, resulting in a decrease in basal glucose concentration. Gluconeogenesis is also inhibited by insulin, whereby it decreases the synthesis of glucose in the liver and kidneys from amino acids, glycerol and lactic acid^{2,29}, preventing an increase in basal glucose concentration.

1.4.2.2 *Insulin defects*

T2DM-induced hyperglycaemia is ultimately a consequence of insulin defects such as with insulin insufficiency, insulin resistance or both³. Insulin resistance manifests when peripheral tissues do not respond normally to secreted or administered insulin³¹. This leads to defective glucose transport into insulin-dependant cells and ongoing liver gluconeogenesis, increasing basal glucose concentration³¹. There are three types of insulin resistance that have been described, type A, B and 'postreceptor'³¹. Type A, also known as 'receptor' type, occurs when there is a decreased amount of insulin receptors on insulin-dependant cells³¹. Type B, or 'pre-receptor' type, is characterized in the presence of auto-antibodies inhibiting the binding of insulin to cell membranes³¹. The 'postreceptor' type is where intracellular signalling defects occur after the binding of insulin to its receptor^{31,33}. Therefore, the search for compounds that may act as insulin mimetics in mediating glucose uptake into insulin-dependent cells are important in the treatment of T2DM.

Obesity is closely related to insulin resistance³⁴, whereby adipose tissue produce and release pro-inflammatory and anti-inflammatory factors known as adipokines². Adipokines cause a decrease of the insulin receptor population, leading to type A resistance². This also initiates the over production of tumor necrosis factor alpha

(TNF- α) and resistin, which alters the enzymatic cascade induced by the binding of insulin, resulting in type B resistance⁴.

On the other hand, the overproduction of insulin occurs when β -cells are sensitized to the presence of glucose³¹. This possibly occurs, due to an increased β -cell mass, a larger number of GLUT2 receptors and a greater expression of hexokinase than glucokinase, which are seen in obese individuals³¹. This partly explains the close association between obesity and the onset of T2DM³¹.

1.4.2.3 *Hyperglycaemia and oxidative stress*

Reactive oxygen species (ROS) are unstable and highly reactive oxygen derivatives with incomplete orbital electron pairs³⁵. These include: singlet oxygen (O_2^*), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl ion (OH^-) and hypochlorous acid (HClO), amongst others³⁵. Naturally, ROS are essential intermediates for immune reactions, detoxification, signaling cascades and the production of energy³⁶. However, excess ROS due to pathological conditions such as hyperglycemia, can cause peroxidation of lipids, deoxyribonucleic acid bases (purine and pyrimidine), structural proteins and enzymes and ultimately lead to biofunctional impairment on a cellular level³⁷.

Hyperglycaemia-induced cellular damage, as mediated through ROS, has been linked to the over-institution of four major biochemical pathways (polyol, hexosamine, protein kinase and glycation) that have been described in detail by Brownlee²⁵ (Figure 4). The consequences of this is associated with an overproduction of ROS, a heightened sensitivity of cells to ROS, defective response

of the antioxidant defense system, biophysical modification of cells and glucotoxicity²⁵. Collectively, these may lead to cellular hypertrophy, apoptosis, necrosis, contractile dysfunction and fibrosis²⁵, all of which may contribute to the secondary complications associated with T2DM (Figure 4).

The four pathways described by Brownlee are linked to the metabolism of glucose in the mitochondria which, is the major source of ROS (O_2^-) production in the body^{20,25}. ROS is generated by mitochondria when cellular glucose stimulates glycolysis²⁵, this leads to an increase of cellular pyruvate concentration, which is carried into the mitochondria, generating reducing agents²⁵. These agents are channeled through the respiratory chain to produce water or ROS²⁵. In the case of chronic hyperglycaemia, this process takes place continuously resulting in an increased ROS load. Furthermore, excess mitochondrial ROS reacts with nitric oxide, producing more ROS, leading to nitrosative stress²⁵. This is associated with the production of toxic substances like peroxynitrate and nitrotyrosine³⁰, both of which may cause micro- and macrovascular complications³⁰.

1.4.2.4 *Antioxidants*

.Antioxidants are substances that significantly delay or prevent the reaction of an oxidizable substrate³⁸. Several types of antioxidants have been identified according to their function against ROS³⁹. These antioxidants neutralize ROS by means of inhibition, reduction, scavenging, forming complexes or quenching³⁶. There are two classes of antioxidants, enzymatic and non-enzymatic. Enzymatic antioxidants such as superoxide dismutase, glutathione peroxidase and catalase are endogenous auto-regulatory antioxidant agents. Examples of non-enzymatic antioxidants include

vitamins, flavonoids, α -lipoic acid, carotenoids, coenzyme Q10, copper, zinc, manganese, selenium and folic acid, amongst others⁴⁰. Synthetic and natural antioxidants are utilized daily, with synthetic antioxidant-concentrates reported to be more active than natural sources due to the impurity of natural antioxidant sources³⁹. However, natural antioxidants are recommended instead of synthetic concentrates due to the toxicity associated with high quantities of antioxidants taken at one time³⁹. In this project natural antioxidant sources are evaluated. Phenols and flavonoids are plant-derived compounds that are known to have antioxidant properties⁴¹⁻⁴⁵.

Another innate protective mechanism against excess mitochondrial ROS is the mitochondrial uncoupling protein system⁴⁶. Uncoupling proteins (UCPs) are mitochondrial proton carriers that are nuclear-encoded in the inner membrane⁴⁶. They are primarily responsible for the leakage of protons across the membrane and modulates energy production and the respiratory electron chain. There are three UCP subtypes⁴⁶, each with a specific function.

The over expression of UCPs has a protective effect in neurons whereby it blocks the production of excess ROS and hyperglycemia-induced cell death⁴⁷. The activation of UCP-2 prevents H₂O₂ damage of β -cells⁴⁸. UCP-3 has a protective effect on myocytes by means of preventing the over-production of mitochondrial ROS⁴⁹. UCPs also exhibit a regulatory role in fatty acid and glucose oxidation, further limiting ROS damage to the mitochondria⁴⁶.

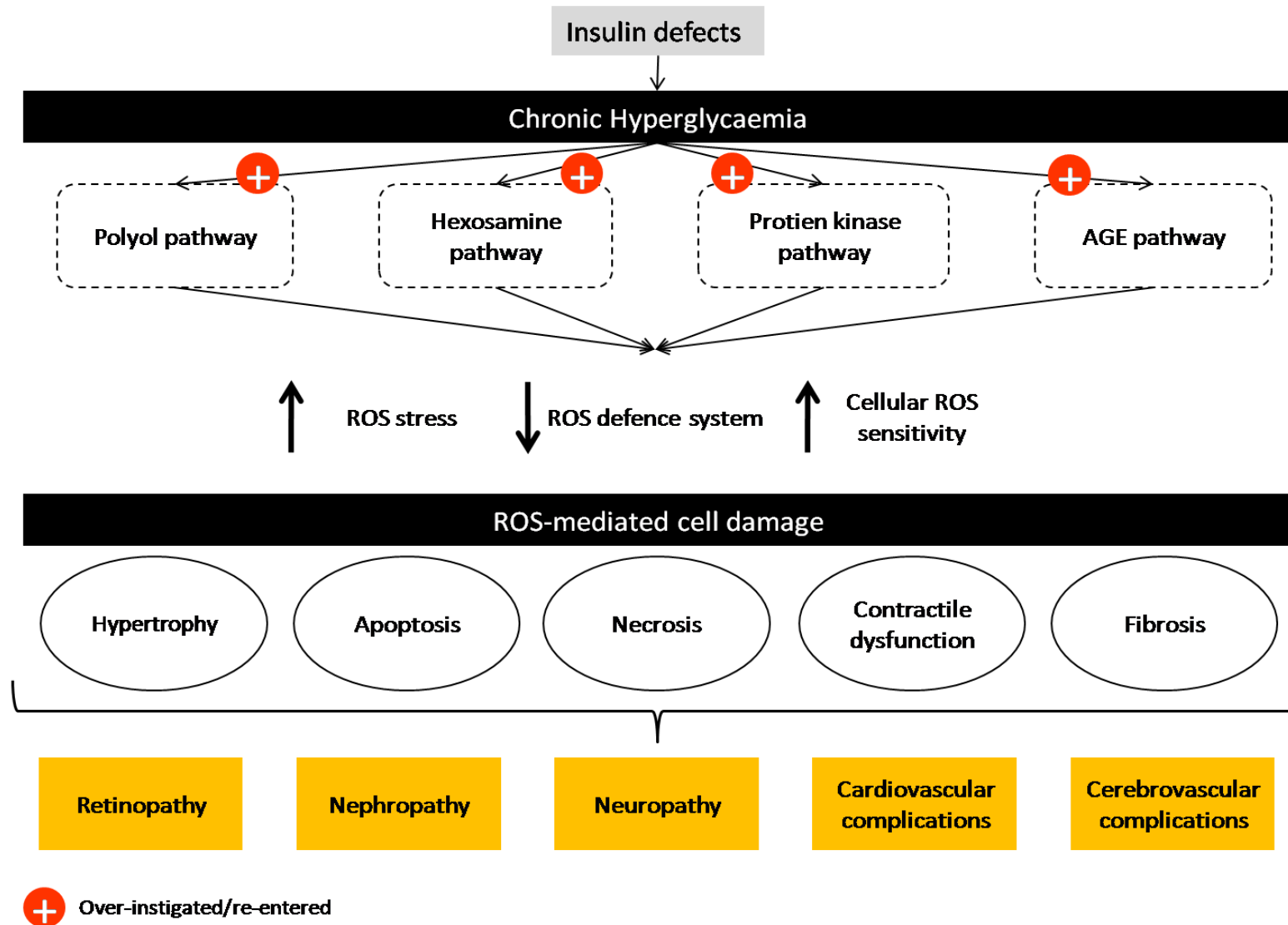


Figure 4: Hyperglycaemia-induced cell damage and associated complications mediated through reactive oxygen species stress. AGE: Advanced Glycation End products.

1.5 Treatment of T2DM

The main target of all T2DM conventional treatment available is the alleviation of chronic hyperglycaemia⁷. Current treatments do not cure T2DM but simply delay the onset of the severe secondary complications associated with it to varying degrees². The majority of T2DM cases can be managed successfully by lifestyle modifications such as regular physical activity and diet modifications^{2,4}. However, most patients do not maintain such lifestyle modifications and therefore rely heavily on pharmacological and/or traditional interventions.

1.5.1 Conventional treatment

There are 10 major non-insulin glucose-lowering classes available⁷. These classes include biguanides, sulfonylureas, meglitinides, thiazolidinediones, α -glucosidase inhibitors, dipeptidyl peptidase-4 (DPP-4) inhibitors, bile acid sequestrant, dopamine-2 agonist, glucagon-like peptide-1 (GLP-1) receptor agonist and amylin mimetics. All of these are associated with unwanted side-effects and/or regimen requirements⁷ (Table 1). Each have a different mechanism by which it induces its hypoglycaemic effect.

Biguanides (metformin), cause a decrease in hepatic glucose production by means of activating adenosine monophosphate (AMP) kinase⁵⁰. Sulfonylureas (glyburide/glibenclamide, glipizide, gliclazide, glimepiride) and meglitinides (repaglinide, nateglinide) increase insulin secretion by closing the adenosine triphosphate-sensitive potassium channels (K_{ATP}) on β -cell plasma membranes⁵¹. Thiazolidinediones (pioglitazone, rosiglitazone), increase insulin sensitivity by

activating the nuclear transcription factor, peroxisome proliferator-activated receptor – γ (PPAR- γ)⁵². α -Glucosidase (Acarbose) inhibitors, slow down carbohydrate digestion and absorption, curbing post-prandial hyperglycaemia⁵³, in the present study, plant extracts will be evaluated for this activity. DPP-4 inhibitors (sitagliptin, vildagliptin, saxagliptin, linagliptin, alogliptin), increase glucose dependent insulin secretion and decrease glucose-dependent glucagon secretion by inhibiting DPP-4 activity and increase glucagon-like peptide-1 GLP-1 and glucose-dependent insulinotropic peptide (GIP) concentrations⁵⁴. Bile acid sequestrant (colesevelam), decreases hepatic glucose production and increases incretin levels by means of increasing bile production and activation of the hepatic farnasoid X factor (FXR)⁵⁵. The exact mechanism of bile acid sequestrants are still under investigation⁷. Dopamine-2 agonist (bromocriptine) regulates the metabolic action controlled by the hypothalamus and increases insulin sensitivity by means of activating dopaminergic receptors⁵⁶. GLP-1 receptor agonists (exenatide, liraglutide), increase insulin secretion, decrease glucagon secretion and retard gastric emptying time by means of activating GLP-1 receptors⁵⁷. Amylin mimetics (pramlintide), decrease glucagon secretion and gastric emptying time and increase satiety by means of activating amylin receptors⁵⁸.

Another class of conventional DM treatment is insulin-based, which include human neutral protamine Hagedorn (NPH), human regular, lispro, aspart, glulisine, glargine, detemir and premixed types⁵⁹. Insulin increases glucose uptake into insulin-dependent cells and decreases basal glucose levels in the liver by means of activating insulin receptors⁷. Each of these drug classes discussed above are provided in Table 1⁷ with their advantages and disadvantages.

Table 1: Advantages and disadvantages of conventional hypoglycaemic drug classes⁷.

Drug Class	Advantages	Disadvantages
Biguanides	Decreases cardiovascular events No increase in weight No hypoglycaemic events Studied extensively	Severe gastrointestinal side-effects Vitamin B12 deficiency Contraindications with chronic kidney disease, acidosis, hypoxia, dehydration
Sulfonylureas	Studied extensively Decreases microvascular complications	Hypoglycaemic events Increases body weight Poorly tolerated
Meglitinides	Multiple dosing concentrations	Frequent dosing Increases body weight Hypoglycaemic events
Thiazolidinediones	No hypoglycaemic events Durable Increases healthy cholesterol Decreases triglyceride concentration	Increase in body weight Myocardial infarction Osteoporosis Increases low density lipoprotein Increased incidence of cancer of the bladder
α-Glucosidase inhibitors	No hypoglycaemia Decreases cardiovascular events	Moderate decrease of HbA _{1c} GIT side effects Frequent dosing
Dipeptidyl peptidase-4 inhibitors	Tolerated well No hypoglycaemic events	Moderate decrease of HbA _{1c} Angioedema Pancreatitis
Bile acid sequestrants	Decreases unhealthy cholesterol No hypoglycaemic events	Moderate decrease of HbA _{1c} Constipation Increases triglyceride load Interacts with other medications

Drug Class	Advantages	Disadvantages
Dopamine-2 agonists	No hypoglycaemic events Decreases incidence of cardiovascular disease	Moderate decrease of HbA _{1c} Dizziness Nausea Tiredness Rhinitis
Glucagon-like peptide-1 receptor agonists	No hypoglycaemic events No increase in body weight	Gastrointestinal side effects Injection and requires training
Amylin mimetics	Decreases postprandial glucose fluctuations Decreases body weight	Moderate decrease of HbA _{1c} Gastrointestinal side effects Hypoglycaemic events Injection Regular dosing
Insulin	Generally effective Decreased microvascular risk	Hypoglycaemic events Increase in body weight Invasive Training is required to self-administer

1.5.2 Herbal treatment

The use of herbal medicine is as ancient as the existence of mankind. According to the World Health Organization (WHO) about 80% of individuals in low to middle income countries rely on herbal remedies daily⁶⁰. Traditional medicine is often the only readily available and affordable source of treatment for the majority of people in developing countries⁶¹. The use of herbal remedies is also increasing steadily⁶².

Diabetes has been treated with herbs long before the westernization of modern medicine⁸. Many plant species have been reported to possess antidiabetic activity⁶³ such as *Catha edulis* Forrsk (Celastraceae; 'khat')^{9,63}. *Vernonia amygdalina* Del.

(Asteraceae; 'bitter leaf')⁶⁴, *Sutherlandia frutescens* Linn. (Fabaceae)^{64,65} and *Leonotis leonurus* L. (Lamiaceae, 'lions tail')^{10,63}. Also, various conventional drugs have been derived either directly or indirectly from herbs, an example of this is metformin, which was isolated from a plant known as *Galega officinalis* in the 1920's¹².

In recent years plants have been studied extensively for their antioxidant properties. Herbally-derived antioxidants have been found to possess the ability to reduce the risk of the onset of T2DM and improve glucose utilization⁶⁶. Polyphenols possess the ability to act against lipid and glucose metabolism disorders associated with T2DM²¹ and hypoglycaemic activity^{67,68}.

1.6 Diabetea

Diabetea is a polyherbal mixture, commercially used to treat diabetes⁶⁹⁻⁷¹. The mixture is prepared as a tea infusion, which is drunk twice daily (1 teaspoon to 1 cup of boiling water left to steep for 10 min). Diabetea, is manufactured by Sing-Fefur organic herbs in South Africa. Sing-fefur is a biodynamic organic farm, based in Cape Town, which grows its herbs indigenously and makes use of wild crafting from surrounding mountains⁷⁰. This polyherbal mixture consists of *Achillea millefolium*, *Agathosma betulina*, *Salvia officinalis*, *Taraxarum officinalis*, *Thymus vulgaris*, *Trigonella foenum-graecum* and *Urtica urens*. Each of these plants were obtained in its dried form from a phytomedicinal company called Natural Products in South Africa.

1.6.1 *Achillea millefolium* L.

Achillea millefolium L. belongs to the Asteraceae family and is a natural exotic plant of South Africa, commonly known as yarrow, milfol, yarrow, *duisendblaar achillea*⁷². Yarrow has a coarse and angular stalk with feather-like leaves that can grow up to 8-10 cm long (Figure 5). Its flowers bloom from June to September and are white and pale lilac in colour. There are fine hairs on the whole plant that gives it a slight white appearance⁷³. Yarrow is widely distributed around the world, and can be found everywhere, from mountain meadows to ordinary gardens⁷³. Yarrow is well known and used as a vulnerary⁷³. Inhabitants in the highlands make use of a yarrow ointment for treating wounds whereas, Orkadians treat melancholy with its tea preparation⁷³. It is also traditionally used for cardiovascular disorders⁷⁵. Yarrow is sometimes referred to as 'nosebleed' since it is used to treat this condition⁷³.

In Brazil and Norway, a tea infusion of yarrow is used to treat joint pain caused by rheumatism as well as fever and lung infections⁷³. The Norwegians use it to treat toothache by chewing the leaves and stalk⁷³. It is especially known for its use as an antipyretic⁷³. Other traditional medicinal uses include antidiuretic, gastrointestinal disturbances, inflammation, spasms, irregular menses, contraceptive, menopausal complaints, measles, kidney diseases, blood detoxification, skin disorders, hyperhidrosis, piles (ointment), lack of appetite, dyspepsia, prevention of baldness (when used as a shampoo) and diabetes^{73,76-85}.

The principal pharmacologically active compounds in *A. millefolium* are flavonoids and phenolcarboxylic acids⁸⁶, which have been shown to have anti-inflammatory⁸⁷ antimicrobial⁸⁸, choleric⁸⁹ and cytotoxic⁹⁰ activities. It has also been shown that *A.*

millefolium exhibits cardio-regulatory activities, supporting its traditional use⁷⁵. Antioxidant properties have been shown per the hydroalcoholic, methanolic, oil and aqueous fractions^{77,86}. The methanolic extract have been found to possess DPPH[•] scavenging activity, correlating with its polyphenolic and flavonoid content^{68,69,81}. Furthermore, it also has anti-inflammatory, spasmolytic, antitumour, antimicrobial, liver protective, gastric anti-secretory and gastro-protective activity^{77,91}. Its medicinal activities are mainly attributed to its high flavonoid content^{78,85}

The major chemical constituents in yarrow have been identified as apigenin, quercetin (flavonoids) and caffeoylquinic acid (phenolic acid)⁹² and other compounds including carbohydrates, proteins, fructose, glucose, sucrose and tre-halose, raffinose, oxalic, quinic, and citric; succinic acid, linoleic acid, palmitic acid, oleic acid, α -tocopherol and ascorbate^{81,93-96}



Figure 5: Illustration of *Achillea millefolium*⁷⁴.

1.6.2 *Agathosma betulina* Bartl. & Wendl.

Agathosma betulina Bartl. & Weidl. (Buchu, Rutaceae) formerly known as *Barosma betulina*, is one of a hundred and fifty indigenous *Agathosma* species and arguably the most popular medicinal herb in South Africa, which is also marketed internationally^{97,98}. Buchu is mainly grown in the south-western area of the Cape⁹⁸. *A. betulina*, also known as ‘short buchu’ grows to a length of between 2.5 to 12 cm, has pale green leaves with oil glands, white flowers and brown fruits (Figure 6). Buchu has a distinct rue- and peppermint-like smell and aromatic taste⁹⁸. Because of the economical importance of buchu in South Africa, the Cape government has ruled that no individuals are allowed to purchase or sell buchu without a license⁹⁸. ‘Buchu’ commercially refers to the collection of three species: *A. betulina*, *A. crenulata* and *A. serratifolia*⁹⁸. In this study, buchu, only refers to *A. betulina*.

Buchu is often taken in the form of a brandy preparation, commonly known as ‘buchu brandy’. This preparation was first introduced by the Hottentots in 1820 and has been introduced as a medicinal preparation in Great Britain⁹⁸. In Great Britain, buchu brandy is mainly used to treat cystitis, urethritis, nephritis and catarrh of the bladder⁹⁸. In the Cape, buchu brandy is used as a tonic and a remedy for gastrointestinal ailments⁹⁸. Other uses reported for Buchu include blood cleanser, diuretic, promotion of sweating, urinary tract infections, diabetes, inflammation, wounds (vinegar preparations), contusions, calculus, gout, rheumatism, prevention of cancer, antispasmodic, antipyretic, haematuria, prostatitis, antibiotic protectant, antifungal, cystitis and nausea^{69,77,78,97,99-103}.

The major constituents of buchu are mucalige, volatile oil and diosphenol⁹⁸, which are known to be antiseptic. Buchu has been found to possess numerous pharmacological activities; antimicrobial activity^{97,105-108}, antioxidant activity^{105,106} and anti-inflammatory activity^{105,109}. The compounds that have been isolated from buchu are: limolene, menthone, diosphenol, *l*-pulegone, (Ψ)-diosphenil, monoterpene triols (responsible for odour), 8-mercapto-*p*-menthan-3-one (flavour and aroma), sulfurated terpanoid, ketones, *p*-menthan-3-one 3-oxo-*p*-menthane-8-thiol acetate, pulegone, sulfur bearing compounds, dithio-*p*-menthane, isomenthone, 4-hydroxydiosphenol, 1-hydroxydiosphenol, bi-, tri-functional monoterpenes and flavonoids¹¹⁰⁻¹¹⁸



Figure 6: Illustration of *Agathosma betulina*¹⁰⁴.

1.6.3 *Salvia officinalis* L.

The genus '*Salvia*', of the Lamiaceae family, originates from the Latin word '*salvere*' which means 'to be saved' due to its healing properties¹¹⁹. Sage, which grows to a height of ~ 30 cm has a thin but firm stalk. The leaves grow in pairs on the stem to a length of between 2.5 to 5 cm and are green-grey in colour (Figure 7). Sage blooms in August, producing a purplish flowers that contain volatile oils responsible for producing the strong scented odour and a bitter taste¹¹⁹.

Salvia officinalis, or 'common sage', grows naturally in areas with an abundance of limestone and very little soil, such as is found in the northern shores of the Mediteranean. Common sage has also been cultivated in areas with extreme winters such as Germany, France and England¹¹⁹.

The main constituent of sage, a green-yellow volatile oil, has a prominent odour and taste and is mainly used as flavouring nowadays, but traditionally was used as an additive to embrocations for rheumatism¹¹⁹. One of the most widely valued methods of using sage (leaves) is in the form of a mouth wash prepared with water, to treat tonsillitis, throat ulcers, sore throat, bleeding gums and to prevent excessive salivation¹¹⁹. In the case of a persistent and very severe throat complaint, a hot vinegar and cold water (1:1) solution has been recommended¹¹⁹. 'Sage tea', made from 28 g of sage and 500 ml boiling water, is used to treat fever, delirium, headache, as a stimulant tonic, to cure gastrointestinal weakness and for blood purification¹¹⁹. The Chinese at times preferred sage tea to their own teas for the treatment of nervous and gastric ailments¹¹⁹. In Sussex, dried leaves are smoked to treat asthma¹¹⁹.

The leaves of sage contain tannins and resin¹¹⁹. *S. officinalis* contains a high phenol and flavonoid content, which is associated with significant antioxidant activity^{69,121}. It has also been shown to activate the PPAR- γ , which is the key regulator of adipogenesis and glucose homeostasis. It is reported to possess the ability to cause insulin-stimulated glucose uptake into insulin-dependent cells¹²². The diterpenoids in *S. officinalis* have been reported to be responsible for its antidiabetic effects¹²³.

The volatile oil of sage contains salvene, a hydrocarbon, and trace compounds such as pinene, cineol, borneol, esters and thujone (a ketone)¹¹⁹. Carbohydrates, proteins, fructose, glucose, sucrose, tre-halose, raffinose, α -tocopherol, ascorbate flavonoids, apigenin, quercetin and many acids (oxalic, quinic, citric, succinic, linoleic, palmitic, oleic phenolic, caffeoylquinic) have been isolated from sage^{81,89,93,95,96}.



Figure 7: Illustration of *Salvia officinalis*¹²⁰.

1.6.4 *Taraxacum officinalis* L.

Taraxacum officinalis (Dandelion, Asteraceae) is colloquially known as the ‘common dandelion’. The genus *Taraxacum* is derived from *taraxos*, and *akos*, meaning ‘disorder’ and ‘cure’, respectively¹²⁴. Dandelion has a thick, dark brown stem that is white on the inside. The long, shiny and jagged leaves mediate rain drops to flow to the base of the shrub ensuring that it stays moistened¹²⁴. The teeth-like shape of the leaves is said to be the reason for the name ‘dandelion’, which comes from French phrase *dent de lion*, meaning teeth of a lion¹²⁴. The flower stems are purplish and shiny with a milky fluid on the inside that causes a brown stain when it comes into contact with skin¹²⁴. Each stem only carries one bright yellow flower¹²⁴. When the yellow flowers are mature they close up and the withered petals are pressed off and replaced with white tufts of hair, which are able to be blown off when fully matured¹²⁴ (Figure 8). Dandelion is native to the northern hemisphere but is naturally grown in the south as well. It is practically found everywhere and can be a great burden to farmers¹²⁴.

Dandelion roots are roasted and used to make coffee that is said to stimulate the whole physiological system, aiding liver and kidney performance and keeping the gastrointestinal tract healthy^{124,125}. In India, dandelion is used to treat liver complaints¹²⁴. The leaves are used as a ‘herb-beer’ and the whole plant as a tincture. Dandelion is also used as a diuretic, blood cleanser, re-mineralizer, tonic, mild laxative, liver tonic, choleric, diuretic, antirheumatic, anti-inflammatory and saluretic^{65,69,78,124,126-128}.

The major constituents in the roots of dandelion are taraxacin and acrylline¹²⁴. Inulin has been found in the root of both the fresh or dried shrub, making up to 24% of the content depending on the season¹²⁴. *In vitro* and *in vivo* research on dandelion has shown activity such as anti-inflammatory, hypoglycaemic, prebiotic, immunomodulator, angiolytic, analgesic and antineoplastic¹²⁵. The leaves of dandelion have been found to contain alkaloids, flavonoids, steroids, saponins, tannins and triterpenoids¹²⁵.

Bioactive compounds identified from dandelion include: luteolin-7-glucoside, apigenin-7-glucoside, quercetin, luteolin, isorhamnetin-3-glucoside and 3,7-diglucoside, caffeic, chlorogenic acids, p-hydroxyphenylacetic acid, p-hydroxyphenylacetic acid, sesquiterpene lactone glucoside, scopoletin, aesculetin, taraxinic acid, 1-glucosyl ester and 11,13-dihydrotaraxinic acid¹³⁰⁻¹³⁵.



Figure 8: Illustration of *Taraxacum officinalis*¹²⁹.

1.6.5 *Thymus vulgaris* L.

T. vulgaris (Lamiaceae), 'common thyme' or 'garden thyme', was originally grown in areas such as Europe, Asia minor, Algeria and Tunisia. Nowadays, it is grown globally. The roots are woody and wiry. Its highly branched stem can grow up to 20 cm long, carrying very small and elliptical leaves (Figure 9), which are dark green with a greyish tint. The flowers are of a lilac colour. The plant grows easily and is especially fond of chalky and dry ground¹³⁶.

Thyme, in the form of a tea or fleshly mixed with honey or syrup is traditionally used to treat a wet cough, sore throat, flu, fever and abdominal discomfort¹³⁶. It is reported to induce sweating and stop abdominal fermentation explaining its application for treating flu and abdominal discomfort¹³⁶. The oil of thyme is used in the treatment of rheumatism and to increase blood flow to the skin. When the smoke of thyme is inhaled it is said to alleviate headaches¹³⁶. Other traditional uses include: antibacterial, inflammation of liver, antiseptic, antibiotic, antidiabetic, antifungal, antispasmodic and antiviral^{69,136-140}.

The most abundant compounds that have been identified in the oil fraction of thyme are thymol and carvacrol¹³⁶. Thymol is said to possess powerful antiseptic activity. Compounds isolated from the oil fraction of thyme are cymene, pinene, menthone, borneol and linalol¹³⁶. A number of other bioactive flavonoids and phenols have been isolated from thyme oil and other extracts; apigenin, naringenin, eriodictyol, cirsilineol, salvigenin, cirsimaritin, thymoine, thymusine, ursolic and oleanolic acids, geraniol, linalool, α -terpineol, transthujan-4-ol and terpinen-4-ol^{136,139-143}.



Figure 9: Illustration of *Thymus vulgaris*¹⁴⁵.

1.6.6 *Trigonella foenum-graecum* L.

Trigonella foenum-graecum (Fabaceae), also known as bird's foot or greek hay-seed, is most commonly called fenugreek. '*Trigonella*' is a Greek word that means 'three-angled' because of the shape of its flower petals. '*foenum-graecum*' means Greek hay for it was used by the Greeks to overcome the odour of old hay. The fenugreek plant reaches a height of up to 60 cm. It has long slender pods on its stem that can host up to twenty yellow-brown seeds. Each seed has two lobes and is said to have a bitter taste¹⁴⁴ (Figure 10).

It has been reported that the Egyptians would take fenugreek in the form of a paste by soaking crushed seeds in water and using it to treat fevers, stomach ailments and diabetes. The antipyretic effect of fenugreek paste is reported to be comparable to that of quinine¹⁴⁴. Fenugreek is traditionally taken in the form of a water-based drink to treat gastrointestinal inflammation and anaemia. Some other traditional uses of fenugreek include: detoxification, wound healing, pancreas cleanser and to promote spleen metabolism^{11,21,146}.

Fenugreek contains ~30% mucilage, 20% proteids (polypeptide containing compounds) and 5% oil. Compounds such as trigonelline, choline, lectin, nuclealbumin, trimethylamine, neurin and betain have been isolated from fenugreek. Neurin and betain are reported to have a stimulatory effect on the nervous system, which causes an increase in appetite and acts as a diuretic¹⁴⁴. Other compounds that have been isolated from fenugreek are: alkaloids, flavonoids, salicylate, nicotinic acid, phenols, apigenin, kaempferol, quercetin glycosides, vitexin, triclin, naringenin, quercetin, triclin 7-O-D-glucopyranoside¹⁴⁷⁻¹⁵².

Pharmacological activities ascribed to fenugreek are hypoglycaemic activity^{21,144,146,153-157}, cholesterol lowering properties^{158,159} and antioxidant activity (attributed to its flavonoid and phenol content)^{160,161}. Furthermore, it also has an anti-glycosuric effect along with reduction in high plasma glucagon and somastatin and a glucose-induced insulin releasing effect¹⁵⁷.

1.6.7 *Urtica urens* L.

Urtica urens (Nettle, Urticaceae) is commonly known as annual nettle, dwarf nettle, small nettle, dog nettle or burning nettle. Its extensive use in South Africa is cited in the material medica¹⁶². Nettle is commonly found in Europe, Asia, Japan, South Africa and Australia. Nettle is also used as a food plant in South Africa due to its nutritious content¹⁶³. The name nettle is derived from the Dutch word *noedle* meaning needle, due to its sharp stinging characteristic. *U. urens* is an ingredient to 12 Herb teas available in the Western Cape namely: arthritea, asthmitea, detoxtea, Diabetea, diatea, energetea, flootea, hangovertea, Hi.Lo.B.P.tea, slimtea, uleratea and voomatea⁶⁹. Nettle can grow up to 90 cm high and has heart shaped, pointy leaves with green flowers that grow in clusters. The stems are also covered in stinging hairs, hence the origin of its name (Figure 11).



Figure 10: Illustration of *Trigonella foenum-graecum*¹⁶⁴.

Since the end of the 19th century *U. urens* and *U. dioica* have been used in the treatment of burns, rashes, kidney stones, agalactia and hemorrhages, to mention a few^{65,69,163}. *U. urens* is commonly used to treat diabetes in Brazil¹⁶⁵. Nettle is traditionally used as a circulatory stimulant, tonic, diuretic, re-mineralizer, detoxifying agent, hypoglycaemic agent, to treat burn wounds, coughs, arthritis, pain, rheumatism and as an antidiabetic¹⁶⁶.

U. urens has been tested and validated as a haemostatic, hypotensive, analgesic and diuretic¹⁶⁷. It also has favourable bronchopulmonary, gynaecological and neurological effects. It possesses antioxidant properties, and protects the liver from the hepatotoxic action of toxic substances¹⁶⁷.

Studies have shown that nettle extracts possess antimicrobial activity¹⁶⁸, chemoprotective effects *in vivo*^{169,170}. Patuletin, a compound isolated from the plant, has significant anti-inflammatory activity¹⁷¹. Bioactive compounds that have been isolated from nettle include: acetylcholine, histamine, 5-hydroxytryptamine (serotonin), small amounts of leukotrienes, shikimic acid derived phenylpropanes, caffeic acid, chlorogenic acid, caffeoylmalic acid as well as the coumarin and scopoletin. Other reports identified the presence of flavonoids, fatty acids, terpenes, protein, vitamins, minerals, kaempferol, isorhamnetin, quercetin, 3-rutinosides and 3-glycosides^{163,172-176}.

All plants evaluated in the present study has been documented to be used traditionally and have been evaluated for pharmacological activity. However, there is no evidence of a combination of all of these tested for its antidiabetic activity.



Figure 11: Illustration of *Urtica urens*¹⁷⁷.

1.7 Aim

The aim of this study was to assess the *in vitro* antidiabetic efficacy of the 'Diabetea' mixture and its individual herbs.

1.8 Objectives

The objectives of this study were to:

- Prepare crude dichloromethane and hot water extracts of the Diabetea mixture and its individual constituents: *A. millefolium* L., *A. betulina* Bartl. & Wendl., *S. officinalis* L., *T. officinalis* L., *T. vulgaris* L., *T. foenum-graecum* L. and *U. urens* L.
- Determine the total polyphenolic and flavonoid content of each extract, using the Folin-Ciocalteu and aluminium trichloride methods, respectively.
- Evaluate the non-cellular antioxidant activity of each extract, using the 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid and 1,1-diphenyl-2-picrylhydrazyl free radical assays.
- Assess the cell-based antioxidant activity against *p*-chloranil induced ROS in Ea.hy926 cells using the fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate.
- Evaluate the cytotoxic potential of each extract in differentiated C2C12 myocytes, Ea.hy926 human endothelial cells and human lymphocytes, using the sulforhodamine B assay.
- Determine the effects of the extracts (as substrates) on α -amylase and α -glucosidase activity using 3,5-dinitrosalicylic acid and *p*-nitrophenyl- α -D-glucopyranoside, respectively.

- Determine the effects of the extracts (as insulin mimetics) on glucose uptake in differentiated C2C12 myocytes using 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-d-glucose.

1.9 Project overview

This is a multifaceted *in vitro* antidiabetic assessment, mainly focused on the potential of plants to overcome three major physiological issues associated with T2DM, namely: oxidative stress, post-prandial hyperglycaemia and insulin deficiency. A schematic overview of this project is provided in Figure 12.

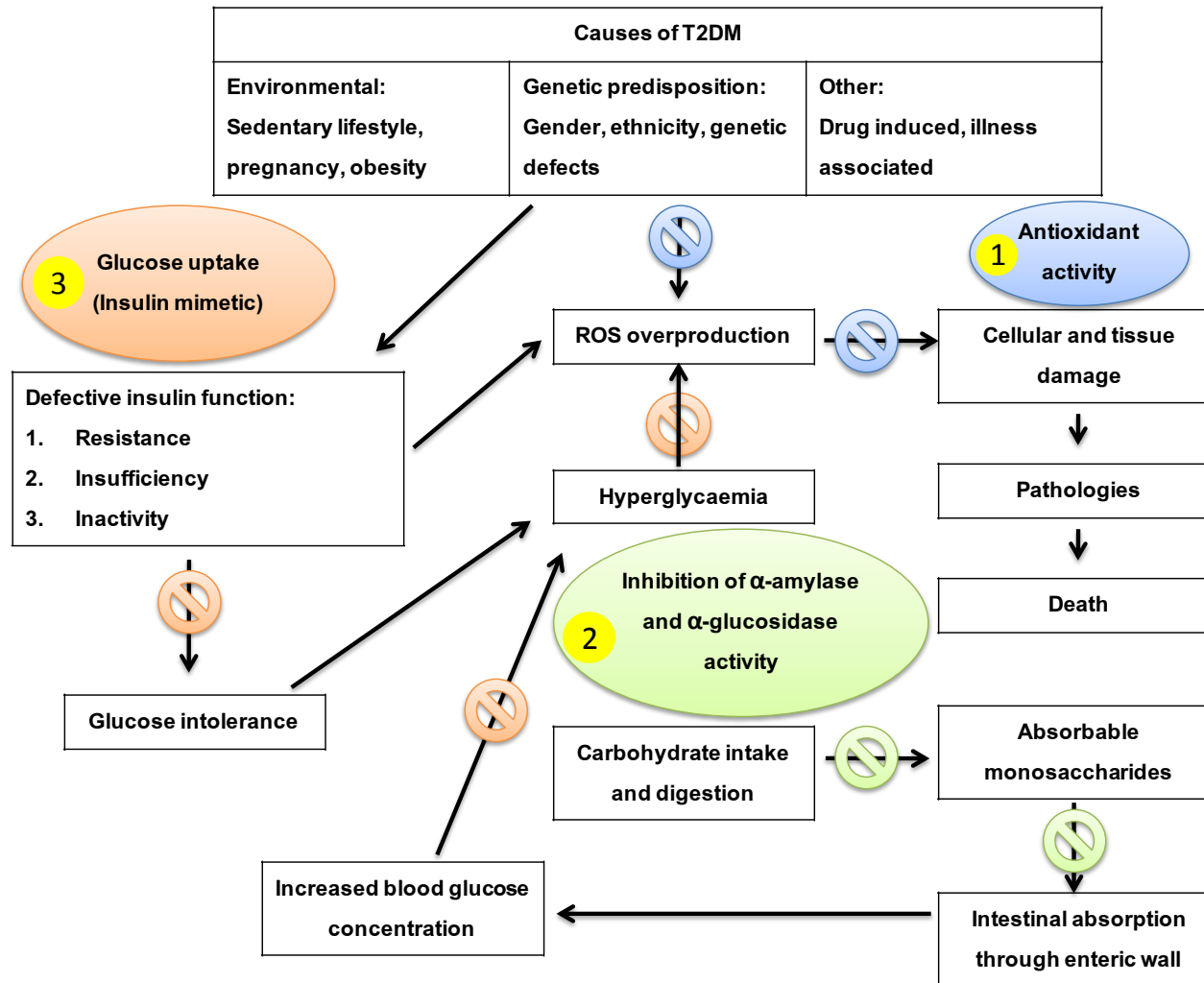


Figure 12: Project overview of the therapeutic targets in the present study: antioxidant activity (blue), prevention of post-prandial hyperglycaemia (green) and glucose uptake into cells (orange) and how it relates to type 2 diabetes mellitus.

CHAPTER 2: EXTRACTION AND CHARACTERIZATION

2.1 Introduction

T2DM is directly associated with the overproduction of ROS which is implicit in the onset and development of life-altering and –threatening secondary complications^{2,30}. The onset of T2DM has also been associated with the presence of chronic elevated ROS levels, down regulation of innate ROS protective mechanisms and heightened sensitivity to ROS²⁵. Therefore the alleviation of oxidative stress is implicit for effective treatment of T2DM. Herbs have been shown to possess antioxidant containing compounds such as phenols and flavonoids^{39,178,179}.

Ea.hy926 (CRL-2922) cells are human endothelial cells harvested from umbilical vein. Hyperglycaemia, the hallmark of T2DM, causes the progression of endothelial damage through the production of ROS^{25,242}. This is the reason why endothelial cells were used to evaluate the cell-based ROS attenuation exerted by each extract.

All of the Diabetea herb ingredients have previously been studied for their radical scavenging activity and polyphenolic content (see section 2.5) and have been reported to exhibit both. However, this is the first account of the antioxidant activity and polyphenolic content of the Diabetea mixture.

2.2 Materials and methods

2.2.1 Materials, chemicals and instrumentation

All reagents and instruments used in the present study are listed in Appendix I (page 157).

2.2.2 Plant ingredients

Individual herbs were obtained from South African Natural Products (Pty) Ltd. The Diabetea mixture was purchased from Sing-Fefur organic herbs. All herbal material was obtained in dried form. The seven ingredients constituting Diabetea are: *A. millefolium* L. (Asteraceae, whole plant), *A. betulina* Bartl. & Wendl. (Rutaceae, leaves), *S. officinalis* L. (Lamiaceae, leaves), *T. officinalis* L. (Asteraceae, aerial plant mixture), *T. vulgaris* L. (Lamiaceae, leaves and stems), *T. foenum-graecum* L. (Fabaceae, seeds) and *U. urens* L. (Urticaceae, whole plant mixture). Plant materials were ground into a fine powder before extraction using a IKA-Werke Yellowline A10 analytical grinder.

2.2.3 Extract preparation

Hot water (HW) and dichloromethane (DCM) were used for the crude herb compound extractions. The HW extraction was done using a decoction method, whereby 2 g homogenous plant powder was mixed with 20 ml distilled water (dH₂O). The solution was left on an electronic shaker (Beckman Coulter, VRN-200) for 1 h and placed in a sonicator (Branson 52 Cleaning Equipment Co.) for another hour, after which the mixtures were macerated for 24 h at 4°C. The following day the

mixtures were left on a shaker for 30 min to reach room temperature before being boiled (Labotec, Büchi Heating Bath, B-490) for 15 min. Mixtures were centrifuged for 30 min at 1000 *g* (Allegra x-22, Beckman Coulter), filtered through a vacuum filtration system (0.22 µm) and then kept at -4°C overnight. The following day the mixtures were concentrated using lyophilization (Freezone 6, Labconco) and stored at -70°C.

The DCM extraction was performed in a similar fashion to that of the HW extraction process up to the centrifugation step, whereafter the supernatant was concentrated by means of *in vacuo* rota-evaporation (Labotec, Büchi Rotavapor, R-200) at 60°C. The concentrate was reconstituted into dimethyl sulfoxide (DMSO, Lab-Scan, Analytical Sciences) and stored as aliquots at -70°C. The extracts were tested at concentrations that are theoretically bioavailable, 1 to 20 µg/ml. The concentration of DMSO, in reaction, was kept at less than 0.05% v/v. The percentage yield of DCM extracts were calculated as follows:

$$\% \text{ Gravimetric Yield} = W \times \frac{V}{DW} \times 100$$

Where,

W: weight of extract solutes in one ml of extract solution (mg/ml)

DW: starting dry weight (mg)

V: volume of solvent used for extraction (ml).

Equation 1: Percentage extraction yield.

2.2.4 Total polyphenolic content

2.2.4.1 Total phenolic content

Total phenolic content (TPC) was determined following a spectrophotometric, 96-well microplate method, using Folin-Ciocalteu's reagent (FCR), as described by Slinkard and Singleton¹⁸⁰, with minor modifications. Gallic acid (GA) was used as the phenol standard to construct a calibration curve at concentrations ranging from 0.1 to 0.8 mM using 20% v/v ethanol (EtOH) as solvent. Experimental wells contained 50 μl of extracts (100 $\mu\text{g}/\text{m}\ell$), 60 μl of FCR and 100 μl of a 3% w/v sodium carbonate (Na_2CO_3) solution. The phytochemical interference was accounted for by wells containing plant extract (50 μl) and distilled water (160 μl). FCR (60 μl) with distilled water (150 μl) served as control while wells with distilled water (210 μl) only served as blank. The plates were incubated in the dark for 1 h and absorbance was measured at 630 nm using a Bio-tech Instruments ELx800_{UV} plate reader. The TPC was expressed in terms of gallic acid equivalence (GAE) (mg GA /g extract \pm SEM), which was calculated as follows:

$$GAE = \frac{[GA] \times \ell(y) \times DF}{g}$$

Where,

[GA]: concentration of gallic acid (mg/m ℓ),

$\ell(y)$: initial volume (m ℓ) yield of extraction,

DF: dilution factor, and

g: total weight (g) of extract.

Equation 2: Gallic acid (or rutin for flavonoid content) equivalents.

2.2.4.2 Total flavonoid content

The total flavonoid content (TFC) was determined following a spectrophotometric, 96-well microplate method, of Quettier-Deleu¹⁸¹, with minor modifications. Rutin was used as the flavonoid standard to construct a calibration curve (20 - 200 µg/ml) with methanol (MeOH) as solvent. The reaction mixture consisted of 40 µl plant extract (100 µg/l), 20 µl of a 3% w/v sodium nitrate (NaNO₃) solution at, 20 µl using aluminium trichloride (AlCl₃) (1% w/v) and 100 µl of sodium hydroxide (NaOH) at 2 M. The plates were incubated in the dark for 10 min, after which absorbance was measured at 450 nm (Bio-tech Instruments, ELx800UV). The wells containing 40 µl sample and 140 µl distilled water served as background control to eliminate phytochemical interference whereas wells with 180 µl distilled water served as blank. The TFC was expressed in terms of rutin equivalence (RE mg/g extract ± SEM), which was calculated using Equation 2 (page 46).

2.2.5 Cell-free antioxidant activity

2.2.5.1 ABTS^{•+} principle

The 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid free radical (ABTS^{•+}) assay was first introduced by Miller and Rice-Evans in 1993 and further modified by Re *et al.*¹⁸². The modification required elimination of the need for an intermediate free radical and the formation of the final ABTS^{•+} free radical without the influence of antioxidants, thus improving accuracy and reproducibility. This improvement allowed for the testing of hydro- and lipophilic antioxidant systems¹⁸².

This assay tests for antioxidants with a hydrogen-donating ability, which makes the reduction of the ABTS^{•+} the foundation of this method¹⁸². ABTS^{•+} is a synthetic blue/green redox-active compound formed by a persulfate oxidation reaction of ABTS. The antioxidant capacity of a compound or solution is determined by the decolouration of the blue/green ABTS^{•+} free radical solution. The extent of the antioxidant capacity of a test compound against ABTS^{•+}, relies on its concentration and duration of the experimental process^{182,183}. The optimal absorption wavelengths of ABTS is 415, 645, 734 and 815 nm¹⁸². Generally, the extent of antioxidant activity is expressed in terms of trolox equivalents, which is why this method is often referred to as the trolox equivalent antioxidant capacity (TEAC) assay¹⁸². In the present study, the attenuation of ABTS^{•+} is reported in terms of percentage scavenging activity instead.

2.2.5.2 ABTS^{•+} assay

The ABTS^{•+} neutralization was determined using a spectrophotometric, 96-well microplate method described by Re *et al.*¹⁸² with minor modifications. The ABTS free radical solution was prepared by incubating a mixture of ABTS (7 mM) and 2.45 mM potassium persulfate (K₂S₂O₈) in dH₂O, in the dark, for 12-16 h at 4°C. Prior to experimentation the ABTS^{•+} solution was diluted with EtOH to a standard absorbance of 0.7 ± 0.02 at 734 nm. The reaction mixture consisted of 50 µl of plant extract (1 - 20 µg/ml) and 150 µl ABTS^{•+}. The plates were incubated at room temperature, in the dark, for 15 min and the absorbance was read at 630 nm. The control wells contained 50 µl of dH₂O and 150 µl ABTS^{•+}. Phytochemical interference was accounted for by wells containing extract (50 µl) with dH₂O (150 µl) whereas dH₂O alone (200 µl) served as blank. Trolox dissolved in pure MeOH

was used as positive control. The antioxidant capacity of each extract was expressed quantitatively in terms of percentage ABTS^{•+} scavenging activity which was calculated as follows:

$$\% \text{ Scavenging activity} = \frac{A(\text{control}) - A(\text{extract})}{A(\text{control})} \times 100$$

Where,

A(extract): absorbance of extract reaction, and

A(control): absorbance of control.

Equation 3: Percentage ABTS^{•+} scavenging activity.

2.2.5.3 DPPH[•] principle

The 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH[•]) assay is a quick, easy and sensitive assay for screening for antioxidant activity in plants¹⁸⁴. DPPH[•] is a stable-synthetic hydrogen/electron-accepting free radical that has an unpaired valence electron on a nitrogen atom (Figure 13)^{185,186}. The reduction of DPPH[•] free radical causes a decolouration from purple to yellow and therefore a decrease in absorption at 515 nm. The extent of antioxidant activity is determined by the number of electrons/hydrogens that are captured by DPPH[•].

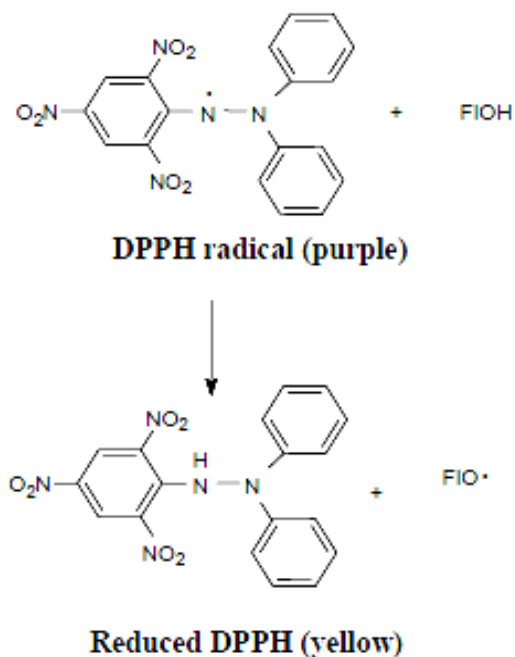


Figure 13: Attenuation of DPPH[•] free radical. FIOH: reducing agent, FIO: oxidized chemical.

2.2.5.4 DPPH[•] assay

The DPPH[•] scavenging activity was determined using a spectrophotometric, 96-well microplate method as described by Gyamfi *et al.*¹⁸⁷ with minor modifications. The DPPH[•] solution was prepared fresh for every experiment, using pure MeOH. Fifty microliters of plant extract (1 - 20 µg/ml) was added to 150 µl of DPPH[•] (100 µM). Each plate was incubated at room temperature, in the dark, for 15 min after which absorbance was read at 570 nm and not at 515 nm due to instrument limitations. Wells containing MeOH (50 µl) and DPPH[•] (150 µl) served as control and wells with MeOH (200 µl) only served as blank. Phytochemical interference was accounted for by wells containing extract (50 µl) and MeOH (150 µl). The antioxidant capacity of each extract was expressed in terms of percentage DPPH[•] scavenging activity, which was calculated using Equation 3 (page 49).

2.2.6 Cell-based antioxidant activity

2.2.6.1 Cell culture propagation and maintenance

Cell-based antioxidant activity of each extract was determined in EA.hy926 cells, which is a human endothelium cell line originating from umbilical vein tissue. This cell line was obtained from Steve Oglesbee (representing Dr. Cora-Jean S. Edgell) at the UNC Lineberger comprehensive cancer center, for the use in non-commercial studies (Appendix II, page 162). All cell work was conducted under strict sterile conditions and incubated in a 5% CO₂ incubator at 37°C in Dulbecco's Modified Eagle's Medium (DMEM). Medium was supplemented with 1% penicillin/streptomycin and 10% foetal calf serum (FCS).

2.2.6.2 DCFH-DA principle

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) is a cell-permeable fluorogenic probe used to determine the relative amount of intracellular ROS concentration^{188,189}. Once DCFH-DA has been absorbed into a cell it is deacetylated by cellular esterase into a non-fluorescent probe, 2',7'-dichlorodihydrofluorescein (DCFH)^{188,189}. DCFH is rapidly oxidized by intracellular ROS into 2',7'-dichlorodihydrofluorescein (DCF), which is highly fluorescent^{188,189}. Therefore, the relative fluorescence intensity (RFI) of a sample represents the extent of intracellular ROS.

2.2.6.3 DCFH-DA assay

The ability of crude plant extracts to attenuate *p*-chloranil-induced ROS was determined using a 96-well microplate method with DCFH-DA as described by

Boissy *et al.*¹⁹⁰, with minor modifications. White plates were pre-seeded with Ea.hy926 human umbilical vein cells at 1.5×10^4 cells/well. Eighty microliters of DMEM-, containing 5 μ M DCFH-DA was added to wells and incubated for 1 h at 37°C in 5% CO₂. The wells were carefully aspirated and 80 μ l of *p*-chloranil (40 μ M) added, which was dissolved in Hank's buffered salt solution (HBSS). Eighty microliters of plant extract (1 - 20 μ g/ml) was added to the experimental wells and the plates were incubated for 2.5 h at 37°C in a 5% CO₂ atmosphere. Trolox was used as positive control at a final concentration of 20 μ g/ml. Wells containing 80 μ l of *p*-chloranil and 80 μ l DMEM- served as control wells. Fluorescence was measured at $\lambda_{\text{ex}} = 485$ nm and $\lambda_{\text{em}} = 540$ nm, with a gain setting of 750 (FLUOstar Optima, BMG Labotech). The results are expressed in terms of RFI.

2.3 Statistical analysis and data representation

All experiments were executed in triplicate (technical and biological, $n = 9$) and the data is presented in the form of bar graphs with each bar representing the mean \pm standard error of the mean (SEM). Statistical analysis between extracts was performed using Tukey's multiple comparisons one-way ANOVA test. Student's *t*-tests were used to compare extracts to the negative and positive controls. The antioxidant activity was further classified as weak (<50%), moderate (>50%, <75%) or strong (>75%). Correlation tests were determined using the Pearson test. The statistical packages used included GraphPad Prism 6.0, STATA 12 and Microsoft Excel 2010. Statistical significance was regarded as $p < 0.05$.

2.4 Results

2.4.1 Extract yields

The extract yields using pure DCM as solvent are represented in

Table 2. *A. millefolium* and *T. vulgaris* had the highest yields of all herbs, followed by Diabetea and *U. urens*. The extracts of *T. officinalis* and *T. foenum-graecum* had the lowest percentage yields. From observation it was evident that the physical properties of the HW and DCM extracts were notably different. The HW extracts were generally pale, brown and viscous, whereas the DCM extracts were bright in colour and volatile. Filtration of the DCM extracts was performed quickly and with great ease, whereas the HW extracts were filtered with greater difficulty due to the viscosity.

Table 2: Mean percentage yields (determined gravimetrically) of DCM herb extracts.

Extract yields	
Herb	% Yield \pm SEM
<i>A. millefolium</i>	26.7 \pm 0.1
<i>A. betulina</i>	11.7 \pm 0.3
<i>S. officinalis</i>	16.7 \pm 0.1
<i>T. officinalis</i>	5.0 \pm 0.1
<i>T. vulgaris</i>	26.7 \pm 0.2
<i>T. foenum-graecum</i>	10.0 \pm 0.2
<i>U. urens</i>	18.3 \pm 0.0
Diabetea	21.7 \pm 0.0

2.4.2 Polyphenolic content

The total phenolic and flavonoid content of each herb extract is represented in Figure 14 and Figure 15, respectively. The HW extracts were rich in both phenols and flavonoids, whereas the DCM extracts hardly contained any phenolics (< 1%) or flavonoids (< 1%). Furthermore, the HW extracts contained more flavonoids than phenolics (Figure 14A and Figure 15A).

The HW extract of *T. vulgaris* contained significantly ($p < 0.05$) more phenolic content (101.3 ± 1.7 mg GAE/ g extract) compared to all the other plant extracts (Figure 14A), with *S. officinalis* possessing the second highest amount of phenolic content (58.3 ± 1.6 mg GAE/ g extract, Figure 14A). The HW extract of the Diabetea contained a noteworthy phenolic content of $\sim 44.4 \pm 1.0$ mg GAE/ g extract (Figure 14A). *T. officinalis* had the lowest phenolic content (22.3 ± 0.8 mg GAE/ g extract, Figure 14A).

The HW extract of *T. vulgaris* also contained significantly ($p < 0.05$) more flavonoids compared to the rest of the extracts (Figure 15A), with *S. officianlis* once again containing the second highest amount of flavonoids (491.7 ± 12.5 RE, Figure 15A). The HW extract of the Diabetea also contained a notable amount of flavonoids (256.6 ± 3.1 RE, Figure 15A). The lowest flavonoid content was observed for the HW extract of *T. foenum-graecum* (147.9 ± 2.6 RE, Figure 15A).

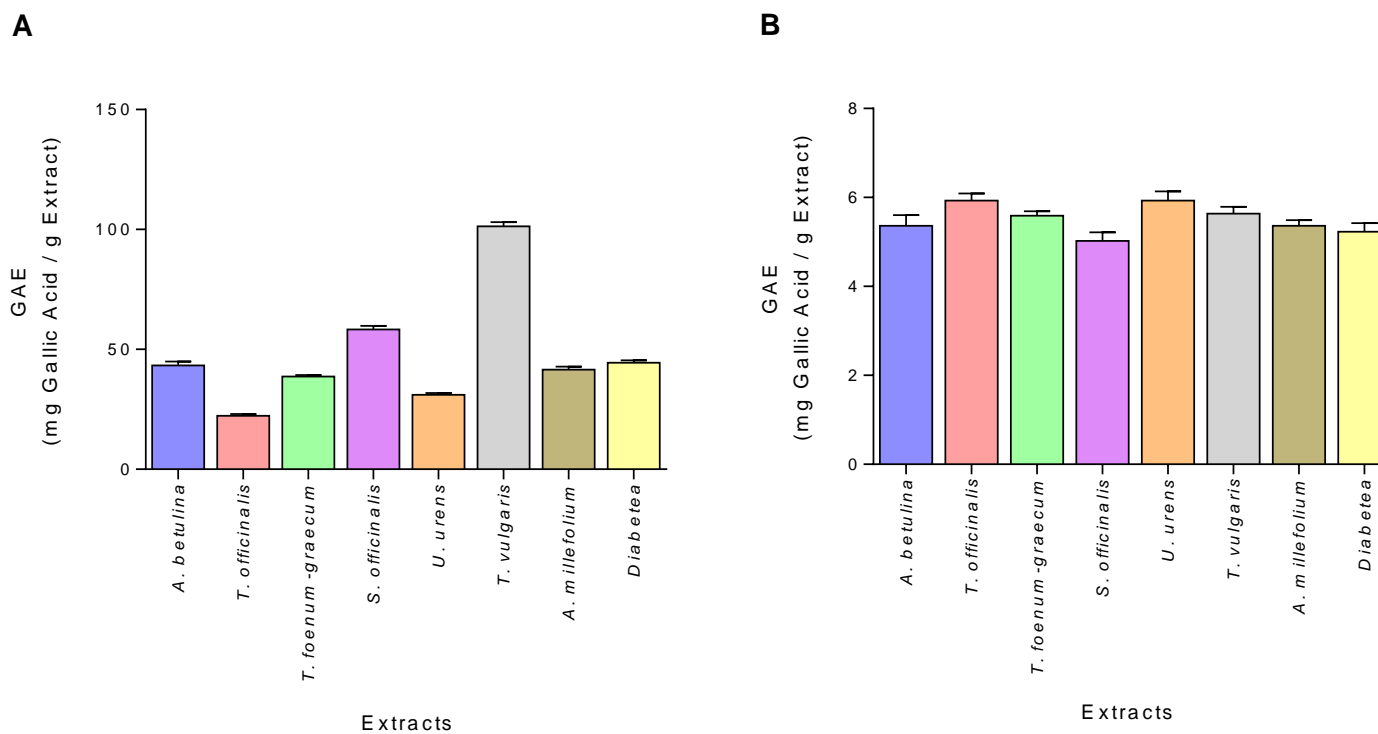


Figure 14: Total phenolic content of (A) hot water and (B) dichloromethane plant extracts. Each extract was tested in triplicate (n = 9) and expressed as mean gallic acid equivalents (GAE) \pm SEM. Note that the scales of the y-axes of graphs A and B are not equal.

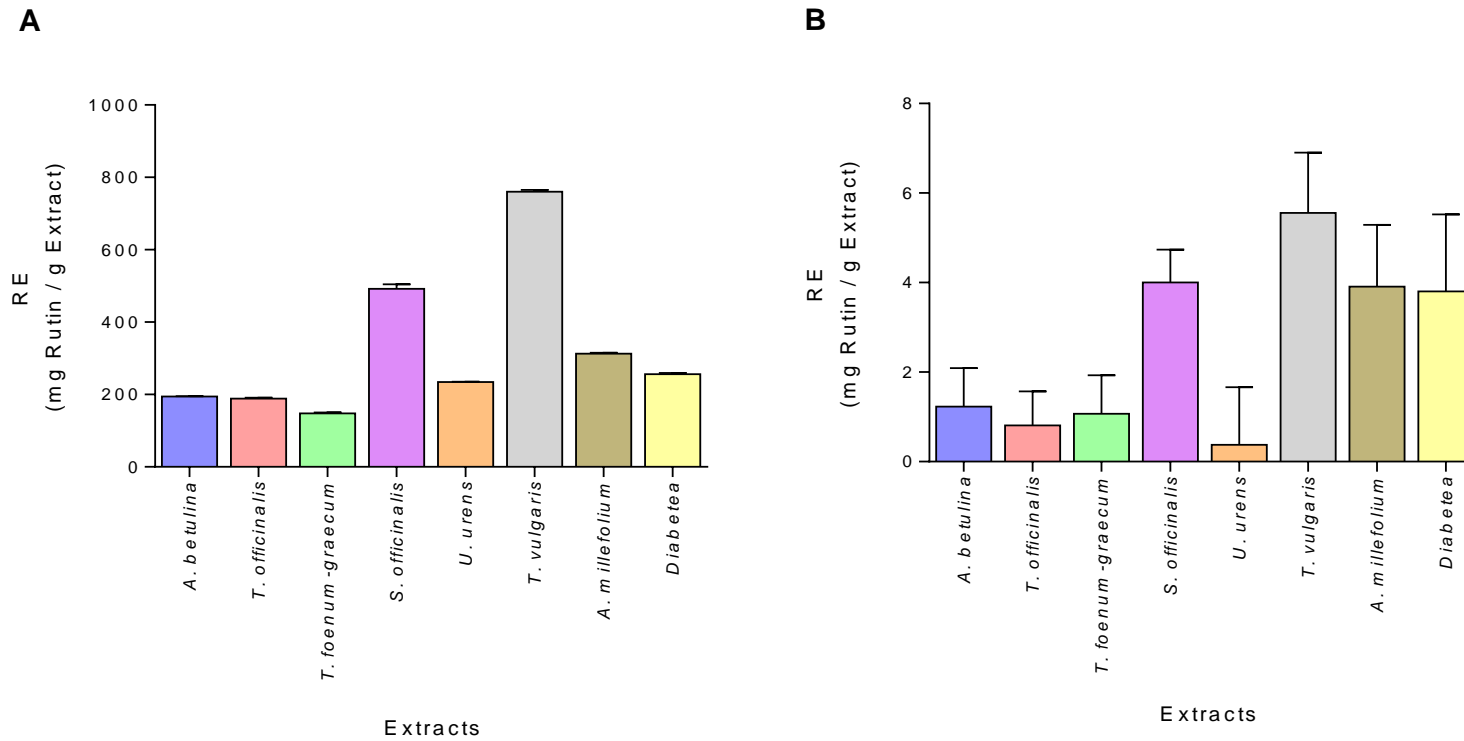


Figure 15: Total flavonoid content of the (A) hot water and (B) dichloromethane plant extracts. Each bar represents the mean rutin equivalents (RE) \pm SEM of triplicate measurements (n = 9). Note that the scales of the y-axes of graphs A and B are not equal.

2.4.3 Cell-free antioxidant activity

All herbs exhibited an antioxidant potential against either ABTS^{•+} or DPPH[•] or both. Both HW and DCM extracts were more active against ABTS^{•+} than DPPH[•] (Figure 16 - Figure 19). In general, the HW extracts displayed a greater antioxidant potential than the DCM extracts. Most of the HW and DCM extracts attenuated ABTS^{•+} and DPPH[•] in a concentration-dependent manner.

2.4.3.1 ABTS^{•+} scavenging activity

T. vulgaris had the most significant ($p < 0.05$) activity against ABTS^{•+}, with a strong (> 75%) and moderate (> 50%, < 75%) scavenging activity exerted by its HW and DCM fractions, respectively (Figure 16 and Figure 17). The antioxidant activity of the HW extract of *T. vulgaris* was comparable to that of trolox at concentrations of 1.25, 2.5 and 5 µg/ml (Figure 16) and its DCM fraction was comparable to trolox at 2.5 µg/ml (Figure 17). The HW extract of *S. officinalis* exhibited a moderate antioxidant activity, which was the second highest ($p < 0.05$) at 10 and 20 µg/ml compared to all other herbs in this study. The only other extract that showed a moderate antioxidant activity was the HW extract of *A. millefolium*, whereas all other extracts displayed low (< 50%) antioxidant activity (Figure 16).

Both the HW and DCM extracts of the Diabetea were active against ABTS^{•+} but showed a low (< 50%) antioxidant activity exerted by both fractions (Figure 16 and Figure 17). The Diabetea displayed the second highest ($p < 0.05$) antioxidant potential of all DCM extracts tested (Figure 17). However, the antioxidant activity of Diabetea was less significantly when compared to the individual herb extracts.

The order of ABTS^{•+} scavenging activity exerted by the HW plant extracts from highest to lowest were: *T. vulgaris*, *S. officinalis*, *A. millefolium*, *U. urens*, Diabetea, *T. officinalis*, *A. betulina* and *T. foenum-graecum*. For the DCM extracts, *T. vulgaris* displayed the highest antioxidant activity followed by Diabetea, *U. urens*, *A. millefolium*, *A. betulina*, *S. officinalis*, *T. officinalis* and *T. foenum-graecum*. The extracts of *T. foenum-graecum* had the poorest antioxidant activity for both extracts.

2.4.3.2 DPPH[•] scavenging activity

The radical scavenging activity of *T. vulgaris* was the most significant ($p < 0.05$) of all plant extracts tested. Despite this being a significant result, it was not comparable to the activity of trolox. HW extract of *S. officinalis* was again the second most potent antioxidant fraction that exerted a moderate antioxidant activity, at the highest concentration tested (20 µg/ml). The HW and DCM extracts of *T. foenum-graecum* displayed the weakest antioxidant activity. The Diabetea also scavenged DPPH[•], although this was low it was significantly ($p < 0.05$) higher than *A. betulina*, *T. officinalis* and *T. foenum-graecum*.

The order of antioxidant activity from highest to lowest of the HW extracts were as follows: *T. vulgaris*, *S. officinalis*, *A. millefolium*, *U. urens*, Diabetea, *A. betulina*, *T. officinalis* and *T. foenum-graecum*. None of the DCM extracts attenuated more than 20% of DPPH[•] and are therefore considered to be poor in antioxidant activity.

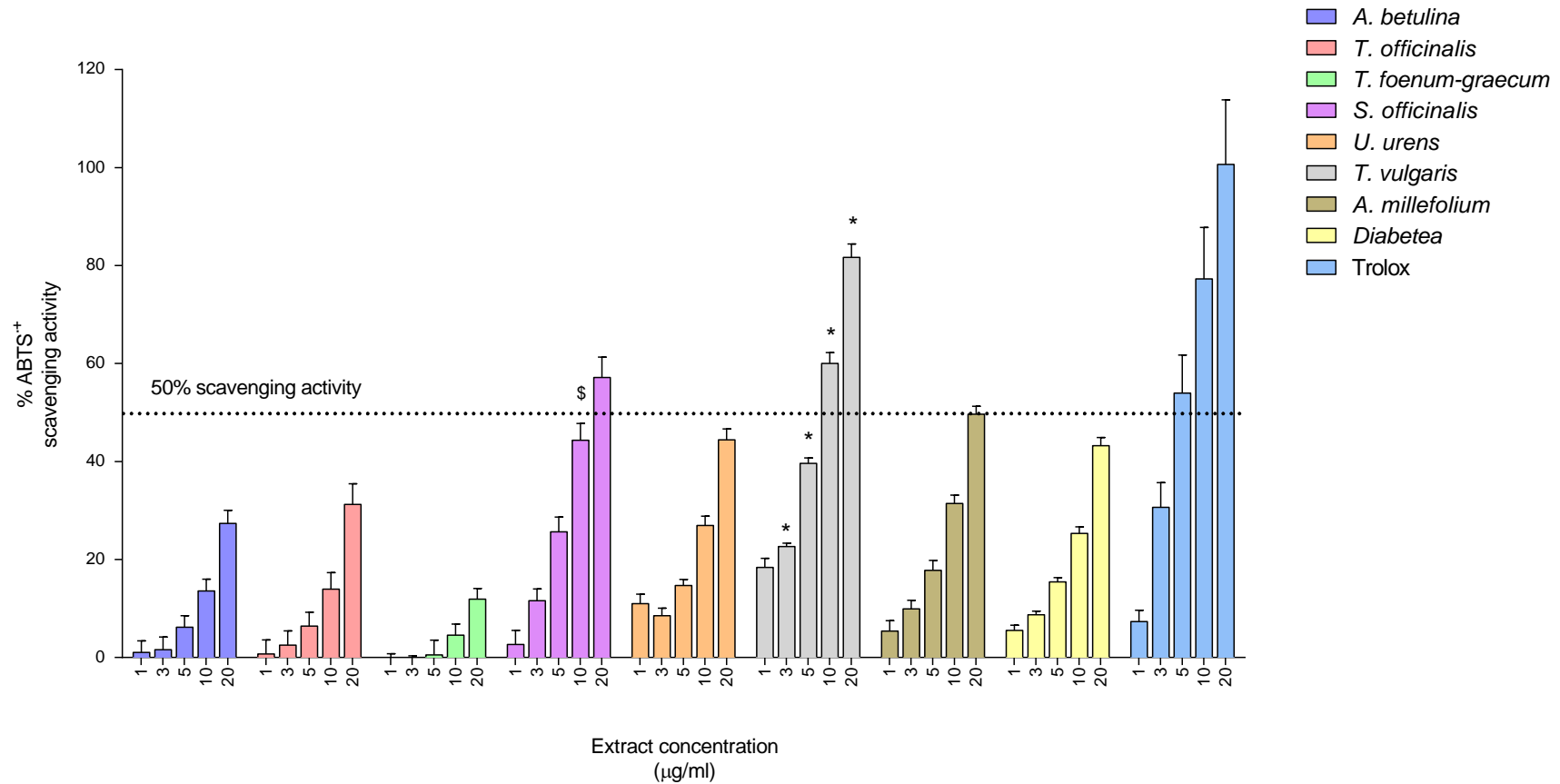


Figure 16: The percentage ABTS⁺ scavenging activity of the hot water plant extracts tested at 1 to 20 µg/ml. Each bar represents the mean percentage ABTS⁺ scavenging activity ± SEM of triplicate experiments (n = 9). Significant ($p < 0.05$) antioxidant activity between extracts (*), and the second most significant (\$) antioxidant activity was determined using Tukey's multiple comparisons two-way ANOVA test. The dotted lines represent the activity of trolox at 20 µg/ml and 50% ABTS⁺ scavenging activity.

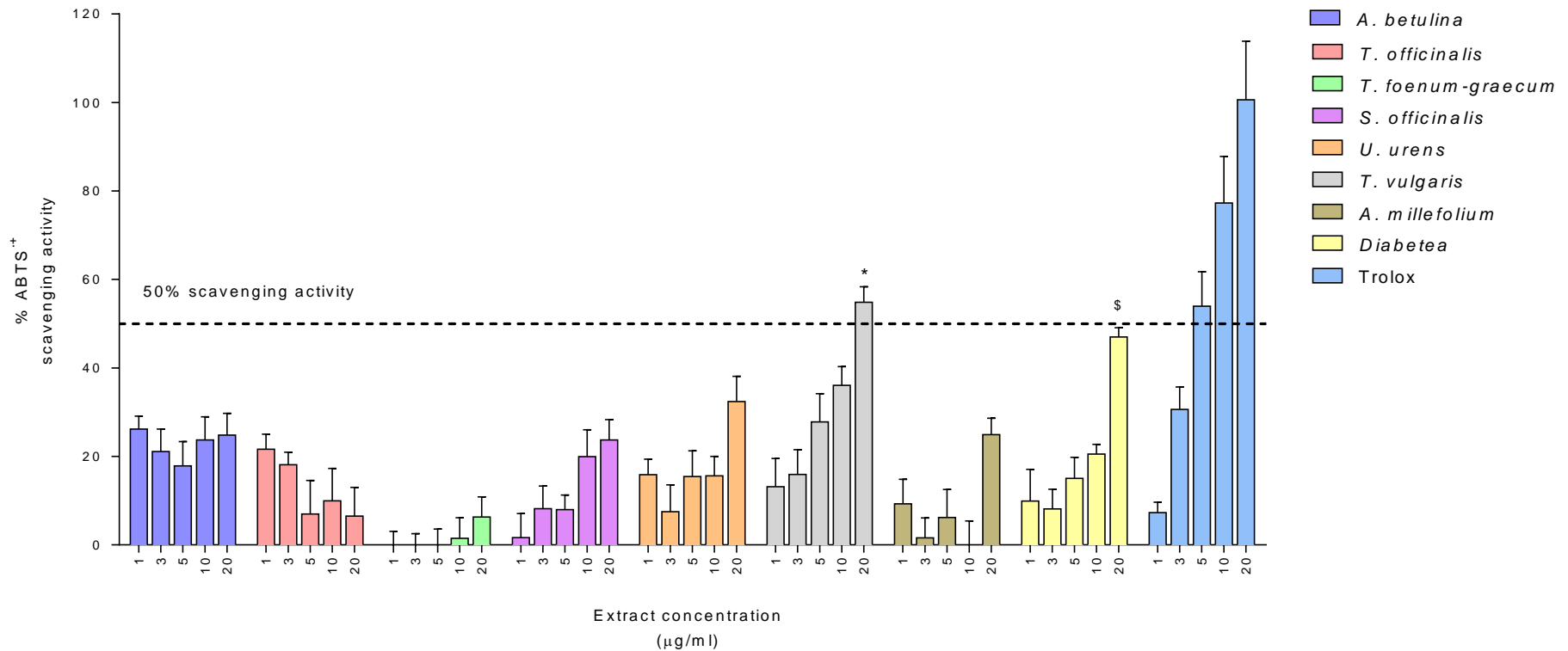


Figure 17: The percentage ABTS^{•+} scavenging activity of the dichloromethane plant extracts tested at 1 to 20 µg/ml. Each bar represents the mean percentage ABTS^{•+} scavenging activity ± SEM of triplicate experiments (n = 9). Significant (p < 0.05) antioxidant activity between extracts (*), and the second most significant antioxidant activity (\$) was determined using Tukey's multiple comparisons two-way ANOVA test. The dotted lines represent the activity of trolox at 20 µg/ml and 50% ABTS^{•+} scavenging activity.

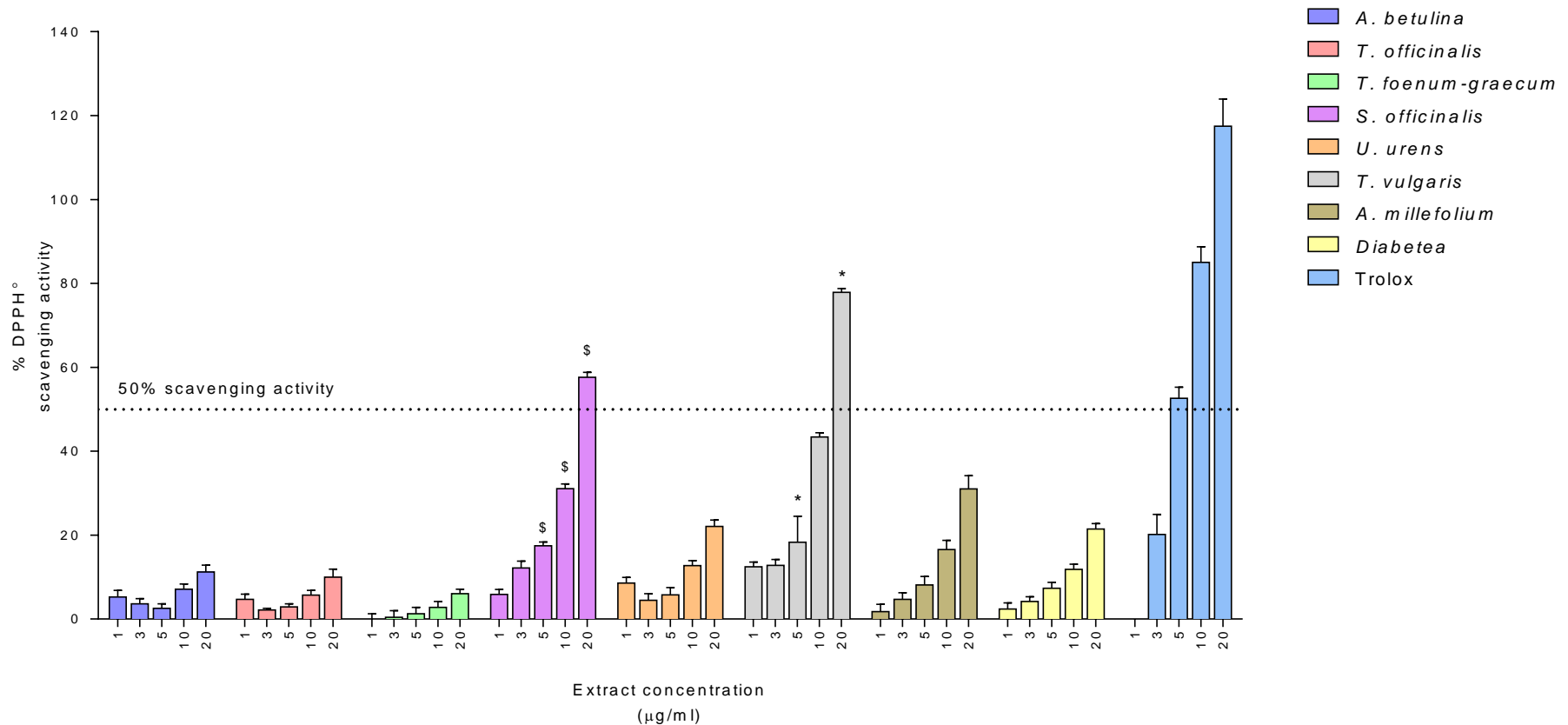


Figure 18: The percentage DPPH[•] scavenging activity of the hot water plant extracts tested at 1 to 20 µg/ml. Each bar represents the mean percentage DPPH[•] scavenging activity ± SEM of triplicate experiments (n = 9). Significant ($p < 0.05$) antioxidant activity between extracts (*), and the second most significant antioxidant activity (\$) was determined using Tukey's multiple comparisons two-way ANOVA test. The dotted lines represent the activity of trolox at 20 µg/ml and 50% DPPH[•] scavenging activity.

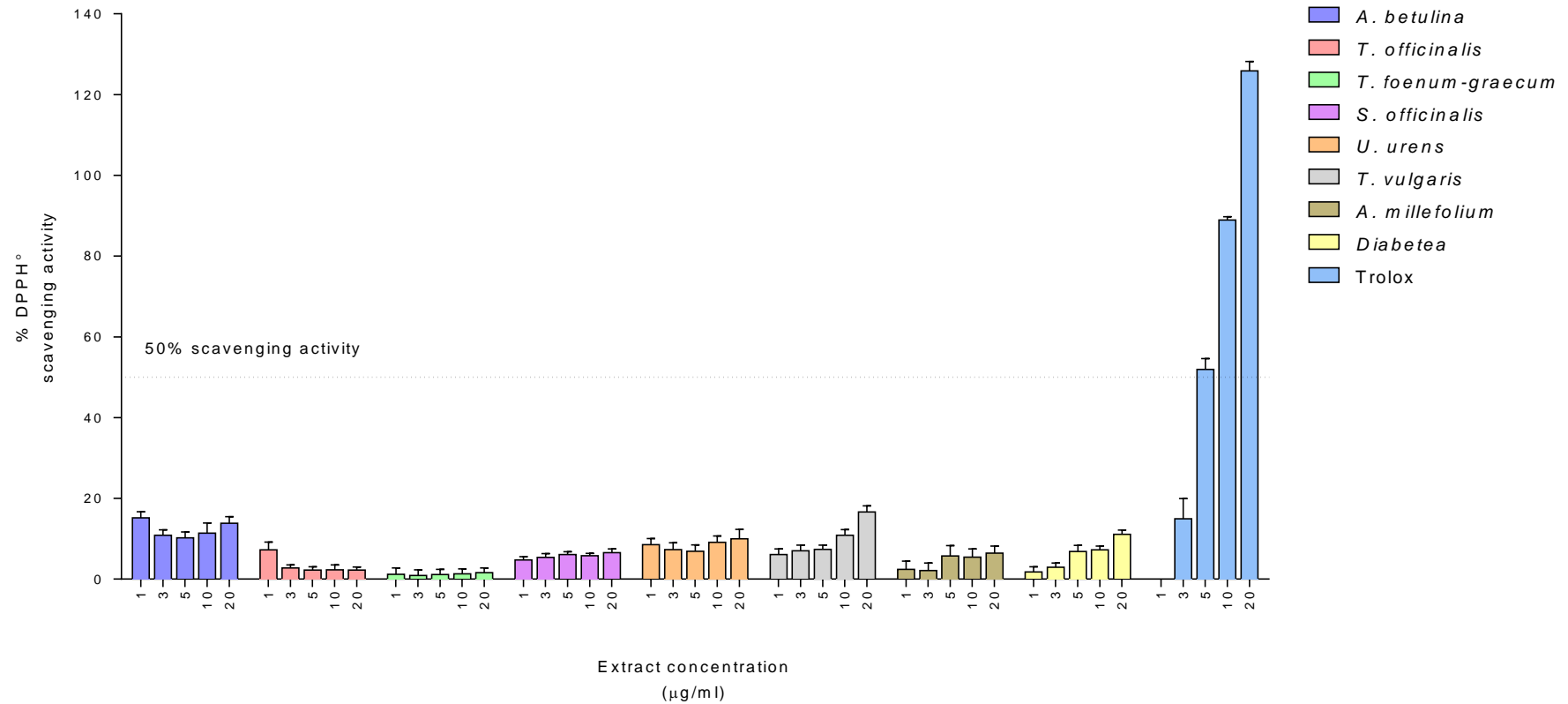


Figure 19: The percentage DPPH[•] scavenging activity of the dichloromethane plant extracts tested at 1 to 20 µg/ml. Each bar represents the mean percentage DPPH[•] scavenging activity ± SEM of triplicate experiments (n = 9). Significant ($p < 0.05$) antioxidant activity between extracts (*), and the second most significant antioxidant activity (\$) was determined using Tukey's multiple comparisons two-way ANOVA test. The dotted lines represent the activity of trolox at 20 µg/ml and 50% DPPH[•] scavenging activity.

2.4.4 Cell-based antioxidant activity

The HW extracts of *T. vulgaris*, *S. officinalis* and *U. urens* significantly ($p < 0.05$) inhibited the production of cellular ROS, whereas none of the DCM extracts displayed intracellular antioxidant activity (Figure 20 and Figure 21). The HW extracts of the Diabetea, *A. betulina*, *T. officinalis* and *T. foenum-graecum* significantly ($p < 0.05$) aggravated the overproduction of ROS (Figure 20) and all the DCM extracts, except for *A. betulina*, caused a significant ($p < 0.05$) increase in cellular ROS (Figure 21). This showed that neither the HW or DCM extracts of the Diabetea displayed any antioxidant activity. The order of antioxidant activity exhibited by the HW extracts was as follows from highest to lowest: *S. officinalis*, *U. urens*, *T. vulgaris* and *A. millefolium*.

2.4.5 Relationship between antioxidant activity and polyphenolic content

There was a significant ($p < 0.05$) correlation between the ABTS^{•+} radical scavenging activity and polyphenolic content observed for the HW fraction (Figure 22). With a greater correlation ($r = 0.93$) between the TFC and ABTS^{•+} scavenging activity (Figure 22B), compared to the relationship between ABTS^{•+} scavenging activity and TPC ($r = 0.79$, Figure 22A). There was no significant correlation between the polyphenolic content and antioxidant activity, with regards to the DCM extracts (Figure 23A-B).

A significant ($p < 0.05$) correlation existed between the polyphenolic content of the HW extracts and their ability to attenuate DPPH[•]. An increase in the TFC of the HW extracts was associated ($r = 0.99$, $p < 0.05$) with a significant increase in the

neutralization of DPPH[•] (Figure 22D), this was strongest correlation found in the present study. Once again, there was a stronger relationship between the TFC of the HW extracts and DPPH[•] scavenging activity ($r = 0.98$, Figure 22D), than observed with the TPC of these extracts ($r = 0.89$, Figure 22C).

There was a significant ($p < 0.05$) correlation between the ABTS^{•+} and DPPH[•] scavenging activities with both HW ($r = 0.94$) and DCM ($r = 0.87$) extracts. Furthermore, a relationship between the cell-free and cell-based antioxidant activities exerted by the HW extracts was also observed (Figure 22E-F), however, this correlation was not present with the DCM extracts (Figure 23E-F). A relationship also existed between the polyphenolic content and cell-based antioxidant activity of the HW extracts (Figure 24A-B), but once more this was not observed with the DCM extracts (Figure 24C-D).

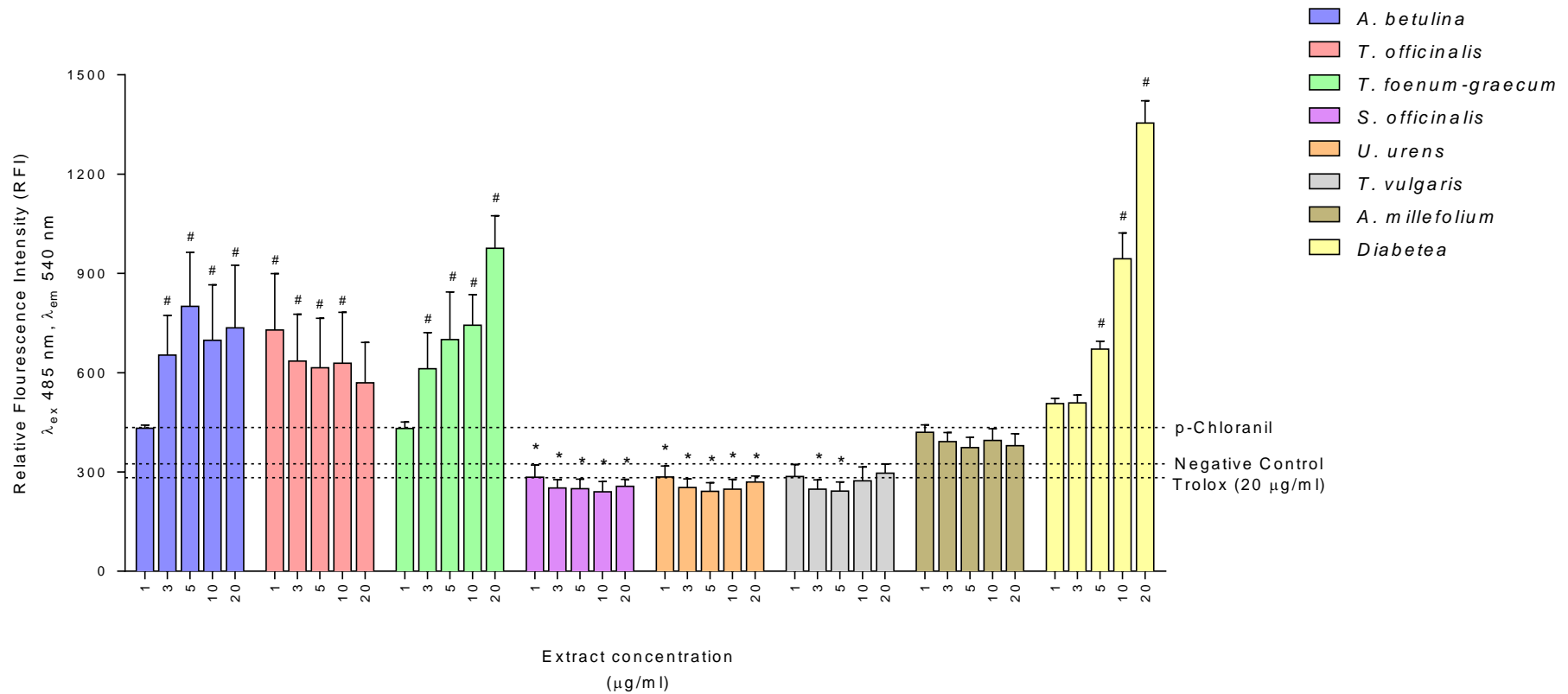


Figure 20: The cell-based antioxidant activity of hot water extracts tested at 1 to 20 µg/ml. Reactive oxygen species (ROS) were generated from Ea.hy926 endothelium cells using *p*-chloranil (20 µM in reaction). Each bar represents the mean fluorescence intensity ± SEM of triplicate (n = 9) tests. The significant ($p < 0.05$) antioxidant activity of plant extract tested against *p*-chloranil (*), and overproduction of ROS compared to the *p*-chloranil (#) was determined using student's *t*-tests. *p*-Chloranil, negative control and trolox are represented by dotted lines.

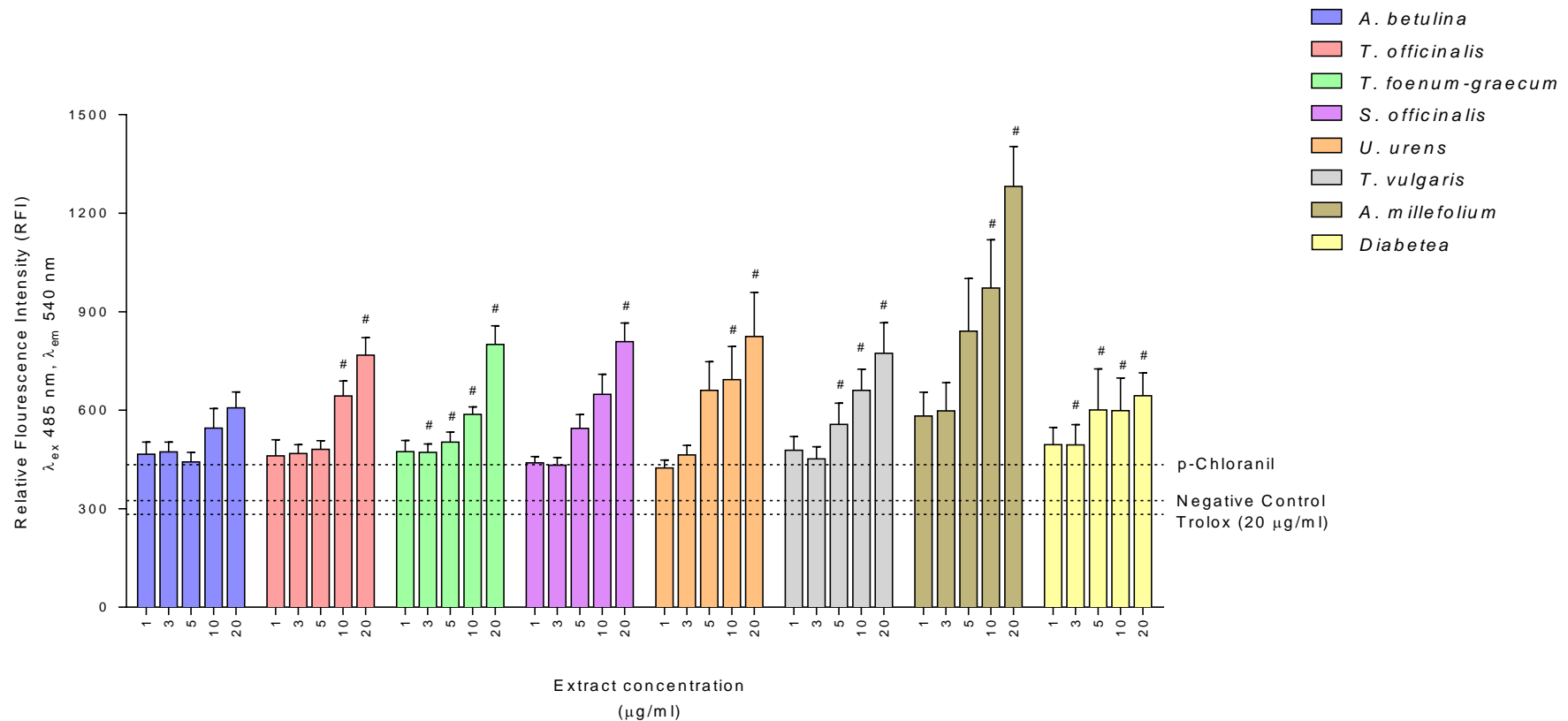


Figure 21: The cell-based antioxidant activity of dichloromethane extracts tested at 1 to 20 µg/ml. Reactive oxygen species (ROS) were generated from Ea.hy926 endothelium cells using *p*-chloranil (20 µM in reaction). Each bar represents the mean fluorescence intensity ± SEM of triplicate (n = 9) tests. The significant ($p < 0.05$) antioxidant activity of plant extract tested against *p*-chloranil (*), and overproduction of ROS compared to the *p*-chloranil (#) was determined using student's *t*-tests. *p*-Chloranil, negative control and trolox are represented by dotted lines.

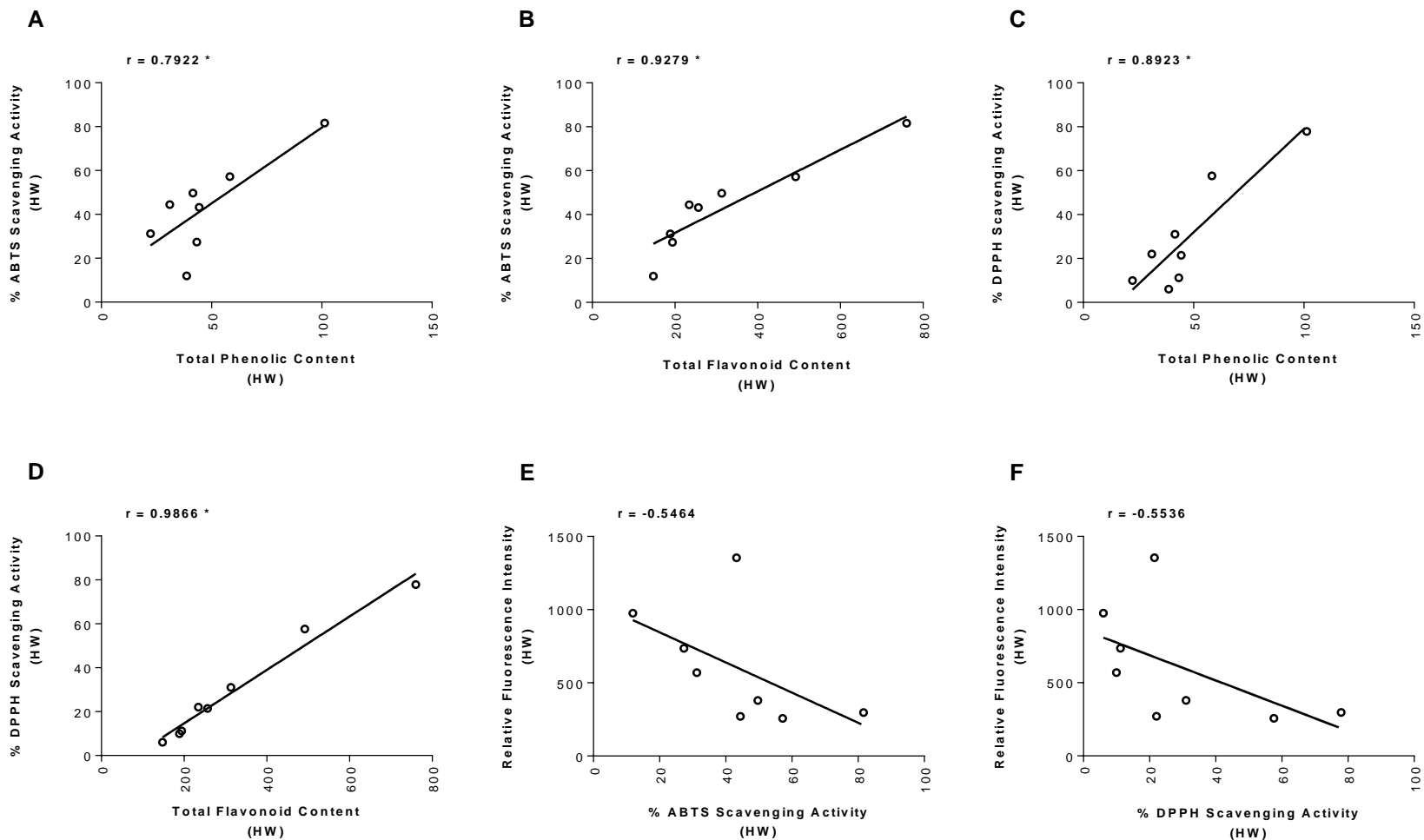


Figure 22: Relationships between (A) ABTS^{•+} scavenging activity and total phenolic content, (B) ABTS^{•+} scavenging activity and total flavonoid content, (C) DPPH[•] scavenging activity and total phenolic content, (D) DPPH[•] scavenging activity and total flavonoid content, (E) cellular reactive oxygen species (ROS) attenuation and ABTS^{•+} scavenging activity and (F) cellular ROS production and DPPH[•] scavenging activity of the hot water (HW) extracts. The data points represent each of the different extracts tested at 20 µg/ml.

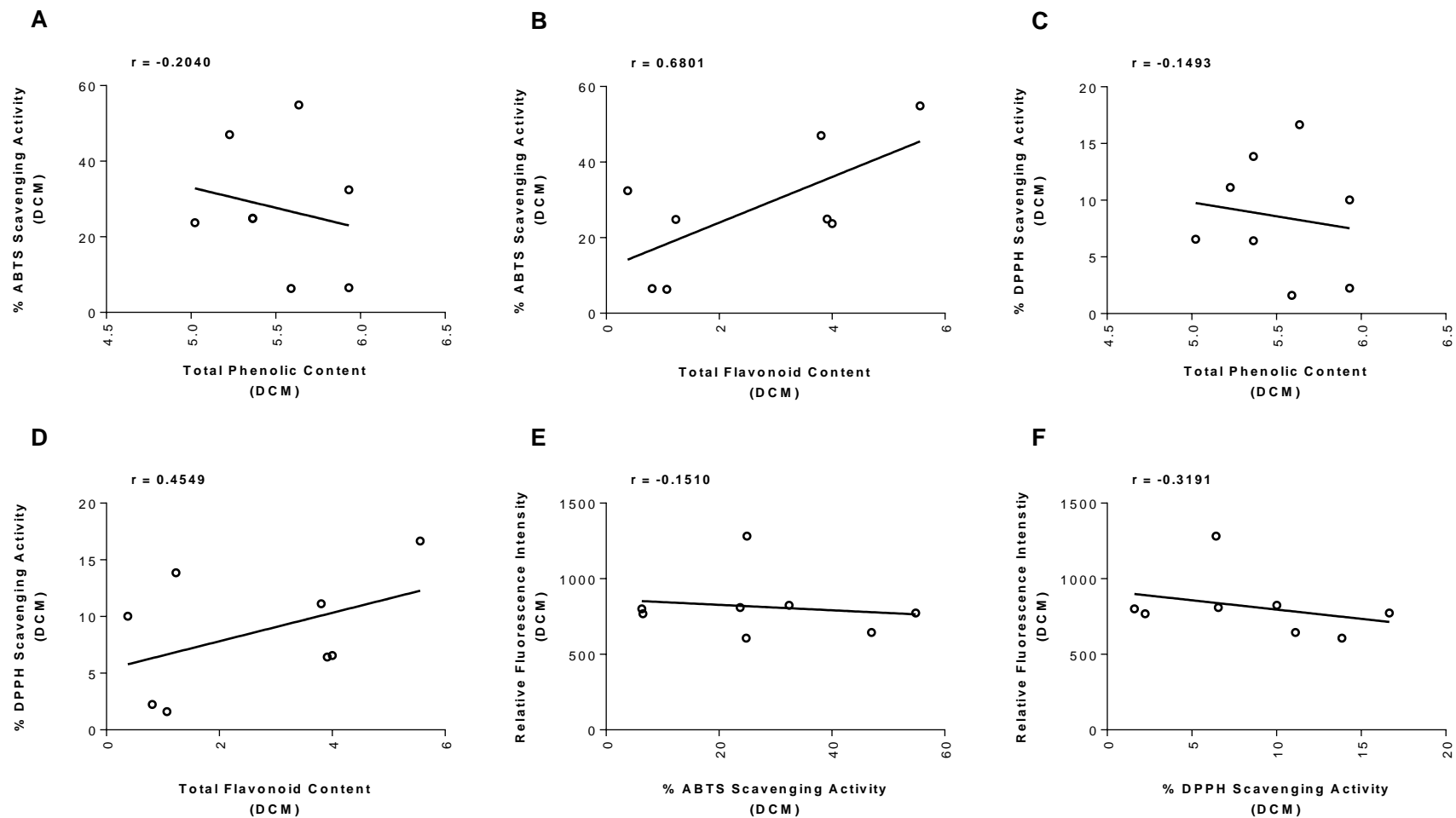


Figure 23: Relationships between (A) ABTS•+ scavenging activity and total phenolic content, (B) ABTS•+ scavenging activity and total flavonoid content, (C) DPPH• scavenging activity and total phenolic content, (D) DPPH• scavenging activity and total flavonoid content, (E) cellular reactive oxygen species (ROS) attenuation and ABTS•+ scavenging activity and (F) cellular ROS production and DPPH• scavenging activity of the dichloromethane (DCM) extracts. The data points represent each of the different extracts tested at 20 µg/ml.

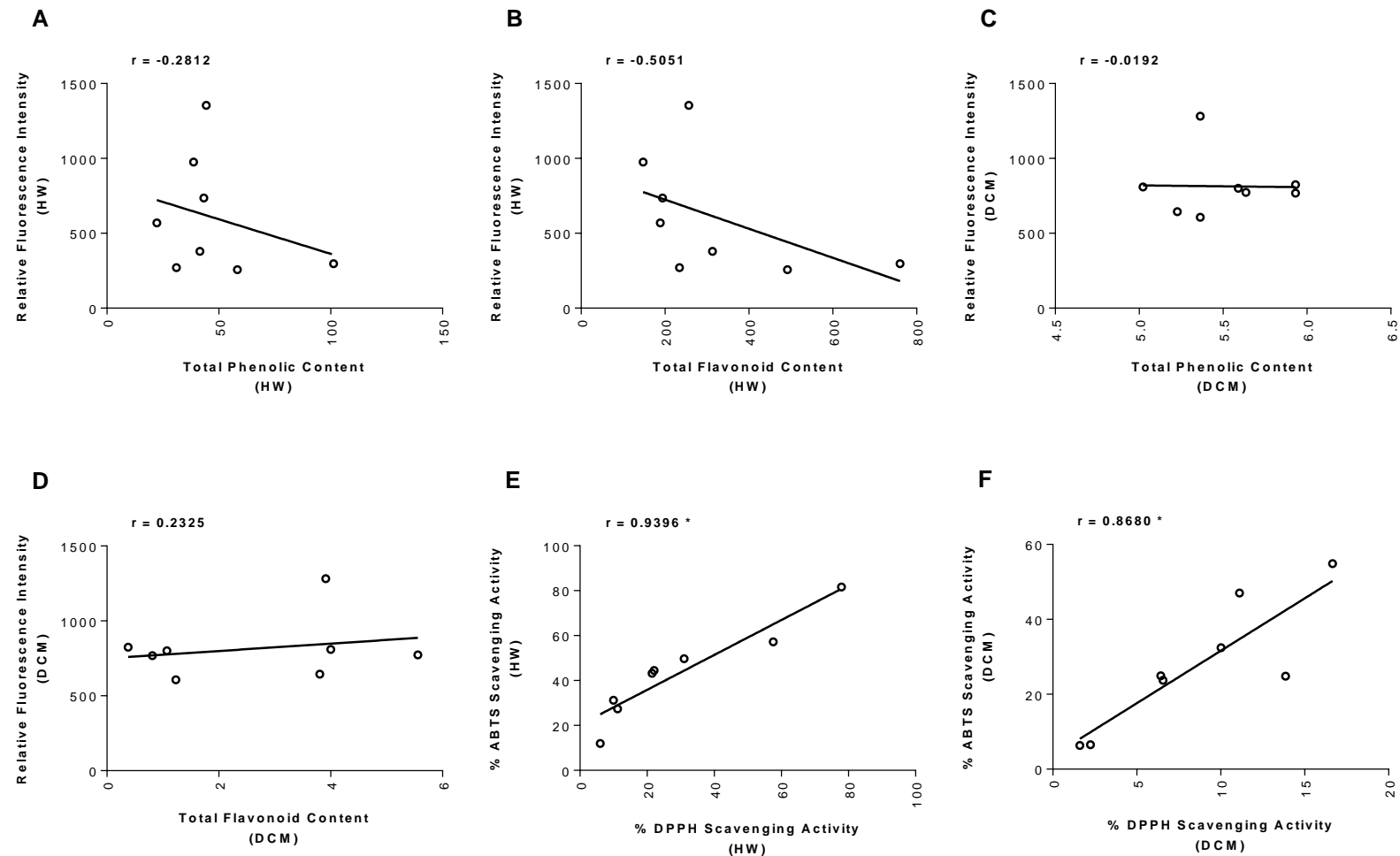


Figure 24: Relationships between (A) cellular ROS production and total phenolic content of the hot water (HW) extracts, (B) cellular ROS production and total flavonoid content of the HW extracts, (C) cellular ROS production and total phenolic content of the dichloromethane (DCM) extracts, (D) cellular ROS production and total flavonoid content of the DCM extracts, (E) ABTS^{•+} and DPPH[•] scavenging activity of the HW extracts and (F) DCM extracts. The data points represent each of the different extracts tested at 20 µg/ml.

2.5 Discussion

2.5.1 Extract yields

There are many factors that influence the percentage yield and type of bioactive compounds extracted from a plant sample, such as the drying method, storing temperature, solvent type, extraction temperature, extraction method, duration of extraction, solvent pH, plant part used, harvest time, environmental climate etc.¹⁹². Therefore, extraction yields of plants using the same solvent can differ between different laboratories due to these factors that influence the extraction yield/process. Furthermore, the differences in percentage yields between different plants are depend on the structural properties of compounds found within the plants, and their affinity and interaction with a specific solvent^{191,193}. This is the reason for the different extract yields, observed between the plants tested in the present study, indicating that some plants such as *T. vulgaris* and *A. millefolium* had more hydrophobic phytochemicals than *T. officinalis* and *T. foenum-graecum*.

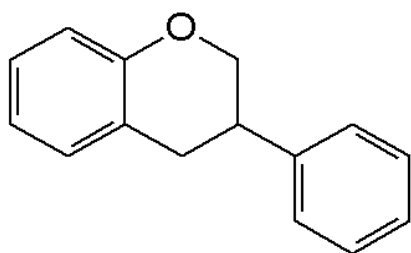
The difference in the extract colour between herbs is also important to note, since it has been shown previously that a relationship between the antioxidant activity (against DPPH[•] and ABTS^{•+}) and colour value of extracts exists¹⁹⁴. It has also been reported that black extracts contain more polyphenolic compounds than pink and yellow extracts, with an increased antioxidant activity associated with the darker coloured extracts¹⁹⁵. This relationship was evident in the present study, where the HW extracts of *T. vulgaris* and *S. officinalis* were dark (green/brown in colour) and exerted a high antioxidant activity, whereas the extracts of *T. foenum-graecum* and

A. betulina, were light (yellow-green in colour), and exerted a low antioxidant activity (Figure 16, Figure 18 and Figure 20).

2.5.2 Polyphenolic content and antioxidant activity

Phenols and flavonoids differ substantially in terms of their chemical structures (Figure 25), causing them to interact differently with HW and DCM. Water has a polarity index (PI) of 9.0, having a strong interaction with electronegative compounds such as flavonoids and phenolics¹⁹³. DCM is less polar (PI = 3.1) forming weak bonds with polyphenolics¹⁹³. This is likely to be the reason for the large amount of polyphenolics extracted using HW, compared to using DCM. Furthermore, flavonoids are more electronegative (containing more oxygen and carbon atoms)¹⁹³ (Figure 25A), compared to phenolics (Figure 25B), which means flavonoids are more hydrophilic, resulting in a stronger dipole-dipole interactions with water^{193,196}. This explains why the HW extracts contained more flavonoids than phenolics.

A



B

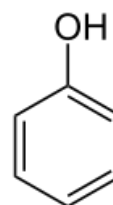


Figure 25: The basic chemical backbone of a (A) flavonoid and a (B) phenol^{196,197}.

ROS are highly reactive molecules derived from metabolized oxygen³⁵. In the case of T2DM, an overproduction of ROS is caused by chronic hyperglycaemia²⁵. The overproduction of ROS is associated with the onset and progression of T2DM and its secondary complications, such as arteriosclerosis, which can lead to heart failure²⁵. This makes the attenuation of excess ROS may prove valuable in the treatment of T2DM. Various flavonoids and phenolics have the ability to attenuate ROS^{39,44} and are therefore important components in determining the antidiabetic profile of a herb extract. In the present study it was found that the antiradical activity of herbs is closely linked to their polyphenolic nature. Flavonoids are a class of polyphenolics that are naturally grouped according to the extent of the oxidation on their C-rings⁴⁴. They are known for their potent antioxidant activity, which have also been shown, in some cases, to be even more effective than that of vitamins C and E¹⁷⁹. The antioxidant activity of flavonoids are attributed to their polyhydroxylated structure and the specific hydroxyl positions on the flavonoid backbone¹⁷⁸(Figure 25A and Figure 26). This is the reason why flavonoids are more active against free radicals than phenolics (which only has one C-ring) and therefore explains why the HW extracts were more active against ABTS^{•+} and DPPH[•] than the DCM extracts. The catechol flavonoid structure (Figure 26) has been identified as the required structure for the presence of antioxidant activity of any flavonoid, containing two phenyl rings and one pyrene ring^{44,178}. Another reason why the HW extracts were more active against ABTS than the DCM extracts is because the ABTS assay is aqueous based making it more hydrophilic whereas³⁰⁴.

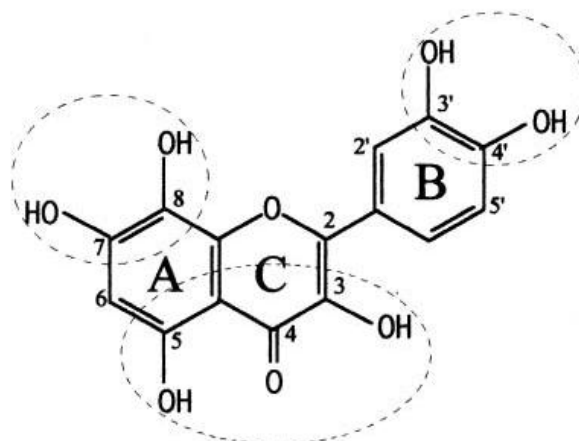


Figure 26: The catechol structure of a flavonoid. Phnyl-rings (A and C) and pyrene C-ring (B). OH – hydroxyl group^{44,178,198}.

It was apparent that all extracts were more active against ABTS^{•+} than DPPH[•]. This could possibly be attributed to the fact that the reaction between ABTS^{•+} and antioxidants occur faster than with DPPH[•]¹⁶¹. It is also apparent that the ABTS^{•+} scavenging assay is more sensitive, compared to the DPPH[•] assay^{182,183}, which was measured at a non-optimum wavelength of 570 nm instead of 515 nm, due to instrument limitations^{182,183}. A similar finding was reported by Kaviarasan *et al.*¹⁶¹ and Moolla *et al.*⁹⁷

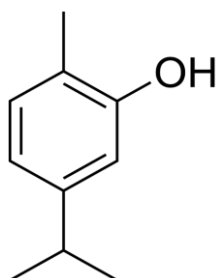
The HW extracts of *A. betulina*, *T. foenum-graecum*, *T. officinalis* and Diabetea had a significant ($p < 0.05$) pro-oxidant activity on Ea.hy926 cells (Figure 20). The overproduction of cellular-ROS is observed when cells are placed under certain biochemical or mechanical stress(es), either caused by unwanted environmental, chemical or pathological conditions¹⁹⁹. The pro-oxidant activity of herb extracts have been linked to large doses/concentrations of antioxidant-type flavonoids and/or the presence of flavonoids containing specific structural modifications on its B-ring²⁰⁰. This could explain the pro-oxidant activity of *A. betulina*, *T. foenum-graecum*, *T.*

officinalis and Diabetea, which are flavonoid-containing (Figure 15A) HW extracts. However, the flavonoid-rich HW extracts of *T. vulgaris* and *S. officinalis* had no pro-oxidant effect on Ea.hy926 cells, which may indicate that the pro-oxidant activity observed with *A. betulina*, *T. foenum-graecum*, *T. officinalis* and Diabetea may not be due to a high concentration of flavonoids but rather due to flavonoids with specific structural modifications exerting pro-oxidant activity. Furthermore, all DCM extracts also had a significant ($p < 0.05$) pro-oxidant activity on Ea.hy926 cells, which could not be attributed to their flavonoid content since they were poor polyphenolic containing extracts (Figure 14 and Figure 15). These findings suggest that further investigation is required to elucidate the exact reason for the pro-oxidant activity observed. Furthermore, this not only shows that these herbs are poor antioxidant containing extracts, but that they could also potentiate hyperglycaemic complications, specifically those with oxidative stress.

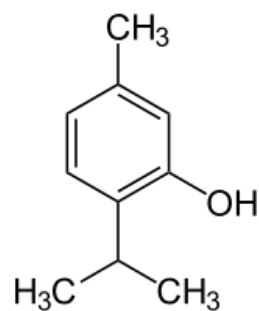
The phenolic content of *T. vulgaris* was observed to be the highest of all extracts in the present study (101.3 ± 1.7 mg GAE/ g extract). This result is supported by the findings of Dorman *et al.*²⁰¹. The authors prepared a HW decoction of *T. vulgaris* for 1 h instead of 15 min, which yielded 95.6 mg GAE/ g extract²⁰¹. The slight difference in phenolic content observed between the present study and that of Dorman *et al.*²⁰¹ could have been caused by the difference in the duration of extraction, which could have been associated with the breakdown of the structural integrity of extracted phenolics. A study carried out by Rababah *et al.*²⁰², using water at 60°C for 1 h, extracted only 11.02 ± 49.8 mg GAE / g extract from *T. vulgaris*, which is approximately a tenth of what was obtained by Dorman *et al.*, when also extracted for 1 h. Rababah *et al.*²⁰² reported that more polyphenolics were extracted at higher

temperatures, which could explain this discrepancy. From these two reports it can be inferred that extraction temperature has a much greater influence on extraction yield than the duration of the extraction process.

The most significant ($p < 0.05$) antioxidant potential was observed with *T. vulgaris*. Carvacrol and thymol are two phenolic compounds that have been isolated from *T. vulgaris*^{141,142,203} (Figure 27), with thymol being a more potent antioxidant^{204,205}. Rababah *et al.*²⁰² found that cravacrol and thyomol are active against DPPH[•], however with IC₅₀ values above 100 µg/ml, which is considered to be of a low antioxidant activity. Since the TPC extracted from *T. vulgaris* was low and the antiradical activity of the main phenolic compounds present in *T. vulgaris* are not potent, it is likely that the antiradical activity observed in the present study was mainly attributed to the flavonoid content of *T. vulgaris*.



Carvacrol



Thymol

Figure 27: Phenolics previously isolated from *Thymus vulgaris*²⁰⁶.

The HW extract of *T. vulgaris* was the highest in flavonoid content compared to other extracts (Figure 15). A number of flavonoids have been isolated from *T. vulgaris*, which have been shown to be naturally occurring antioxidant compounds (Figure 28). Some of these include luteolin²⁰⁷, apigenin²⁰⁸, naringenin²⁰⁹, eriodictyol²¹⁰, cirsilin²¹¹, salvigenin²¹² and cirsimaritin^{140,213} (Figure 28). Luteolin has been shown to exert an antioxidant activity²⁰⁷, equivalent to that of quercetin (the most widely studied flavonoid and a potent natural antioxidant)²¹⁴. Therefore, the TFC of *T. vulgaris*, extracted in the present study, may be the cause for its significant ($p < 0.05$) cell-free and cell-based antioxidant activities.

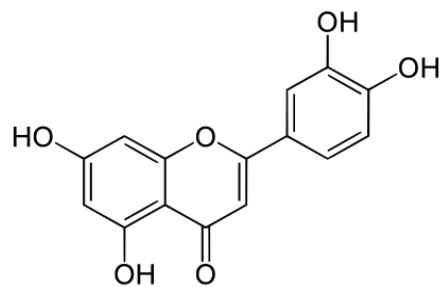
Another group of naturally occurring antioxidants found in *T. vulgaris* are called phenylpropenes (such as eugenol and 4-allylphenol), which possess significant antioxidant capacities comparable to known antioxidants such as α -tocopherol and butylated hydroxy toluene²¹⁵. This could further explain the potent antioxidant activity exerted by *T. vulgaris*.

The HW extract of *S. officinalis* was found to contain large amounts of flavonoids but was not rich in phenolics, which is supported by the findings of Cazzola *et al.*²¹⁶. *S. officinalis* is known to contain bioactive antioxidant flavonoids that are also found in *T. vulgaris*, such as: salvigenin, nevadensin, apigenin, cirsilin and cirsimaritin²¹⁶ (Figure 28). Previously, *S. officinalis* was reported to inhibit DPPH[•] by 76%²¹⁶, this finding compared to the observation in the present study (~60%). The reason why Cazzola *et al.*²¹⁶ observed a slightly higher antioxidant activity is possibly because these authors used a maximum concentration of 1 mg/mL versus 20 μ g/mL used in the present study. The antioxidant activity of *S. officinalis* has been attributed to

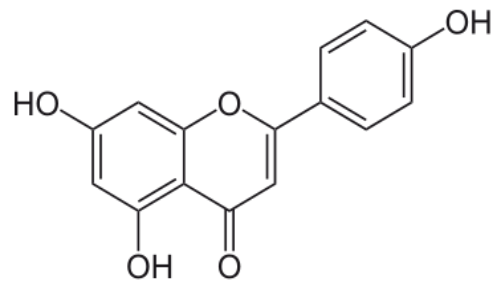
flavonoid compounds such as carnosic acid and carnosol, as well as rosmarinic acid^{217,218} (Figure 29). Although the HW extracts of *S. officinalis* was not as active against cell-free radicals as *T. vulgaris*, it significantly ($p < 0.05$) attenuated cell-based ROS at all concentrations tested (Figure 20), which could be attributed to its TFC.

The phenolic content *A. millefolium* (HW extract) in the present study is similar to the findings of Eghdami *et al.*²¹⁹. With regards to the flavonoid content, no study could be found that reported the TFC of *A. millefolium* in terms of RE. However, a study carried out by Eghdami *et al.*²¹⁹, showed that an aqueous extract of *A. millefolium* contained approximately 13.2 ± 1.8 mg quercetin equivalents (QE)/ g extract²¹⁹. The HW extract of *A. millefolium* was only moderately active against both ABTS^{•+} and cell-based ROS (Figure 16 and Figure 20) having no other favourable activity.

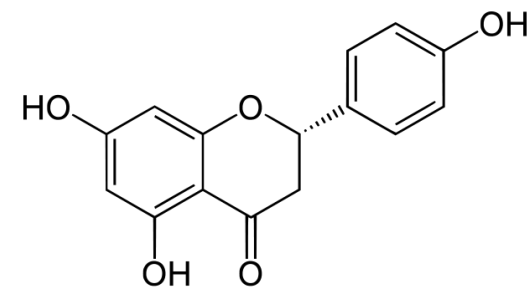
The low antioxidant activity of *A. millefolium* against ABTS^{•+} and DPPH[•] observed in the present study is supported by previous studies^{220,221}. Irrespective of the present observations, it has been shown that a hydro-alcoholic fraction of *A. millefolium* was active against superoxide and hydroxyl radicals with IC₅₀ values of 0.82 and 0.26 µg/ml, respectively²²². It has also been shown that *A. millefolium* contained potent antioxidant compounds such as caffeic and *p*-coumaric acid (Figure 30)²²³. The reason for the discrepancies found between the present study and those carried out previously could be attributed to the different assays used as well as the type of extract.



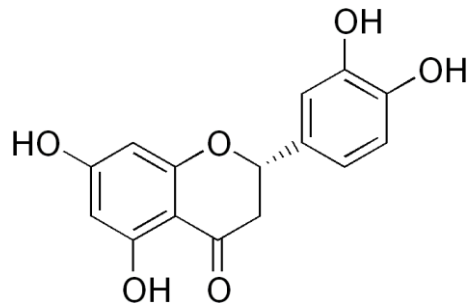
Luteolin



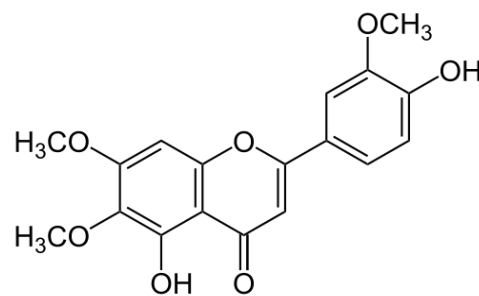
Apigenin



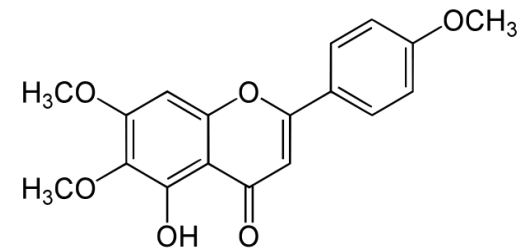
Naringenin



Eriodictyol

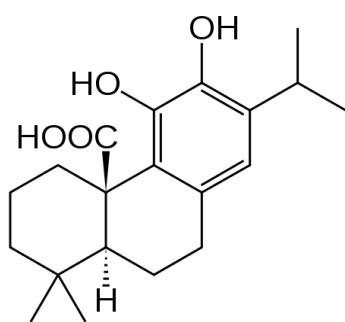


Cirsilineol

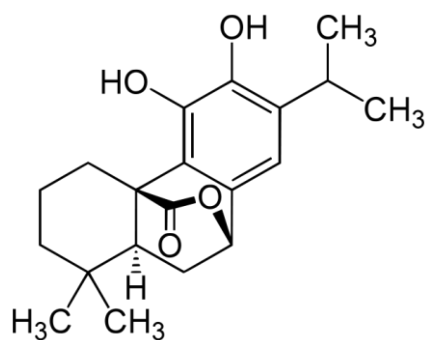


Salvigenin

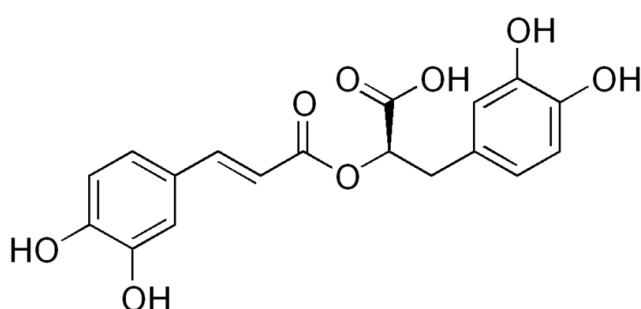
Figure 28: Flavones isolated from *Thymus vulgaris* that possesses antioxidant activity^{198,224-227}.



Carnosic acid

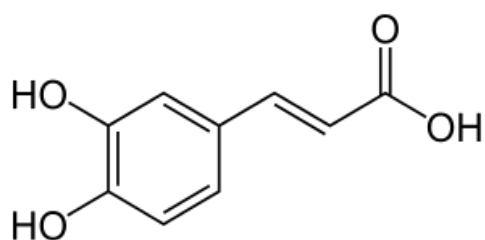


Carnosol

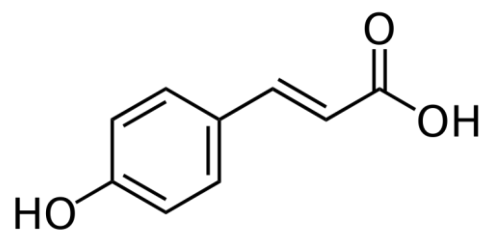


Rosmarinic acid

Figure 29: Antioxidants in *Salvia officinalis*^{227,227,228}.



Caffeic acid

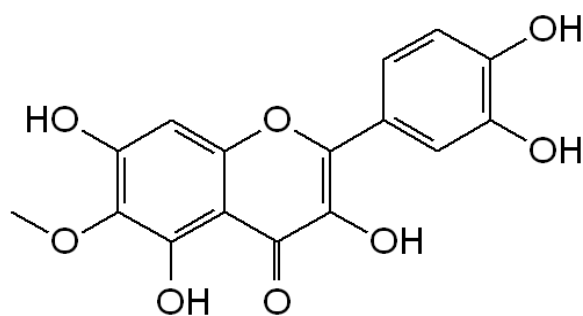


p-coumaric acid

Figure 30: Hydroxycinnamic acids, antioxidant compounds of *Achillea millefolium*²²³.

The extracts of *U. urens* were not rich in polyphenolics compared to other extracts, which are similar to the findings of Manu Kumar *et al.*²²⁹. This may be the reason for

its low cell-free antioxidant capacity. However, *U. urens* displayed a significant cell-based ROS attenuating activity at all concentrations tested. This may be due to potent antioxidant polyphenolics (active at low concentrations) found in *U. urens* such as patuletin (Figure 31). Patuletin is a flavonoid, which has been isolated from *U. urens*, with potent antioxidant activity *in vivo*²³⁰. The antioxidant activity of *U. urens* has been suggested to be responsible for its hepatoprotective effects^{169,170}. Manu Kumar *et al.*²²⁹ found an aqueous extract of *U. urens* to have approximately 40% scavenging activity against DPPH[•] and 60% scavenging activity against ABTS^{•+}. The reason these authors observed a higher antioxidant activity, compared to the present study, may be attributed to the fact they tested the extract at more than double the concentration (50 µg/ml versus 20 µg/ml).



Patuletin

Figure 31: O-methylated flavonol in *Urtica urens*²³¹.

Hu *et al.*¹³⁷ found that a 70% ethanol extract of *T. officinalis* contained approximately 195 mg/g GAE¹³⁷, which was comparable to the findings of the present study (188 mg/g GAE). Furthermore, it has been shown that *T. officinalis* contains several flavonoids including caffeic acid, chlorogenic acid, luteolin, and luteolin 7-

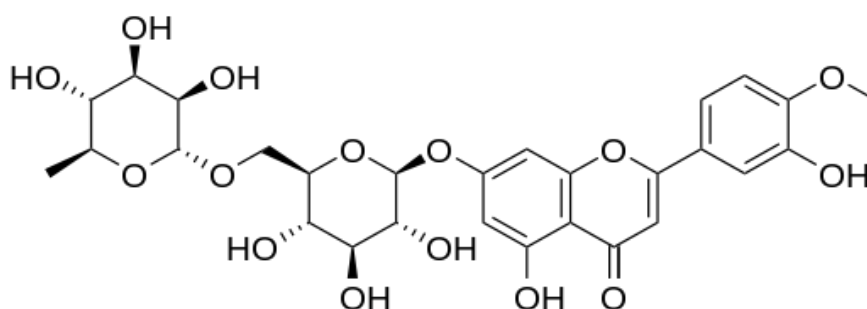
glucoside¹³⁵, which are all known for their antioxidant activity. Water and ethyl acetate extracts of *T. officinalis* flowers have been shown to inhibit DPPH[•] activity in a concentration-dependant manner, and their antioxidant activities were comparable to that observed in the present study²³². In the present study it was apparent that *T. officinalis* had a low antioxidant activity and polyphenolic content, which is supported by previous studies^{137,223,232}.

The extracts of *T. foenum-graecum* had a low polyphenolic content and antioxidant activity, which is supported by Kaviarasan *et al.*¹⁶¹. However, in an *in vivo* study, *T. foenum-graecum* seeds fed (5% w/w) to diabetic mice were shown to cause a normalization of antioxidant defense response and a decrease in peroxidative damage²³³. This indicates that even though the present study observed a low antioxidant activity *in vitro*, *in vivo* antioxidant activity may still be possible. However, these authors²³³ used seed powder, instead of seed extract, which makes a true comparison difficult.

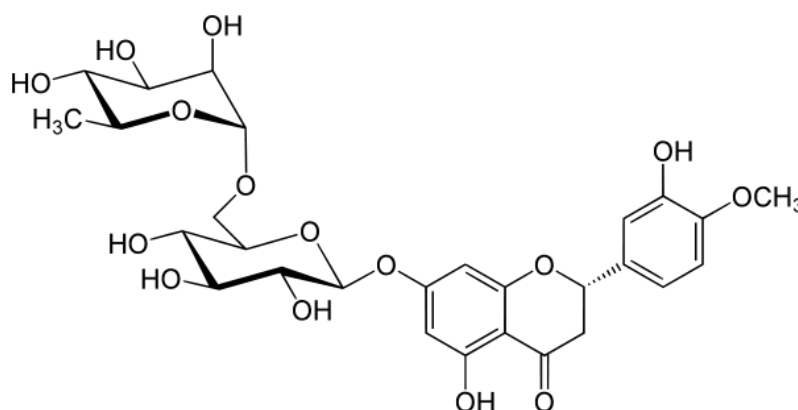
The extracts of *A. betulina* contained a low polyphenolic content, with more flavonoids than phenolics, and a low antioxidant activity. Diosmin and hesperidin are two main flavonoid compounds that have been isolated from the essential oil of *A. betulina* (Figure 32), which have been proven to act as diuretic, antihypertensive, hypolipidaemic, anticancer, antioxidant, and anti-inflammatory agents¹¹⁸. These flavonoids have been tested in the form of a micronised purified flavonoid fraction called Daflon 500 (90% diosmin and 10% hesperidin), and were found to have a long term hypoglycaemic, antiglycating and antioxidant action on type 1 diabetic patients²³⁴⁻²³⁶. Furthermore, a study done on streptozotocinnicotinamide-induced

diabetic rats using diosmin showed an increase in insulin secretion from pancreatic β -cells²³⁴. Although the present study does not report a noteworthy antioxidant activity exerted by *A. betulina in vitro*, it may not be indicative of its *in vivo* and clinical activity.

Diabetea also had a low antioxidant activity against ABTS^{•+} and DPPH[•] for both DCM and HW extracts. It also caused a significant overproduction of ROS, indicating that it could be more harmful when taken in the form of its traditional preparation. However, this may give no indication of its *in vivo* activity, which needs to be investigated further.



Diosmin



Hesperidin

Figure 32: Main flavones isolated from *Agathosma betulina*^{237,238}.

2.5.3 Relationships between antioxidant activity and polyphenolic content

A significant ($p < 0.05$) relationship between antioxidant activity and polyphenolic content was observed with the HW extracts, but this was not observed with the DCM extracts, which indicates that this association may be dependent on the type of solvent used for extraction. However, the DCM extract of *T. vulgaris* had a moderate cell-free antioxidant activity but low polyphenolic content. This shows that the antioxidant activity exerted by plant extracts may not exclusively be attributed to phenolics or flavonoids. Furthermore, there was a significant ($p < 0.05$) correlation between ABTS^{•+} and DPPH[•] scavenging activity per both DCM and HW extracts, showing that the ABTS^{•+} scavenging activity of an extract may be indicative of DPPH[•] scavenging activity.

The TFC significantly ($p < 0.05$) correlated with the activity of ABTS^{•+} and DPPH[•] (HW extracts), which was stronger than the relationship between the TPC and cell-free antioxidant activity.

A correlation was observed between the cell-free and cell-based antioxidant activity with regards to the HW extracts, however, this was not the case with the DCM extracts. There was also a positive correlation between the cell-based antioxidant activity and polyphenolic content with HW extracts. This may be due to the activity of unmetabolized free-floating polyphenolics compared to intracellular modifications that take place with absorbed phenolics and flavonoids²³⁹. In addition, the cell-based antioxidant activity of polyphenolics depend on their ability to move across the cell membrane, the action of which is selective²³⁹.

2.6 Chapter summary

There are various factors that influence the extraction yield of a plant. The extract colour is important to note since it has previously been linked to the amount of polyphenolics and antioxidant activity of an extract, which was also the case in the present study.

Flavonoids are more electronegative than phenolics and are therefore more readily miscible into polar solvents such as water. Flavonoids are more active against cell-free radicals than phenolics because of the greater amount of reducing molecules. DCM extracts were associated with an overproduction of ROS, for reasons that could not be deduced in this study. The extracts were more active against ABTS^{•+} than DPPH[•], possibly due to discrepancies regarding the reaction rate, antiradical specificity and assay sensitivity.

The traditional use of *T. vulgaris*, *S. officinalis*, *U. urens* as antidiabetic herbs is supported in the present study in terms of their significant ($p < 0.05$) antioxidant capacity *in vitro*. The most significant ($p < 0.05$) of these was the HW extract of *T. vulgaris*. The HW extract of *A. millefolium* was also active against cell-free radicals and cell-based radicals but this result was not significant or showed >50% attenuating activity. The extracts of *T. foenum-graecum*, *T. officinalis* and *A. betulina* as well as Diabetea were found to be weak *in vitro* radical scavenging extracts and were associated with the overproduction of cellular ROS. This shows that the *in vitro* application of Diabetea, in its traditional form, may be unsuitable as an antidiabetic preparation. Some of the single-herb extracts outperformed the activity of Diabetea

and this indicates that the polyherb mixture is not more active than compared to its individual constituents.

The relationship between polyphenolic content and antioxidant activity was shown to be dependent on the solvent type used. The cell-free antioxidant activity could not be exclusively attributed to the polyphenolic content of an extract and therefore, other types of compounds may be involved in this activity.

From the correlation data between ABTS^{•+} and DPPH[•] assays it was evident that if an extract has activity against ABTS^{•+}, it is likely to attenuate DPPH[•] as well, and vice versa. There was also a relationship between cell-based and cell-free antioxidant activity with the HW extracts but not with the DCM extracts, which was expected since the HW extracts were more active against ABTS^{•+} and DPPH[•] than the DCM extracts.

CHAPTER 3: TOXOLOGICAL PROFILING OF EXTRACTS

3.1 Introduction

According to the WHO, about 80% of individuals in low- to middle-income countries rely on herbal remedies for primary healthcare^{60,61}. The 'all natural' approach is a growing body within the westernized world and is often associated with being 'naturally' safe. Based on the wide usage and general misunderstanding surrounding the safety of plant-based treatments, the need for toxicological profiling of traditional herbs is essential. Cells are intrinsically sensitive to compounds and therefore it is necessary to make use of a variety of cell types, both primary and perpetual cell lines, when testing the toxicity of an extract. In the present study the cytotoxicity of extracts were tested against mouse myotubes, human lymphocytes and human endothelial cells.

The C2C12 cell line is a subclone of mouse myoblast cells²⁴⁰, which readily differentiates into myotubes under the correct conditions. Myotubes were selected for the glucose uptake assay in the present study, as muscle tissue relies heavily on glucose as a source of energy and requires insulin to absorb glucose^{1,2}. Human lymphocytes (HL) or peripheral blood mononuclear (PBMNC) cells, were selected as primary cells to provide pre-clinical data on the safety of each extract. PBMNC cells are also readily isolated from blood drawn from healthy volunteers. Resting human lymphocytes are small lymphocytes in the G₀ stage of the cell cycle and stimulated or activated lymphocytes are lymphocytes that have undergone blastogenesis forming lymphoblasts²⁴¹.

Hyperglycaemia, the hallmark of T2DM, causes the progression of endothelial damage through the production of ROS^{25,242}, which is the reason why endothelial cells was evaluated in the present study.

Sulforhodamine B (SRB) is an aminoxanthene dye that stains cellular proteins in fixed cells²⁴³. It is used in various cell numeration assays due to this property and is especially employed in cytotoxicity tests *in vitro*²⁴³. The SRB assay is easy, practical, sensitive and does not require cellular metabolism to work, as is the case with tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)^{243,244}. MTT can only stain viable cells whereas SRB stains both viable and dead cells, making no distinction²⁴³. However, previous SRB results have been shown to compare well with that obtained from using MTT^{245,246}. Furthermore, SRB has been used extensively in previous cytotoxicity studies involving the use of natural compounds and/or herb extracts²⁴⁷⁻²⁴⁹.

3.2 Materials and methods

3.2.1 Propagation of cells

Ethical approval for the use of C2C12 (ATCC CRL-1772) myocytes and Ea.hy926 cells were obtained from the University of Pretoria (Appendix III, page 162). The endothelial cell line, Ea.hy926, procured from umbilical vein cells, was obtained from Steve Oglesbee (representing Dr. Cora-jean S. Edgell) at the UNC Lineberger comprehensive cancer center (Appendix II, page 163). Human lymphocytes were procured by means of blood collection from volunteers using the blanket ethics approval obtained from the University of Pretoria, ethics committee (Appendix IV,

page 163). All cell work was conducted under strict sterile conditions and incubated in a 5% CO₂ incubator at 37°C in DMEM. Medium was supplemented with 1% penicillin/streptomycin and 10% FCS.

3.2.2 C2C12 differentiation

C2C12 myoblasts were differentiated into myotubes according to the method described by Burattini *et al.*²⁵⁰. The myocytes were cultured in flasks until they were 50% confluent. The flasks were rinsed with 1 ml sterile phosphate buffered saline (PBS) and spent medium replaced with 4 ml of DMEM+ containing 1% FCS and incubated at 37°C and 5% CO₂ for 7 days. The medium was changed daily until the cellular morphology resembled that of myotubes when observed under a microscope (Olympus Optical Microscope 1X70, A.R. Instruments).

3.2.3 Cell harvesting and seeding

Prior to performing cytotoxicity evaluations, the medium was removed and cells were washed with DMEM- (for C2C12 and Ea.hy926) or RPMI-1640 (for lymphocytes). The differentiated C2C12 myotubes were harvested with 1 ml TrypLE Express in EDTA for 3 min at 37°C, 5% CO₂. Ea.hy926 cells were harvested using 1 ml trypsin (The Scientific Group, Gauteng, South Africa) for 1 min at 37°C, 5% CO₂. Ten millilitres of DMEM+ with 2% FCS was added to the dissociated cells and the suspension decanted into a 15 ml polypropylene (PP) tube. The homogenous cell suspension was centrifuged for 5 min at 200 g (Allegra X-22, Beckman Coulter centrifuge). Thereafter, the medium was decanted and replaced with 1 ml of DMEM+ and the cell pellet was carefully suspended into the medium. Twenty

microliters of the cell solution was added to 180 μl of filtered (0.22 μm) trypan blue which was prepared in PBS (0.4% w/v). The cell density was determined by making use of a haemocytometer. The cells were seeded (80 μl) at densities of 1.2×10^4 cells/well (C2C12), 1.5×10^4 cells/well (Ea.hy926) and 2.6×10^5 cells/well (HL).

Human lymphocytes were isolated following the method described by Böyum *et al.*²⁵¹ with minor modifications. Thirty millilitres of heparinized blood (1 ml of refrigerated heparin for every 10 ml of blood) was carefully loaded onto 15 ml of Histopaque 1077 and spun down for 25 min at 650 g at 4°C. The top plasma layer was removed and the PBMNC layer was transferred to sterile 50 ml tubes. The tubes were filled with RPMI-1640 and spun down for 15 min at 200 g in order to eliminate coagulated platelets. The supernatant was discarded and the tubes filled with RPMI-1640 and spun down for 10 min at 200 g, after which the supernatant was discarded and tubes filled with cold sterile ammonium chloride (NH_4Cl) solution, which was kept on ice for 10 min in order to lyse contaminating red blood cells. The NH_4Cl solution contained 8.3 g chloramine (NH_2Cl), 1 g sodium bicarbonate (NaHCO_3) and 74 mg ethylenediaminetetra-acetic acid (EDTA) in 1 l dH_2O . The tubes were centrifuged for 10 min at 200 g and the discarded supernatant replaced with RPMI-1640. The tubes were centrifuged for 10 min at 200 g, thereafter the medium was decanted and replaced with 1 ml of RPMI+1640 and the cell pellet was carefully suspended into the medium supplemented with 2% v/v FCS.

3.2.4 Cytotoxicity

The cytotoxicity analysis was done following a colorimetric, 96-well, microplate method, using SRB, as described by Vichai and Kirtikara²⁴³. Pre-seeded plates were

incubated (1 h for HL and 24 h for C2C12 and Ea.hy926) before exposure to extracts to allow cells to acclimatise. Eighty microliters of extracts were added to respective experimental wells (final concentrations of 0.02 - 100 $\mu\text{g}/\text{m}\ell$). Phytohaemagglutinin (PHA) was used to obtain stimulated HL cells. Eighty microliters of DMEM+ was added to negative control wells and 160 $\mu\ell$ of DMEM+ to medium control wells. The wells with 80 $\mu\ell$ extract and 80 $\mu\ell$ DMEM+ was used to control for phytochemical interference from the plant extracts. The plates were incubated (72 h for HL and only 24 h for C2C12 and Ea.hy926, due to the fast proliferation rate of these cells) at 37°C in a 5% CO₂ ambiance. Following exposure, 80 $\mu\ell$ of trichloroacetic acid (TCA) (30% w/v) was added and the plates incubated for 72 h at 4°C to fix cells. Plates were then washed three times using tap water and left to dry. One hundred microliters of SRB (0.057% w/v) dissolved in 1% v/v acetic acid (AcOH) solution was added to each well and left for 30 min at room temperature. The plates were rinsed three times with 1% acetic acid to remove excess dye. Bound dye was dissociated with 100 $\mu\ell$ of a 10 mM trisaminomethane (TRIS) solution at pH 10.5 and the absorbance read using a spectrophotometer at 540 nm with a reference wavelength (λ_{re}) 630 nm. Saponin, at 100 $\mu\text{g}/\text{m}\ell$ final concentration, was used as positive control.

3.3 Statistical analysis and data representation

All experiments were executed in triplicate (technical and biological, $n = 9$) and the percentage cell density presented in the form of bar graphs, as grouped by concentration. The extent of toxicity was considered as: toxic (<50% cell density), moderate toxicity (>50% but <75% cell density), growth inhibition/possible low

toxicity (>75% cell density) and, in the case of a commercial cell line, possible stimulation of cell growth (>100% cell density). In the case with human lymphocytes, >100% cell density, may indicate an increase in protein content but not cell proliferation.

3.4 Results

3.4.1 C2C12 differentiation

The myocyte differentiation into myotubes took 7 days. On day 0, the flasks were harvested and added to cell culture flasks and differentiation started by adding medium that contained 1%, instead of 10%, FCS. Undifferentiated cells were round, small, either isolated or found in colonies (Figure 33A). On the 5th day the cells were bigger, stellar shaped, inter-connected and the monolayer covered more of the flask surface area (Figure 33B). On the final day of differentiation (day 7) the cells were longer and more tube-like. By this time flasks had usually reached almost 100% confluency (Figure 33C).

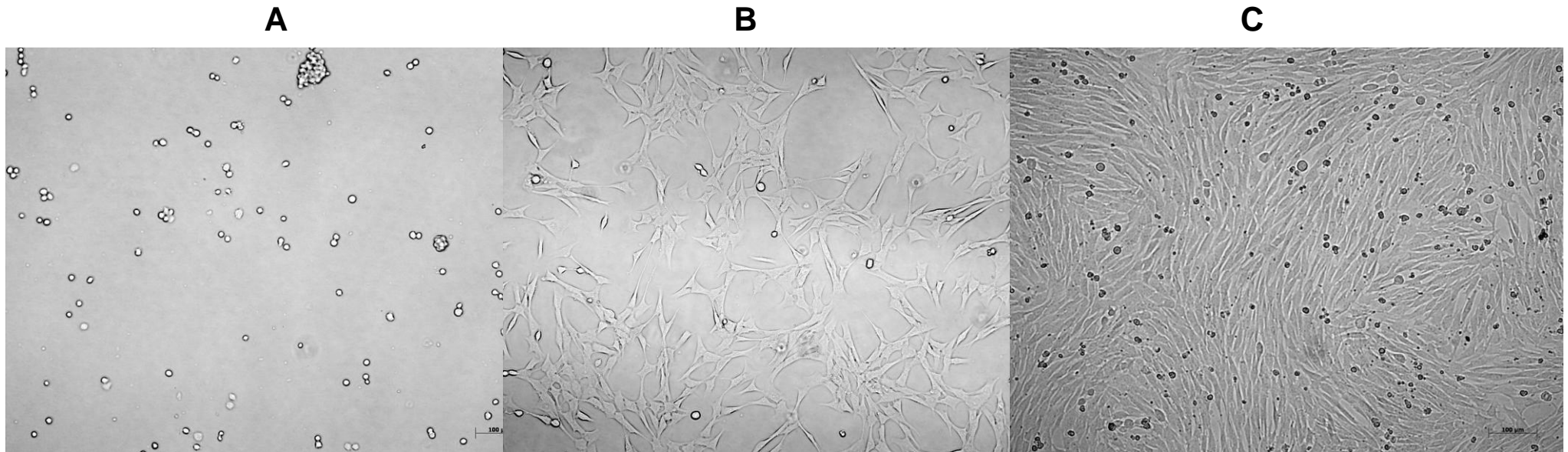


Figure 33: C2C12 myocyte to myotube differentiation process (10x magnification), using 1% FCS. (A) Day 0 of differentiation, where medium containing 10% FCS was replaced with 1% FCS to initiate the differentiation process, (B) day 5 of differentiation and (C) day 7, the final day of differentiation and on which cells were harvested for experimental use.

3.4.2 Cytotoxicity

In general the DCM extracts were more toxic than the HW extracts (Figure 34–Figure 41). None of the HW extracts were toxic except for *T. foenum-graecum* at 100 µg/ml tested against stimulated HL cells (Figure 37). The stimulated HL cells were generally more sensitive than resting HL cells (Figure 36 - Figure 39). The C2C12 myotubes were most sensitive to the DCM extracts (Figure 35). None of the extracts were toxic against any of the cell lines tested at 25 µg/ml, which is relevant to the highest concentration used for antidiabetic tests in the present study (of 20 µg/ml). HL cells do not proliferate after isolation and seeding, therefore a perceived increased cell density (>100% cell density) in the present study is not due to cellular proliferation but a possible stimulation of cellular protein synthesis, stained by SRB.

The DCM extract of *A. betulina* was toxic against stimulated HL cells (Figure 39), and possibly caused proliferation of C2C12 cells and stimulated protein proliferation in resting HL cells at all concentrations (Figure 35 and Figure 38), except for 100 µg/ml, where it showed a low toxicity. The HW extracts of *A. betulina* was not toxic against any cell line tested, however, it did show low toxicity against C2C12 (Figure 34), and stimulated HL cells (Figure 37) as well as Ea.hy926 cells (Figure 40). This extract also may have stimulated cellular protein synthesis in the resting HL cells in a concentration-dependent manner (Figure 36), associated with a greater cell mass.

The DCM extract of *T. officinalis* was not highly toxic to any of the cell types tested; however it did have a mild and low toxic effect on stimulated HL (Figure 39) and Ea.hy926 cells (Figure 41), respectively. Furthermore, it stimulated the growth C2C12 (Figure 35) cells and the proliferation of protein content in resting HL cells

(Figure 38) at all concentrations tested. The HW extract of *T. officinalis* did not have a toxic effect on any of the cells tested. However it did have a low toxic effect on C2C12 (Figure 34) and Ea.hy926 cells (Figure 40), and was mildly toxic against stimulated HL (Figure 37). The HW extracts showed a possible stimulation of protein synthesis in resting HL (Figure 36) and proliferation of Ea.hy926 cells (Figure 40).

The DCM extract of *T. foenum-graecum* appears to have stimulated the growth of C2C12 cells (Figure 35) and caused a stimulation of protein synthesis in resting HL cells (

Figure 38) at all concentrations. It also had a mild and low toxic effect on stimulated HL (Figure 39) and Ea.hy926 cells (Figure 41), respectively. The HW extract of *T. foenum-graecum* had an increasing effect on the protein cell mass of resting HL cells (Figure 36) at all concentrations. It also had a low toxicity against C2C12 (Figure 34), stimulated HL (Figure 37) and Ea.hy926 cells (Figure 40).

The DCM extract of *S. officinalis* was toxic against C2C12 cells at 100 µg/ml (Figure 35) and a growth stimulatory effect at all other concentrations (Figure 35). This extract was non-toxic (~100%) against Ea.hy926 cells (Figure 41). It also had a slight growth/protein producing stimulatory effect at 25 µg/ml against Ea.hy926 cells (Figure 41). The DCM extract of *S. officinalis* had a mildly toxic effect on stimulated HLs (Figure 39) and an increase in cellular protein on resting HL, except at 25 µg/ml where it was shown to be mildly toxic (Figure 38). The HW extracts of *S. officinalis* also had an increasing effect on the protein mass in resting HL at all concentrations (Figure 36). This extract had a low toxicity against C2C12, which was concentration

dependent (Figure 34). Furthermore, it had a non-toxic (~100) effect on Ea.hy926 (Figure 40) and stimulated HL cells (with an increase in cell protein HL cells 0.4-6.3 and 100 µg/ml) (Figure 37).

U. urens, as a DCM extract preparation, was non-toxic against resting HL cells and caused an increase in cell protein content at lower concentrations (0.1 – 25 µg/ml) (

Figure 38). The latter was also observed in stimulated HL cells at 0.4 to 25 µg/ml, and was non-toxic (~100% cell density) at 0.1 and 100 µg/ml (Figure 39). A similar finding was observed in the Ea.hy926 cells at 0.1 to 25 µg/ml (Figure 41). However, *U. urens* was toxic against Ea.hy926 cells at 100 µg/ml (Figure 41). It also had a stimulatory effect on the growth/protein content of C2C12 cells at all concentrations except for 100 µg/ml where it was mildly toxic (Figure 35). The HW extract of *U. urens* had a concentration dependent cytotoxic (low to mild) effect on HL from 0.1 to 25 µg/ml, yet showed a decrease in toxicity at 100 µg/ml (Figure 37). It was also non-toxic to Ea.hy926 and C2C12 cells, with some growth/protein density stimulation of Ea.hy926 cells at 25 µg/ml and resting HL cells at all concentrations except for 25 µg/ml, where it showed low toxicity (Figure 34, Figure 36 and Figure 40).

The DCM extract of *T. vulgaris* was toxic to C2C12 at 100 µg/ml and was non-toxic at all other concentrations except for 25 µg/ml where low toxicity was found (Figure 35). This extract had a mixed (increase in protein content, low and mild toxic) effect at various concentrations on resting HL cells. This trend was also observed for the DCM of *A. millefolium*, *Diabetea* and *S. officinalis* (Figure 38). The exact reason for

this 'pattern' is not known, however, it may be due to experimental error or an unexplained interaction between the extracts and HL cells. Furthermore, the DCM extract of *T. vulgaris* had a low toxicity against stimulated HL and Ea.hy926 (at 100 µg/ml with no toxic effect at all other concentrations), it also caused a possible increase in cellular protein in stimulated HL cells at 100 µg/ml (Figure 39 and Figure 41). On the other hand, the HW extracts of *T. vulgaris* had a dose-dependent low- to mild cytotoxic effect on C2C12s (Figure 34). It also caused a possible increase in protein structures in resting HL cells and had a low toxicity to stimulated HL at all concentrations (Figure 36 and Figure 37). Furthermore, this extract had no toxic effect on Ea.hy926 cells (Figure 40).

A. millefolium, as a DCM preparation, was found to be toxic to C2C12 and Ea.hy.926 cells at 100 µg/ml, but had a non-toxic and growth/increase in protein content at all other concentrations (Figure 35 and Figure 41). This extract was low- to non-toxic against stimulated HL (Figure 39) and non-toxic to Ea.hy926 at all concentrations tested except for 100 µg/ml (Figure 41). The HW extract of *A. millefolium* was low to non-toxic against C2C12 and Ea.hy926 (Figure 34 and Figure 40). This caused a possible increase in cell protein content in resting HL cells except at 0.4 µg/ml (Figure 36). It also had a concentration-dependent mildly toxic effect on stimulated HL, which decreased at 100 µg/ml (Figure 37).

The DCM extract of Diabetea was the most toxic of all extracts at 100 µg/ml against C2C12 (Figure 35). However, it caused an increase in cell/protein growth/increase at all other concentrations tested (Figure 35); this was also observed against stimulated HL (Figure 39). This extract displayed a low to no toxicity against

Ea.hy926 cells at all concentrations except for 100 µg/ml where it was mildly toxic (Figure 41). The HW extract of *Diabetea* had a low to non toxic effect on C2C12 (Figure 34) and stimulated HL (Figure 37). It also caused a possible increase in cellular protein content in resting HL at all concentrations (Figure 36). Furthermore, this extract was non-toxic to Ea.hy926 (Figure 40).

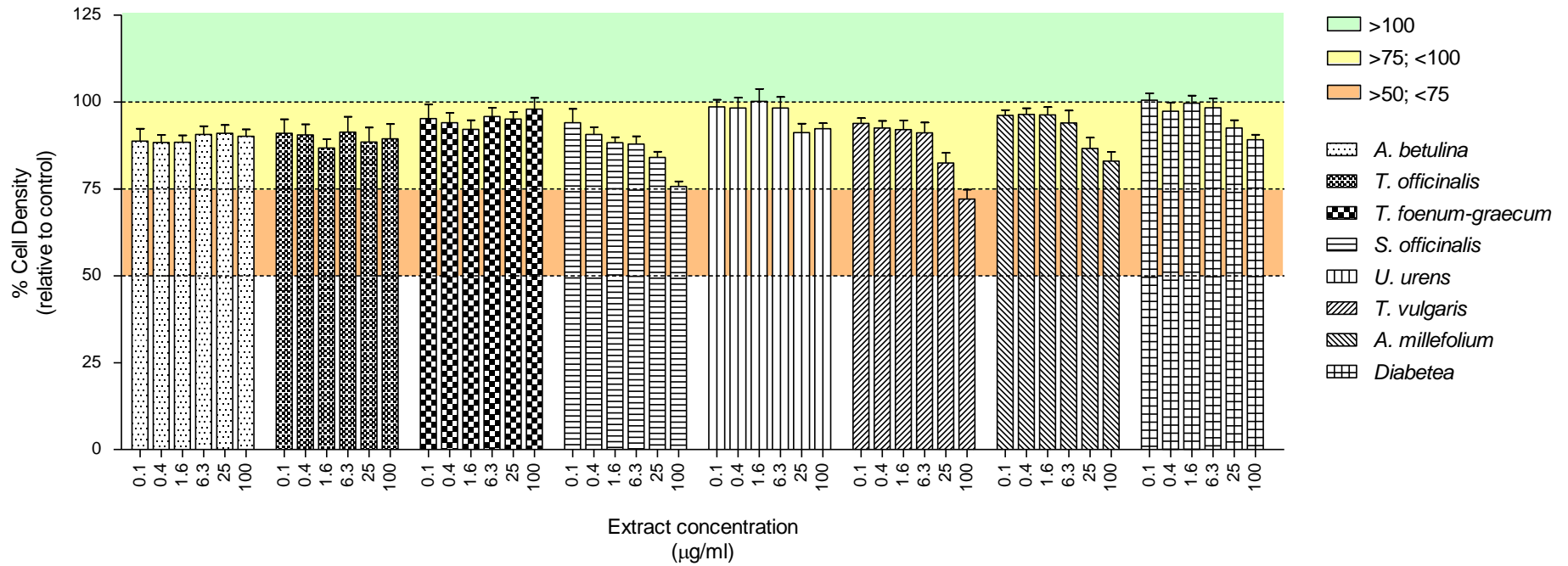


Figure 34: The effect of hot water herb extracts, tested at 0.1 to 100 µg/ml, on differentiated C2C12 measured using the sulforhodamine B uptake assay after 24 h incubation.

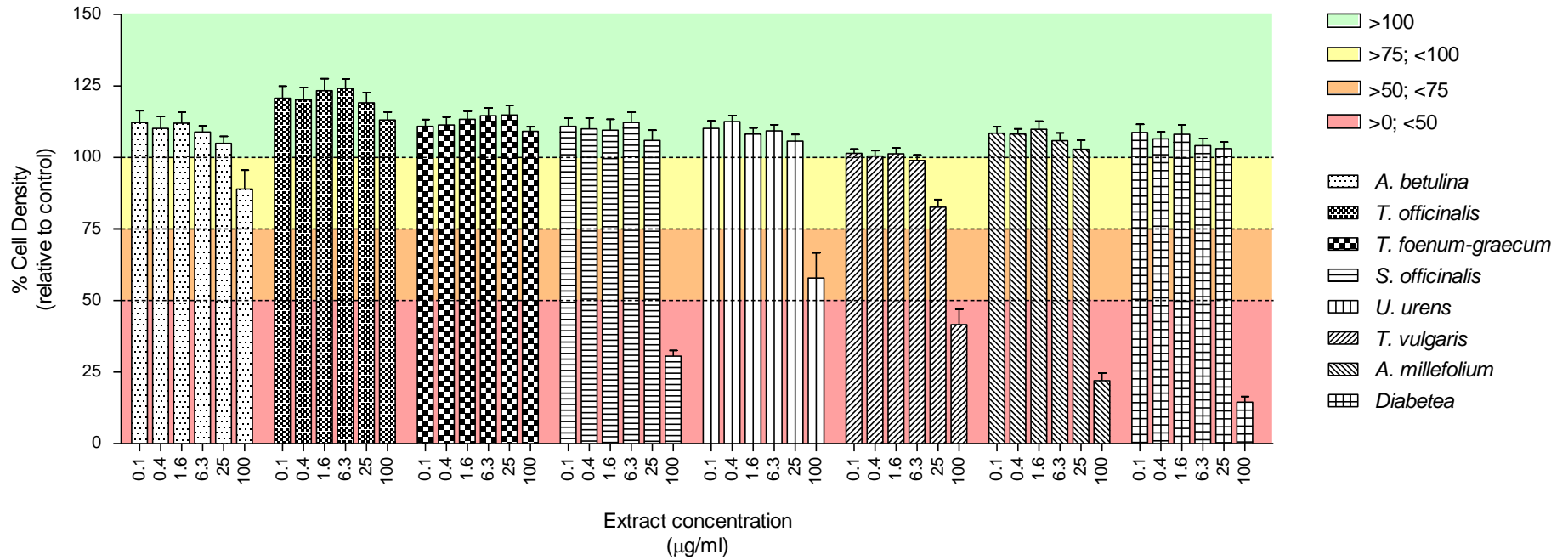


Figure 35: The effect of dichloromethane extracts on C2C12, tested at 0.1 to 100 µg/ml (n = 9), measured using the sulforhodamine B uptake assay after 24 h incubation.

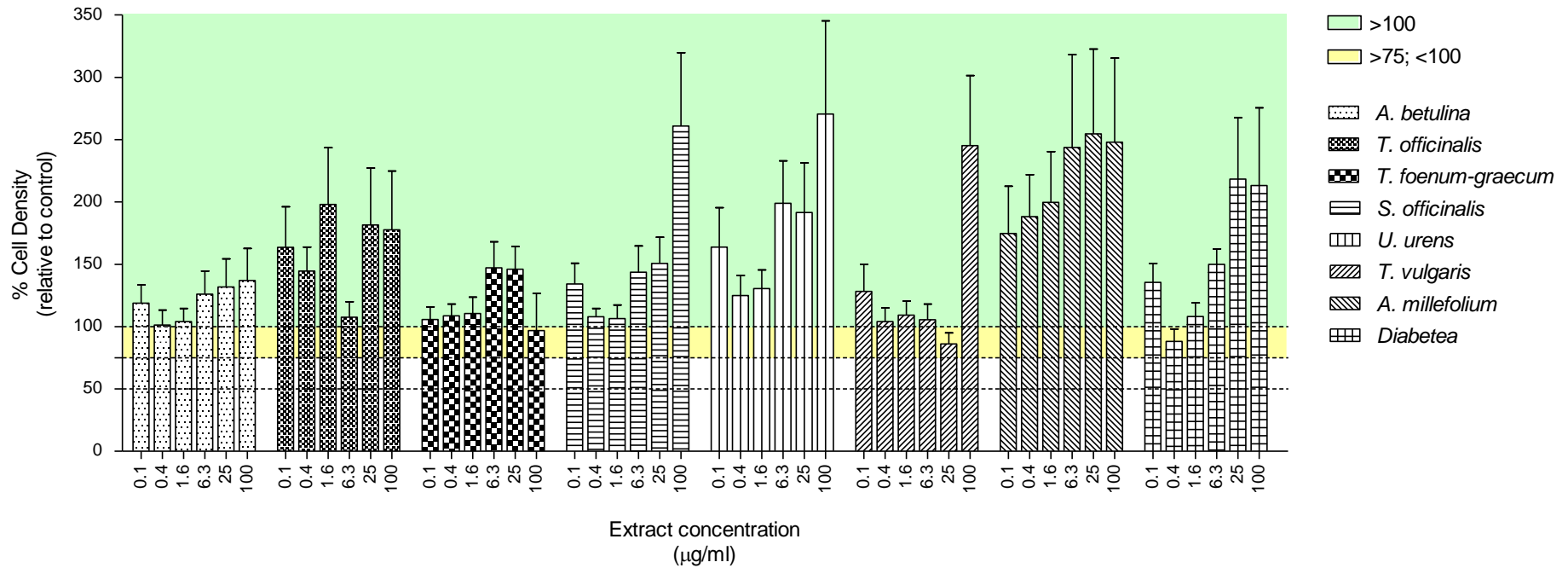


Figure 36: The effect of hot water herb extracts on resting human lymphocytes, tested at 0.1 to 100 µg/ml (n = 9), measured using the sulforhodamine B uptake assay after 72 h incubation.

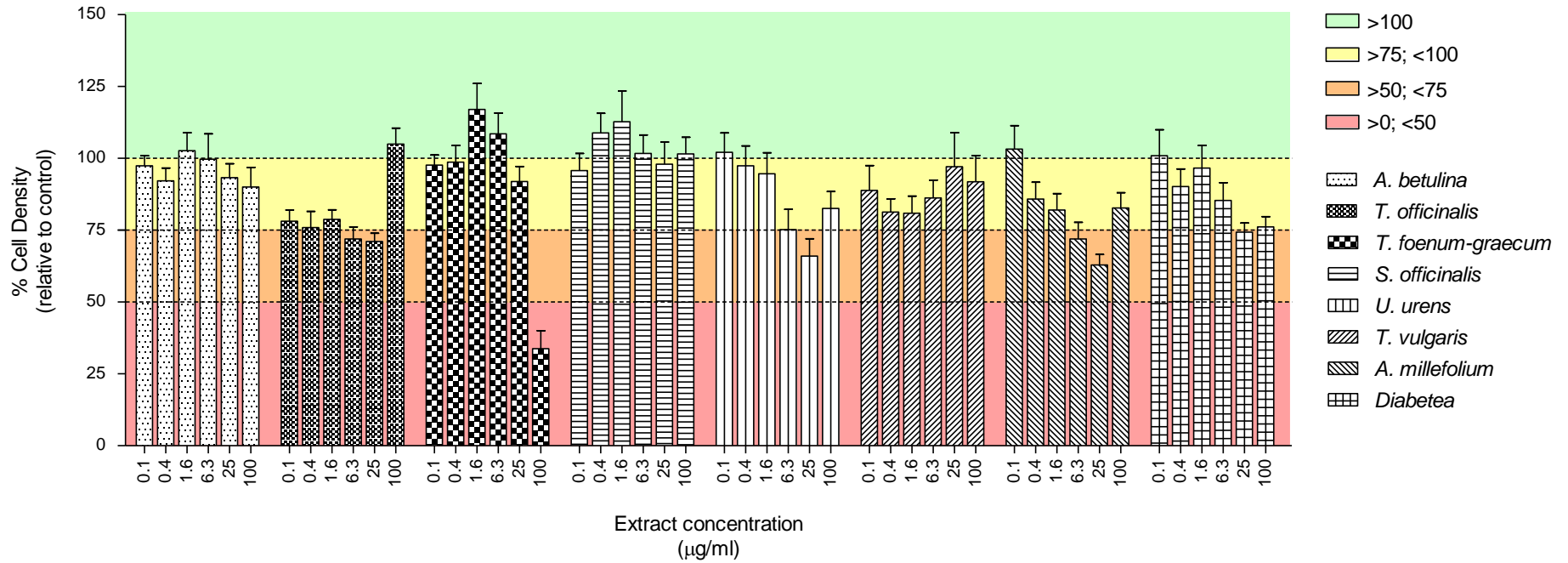


Figure 37: The effect of hot water herb extracts on phytohaemagglutinin stimulated human lymphocytes, tested at 0.1 to 100 µg/ml (n = 9), measured using the sulforhodamine B uptake assay after 72 h incubation.

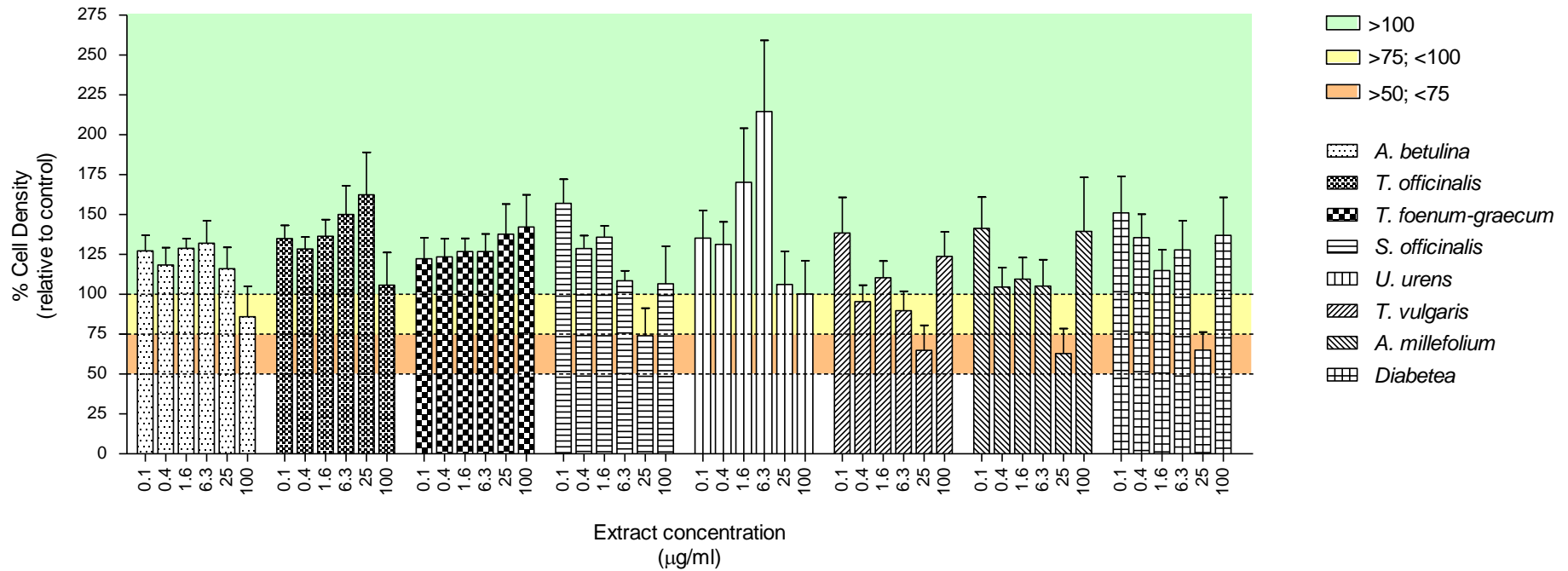


Figure 38: The effect of dichloromethane herb extracts on resting human lymphocytes, tested at 0.1 to 100 µg/ml (n = 9), measured using the sulforhodamine B uptake assay after 72 h incubation.

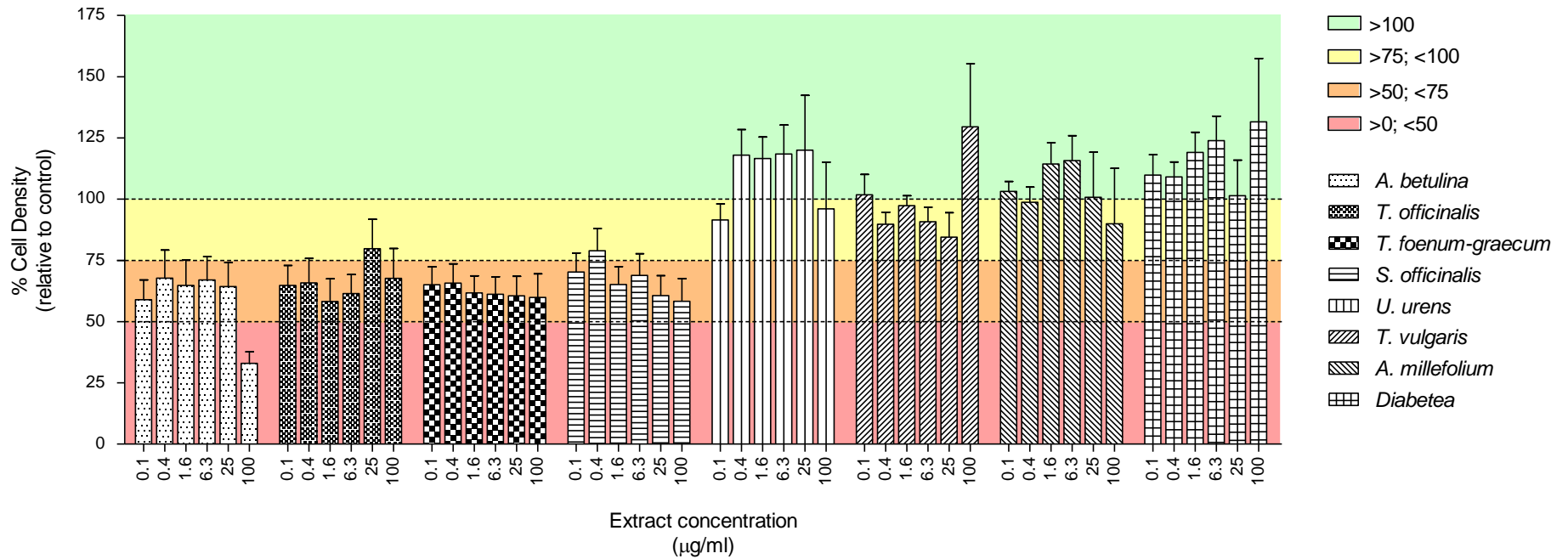


Figure 39: The effect of dichloromethane herb extracts on phytohaemagglutinin stimulated human lymphocytes, tested at 0.1 to 100 µg/ml (n = 9), measured using the sulforhodamine B uptake assay after 72 h incubation.

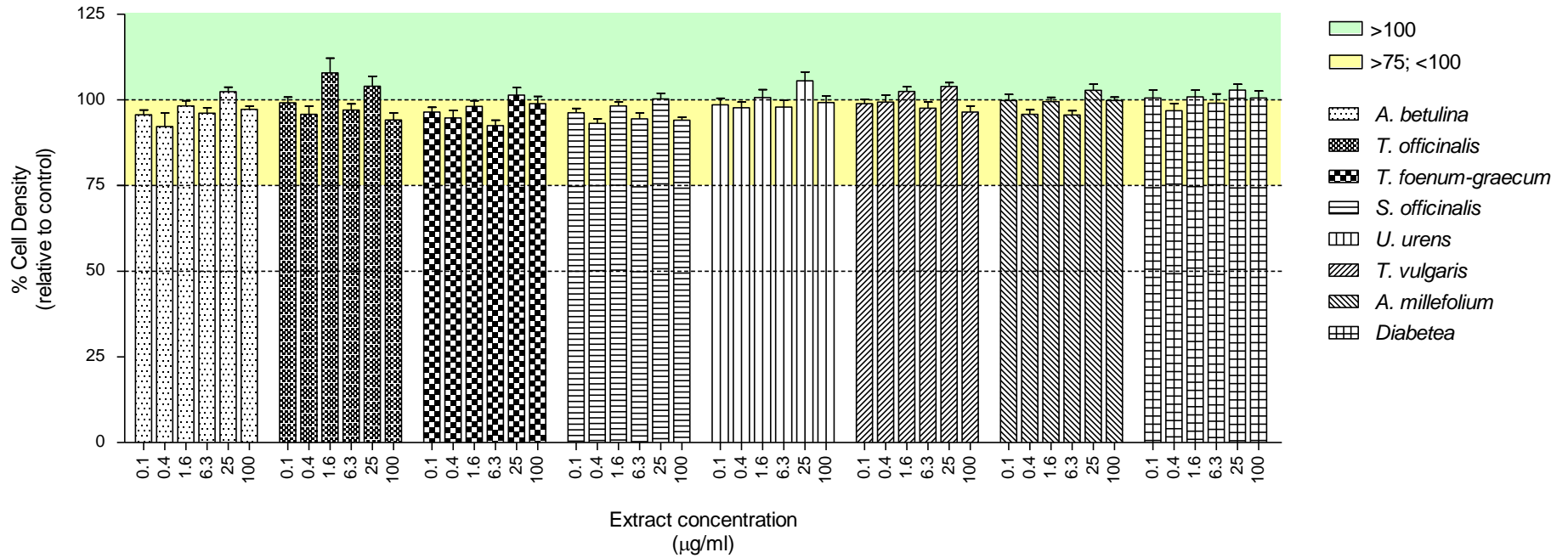


Figure 40: The effect of hot water extracts on Ea.hy926 cells, tested at 0.1 to 100 µg/ml (n = 9), measured using the sulforhodamine B uptake assay after 24 h incubation.

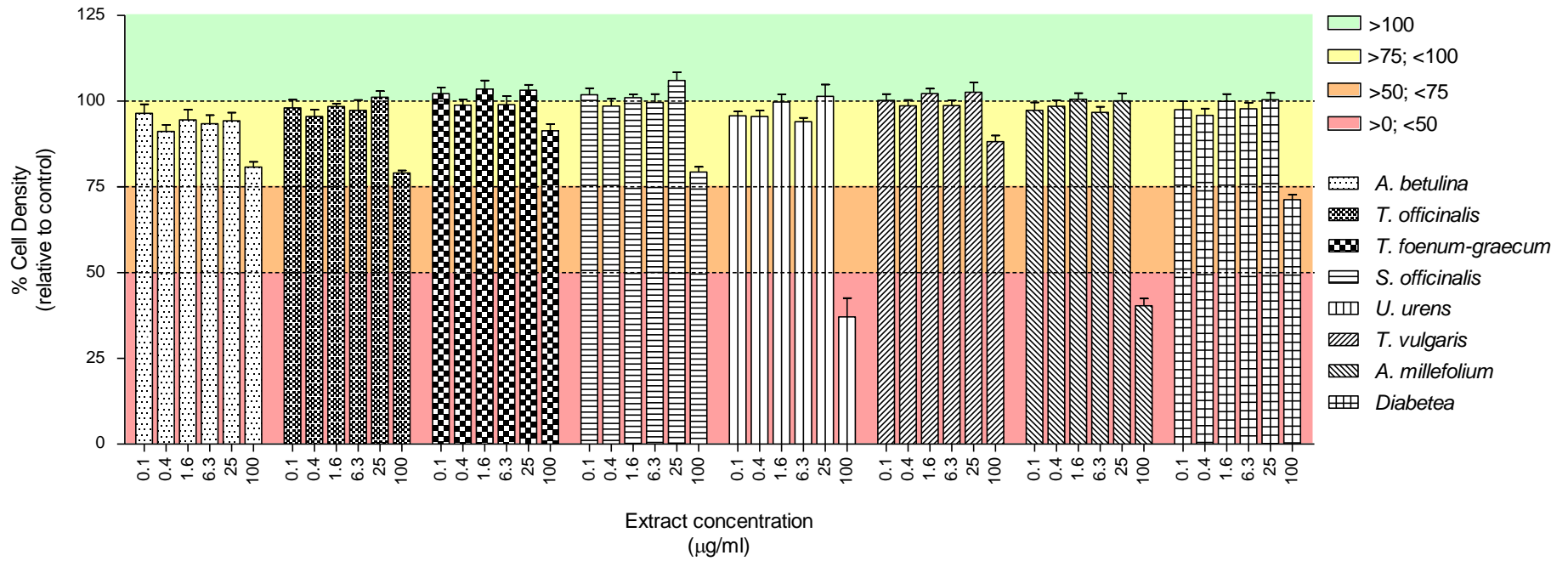


Figure 41: The effect of dichloromethane extracts on Ea.hy926 cells, tested at 0.1 to 100 µg/ml (n = 9), after 24 h incubation, measured using the sulforhodamine B uptake assay after 24 h incubation.

3.5 Discussion

All herbs tested in the present study have previously been screened for toxicity. However, the exact cell type and extract solvent used in the present study could not be found in previous literature for each plant. Furthermore, as this is the first study to test the toxicity of the commercial Diabetea mixture, no literature was found for comparison of data.

The different cell types used in the present study were found to have varying results with the extracts tested, which supports the importance of using a variety of cell types for toxicological profiling. There are various factors that influence the cytotoxic potency of extracts such as the solvent used for extraction, the mechanism of extraction, temperature and duration the extracts were prepared for as well as the plant part used^{192,252}.

In this study it was evident that the results of the cytotoxic activity for DCM and HW extracts were notably different. It was evident that the DCM extracts had more toxic profiles than the HW extracts. It has also been proven previously that heated plant constituents decrease in toxicity^{253,254}, this could explain why the HW extracts were less toxic. It was also evident that most of the extracts had a concentration-dependent cytotoxic effect. However, it is necessary to perform compound isolation and characterization in order to ascertain the compounds that are specifically responsible for the toxic effects. None of the extracts were toxic to the myocytes, HL and Ea.hy926 cells at a concentration range between 1 to 25 $\mu\text{g}/\text{m}\ell$, indicating that all the plants tested in the present study were not toxic at concentrations tested for antidiabetic activity. Toxic activities were only observed at concentrations >100

$\mu\text{g}/\text{m}\ell$. This provides evidence of the preclinical safety of these plant extracts and Diabetea at theoretically viable concentrations.

The most toxic plant extracts were that of *A. betulina* (stimulated HL), *T. foenum-graecum* (stimulated HL), *U. urens* (C2C12), *T. vulgaris* (C2C12), *A. millefolium* (C2C12 and Ea.hy926) and Diabetea (C2C12). All these plants were toxic only at 100 $\mu\text{g}/\text{m}\ell$ and most of them were of the DCM fraction except for *T. foenum-graecum*.

The toxicity at 100 $\mu\text{g}/\text{m}\ell$, observed from the DCM extract of *A. betulina*, was not surprising since toxic compounds such as quinine and *cis*-isopulegone^{97,109} have been isolated from its essential oil fraction. The non-toxic activity of the HW extract of *A. betulina* is supported by the study of Steenkamp *et al.*¹²¹ where a 10 $\text{mg}/\text{m}\ell$ aqueous extract of *A. betulina* was found to be non-toxic to resting HL cells. Furthermore, a methanol:dichloromethane extract of *A. betulina* has been reported to be non-toxic against kidney epithelial cells up to 100 $\mu\text{g}/\text{m}\ell$ ^{97,109}.

The toxicity of *T. foenum-graecum* has previously been studied and it was found that an interperineal administration of *T. foenum-graecum* was not highly toxic to mice liver with a LD_{50} of 1.9 g/kg ²⁵⁵. Abdel-Barry *et al.*²⁵⁵ reported that an oral administration of *T. foenum-graecum* was non-toxic to all mice organs with a LD_{50} of 10 g/kg ²⁵⁵. No reports on the effect of the HW extract of *T. foenum-graecum* against HL cells could be found.

Previously *U. urens* has been shown to be non-toxic to brine shrimp with $IC_{50} >10,000 \mu\text{g}/\text{m}\ell$ (water extract) and $>200 \mu\text{g}/\text{m}\ell$ (EtOH extract)²⁵⁶. In a study by Abu-Dahab it was shown that *U. urens* was non-toxic to MCF-7 cells²⁵⁷. These findings contradict the observations of the present study, where *U. urens* was found to be toxic at $100 \mu\text{g}/\text{m}\ell$ in C2C12 cells. This discrepancy may be caused by the different cell lines, model and extract solvents used.

The essential oil of *T. vulgaris* has been reported to be non-toxic in Vero cells²⁵⁸. Also, aqueous extract of *T. vulgaris* has been shown to have an IC_{50} value of $407 \mu\text{g}/\text{m}\ell$ in MCF-7 cells which is considerably higher than the concentration tested in the present study²⁵⁹. These findings do not support the toxic effect of *T. vulgaris* against C2C12 cells reported in the present study, which suggests that *T. vulgaris* shows different effects on cell density depending on the cell type used.

The non-toxic effect of the HW extract of *A. millefolium* in the present study is supported by findings of studies done using different cell lines and in rats^{81,260}. The aqueous extract of *A. millefolium* has been shown to be non-toxic to the hepatic, nephrotic and haematological systems when administered chronically for 90 days⁷⁹, supporting the non-toxic effect of the HW extract on Ea.hy926 and C2C12 cells. However, the ethanolic extract of *A. millefolium* has been reported to be toxic to tumour cells lines due to sesquiterpene lactones and flavonols⁸¹. The DCM extract has previously been tested on brine shrimp and reported to have a LC_{50} of $144 \mu\text{g}/\text{m}\ell$ ²⁶¹, which may support the toxicity noted for the DCM extract of *A. millefolium* observed in the present study. The DCM extract of this plant was found to be much

more potent to C2C12 and Ea.hy926 cells than what was observed in brine shrimp, possibly due to the natural protective mechanisms of brine shrimp against toxins²⁶¹.

T. officinalis is reported to be non-toxic^{262,263}, and said to be safe even when taken in large quantities and used in patent medicines¹²⁴. In the present study, *T. officinalis* was mostly non-toxic. This finding is supported by studies carried out previously where the water extract were found to be non-toxic to RAW 264.7 murine macrophage cells²⁶⁴, MV-4-11 cells²⁶⁵, murine peritoneal macrophages²⁶⁶ and HepG2 cells²⁶⁷.

The non-toxic profile of *S. officinalis* observed in the present study has also been reported by previous studies. Kozics *et al.*¹⁹² showed a 40% hexane extract of *S. officinalis* to be non-toxic below 1 mg/ml with an IC₅₀ of 5.7 mg/ml in HepG2 (in human hepato-cellular carcinoma) cells. The IC₅₀ value of *S. officinalis* was reported to be even higher than that by Aherne *et al.*²⁶⁸ in caco-2 cells.

The DCM and HW extracts of the Diabetea mixture was not toxic to C2C12, Ea.hy926 and HL cells at concentrations below 100 µg/ml. It was evident that the effect of Diabetea on cell density was concentration-dependent. The DCM extract of Diabetea had a greater effect on cell density than the HW extract. The present study provides initial evidence for the possible safety of Diabetea at concentrations below 100 µg/ml, which may support its traditional use. However, further investigation is necessary to validate this finding.

3.6 Chapter summary

The cytotoxic potential of both the HW and DCM extracts of *A.millefolium*, *A. betulina*, *S. officinalis*, *T. officinalis*, *T. vulgaris*, *T. foenum-graecum*, *U. urens* and Diabetea was determined using the SRB assay. A greater decrease in cell density was observed with the DCM (non-polar) extracts, compared to the HW (polar) extracts. For concentrations at which each extract was tested for its antidiabetic potential in the present study, no toxic effect was observed. Toxic effects were only observed at a concentration as high as 100 µg/ml, which was exerted by the extracts of *A. betulina*, *T. foenum-graecum*, *U. urens*, *T. vulgaris*, *A. millefolium* and Diabetea in specified cells. It was evident that the toxicity of each extract was concentration-dependent. Furthermore, all the HW extracts were non-toxic to Ea.hy926 cells.

Each herb tested in the present study is traditionally used (Section 1.6, Chapter 1, page 20), which assumes its apparent 'safety'. The present study provides *in vitro* evidence of the safety of each herb tested at concentrations below 100 µg/ml in C2C12 myotubes, Ea.hy926 umbilical vein endothelial cells and human lymphocytes (resting and stimulated).

CHAPTER 4: *IN VITRO* ANTIDIABETIC EFFICACY

4.1 Introduction

The prevention and/or alleviation of chronic hyperglycaemia is the key therapeutic approach to T2DM because of its direct association with co-morbidities and pathologies (see sections 1.4, Chapter 1, page 5). All available/current conventional treatments target the alleviation of hyperglycaemia (see section 1.5, Chapter 1, page 15). When hyperglycaemia is managed well, it is associated with a decreased progression of diabetes, hypertension and myocardial infarction^{269,270}.

Two main enzymes, α -amylase and α -glucosidase, are responsible for carbohydrate metabolism, the action of which, is associated with postprandial hyperglycaemia in T2DM individuals (see section 1.4.1.1, Chapter 1, page 7). The inhibition of α -amylase and α -glucosidase is associated with decreased postprandial plasma glucose levels and therefore postprandial hyperglycaemia in T2DM patients, as is seen with the action of acarbose⁵³. α -Amylase and α -glucosidase have been studied as potential drug targets to control diabetes since the 1960s^{271,272}. α -Amylase is responsible for the hydrolysis of polysaccharides into di- and tri-saccharides, whereas α -glucosidase breaks these down into monosaccharides, which are eventually absorbed into the circulatory system through the enteric wall by glucose transporters or passive diffusion².

Enzyme catalysed reactions are described using a lock-and-key model¹⁹³. Whereby, an enzyme possesses an active site (lock) which interacts with a specific

substrate/ligand (key), forming an enzyme-substrate (lock-and-key) complex, which is dissociated into products¹⁹³. The active site of an enzyme is highly selective to its substrate¹⁹³. The inhibition of the action of an enzyme occurs when a compound, other than the substrate, interacts with the enzyme. This interaction can include the binding of a compound to the active site (competitive inhibitor), a site other than the active site (noncompetitive or mixed inhibitor) or to the already formed enzyme-substrate complex (uncompetitive inhibitor)^{193,273}.

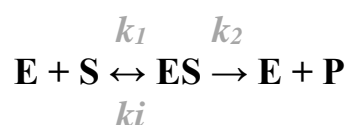
The types of inhibition exerted on enzymes are important to note, since inhibitors interact differently with enzymes. Knowing the type of inhibition exerted by an inhibitor gives insight into the physiological conditions that are either conducive to its action or not¹⁹³. For instance, the action of a competitive inhibitor can be overcome with an increase in substrate concentration, which is not so with a non-competitive/mixed inhibitor¹⁹³. Also, some inhibitors such as the uncompetitive inhibitors only act once the enzyme-substrate complex has formed, implying that it does not matter when this type of inhibitor is added to the system, as long as the substrate is present. In the present study, the type of inhibition was determined using the Michaelis-Menten kinetics model described below.

Furthermore, the alleviation of hyperglycaemia by means of absorption of circulatory glucose into muscle and adipose tissue, as mediated by the action of insulin, is the main mechanism of action of various conventional treatments such as glibenclamide, rosiglitazone, vildagliptin, liraglutide, lispro etc.^{51,52,54,57,59}. This is achieved by means of either stimulating insulin secretion, increasing insulin sensitivity of cells or acting as insulin mimetics⁷.

Two of the three key antidiabetic aspects evaluated in the present study, on Diabetea and its constituents, are disclosed in this chapter namely: prevention of postprandial hyperglycaemia by means of inhibition of carbohydrases (α -amylase and α -glucosidase) and hypoglycaemic activity by means of acting as insulin mimetics.

4.1.1 Michaelis-Menten enzyme kinetics model

The Michaelis-Menten model is described below as layed out by Campbell *et al.* and White *et al.*^{193,273}. This model describes enzyme-catalyzed reactions or behaviours based on the following reaction equation¹⁹³:



E, S, ES and P represent the enzyme, substrate, enzyme-substrate complex and product, respectively. The kinetics of this reaction involves three important rate constants: k_1 formation of ES complex, k_2 dissociation of ES into E + P and k_{-1} the dissociation of ES complex into E + S. This model assumes that P is never converted back to S and therefore only the initial velocity (V_0 or V_{init}) of the reaction is measured to avoid the reaction reaching steady-state. Steady-state is when the reaction rate/velocity (V) by which ES is formed and broken down are equal, i.e. $k_1 = k_{-1}$, thus resulting in the reconversion of P back to S. Since the V depends on the substrate concentration ($[S]$), the rate of the reaction is measured after a short period of time (t , min) such as 5 min, to ensure that V_0 is observed. At low $[S]$, V is

dependent on the $[S]$, following first order kinetics. However, at higher $[S]$, V is independent of the $[S]$ and the reaction follows zero-order kinetics.

The moment E is saturated with S , V reaches its maximum (V_{max}). The $[S]$ at which V_{max} is halved represents enzyme-to-substrate affinity (K_M , Michaelis-Menten constant). A low K_M value indicates a high E -to- S affinity and vice versa. These reaction descriptives (V_{max} and K_M) are determined by plotting $[S]$ against V (Figure 42).

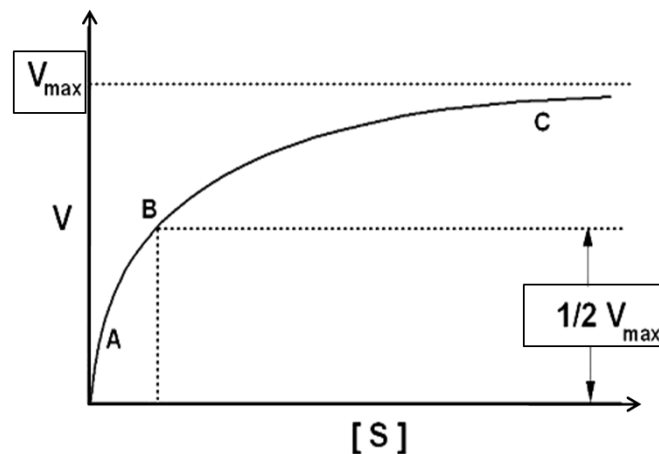


Figure 42: Michaelis-Menten graph representing substrate concentration $[S]$ and reaction velocity (V); A: first order kinetics, B: K_M at half of V_{max} and C: zero order kinetics.

The Michaelis-Menten parameters are normally used to describe the kinetics of an enzyme reaction, but it can also be employed to describe the mode of inhibition in the presence of a reversible inhibitor¹⁹³. These are competitive, non-competitive, uncompetitive and mixed inhibitors and are determined by the relationship between V_{max} and K_M , using a Lineweaver-Burk (LW-B) plot (Figure 43). A LW-B plot is a double reciprocal plot of the reaction graph.

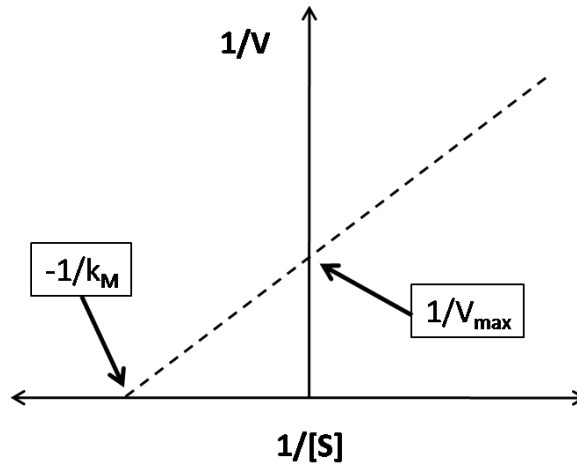


Figure 43: Lineweaver-Burk plot. V_i : initial velocity, K_M : Michaelis-Menten constant, V_{max} : maximum reaction velocity.

A competitive inhibitor competes with the substrate for the active site of the enzyme. When a competitive inhibitor is present, the slope and x-intercept of the LW-B plot changes but the y-intercept stays the same when compared to the uninhibited reaction (Figure 44). The change in slope determines the extent of inhibition (which correlates with the % inhibition), whereas the x- and y-intercepts represent the inverse effect that the inhibitor has on the $[S]$ and V . In the presence of a competitive inhibitor V_{max} stays the same but K_M increases, indicating a decrease in substrate-to-enzyme affinity. This indicates that an increased $[S]$ is needed to obtain the same V than in an uninhibited reaction. The action of a competitive inhibitor can be deterred by increasing $[S]$ significantly.

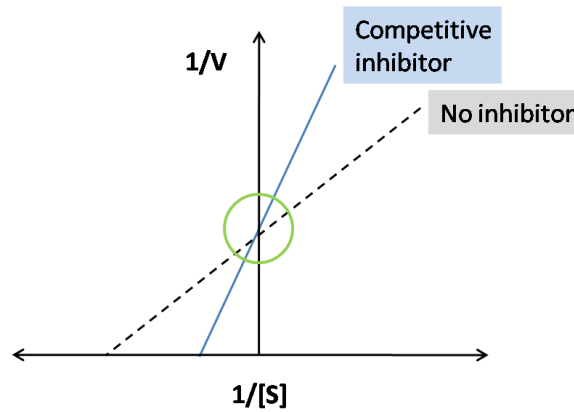


Figure 44: Lineweaver-Burk plot of an enzyme reaction in the presence of a competitive inhibitor.

A non-competitive inhibitor binds to a site other than the active site on an enzyme and its effect cannot be eliminated by simply increasing the $[S]$. On a LW-B plot the y-intercept and slope changes but the x-intercept stays the same, where V_{max} decreases and K_M remains constant (Figure 45), indicating that the enzyme does not affect the binding site and therefore has no effect on the enzyme-to-substrate binding affinity but decreases the maximal reaction velocity.

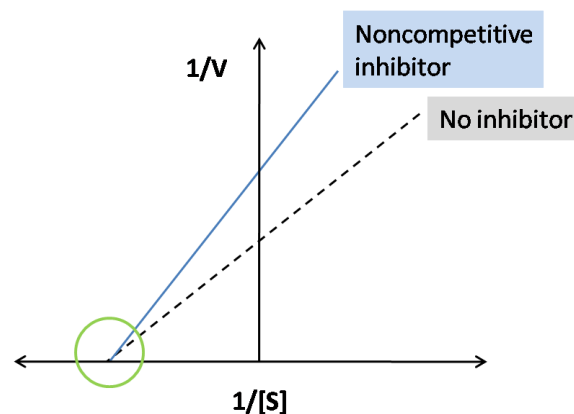


Figure 45: A Lineweaver-Burk plot of an enzyme reaction on the presence of a non-competitive inhibitor.

Uncompetitive inhibition occurs in extreme cases when the inhibitor binds to the ES complex but has no effect on unbound E. This inhibitor decreases both V_{max} and K_M , resulting in parallel lines seen on a LW-B plot (Figure 46, left), increasing E-to-S affinity and decreasing reaction velocity. Mixed inhibition occurs when an inhibitor binds to a site other than the active site with an effect on the active binding site decreasing the E-to-S binding affinity and reaction rate. Mixed inhibition is a type of non-competitive inhibition, where K_M does not remain constant. On a LW-B plot, the lines representing the normal reaction and inhibitory reaction intersect in the upper left quadrant (Figure 46).

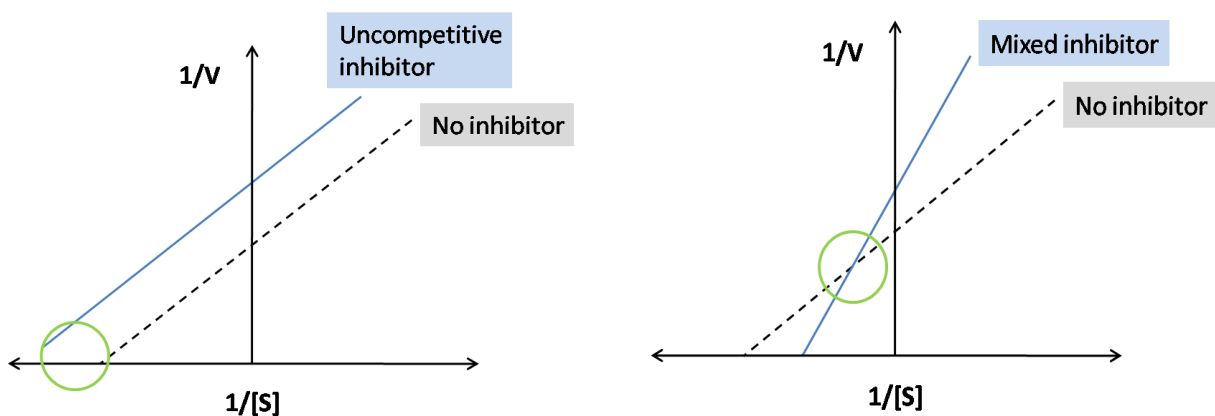


Figure 46: Lineweaver-Burk plots of enzyme reactions in the presence of an uncompetitive inhibitor (left) and a mixed inhibitor (right).

There is no inhibitor that causes an increase in reaction rate, but inhibitors always cause a decrease in maximal reaction velocity, however, they do have varying (increasing or decreasing) effects on the E-to-S affinity. If a substance has an increasing effect on reaction velocity, it does not have any inhibitory but a stimulatory effect, which the Michaelis-Menten model does not describe in detail and

needs to be understood by different reaction models, which will not be discussed in the present study.

4.2 Materials and methods

4.2.1 Enzyme activity

4.2.1.1 α -Amylase assay principle

The α -amylase activity was determined in this study following the chromogenic method, using 3,5-dinitrosalicylic acid (DNSA), as described by Bernfeld *et al.*²⁷⁴. Pancreatic α -amylase mediates the hydrolysis of the internal 1,4- α -glycosidic linkages on a long chain carbohydrate, yielding reducing sugars such as maltose. This assay tests the presence of such reducing sugars whereby DNSA (yellow) is reduced to 3-amino-5-nitrosalicylic acid (ANSA) (orange - red). The darker the solution over time, the greater the amount of reducing sugar released from the substrate in the reaction. Below is a diagram representing the principle of this assay (Figure 47). α -Amylase has its activity by means of acid catalytic hydrolysis via donating H^+ ions to the carbon-chain¹⁹³.

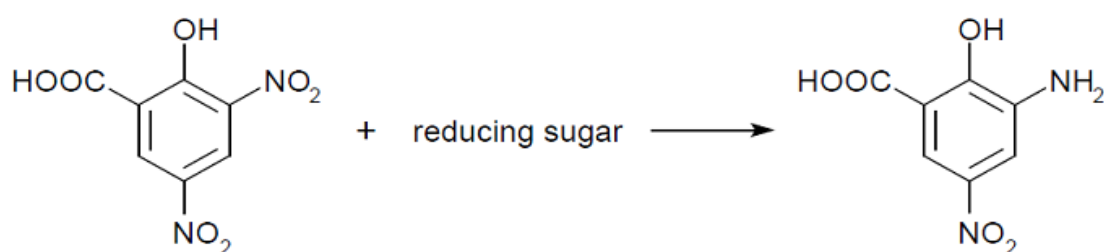


Figure 47: The reduction of DNSA yellow (left) to ANSA orange-red (right)²⁷⁵.

4.2.1.2 α -Amylase assay

This method is normally performed using tubes. In the present study it was adapted to a two-step 96-well plate method which involved decreasing the amount of reagents used, making use of PP 96-well plates (which can withstand a temperature of 90°C), omitting the DNSA dilution steps and decreasing the time it takes to transfer reagents. This method is especially useful for testing a large quantity of samples concurrently.

A stock solution of porcine pancreatic α -amylase was prepared at 0.2 U/ml using a sodium phosphate buffer (9.66 mM sodium chloride (NaCl), pH 6.9) and kept on ice for the duration of the experiment. Potato starch was used as the substrate (0.5 %w/v) and was prepared in dH₂O using a glass beaker covered with perforated parafilm and left on a magnetic stirrer for 15 min at 60°C.

The substrate was tested at eight different concentrations (0.1 - 5 mg/ml) to ensure that saturation of α -amylase occurred. The DNSA colour reagent was prepared at 80°C by mixing 5 M potassium tartrate (K₂C₄H₄O₆, dissolved in 2 M NaOH) and 96 mM DNSA (dissolved in DI H₂O) at a ratio of 2:5. The colour reagent was diluted further with DI H₂O at a ratio of 1:0.7 and stored in a dark amber container.

Thirty microliters of extract (20 μ g/ml, final concentration) and 60 μ l of potato starch were added to a PP 96-well plate and the reaction was initiated by the addition of 30 μ l of α -amylase, which was incubated for 5 min at 25°C. The reaction was terminated by incubating the plates on a waterbath at 90°C for 15 min (Figure 48), before which 60 μ l of DNSA colour reagent was added to each well. The plates were

left to cool down and contents were transferred to a polystyrene (which cannot withstand temperatures above 85°C) 96-well plate using a multichannel pipette.

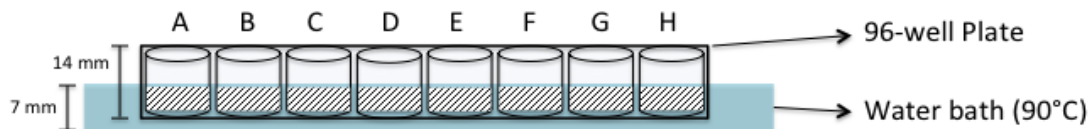


Figure 48: α -Amylase plate method by submerging plate into water bath. A to H: wells, mm: millimeter.

Acarbose (20 $\mu\text{g}/\text{mL}$) was used as positive control and the wells containing 120 μl of buffer with 60 μl of colour reagent served as blank. Phytochemical interference was accounted for by the wells containing 90 μl buffer, 30 μl extract and 60 μl DNSA colour reagent. Wells containing 30 μl buffer, 30 μl α -amylase, 60 μl substrate and 60 μl colour reagent served as control. The plates were read at 540 nm (Bio-tech Instruments, ELx800UV). The results were expressed in terms of the mode of inhibition (making use of Lineweaver-Burk plots) and the percentage inhibition of enzyme activity, which was calculated as follows:

$$\% \text{ Enzyme inhibition} = \frac{A(\text{control}) - A(\text{extract})}{A(\text{control})} \times 100$$

Where,

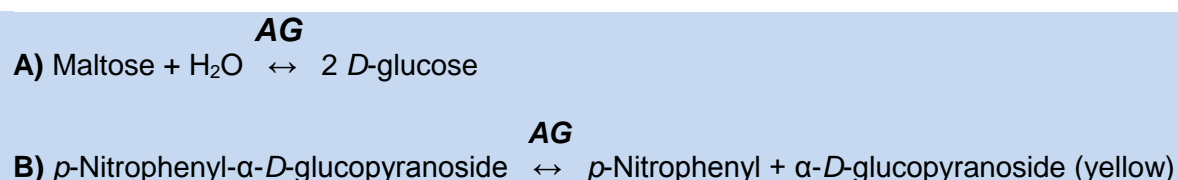
A(extract): absorbance of extract reaction and

A(control): absorbance of control.

Equation 4: Percentage enzyme inhibition.

4.2.1.3 α -Glucosidase assay principle

The α -glucosidase, sourced from *Bacillus stearothermophilus* (Sigma Aldrich, St. Louis, USA), activity was determined using the spectrophotometric, 96-well microplate method described by Collins *et al.*²⁷⁶ with modifications. α -Glucosidase catalyses the hydrolysis of external 1,4- α -D-glucose linked residues of oligosaccharides from non-reducing ends in the microvilli of the enterocytes, resulting in the release of β -glucose². In this assay *p*-nitrophenyl- α -D-glucopyranoside (*p*-NPG) was used as the substrate, instead of maltose (Equation 5 Equation 5A). The digestion of *p*-NPG yields a yellow metabolite upon reacting with its solvent, sodium phosphate buffer (0.01 M, pH 6.9). The hydrolysis of *p*-NPG to *p*-nitrophenyl and α -D-glucopyranoside (Equation 5B) represents the normal hydrolysis mediated by α -glucosidase activity (Equation 5A). Therefore, the enzyme activity is determined by the colour of the solution, the more yellow the solution the greater the activity of the enzyme.



Equation 5: (A) Normal biological α -glucosidase (AG) activity and (B) *p*-NPG assay reaction.

4.2.1.4 α -Glucosidase assay

A stock solution of α -glucosidase at 0.1 U/ml (tested as the optimal concentration that saturated the enzyme) was prepared using a 0.01 M sodium phosphate buffer (pH 6.9) and kept on ice for the duration of experiment. The substrate, *p*-NPG, was

prepared in sodium phosphate buffer (0.01 M, pH 6.9) and glycine (pH 10) was used to terminate the reaction.

Thirty microliters of plant extract (20 $\mu\text{g}/\text{mL}$, final concentration) and 60 μl of *p*-NPG was pipetted into the 96-well plate. The reaction was initiated by adding 30 μl of α -glucosidase and incubated for 5 min at 25°C. Acarbose was used as positive control at 20 $\mu\text{g}/\text{mL}$. Glycine (60 μl) was added and the plates were read at 405 nm (Bio-tech Instruments, ELx800_{UV}). Reactions containing 30 μl buffer, 30 μl α -glucosidase solution, 60 μl substrate and 60 μl glycine served as negative control. The reactions containing 30 μl plant extract, 90 μl buffer and glycine served as background control in order to eliminate phytochemical interference. The mode of inhibition was determined using Lineweaver-Burk plots and the percentage inhibition against α -glucosidase activity was determined using Equation 4, page 120.

4.2.2 Glucose uptake activity

4.2.2.1 2-NBDG principle

Insulin-mediated glucose uptake into myotubes was determined using a fluorescent *D*-glucose analogue, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-*D*-glucose (2-NBDG). 2-NBDG is actively transported across the cell membrane via GLUT transporters²⁷⁷. The uptake of 2-NBDG is very sensitive and inhibited especially by *D*-glucose as well as other glucose molecules^{277,278}. Once inside the cell, the 2-NBDG analogue gets trapped within the cell and can be detected fluorometrically at $\lambda_{\text{ex}} = 460 \text{ nm}$ and $\lambda_{\text{em}} = 544 \text{ nm}$. The greater the fluorescent signal is, the higher the relative rate of glucose uptake.

4.2.2.2 2-NBDG assay

The hypoglycaemic activity of plant extracts was determined using a fluorescence, 96-well microplate, method described by Zou *et al.*²⁷⁹. The C2C12 myotubes were prepared and counted as described in Section 3.2.3 (page 88). C2C12 myotubes (80 μl) were seeded into white plates at a density of 1.5×10^4 cells/well and incubated for 48 h at 37°C with 5% CO₂. The medium was changed after 24 h. Fifty microliters of glucose free DMEM+, containing 400 μM of 2-NBDG and 80 μl of plant extracts (1 - 20 $\mu\text{g}/\text{m}\ell$) was added and incubated at 37°C in 5% CO₂ for 1 h. The medium was then removed using a multichannel pipette and 50 μl HBSS was added to each well. Metformin and insulin were used separately as positive controls (20 $\mu\text{g}/\text{m}\ell$ in reaction). Wells containing 50 μl HBSS and no cells served as blank and pre-seeded wells treated with 2-NBDG and glucose free DMEM served as control. The plates were read fluorometrically at $\lambda_{\text{ex}} = 460$ nm and $\lambda_{\text{em}} = 544$ nm. The results were expressed in terms of RFI.

4.3 Statistical analysis and data representation

All experiments were executed in triplicate (technical and biological, $n = 9$) and the data is presented in the form of bar graphs with each bar representing the mean \pm SEM. Statistical analysis between extracts was performed using a one-way ANOVA test with Dunnet's multiple comparison. Student's *t*-tests were used to determine the significance of extracts compared to the negative and positive controls. V_{max} and K_M were determined using Michaelis-Menten non-linear regression analysis. The

statistical packages that were used included GraphPad Prism 6.0, STATA 12 and Microsoft Excel 2010. Statistical significance is regarded at $p < 0.05$.

4.4 Results

4.4.1 Enzyme activity

The results are presented in four ways, with each indicating essential information about the enzyme activity. These are: V_{\max} bar graphs, percentage inhibition, Michaelis-Menten line-graphs and Lineweaver-Burk plots. The V_{\max} bar graphs indicate the presence of inhibition or no-inhibition, the percentages inhibition shows the extent of the inhibition/stimulation observed. The Michaelis-Menten graphs show the significance of the enzyme reaction at different substrate concentrations compared to the negative control and the LW-B plots represent the type of inhibition. Only significant results are presented (% inhibition, V_{\max} bar graphs and LW-B plots) in this chapter and all other results, including Michaelis-Menten reaction graphs, can be found in Appendix V (page 161).

4.4.1.1 α -Amylase

None of the HW extracts inhibited the activity of α -amylase (Table 3), instead all of them except for *T. foenum-graecum* caused a significant ($p < 0.05$) increase in the amount of product formed over time (Table 3) by increasing reaction velocity (Figure 49A).

The DCM extract of *U. urens* inhibited the activity of α -amylase (Figure 49B), and significantly ($p < 0.05$) decreased the amount of product formed (Table 3). The DCM extract of *U. urens* displayed inhibitory activity similar to that of acarbose (Table 3).

U. urens inhibited α -amylase uncompetitively (Figure 50) whereas acarbose is a known competitive inhibitor²⁸⁰.

Table 3: The effect of hot water (HW) and dichloromethane (DCM) extracts (n = 9) on α -amylase activity, in relation to the control. Significance was determined using Student's *t*-test, with $p < 0.05$.

Percentage α -amylase activity		
Herb	HW	DCM
<i>A. betulina</i>	-50.7 \pm 9.4 [#]	-1.1 \pm 10.3
<i>T. officinalis</i>	-29.7 \pm 10.7 [#]	10.6 \pm 5.8
<i>T. foenum-graecum</i>	-11.1 \pm 11.7	-5.2 \pm 7.9
<i>S. officinalis</i>	-27.6 \pm 8.0 [#]	10.3 \pm 7.3
<i>U. urens</i>	-31.9 \pm 7.0 [#]	52.8 \pm 12.1*
<i>T. vulgaris</i>	-53.1 \pm 10.6 [#]	10.6 \pm 4.8
<i>A. millefolium</i>	-51.5 \pm 8.5 [#]	12.5 \pm 7.6
Diabetea	-31.8 \pm 9.5 [#]	0.8 \pm 10.7
Acarbose	57.0 \pm 7.4*	57.0 \pm 7.4*
* Significant inhibition # Significant stimulation		

4.4.1.2 α -Glucosidase

A. betulina and *T. vulgaris* were the only HW extracts that significantly ($p < 0.05$) inhibited α -glucosidase activity (Table 4). Both *A. betulina* and *T. vulgaris* inhibited glucosidase in a mixed manner (Figure 52).

The DCM herb extracts exhibited a greater inhibitory activity against α -glucosidase than the HW extracts (Table 4). All DCM extracts were active against the activity of α -glucosidase except for *A. millefolium*. The DCM extracts of *A. betulina*, *S. officinalis* and *T. vulgaris* significantly ($p < 0.05$) inhibited α -glucosidase activity in a non-competitive, mixed and uncompetitive manner, respectively (Figure 53).

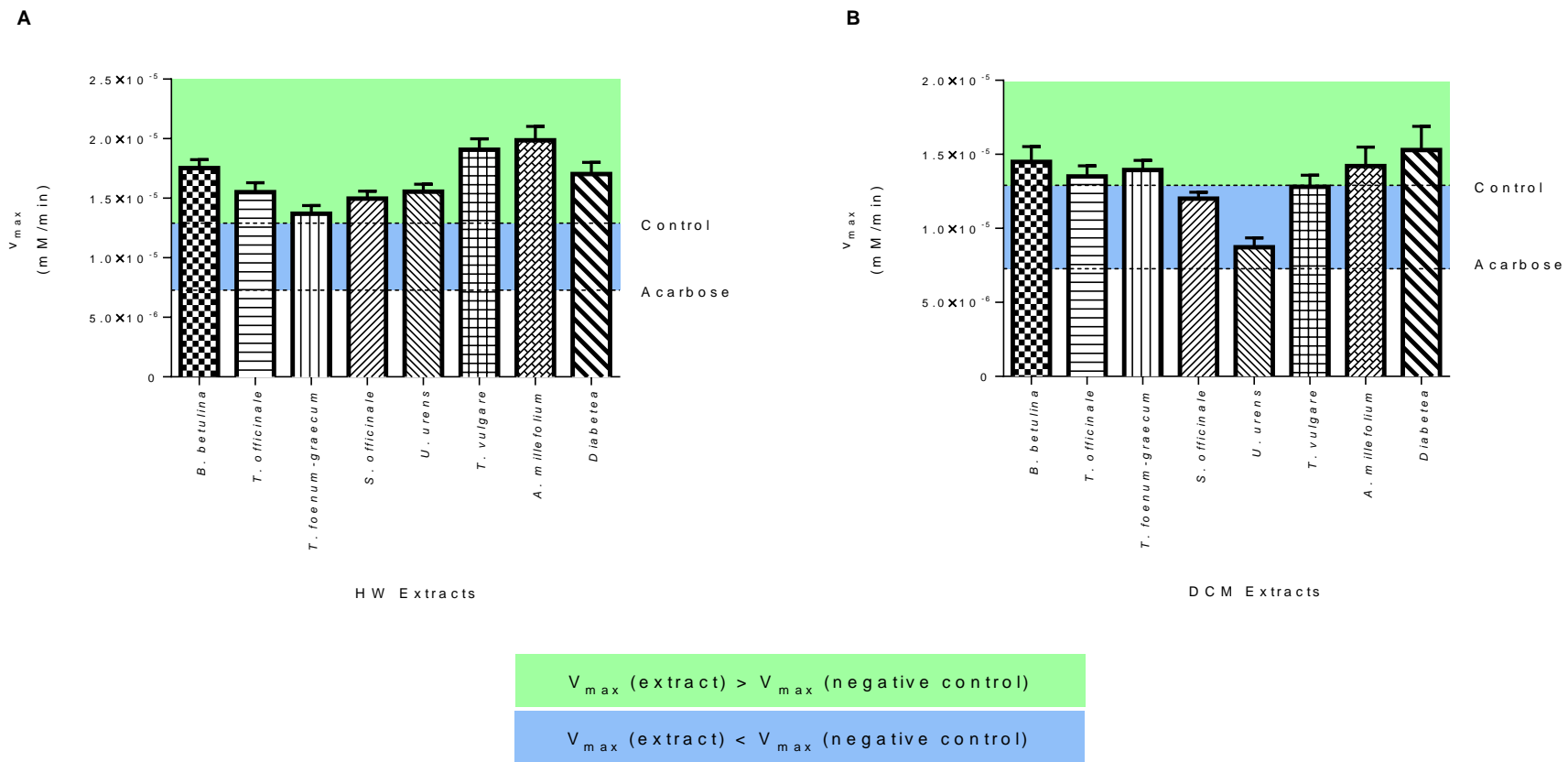


Figure 49: The effect of (A) hot water (HW) and (B) dichloromethane (DCM) extracts (n = 9) on the maximal reaction velocity (V_{max}) of α -amylase. Extracts that caused an increase of V_{max} (green), relative to the control, did not inhibit α -amylase activity whereas extracts that decreased V_{max} (blue) inhibited α -glucosidase activity. The negative and positive (acarbose) controls are represented by dotted lines. The y-axis between graphs A and B are different.

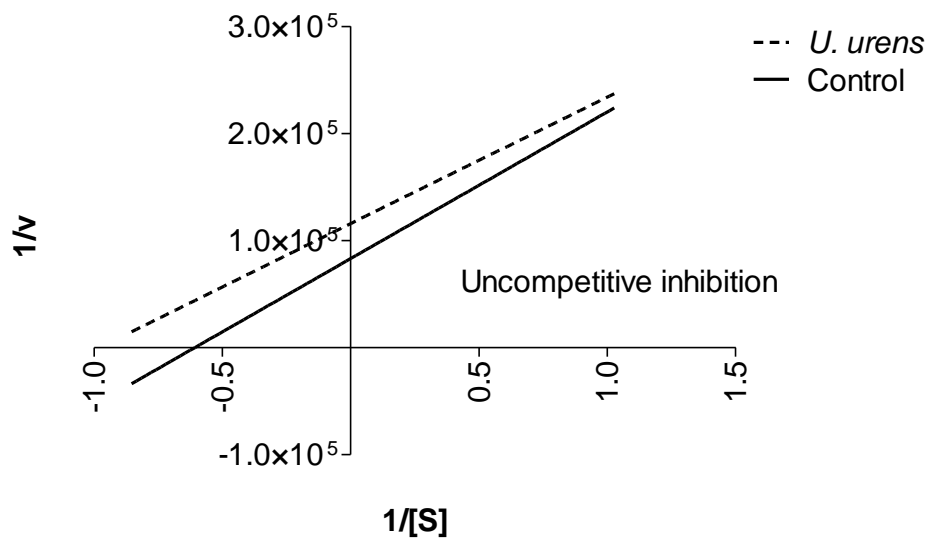


Figure 50: Lineweaver-Burk plot of the reaction of the dichloromethane extract of *U. urens* against α -amylase activity.

Table 4: The effect of HW and DCM extracts (n = 9) on α -glucosidase activity, in relation to the control. Significance was determined using Student's *t*-test, with $p < 0.05$.

Percentage α -glucosidase activity		
Herb	HW	DCM
<i>A. betulina</i>	42.6 \pm 5.5*	93.8 \pm 0.8*
<i>T. officinalis</i>	-11.9 \pm 6.6	10.9 \pm 2.3
<i>T. foenum-graecum</i>	-2.7 \pm 4.9	3.9 \pm 4.8
<i>S. officinalis</i>	0.3 \pm 6.2	52.7 \pm 4.7*
<i>U. urens</i>	-12.5 \pm 6.0	10.4 \pm 3.5
<i>T. vulgaris</i>	24.5 \pm 4.1*	46.1 \pm 5.1*
<i>A. millefolium</i>	-2.0 \pm 6.1	-3.3 \pm 1.7
Diabetea	2.02 \pm 5.9	8.2 \pm 7.6
Acarbose	100.0 \pm 0.6*	100.0 \pm 0.6*
* significant inhibition		

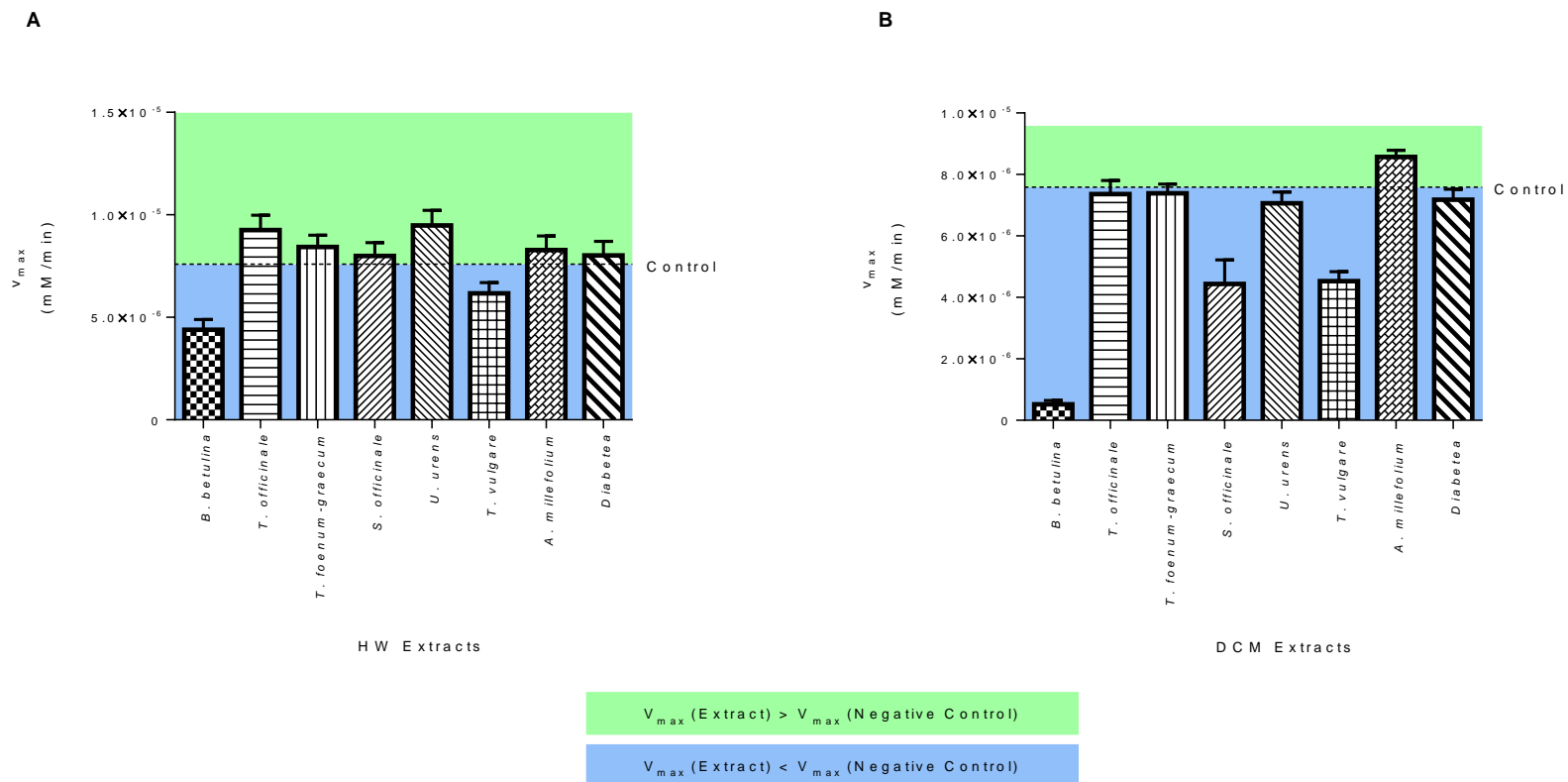


Figure 51: The effect of (A) hot water (HW) and (B) dichloromethane (DCM) extracts (n = 9) on the maximal reaction velocity (V_{max}) of α -glucosidase. Extracts that caused an increase of V_{max} (green) relative to the control, did not inhibit α -glucosidase activity. Extracts that decreased V_{max} (blue), inhibited α -glucosidase activity. The negative and positive (acarbose) controls are represented by dotted lines. The y-axis between graphs A and B are different.

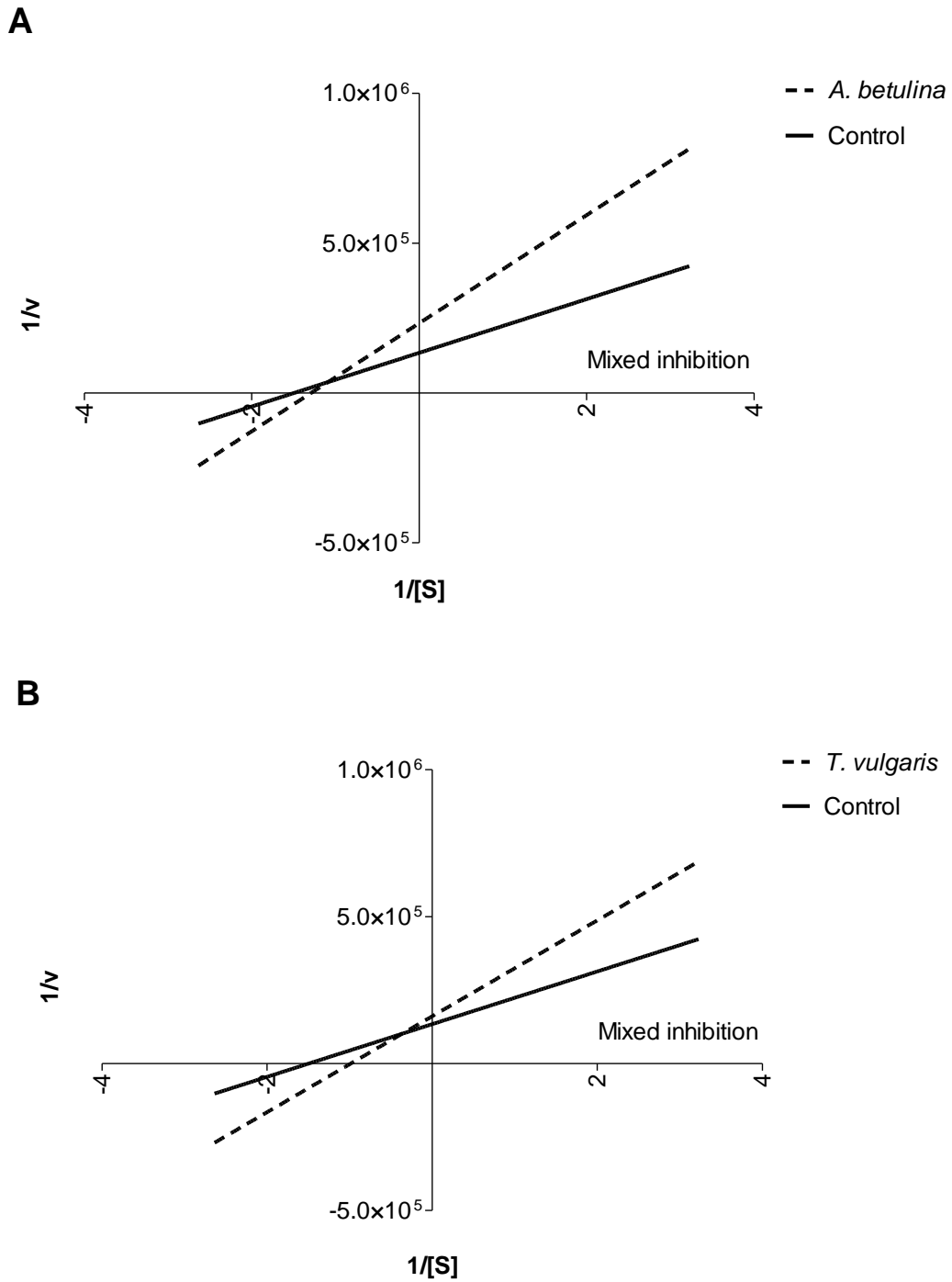


Figure 52: Lineweaver-Burk plots of the hot water extracts of (A) *A. betulina* and (B) *T. vulgaris* showing a mixed inhibitory effect against the activity of α -glucosidase.

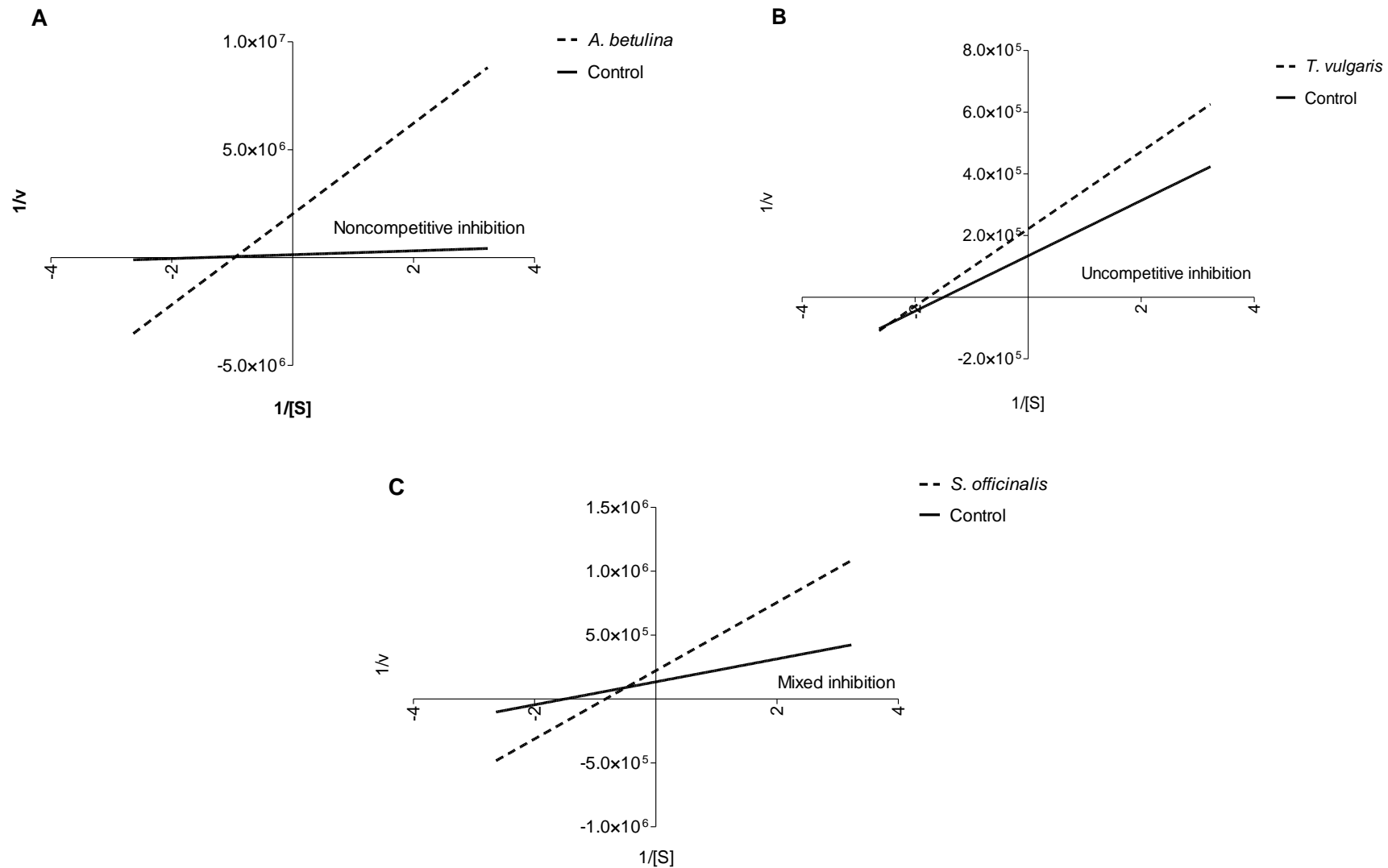


Figure 53: Lineweaver-Burk plots of the dichloromethane extracts of (A) *A. betulina*, (B) *T. vulgaris* and (C) *S. officinalis* showing a non-competitive, uncompetitive and mixed inhibitory effect against the activity of α -glucosidase, respectively.

4.4.2 Glucose uptake activity

The HW extracts of *A. betulina*, *T. officinalis*, *T. foenum-graecum*, *S. officinalis* and *U. urens* significantly ($p < 0.05$) increased the amount of glucose absorbed into the myotubes compared to the control (Figure 54). The activity of the HW extract of *S. officinalis* was more significant ($p < 0.05$) to the activity of insulin (Figure 54) at 3 $\mu\text{g}/\text{m}\ell$. Furthermore, the HW extract of *A. millefolium* (Figure 54) significantly ($p < 0.05$) inhibited normal glucose uptake.

All DCM extracts caused an increase in glucose uptake except for *A. betulina* at 20 $\mu\text{g}/\text{m}\ell$ (Figure 55). The DCM extracts of *T. officinalis*, *U. urens*, *T. vulgaris*, *A. millefolium* and Diabetea also significantly ($p < 0.05$) increased the amount of glucose uptake (Figure 55). The glucose uptake induced by the DCM extract of *T. vulgaris* was significantly ($p < 0.05$) higher than that of insulin at 20 $\mu\text{g}/\text{m}\ell$ (Figure 55).

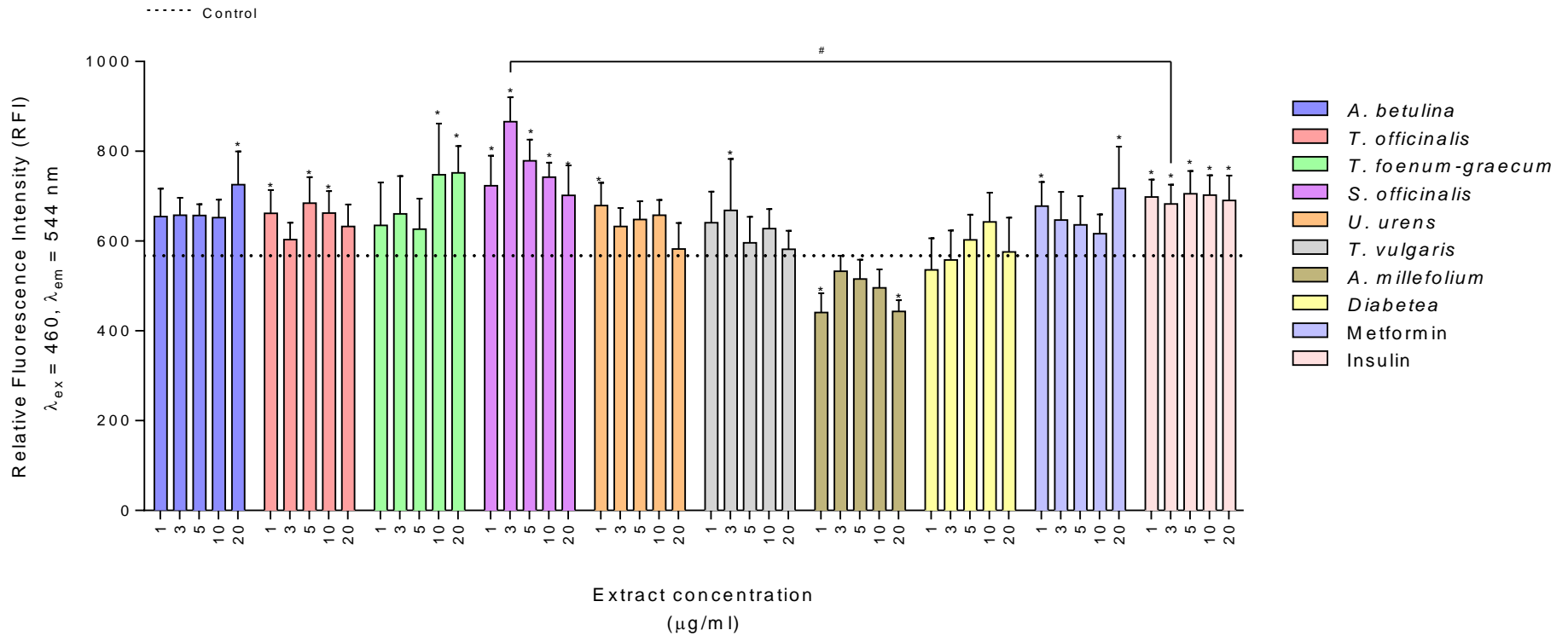


Figure 54: The ability of hot water herb extracts ($n = 9$) to mediate glucose uptake into C2C12 myotubes. Significance ($p < 0.05$) was determined against control (*) and positive controls (#) using the student's t -test. Metformin and insulin served as positive controls.

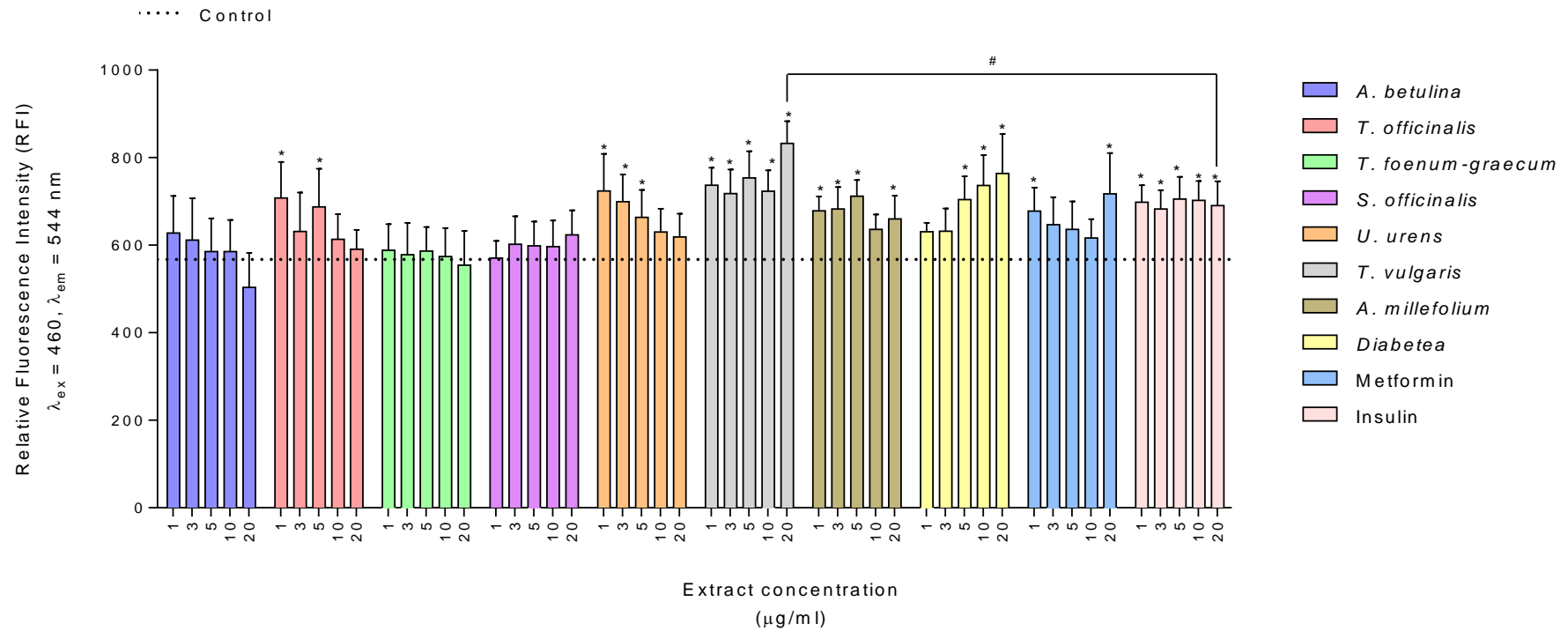


Figure 55: The ability of dichloromethane herb extracts to mediate glucose uptake into C2C12 myotubes. Significance ($p < 0.05$) was determined against control (*) and positive controls (#) using the student's t -test. Metformin and insulin served as positive controls.

4.5 Discussion

4.5.1 α -Amylase and α -glucosidase activity

There was no inhibitory activity exhibited by any of the HW extracts against α -amylase. However, there was an interaction between the extracts and this enzyme since all of the herbs except for *T. foenum-graecum* caused a significant ($p < 0.05$) increase in the reaction velocity, indicating a stimulatory effect on α -amylase activity (Table 3). Porcine α -amylase has one active site with five subunits for the binding of ligands. The efficacy of α -amylase is enhanced with an increase in chain length of a ligand²⁸¹. The active site of α -amylase contains Asp197, 300 and Glu233 in close proximity with a network of H₂O molecules within the cleft²⁸¹. The amino acid residues in the active site have polar side chains and interact via hydrogen-bonding. The inhibition of α -amylase increases with increasing molecular weight of the inhibitor²⁸¹. The interaction at the active site has been described using general acid-base mechanism with the general acid hydrolyzing/nucleophilic substitution activity²⁸¹. This indicates that the catalytic reaction mediated by α -amylase occurs with the addition of water molecules to the substrate, which could be more favourable to hydrophilic substrates. This may be the reason why the HW extracts (being more hydrophilic) caused a significant increase in the rate of substrate hydrolysis. Furthermore, it has been shown that lignin also activates the activity of α -amylase, and has an even greater activating activity on amylase than chloride³⁰². Lignin is a type of dietary fibre abundant in plant material that is immune to enzyme digestion³⁰². This could also be the reason for the stimulatory effect observed in the present study.

Furthermore, the HW extracts tested did not contain any excess starch, as verified with phytochemical background controls indicating that the activation was not brought about by the presence of excess substrate as in the case of a first order kinetics reaction (see section 4.1.1., page 113). However, this result is not necessarily undesired, since the inhibition of α -amylase has been associated with abdominal discomfort due to polysaccharide fermentation^{282,283}. This implies that a weak α -amylase inhibitor in the presence of a potent α -glucosidase inhibitor may be preferred. In the present study the stimulated hydrolysis of polysaccharides may be favourable in preventing the fermentation of higher sugars, with the inhibition of the absorption of monosaccharides, decreasing postprandial hyperglycaemia. The preferred inhibition of α -glucosidase is that of mediated by a non-competitive, mixed or uncompetitive inhibitor, due to the first order kinetic nature of a competitive inhibitor.

The DCM herb extracts exerted a greater inhibitory activity against α -glucosidase than that of the HW extracts. A study done by Yan Qin Li *et al.*²⁸⁴, suggested that the interaction between the active site on α -glucosidase and inhibitors are mainly hydrophobic. This could be the reason why DCM extracts (which are more hydrophobic than HW extracts) were more active against α -glucosidase activity in the present study.

Of all extracts tested, the DCM extract of *U. urens* had the strongest inhibitory activity against α -amylase, which was comparable to the activity of acarbose (Table 3). Jianbo Xiao *et al.*³⁰¹ has shown that hydroxylated flavonoids are associated with a stronger α -amylase inhibition activity. The presence patuletin, which is a

hydroxylated flavonoid, in *U. urens* is possibly the reason for the potent inhibition observed with *U. urens*. *U. urens* inhibited α -amylase uncompetitively (Figure 50), whereas acarbose is a competitive inhibitor. This means that *U. urens* inhibits the activity of α -amylase when the enzyme-substrate complex has formed, decreasing maximal reaction velocity and increasing E-to-S affinity. No supporting data could be found for the present activity observed with the DCM extract of *U. urens*. A study done on a 50% methanol-water extract of *U. urens* showed no inhibitory potential against α -amylase activity with a percentage inhibition of -7.1%²⁷¹, indicating a stimulatory effect supporting the result with the HW extract of *U. urens* in the present study. In the present study *U. urens* stimulated the activity of α -amylase by -31.9%, which is almost four times higher than observed by Hamdan *et al.*²⁷¹. This discrepancy may be due to the difference in extract solvent used, in the present study the extract was theoretically more polar than that used by Hamdan *et al.*²⁷¹, this may further support the notion that more polar/hydrophilic substrates/compounds have a greater stimulatory effect on α -amylase activity.

The extracts of *A. millefolium* were not active against the α -amylase or α -glucosidase activity in the present study. Previously, a 60% ethanol extract of *A. millefolium* was shown to inhibit the activity of α -glucosidase by ~52.3%²⁸⁵. This contradiction to the present results is most probably due to the high concentration (1 mg/ml) of *A. millefolium* tested, which is five times higher than the highest concentration tested in the present study. Another probable reason for this incongruity is the difference in extract solvent used.

The HW and DCM extracts of *A. betulina* were the most potent against α -glucosidase activity of all extracts tested (Table 3 and Table 4). The HW extract of *A. betulina* inhibited α -glucosidase in an uncompetitive manner, whereas the DCM extract showed a mixed inhibition, indicating that the active compound(s) inhibited α -glucosidase activity by binding to the enzyme at a site other than the active site, causing a decrease in its reaction rate in both cases and an increase (uncompetitive) and decrease (mixed) in E-to-S affinity. This resulted in a decreased product formation over time. This type of inhibition described by a linear reaction model such as Michaelis-Menten is stable and not influenced by the amount of substrate added. The inhibition exerted by the DCM extract was comparable the activity of acarbose. No supporting literature regarding the activity of *A. betulina* against α -glucosidase could be found.

The HW extract of *T. foenum-graecum* did not have any favourable antidiabetic effect against α -glucosidase and α -amylase and no supporting data of this particular result could be found.

The HW and DCM extracts of *T. vulgaris* significantly ($p < 0.05$) inhibited α -glucosidase activity. The mode of inhibition exerted by both extracts were mixed, resulting in a decrease in reaction rate and E-to-S affinity. Another study done on the essential oil of *T. vulgaris* also showed an inhibitory activity against both α -amylase and α -glucosidase²⁹⁰. Furthermore, the inhibitory activity of *T. vulgaris* is subject to the source of enzyme tested²⁹¹. A flavonoid isolated from *T. vulgaris* known as luteolin has been found to inhibit α -glucosidase by 36% at 0.5 mg/ml²⁹²,

which is within the range of the inhibitory activity of *T. vulgaris* (HW and DCM) observed in the present study (between 24 – 47% inhibition).

The DCM extract of *S. officinalis* significantly ($p < 0.05$) inhibited α -glucosidase in a mixed manner (Figure 53). No literature could be found that tested the effect of a DCM extract of *S. officinalis* on α -glucosidase activity. However, other extracts, such as HW and methanol, have been shown to inhibit rat intestinal α -glucosidase activity by approximately 30 and 18%, respectively²¹⁶. This supports the inhibitory activity observed in the present study (HW ~53%). The different degrees by which α -glucosidase was inhibited could be accounted for by the different sources of α -glucosidase used.

Cazzola *et al.*²¹⁶ reported that a HW and methanol extract of *S. officinalis* inhibited 25 and 15% of the activity of porcine α -amylase activity, respectively²¹⁶. This result by Cazzola *et al.* using the HW extract was contrary to that reported in the present study (HW -27%), where it caused a significant stimulatory activity of α -amylase (Table 3). The reason for the discrepancy could be due to the difference in duration of extraction time, whereby Cazzola *et al.* boiled the extracts until 100 ml of water evaporated instead of for 15 min, and they stirred the extract for 24 h instead of 1 h.

The extracts of *T. officinalis* were not active against α -amylase or α -glucosidase. Previously, Onal *et al.*²⁹⁵ reported that a HW extract of *T. officinalis* inhibited 50 – 80% of α -glucosidase activity, depending on the source of α -glucosidase used²⁹⁵. However, these authors did not investigate α -glucosidase from *Bacillus stearothermophilus*, which could be the reason for the discrepancy between the

report of Onal *et al.*²⁹⁵ and the present study. The aqueous extract of *T. officinalis* at 1, 3, and 5 mg/ml has been found to inhibit α -amylase activity by 25, 43, 72% respectively, *in vitro*²⁹⁶. Although these authors observed an inhibitory activity with this extract, their extracts were tested at five to twenty-five times the concentration used in the present study.

4.5.2 Glucose uptake mediated by insulin mimetic action

All HW extracts were rich in polyphenolic compounds. It has been shown previously that polyphenols, especially flavonoids, are active in carbohydrate homeostasis in various ways. One of which is the increase in glucose uptake into insulin-dependent cells³⁰³. Since each of the HW extracts were rich in polyphenols, this may have been instrumental in the significant glucose uptake activity.

Both the HW and DCM extracts of *U. urens* significantly ($p < 0.05$) increased the amount of glucose absorbed into the myotubes (Figure 54 and Figure 55). However, this was not at all concentrations tested and there was a concentration-dependent decrease in its glucose uptake activity, which may indicate an inhibitory effect on absorption of 2-NBDG. The mechanism of this phenomenon is unclear, and could possibly be because *U. urens* contained compounds that either interact with 2-NBDG, interfere with glucose receptors or it may have caused an increase in the metabolic activity of the cells thereby causing faster degradation of 2-NBDG. No other literature could be found for the glucose uptake activity mediated by *U. urens*.

The HW extract of *A. millefolium* was the only herb to significantly ($p < 0.05$) inhibit normal cellular glucose uptake (Figure 54). The possible reason for the significant (p

< 0.05) inhibition of glucose uptake is unclear. These results suggest that the HW extract of *A. millefolium* may be ineffective as a hypoglycaemic preparation. Irrespective of this result, the DCM extract of *A. millefolium* significantly ($p < 0.05$) increased glucose uptake into myotubes (Figure 55). *A. millefolium* has been studied extensively and is known to exert a hypoglycaemic effect, both *in vitro* and *in vivo*^{286,287}. A commercial drug combination known as Liv.52, includes *A. millefolium* as one of its main ingredients, which act as an insulin mimetic for glucose uptake into steatotic HepG2 cells²⁸⁶. Another study showed that both aqueous and methanolic extracts of *A. millefolium* have hypoglycaemic activity in rats, due to possible insulin secretory effects or direct insulin mimetic action²⁸⁷. These results do not support the findings of the present study; however, there are fundamental discrepancies with regards to the type of assays, extraction methods, and concentrations used.

The HW extract of *A. betulina* significantly increased the amount of glucose absorbed into the myotubes at 20 $\mu\text{g}/\text{ml}$ (Figure 54). However, there was no concentration-dependent relationship or stimulation observed at the other concentrations tested. There is no supporting literature regarding the glucose uptake activity of HW or DCM extracts of *A. betulina*. However, important biological flavonoids, such as diosmin and hesperidin, have been isolated from its essential oil. These have been shown to function as diuretics, antihypertensives, hypolipidaemics, anticancer agents, antioxidants and inflammatory mediators¹¹⁸. Furthermore, a micronised purified flavonoid fraction called Daflon 500 (90% diosmin and 10% hesperidin), has been tested in a long term study for its antidiabetic activities and was found to be hypoglycaemic, antiglycating and possess antioxidant action in type

1 diabetic patients²³⁴⁻²³⁶. A study done on streptozotocinnicotinamide-induced diabetic rats using diosmin showed that it caused an increase in insulin secretion from pancreatic β -cells²³⁴. The findings in the present study rather support insulin mimetic function exerted by the extract, rather than insulin secretary activities. The present findings suggest that the HW extract of *A. betulina* contains active compounds that may act as insulin mimetics, which may be due to active compounds previously isolated, tested and described in literature^{234,288}.

The HW extract of *T. foenum-graecum* caused a significant ($p < 0.05$) glucose uptake at 10 and 20 $\mu\text{g}/\text{m}\ell$ (Figure 54). Major compounds such as nicotinic acid, nicotinamide and coumarin have been isolated from *T. foenum-graecum* seeds and have been shown to be hypoglycaemic agents in alloxan-diabetic rats²⁸⁹. The aqueous extract of *T. foenum-graecum* leaves has also been shown to significantly decrease blood glucose in alloxan-diabetic rats at a minimum of 0.2 g/kg after 1 h²⁵⁵. The exact mechanism of action has not been established in these studies but could be supported by the *in vitro* hypoglycaemic activity of *T. foenum-graecum* observed in the present study.

The glucose uptake activity induced by the DCM and HW extracts of *T. vulgaris* was significant ($p < 0.05$) (Figure 54 and Figure 55). This result is supported by a study carried previously on a DCM extract of aerial parts of *T. vulgaris*, that significantly ($p < 0.05$) stimulated glucose uptake into 3T3-L1 adipocytes¹²³. In addition, the DCM extract of *T. vulgaris* was significantly ($p < 0.05$) more active than insulin at 20 $\mu\text{g}/\text{m}\ell$ (Figure 55) in the present study.

The HW extract of *S. officinalis* significantly ($p < 0.05$) increased glucose uptake at all concentrations tested and was significantly ($p < 0.05$) higher than the activity of insulin at 2.5 $\mu\text{g}/\text{m}\ell$ (Figure 54). Previously, a 15% ethanol-water extract was shown to have hypoglycaemic activity in normal and mild aloxan-diabetic mice in the presence of insulin²⁹³. This is contrary to results from the present study, since the HW extract of *S. officinalis* did not need the presence of insulin to function. However, Cristovao *et al.*²⁹⁴ found no hypoglycaemic activity in diabetic rats with the administration of a HW extract, *ad libitum* for 14 days. This variation could be due to the difference in administration and extract preparation used.

The HW and DCM extracts *T. officinalis*, significantly ($p < 0.05$) increased the amount of glucose absorbed into the myotubes (Figure 54 and Figure 55). However, this *in vitro* result is not supported by previous *in vivo* studies, where the HW and alcoholic extract of *T. officinalis* showed no hypoglycaemic activity in male Swiss mice²⁹⁷. This was also observed by Swanston-Flatt *et al.*²⁹⁸.

Although Diabetea did not show any significant antidiabetic activity with regards to the enzyme activity, its DCM extract significantly ($p < 0.05$) mediated the absorption of 2-NBDG into C2C12 myotubes (Figure 55). This is the first time this tea has been investigated for its hypoglycaemic activity. The Diabetea as a HW extract had no direct *in vitro* antidiabetic activity as tested in the present study, which may not support its traditional use.

4.6 Chapter summary

The DCM extract of *U. urens* significantly ($p < 0.05$) inhibited α -amylase activity in an uncompetitive manner, which was comparable to the percentage inhibition

exerted by the commercial drug, acarbose. Both the HW and DCM extracts of *U. urens* caused a significant ($p < 0.05$) increase in glucose uptake into C2C12 myotubes. The HW extract of *T. vulgaris* had a significant ($p < 0.05$) inhibitory activity against α -glucosidase (mixed). It also caused the uptake of glucose into C2C12 myotubes, which was significantly ($p < 0.05$) more active than insulin. *S. officinalis* (DCM extract) also inhibited α -glucosidase activity ($p < 0.05$) in a mixed manner. Its HW extract displayed potent hypoglycaemic potential by causing glucose uptake into C2C12 myotubes, which was more significant ($p < 0.05$) than the activity of the positive control, insulin. The DCM extract of *A. betulina* was active against α -glucosidase (non-competitive), which was comparable to the activity of acarbose. Its HW extract also showed a significant ($p < 0.05$) glucose uptake activity. Furthermore, the DCM extracts of *T. officinalis*, *A. millefolium*, Diabetea and HW extracts of *T. foenum-graecum* and *T. officinalis* also caused a significant ($p < 0.05$) increase in glucose uptake into C2C12 myotubes.

CHAPTER 5: CONCLUSION

The aim of this study was to assess the *in vitro* antidiabetic efficacy of the crude DCM and HW extracts of Diabetea and its individual constituents, in terms of alleviating oxidative stress, preventing post-prandial hyperglycaemia and acting as insulin mimetics.

None of the DCM extracts were active antioxidant preparations, whereas the HW extracts exerted antioxidant activity, which correlated with its polyphenolic content. The most potent antioxidant activities were observed with *T. vulgaris*, *S. officinalis* and *U. urens*, which have previously been attributed to bioactive polyphenolics such as thymol²⁰¹, salvigenin²¹⁶ and patuletin²³⁰ (amongst others), respectively. The antioxidant activity exerted by these plants may have been by means of either inhibiting the action of *p*-chloranil (in the cell-based system) or by attenuating ROS by means of reducing, scavenging, complexing or a quenching activity. All DCM extracts caused an increase in intracellular ROS levels. The reason for this could not be ascertained in the present study. Furthermore, this was also observed with most of the HW extracts, which may be linked to flavonoids with specific structural modifications on its B-ring.

None of the extracts tested in the present study were toxic to muscle cells, endothelial cells and human lymphocytes at the concentrations evaluated for its antidiabetic efficacy (1 – 20 µg/ml), suggesting that these extracts may present with favourable safety profiles. However, all DCM extracts caused a significant

overproduction of cellular ROS, as did the HW extracts of *A. betulina*, *T. officinalis*, *T. foenum-graecum* and Diabetea, which may produce adverse effects *in vivo*.

U. urens (DCM) was the only potent inhibitor of α -amylase activity. This plant exerted an activity that was comparable with acarbose and showed an uncompetitive inhibitory activity. This implies that the best time to consume this type of inhibitor is shortly after a meal to prevent hyperglycaemia. This type of inhibition was also observed with the DCM extract of *T. vulgaris* against α -glucosidase activity. All HW plant extracts, except for *T. foenum-graecum*, caused a prominent activation of α -amylase activity, which could be attributed to the hydrophilic catalytic nature of α -amylase as it interacted with the HW extracts.

The extracts of *T. vulgaris* (HW), *S. officinalis* (DCM) and *A. betulina* (HW) were potent inhibitors of α -glucosidase activity. This activity of *T. vulgaris* is supported in literature^{290,292} and shown to be mediated by luteolin²⁹². All three of these extracts showed a mixed inhibition of α -glucosidase. This implies that the inhibitor bound to an allosteric site (other than its active cleft), causing a decrease in the enzyme's affinity for the substrate, indicating that this type of inhibitor could be taken before or after a meal. Furthermore, the DCM extract of *A. betulina* inhibited α -glucosidase in a non-competitive manner. The extent of this activity was comparable to that of acarbose. Since the action of non-competitive and mixed inhibitors cannot be overcome with an increase in substrate concentration, it can be administered either before or after a meal to be effective in preventing hyperglycaemia.

All plants as well as Diabetea caused an increase in glucose uptake into muscle cells. The mechanism of this activity is hypothesized to be an insulin mimetic action, which instigates the translocation glucose transporters to the cell membrane, causing a glucose influx. This activity by *T. vulgaris* (DCM) and *S. officinalis* (HW) was similar to that observed with commercial insulin. The hypoglycaemic activity of *T. vulgaris*, *A. betulina* (HW) and *A. millefolium* (DCM) is supported by previous studies^{286,288,300}. However, no literature could be found for the activity of *T. officinalis* (DCM), *U. urens* (HW and DCM) and *S. officinalis* (HW), making this the first study to assess this specific hypoglycaemic activity of these extracts.

In conclusion, the present *in vitro* study does not support the traditional preparation of Diabetea as an antidiabetic mixture. The most potent *in vitro* antidiabetic preparations were that of *T. vulgaris*, *S. officinalis*, and *U. urens* due to their multifaceted activity, whereby they were active in all three antidiabetic categories assessed. These plants warrant further investigation into their antidiabetic activity.

CHAPTER 6: SUMMARY

DM is an ever-increasing global health threat, estimated to affect 382 million individuals. The most common type of DM is T2DM, affecting 85-95% of DM patients. T2DM is a non-insulin dependent DM that generally affects adults. Its onset has been associated with obesity, a sedentary life style and a poor diet. There is no cure for T2DM, although there are a wide variety of conventional treatments currently available, which are associated with side effects and unwanted regimen requirements. The hallmark of T2DM is chronic hyperglycaemia, which is directly associated secondary pathologies. The onset and progression of T2DM has been linked to chronic hyperglycaemia-induced oxidative stress. Therefore, the main approach in the treatment of T2DM is alleviation of chronic post-prandial hyperglycaemia. The inhibition of enzymes, involved in carbohydrate digestion and absorption, such as α -amylase and α -glucosidase, have been shown to mediate a hypoglycaemic effect. Herbal remedies have been documented and proven to possess this activity as well as antioxidant capacity exerted by their polyphenolic compounds. Herbal remedies are widely used without scientific validation of its efficacy or safety.

A traditional tea mixture, Diabetea, is commercially available in South Africa for the treatment of diabetes without pharmacological validation of its efficacy or safety. It is a polyherbal mixture containing seven plants namely: *Achillea millefolium* L. (Yarrow), *Agathosma betulina* Bartl. & Wendl. (Buchu), *Salvia officinalis* L. (Sage), *Taraxacum officinalis* L. (Dandelion), *Thymus vulgaris* L. (Thyme), *Trigonella*

foenum-graecum L. (Fenugreek) and *Urtica urens* L. (Nettle), all of which have been documented to possess traditional medicinal value. The aim of this study was to assess the *in vitro* antidiabetic efficacy of the crude dichloromethane (DCM) and hot water (HW) extracts of Diabetea and its individual herbs. This study was designed to assess three major antidiabetic targets namely, alleviating oxidative stress, preventing post-prandial hyperglycaemia and hypoglycaemic activity by insulin mimetic activity.

The polyphenolic content of each extract was determined by means of Folin-Ciocalteu and aluminium trichloride methods. The antioxidant activity was assessed by means of cell-free methods using 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. The cell-based antioxidant activity of each extract was determined against *p*-chloranil-induced reactive oxygen species (ROS) in Ea.hy926 cells, using the fluorescent dye, 2',7'-dichlorofluorescein-diacetate (DCFH-DA). The toxicity of each extract was determined in C2C12 myotubes, Ea.hy926 umbilical vein endothelial cells and peripheral blood mononuclear cells (PMNC) / human lymphocytes (HL) using the sulforhodamine B (SRB) assay. The prevention of post-prandial hyperglycaemia was evaluated against α -amylase and α -glucosidase activities using 3,5-dinitrosalicylic acid (DNSA) and *p*-nitrophenyl- α -D-glucopyranoside (*p*-NPG), respectively. The type of inhibition exerted on these enzymes was determined using the Michaelis-Menten enzyme kinetics model and was expressed in terms of mixed, competitive, non-competitive or uncompetitive. The hypoglycaemic activity of each extract and Diabetea was determined by means of their glucose uptake activity into C2C12

myotubes using the 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-*D*-glucose (2-NBDG) fluorescent glucose analogue.

The HW extracts were rich in polyphenolic content, whereas the DCM extracts were poor polyphenolic containing preparations. The HW extract of *T. vulgaris* had the highest quantity of polyphenolics and most significant ($p < 0.05$) cell-free antioxidant activity. Flavonoids were more active against cell-free radicals than phenolics. Extracts were more active against ABTS^{•+} than DPPH[•]. The most potent (>50% scavenging activity) cell-free antioxidant activity was observed with the HW extracts of *T. vulgaris* and *S. officinalis*. It was found that the HW extracts of *T. vulgaris*, *S. officinalis* and *U. urens* possessed significant cell-based ($p < 0.05$) antioxidant activity. All DCM extracts were associated with an overproduction of ROS. This was also seen with the HW extracts of *T. foenum-graecum*, *T. officinalis*, *A. betulina* and Diabetea. A significant ($p < 0.05$) correlation was found to exist between the antioxidant activity of the HW extracts and their polyphenolic content. The ABTS^{•+} and DPPH[•] assays yielded similar results. There was also a relationship between cell-based and cell-free antioxidant activity with the HW extracts.

No toxic effect was exerted by the extracts on myotubes, endothelial cells or human lymphocytes at concentrations of 1 to 20 µg/ml. Toxic effects were only observed at a concentration as high as 100 µg/ml for the extracts of *A. betulina*, *T. foenum-graecum*, *U. urens*, *T. vulgaris*, *A. millefolium* and Diabetea. Furthermore, the toxicity of each extract was concentration-dependent. There was also a prominent cell growth observed in C2C12 cells as well as protein stimulation in human lymphocytes.

The DCM extract of *U. urens* was the only preparation to significantly ($p < 0.05$) inhibit the activity of α -amylase, resembling the activity of an uncompetitive inhibitor. All HW extracts, except for *T. foenum-graecum* caused a significant stimulation of α -amylase activity. The HW extract of *T. vulgaris* had a significant ($p < 0.05$) inhibitory activity against α -glucosidase (mixed). *S. officinalis* (DCM) also inhibited α -glucosidase activity ($p < 0.05$). The DCM extract of *A. betulina* was active as an inhibitor of α -glucosidase (non-competitive), which was comparable to the activity of acarbose.

Both the HW and DCM extracts of *U. urens* caused a significant ($p < 0.05$) increase in glucose uptake into C2C12 myotubes. The HW extract of *T. vulgaris* caused the uptake of glucose into C2C12 myotubes, which was significantly ($p < 0.05$) more active than insulin. The HW extract of *S. officinalis* displayed potent hypoglycaemic potential by causing glucose uptake into C2C12 myotubes, which was more significant ($p < 0.05$) than the activity of the positive control, insulin. The HW extract of *A. betulina* also showed a significant ($p < 0.05$) glucose uptake activity. Furthermore, the DCM extracts of *T. officinalis*, *A. millefolium*, Diabetea and HW extracts of *T. foenum-graecum* and *T. officinalis* also caused a significant ($p < 0.05$) increase in glucose uptake into C2C12 myotubes. The antidiabetic results are summarized in Figure 56.

HW extracts have a more polar and electronegative makeup than DCM extracts, therefore more polyphenolics (polar compounds) were extracted with HW than with DCM. Various flavonoids and phenolics have been isolated from *T. vulgaris* and have been proven to possess potent antioxidant activity, which is the most probable

reason for its observed antioxidant activity. The polyhydroxylated structure of flavonoids exert a higher reducing potential than phenolics, making them more active against free radicals. The extracts were more active against ABTS^{•+} than DPPH[•], which is ascribed to differences in reaction rate, assay sensitivity and antioxidant selectivity. The antioxidant activity of *T. vulgaris*, *S. officinalis* and *U. urens* has been linked to the bioactivity of thymol, salvigenin and patuletin, amongst others. The antioxidant activity of the HW extracts was attributed to their high polyphenolic content. If an extract exerted activity against ABTS^{•+} it was likely to also have activity against DPPH[•] due to a significant correlation found between these assays. The antioxidant activity exerted by these plants may have been by means of either inhibiting the action of *p*-chloranil (in the cell-based system) or by attenuating ROS through reducing, scavenging, complexing or quenching its activity. The exact reason for the overproduction of ROS exhibited by the DCM extracts could not be ascertained in the present study. However the overproduction of ROS mediated by the HW extracts could be attributed to the presence of flavonoids with specific modifications to their B-ring.

No toxic effect was observed with all extracts between 1 to 20 µg/ml (in reaction) for C2CC12 myotubes, Ea.hy926 umbilical vein endothelial cells and human lymphocytes (resting and stimulated). This is a physiologically viable concentration range and therefore shows the possible safety of these extracts. The exact cause of growth stimulation of myotubes and protein synthesis in human lymphocytes observed in the present study was not assessed.

U. urens was the only plant to significantly ($p < 0.05$) inhibit the activity of α -amylase. This was mediated by uncompetitive inhibition, which is only active in the presence of an enzyme-substrate complex, implying that this inhibitor is best active taken shortly after a meal. The significant ($p < 0.05$) stimulation of α -amylase activity mediated through the HW extracts could be explained by the nucleophilic substitution activity and hydrophilicity of α -amylase, which could have a stronger interaction with the HW extracts, stimulating its activity by perceived increase in substrate concentration. The inhibitory (mixed) activity of *T. vulgaris* against α -glucosidase has been shown to be mediated by the flavonoid luteolin. A mixed inhibitor binds to an allosteric site of the enzyme, causing a decrease in substrate-to-enzyme affinity, which was also observed with *S. officinalis*. The activity of *A. betulina* against α -glucosidase was non-competitive, and, since the action of non-competitive and mixed inhibitors cannot be overcome with an increase in substrate concentration, it can be administered either before or shortly after a meal to be effective in preventing hyperglycaemia.

The mechanism of glucose uptake activity of all plants is hypothesized to be an insulin mimetic action, leading to the translocation of glucose transporters to the cell membrane, causing a glucose influx. The hypoglycaemic activity of *T. vulgaris*, *A. betulina* (HW) and *A. millefolium* (DCM) is reported previously, however, no literature could be found for the activity of *T. officinalis* (DCM), *U. urens* (HW and DCM) and *S. officinalis* (HW), making this the first study to assess this specific hypoglycaemic activity of these extracts.

In conclusion, the HW extract of *Diabetea* showed insignificant antioxidant activity, prevention of post-prandial hyperglycaemia and hypoglycaemic activity. It also caused an overproduction of ROS in Ea.hy926 cells, raising concerns about its safety. Therefore, the findings of the present study do not support *Diabetea* for use as an antidiabetic preparation, pending *in vivo* safety studies. *T. vulgaris*, *S. officinalis* and *U. urens*, were the only plants that had significant activity in all three antidiabetic targets indicating that they possess multi-faceted antidiabetic activities *in vitro*. The significance of these findings warrants further investigation.

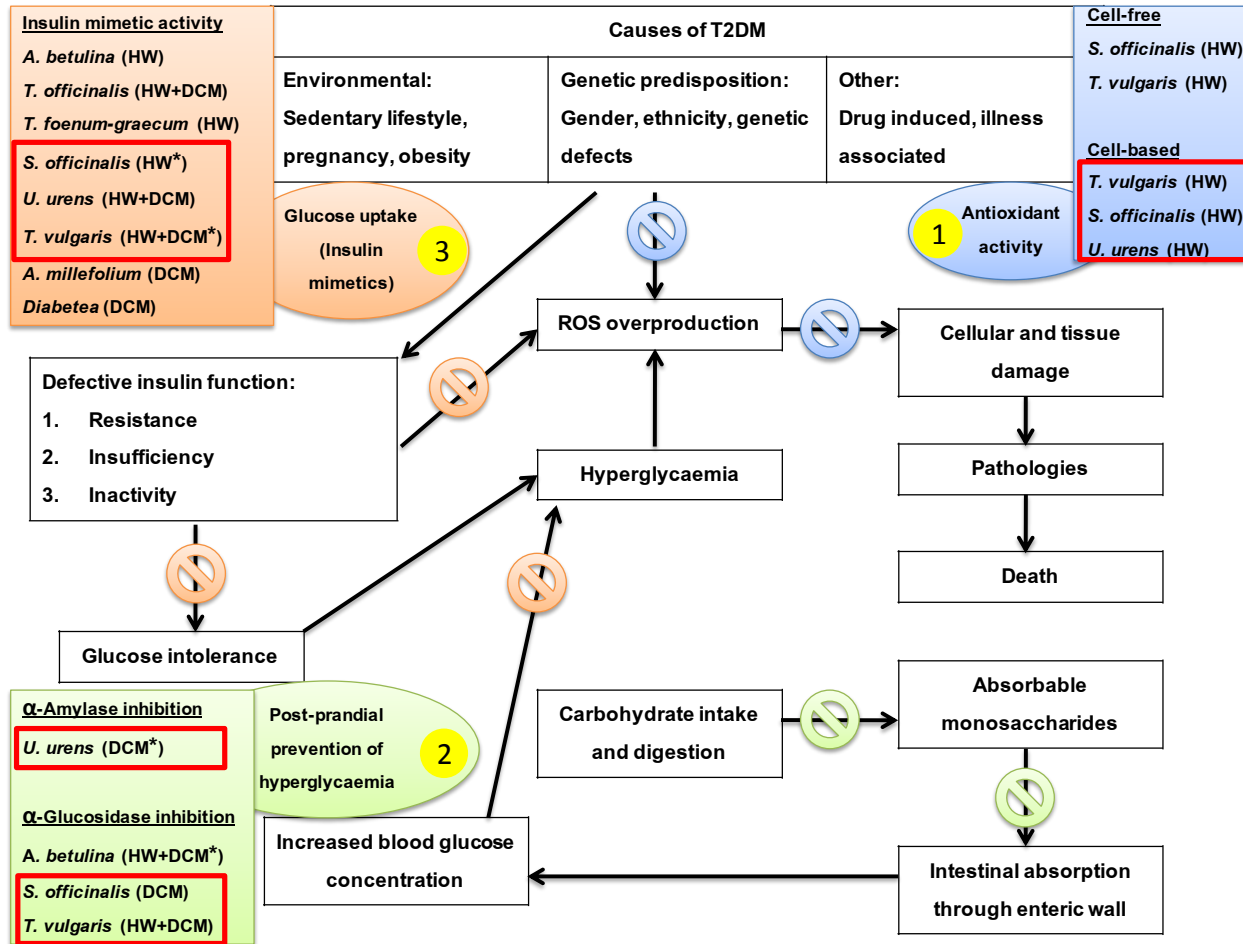


Figure 56: Graphic summary of the significant ($p < 0.05$) results observed of all hot water (HW) and dichloromethane (DCM) plant extracts tested. The three major antidiabetic *in vitro* targets evaluated in the study are indicated by the blue, orange and green boxes. Results, which are comparable to the positive controls are indicated by *. The red blocks indicate plants that have activity in all three antidiabetic categories evaluated.

LIMITATIONS OF THIS STUDY

- Claims of the antidiabetic efficacy in the present study are only pertaining to *in vitro* systems, which does not take into account *in vivo* absorption, distribution, metabolism and excretion of extracts.
- The quantity of each extract comprising the Diabetea mixture is unknown and highly subjective; after a telephonic conversation with the supplier of this tea it was evident that the herbs were added together as led by “the spirits of the plants” and therefore no comment on possible synergistic activity could be made.
- The South African Natural Products (Pty) Ltd obtain their herbs from other countries (such as Germany) and is not grown indigenously as with the ingredients that are used for the tea mixture; this may have an effect on the degree of activity exerted by each plant.
- Batch differences, quality control and storage effects.

FUTURE WORK

- Test combinations of extracts for synergistic activity and to target multifaceted antidiabetic activity in combination preparation.
- Compound isolation to elucidate bioactive compounds as well as test previously known isolated compounds of plants.
- Antiglycation assay to assess the potential of preventing further T2DM complications.
- Additional antidiabetic *in vitro* tests to gain more information on the antidiabetic profile of a plant.
- *In vivo* antidiabetic analysis on diabetic mice/rats.
- Toxicity on hepatocytes due to the integral function of hepatocytes in the detoxification process.
- Hypoglycaemic activity on adipocytes, which are also insulin-dependent for GLUT-4 transport of glucose.

APPENDIX I: REAGENTS AND INSTRUMENTATION

List of reagents

Reagent	Supplier
2-NBDG	Invitrogen molecular probes, USA
ABTS	Sigma-Aldrich, St. Louis, USA
Acarbose	Sigma-Aldrich, St. Louis, USA
AcOH	Merck Chemicals (Darmstadt, Germany)
α -Glucosidase from <i>Bacillus stearothermophilus</i>	Sigma-Aldrich, St. Louis, USA
AlCl ₃	Sigma-Aldrich, St. Louis, USA
C ₂ C ₁₂ myocytes (CRL-1772)	American tissue culture collection (ATCC)
DCM	Merck Chemicals, Darmstadt, Germany
DCFH-DA	Sigma-Aldrich, St. Louis, USA
dH ₂ O	ELGA, pure lab ultra, LABOTEC, RSA
DI H ₂ O	ELGA, pure lab ultra, LABOTEC, RSA
Diabetea	Sing-fefur organic herbs, RSA
DMEM	Sigma-Aldrich, St. Louis, USA
DMSO	Lab-Scan, Analytical Sciences, Poland
DNSA	FLUKA, India
DPPH	Sigma-Aldrich, St. Louis, USA
Ea.hy926	Obtained from Dr CJ Edgell, the originator of the cell line, University of North Carolina, USA
EDTA	Sigma-Aldrich, St. Louis, USA

EtOH	Merck Chemicals, Darmstadt, Germany
FCr	Sigma-Aldrich, St. Louis, USA
FCS	The Scientific Group, Gauteng, RSA
Gallic acid	Sigma-Aldrich, St. Louis, USA
Glycine	Sigma-Aldrich, St. Louis, USA
HBSS	Biochrom, Berlin
Heparin	Sigma-Aldrich, St. Louis, USA
Histopaque 1077	Sigma-Aldrich, St. Louis, USA
Insulin	Sigma-Aldrich, St. Louis, USA
$K_2C_4H_4O_6$	Sigma-Aldrich, St. Louis, USA
$K_2S_2O_8$	Sigma-Aldrich, St. Louis, USA
MeOH	Sigma-Aldrich, St. Louis, USA
Metformin	Sigma-Aldrich, St. Louis, USA
Na_2CO_3	Sigma-Aldrich, St. Louis, USA
NaCl	Sigma-Aldrich, St. Louis, USA
$NaHCO_3$	Sigma-Aldrich, St. Louis, USA
$NaNO_3$	Sigma-Aldrich, St. Louis, USA
NaOH	Sigma-Aldrich, St. Louis, USA
NH_2Cl	Sigma-Aldrich, St. Louis, USA
NH_4Cl	Sigma-Aldrich, St. Louis, USA
p-Chloranil	Merck Chemicals, Darmstadt, Germany
Penicillin	Lonza, Basel, Switzerland
PHA	Thermo Fischer Scientific, RSA
p-NPG	Sigma-Aldrich, St. Louis, USA
Potato starch	Sigma-Aldrich, St. Louis, USA

PPA	Sigma-Aldrich, St. Louis, USA
RPMI 1640	Adcock Ingram Scientific, RSA
Rutin	Sigma-Aldrich, St. Louis, USA
SRB	Sigma-Aldrich, St. Louis, USA
TCA	Merck Chemicals, Darmstadt, Germany
TRIS	Sigma-Aldrich, St. Louis, USA
Trolox	Sigma-Aldrich, St. Louis, USA
Trypan blue	Sigma-Aldrich, St. Louis, USA
Trypsin	Highveld biological, RSA
TrypLEexpress	Gibco, RSA

List of instruments

Instrument	Manufacturer
Centrifuge (Allegra x-22)	Beckman Coulter, RSA
Electronic shaker (VRN-200)	Beckman Coulter, RSA
Fluorometer (FLUOstar Optima)	BMG Labtech, Germany
Haemocytometer	Marienfeld, Germany
Heating bath (Büchi, B-490)	BMG Labtech, Germany
Homogenizer (Yellowline A10)	IKA-Werke, Germany
Incubator	Binder, USA
Lyophilizer (Freezone 6)	Labconco, Kansas
Magnetic stirrer	Thermo Fischer Scientific, Denmark
Microscope (Olympus 1X70)	A.R. Instruments, India

Multichannel pipette (Finnpipette)	Thermo Fischer Scientific, Denmark
pH meter	Crison, Spain
Plate reader (ELX800UV)	BioTek Instruments, Germany
Rotavaporator (Büchi, R-200)	BMG Labtech, Germany

List of materials

Material	Supplier
96-well clear plates	NUNC, Thermo Fischer Scientific, Denmark
96-well polypropylene plates	NUNC, Thermo Fischer Scientific, Denmark
96-well polystyrene plates	Costar, 3789, USA
96-well white plates	NUNC, Thermo Fischer Scientific, Denmark
Parafilm	Lasec, RSA
Syringe filters	Lasec, RSA
Polypropylene tubes (5ml)	J-plast, RSA
Culture flasks	Cellstar, Greiner bio-one, USA
Sterile 96-well plates	Cellstar, Greiner bio-one, USA
Syringe filters (non-sterile)	Lasec, RSA

APPENDIX II: PERMISSION FOR USAGE OF EA.HY926 CELLS



AGREEMENT For Single User Distribution of EA.hy926 CELLS

FROM: Steve Oglesbee representing Dr. Cora-Jean S. Edgell
TO: Dr AD Cromarty of the Pharmacology Department of the University of Pretoria, Pretoria, South Africa.
DATE: 30 January 2013
RE: General Terms for the Transfer of the EA.hy926 Cell Line

In order to avoid conflicts of interest, and because of potential commercial applications for this cell line, each investigator who requests EA.hy926 cells must agree to the conditions as specified below.

Please sign, make a copy for your own records, and return the original to the Tissue Culture Facility, attention Steve Oglesbee.

The EA.hy926 cells will be used in this lab for studies regarding:

cytotoxicity and angiogenesis studies.

The University of North Carolina at Chapel Hill and the Tissue Culture Facility are not-for-profit, and the use of this cell line or its derivatives are not allowed to be used for commercial purposes without prior successful negotiation and a signed licensing agreement between the appropriate party(s) and UNC. Any such agreement will be negotiated with the UNC Office of Technology Development (OTD). Use of this cell line by for-profit institutions for basic research and NOT FOR USE IN COMMERCIAL APPLICATIONS can also be accommodated but an additional fee will be imposed. (Please inquire about this specific situation).

I, Dr AD Cromarty, understand and agree with the above restrictions for use and subsequent distribution and I will be responsible for preventing this cell line from being passed on to other investigators outside my authority.

A handwritten signature in black ink that reads "AD Cromarty".

Signature:
Research Coordinator: Pharmacology Department University of Pretoria

Date: 30 January 2013

APPENDIX III: PERMISSION FOR USAGE OF COMMERCIAL CELL LINES

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- * FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- * IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.



Universiteit van Pretoria
University of Pretoria

Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiek Komitee
DATE: 1/10/2012

NUMBER	176/2012 - Cell Line
TITLE OF THE PROTOCOL	Determination of the <i>in vitro</i> anti-diabetic potential of a commercial tea
PRINCIPAL INVESTIGATOR	Mrs V Paddy Dept: Pharmacology; University of Pretoria. Cell: 0733080396 E-Mail: veronica.paddy@gmail.com
SUB INVESTIGATOR	Not applicable
STUDY COORDINATOR	Not applicable
SUPERVISOR (ONLY when STUDENTS)	Prof V Steenkamp E-Mail: vanessa.steenkamp@up.ac.za
STUDY DEGREE	MSc
SPONSOR COMPANY	Not applicable
CONTACT DETAILS OF SPONSOR	Not applicable
SPONSORS POSTAL ADDRESS	Not applicable
MEETING DATE	26/09/2012

The **Commercially purchased cell lines** was approved on **26/09/2012** by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. The approval is valid for 5 years **period [till the end of December 2017]**, and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Members of the Research Ethics Committee:

Prof M J Bester	(female) BSc (Chemistry and Biochemistry); BSc (Hons)(Biochemistry); MSc(Biochemistry); PhD (Medical Biochemistry)
Prof R Delpoit	(female) BA et Scien. B Curatiosis (Hons) (Intensive care Nursing), M Sc (Physiology), PhD (Medicine), M Ed Computer Assisted Education
Dr NK Likibi	MBB HM – Representing Gauteng Department of Health) MPH
Dr MP Mathebula	(female) Deputy CEO: Steve Biko Academic Hospital; MBChB, PDM, HM
Prof A Nienaber	(female) BA(Hons)(Wits); LLB; LLM; LLD(UP); PhD; Dipl.Datametrics(UNISA) – Legal advisor
Mrs MC Nzeku	(female) BSc(NUL); MSc(Biochem)(UCL, UK) – Community representative
Prof L M Ntlhe	MbChB (Natal) FCS (SA)
Snr Sr J Phatoli	(female) BCur(Eet.A); BTec(Oncology Nursing Science) – Nursing representative
Dr R Reynders	MBChB (Prêt), FCPaed (CMSA) MRCPCH (Lon) Cert Med. Onc (CMSA)
Dr T Rossouw	(female) MBChB (cum laude); M.Phil (Applied Ethics) (cum laude), MPH (Biostatistics and Epidemiology (cum laude), D.Phil
Dr L Schoeman	(female) B.Pharm, BA(Hons)(Psych), PhD – Chairperson: Subcommittee for students' research
Mr Y Sikweyiya	MPH; SARETI Fellowship in Research Ethics; SARETI ERCTP; BSc(Health Promotion) Postgraduate Dip (Health Promotion) – Community representative

Dr R Sommers	(female) MBChB; MMed(Int); MPharmMed – Deputy Chairperson
Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path), PGCHE – School of Dentistry representative
Prof C V van Staden	MBChB; MMed (Psych); MD; FCPsych; FTCL; UPLM - Chairperson

DR R SOMMERS; MBChB; MMed(Int); MPharmMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

APPENDIX IV: BLOOD COLLECTION ETHICS



Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 27/08/2009

Prof V Steenkamp
Department of Pharmacology
University of Pretoria

Best Prof Vanessa Steenkamp

RE.: Application for Blood Collection utilizing lymphocytes, macrophage, neutrophils and plasma.

Herewith acknowledgement that the above Application for blood collection has been received and tabled on 26/08/2009, and found to be acceptable by the Faculty of Health Sciences Research Ethics Committee.

With regards

DR R SOMMERS; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

APPENDIX V: ENZYME KINETICS RESULTS

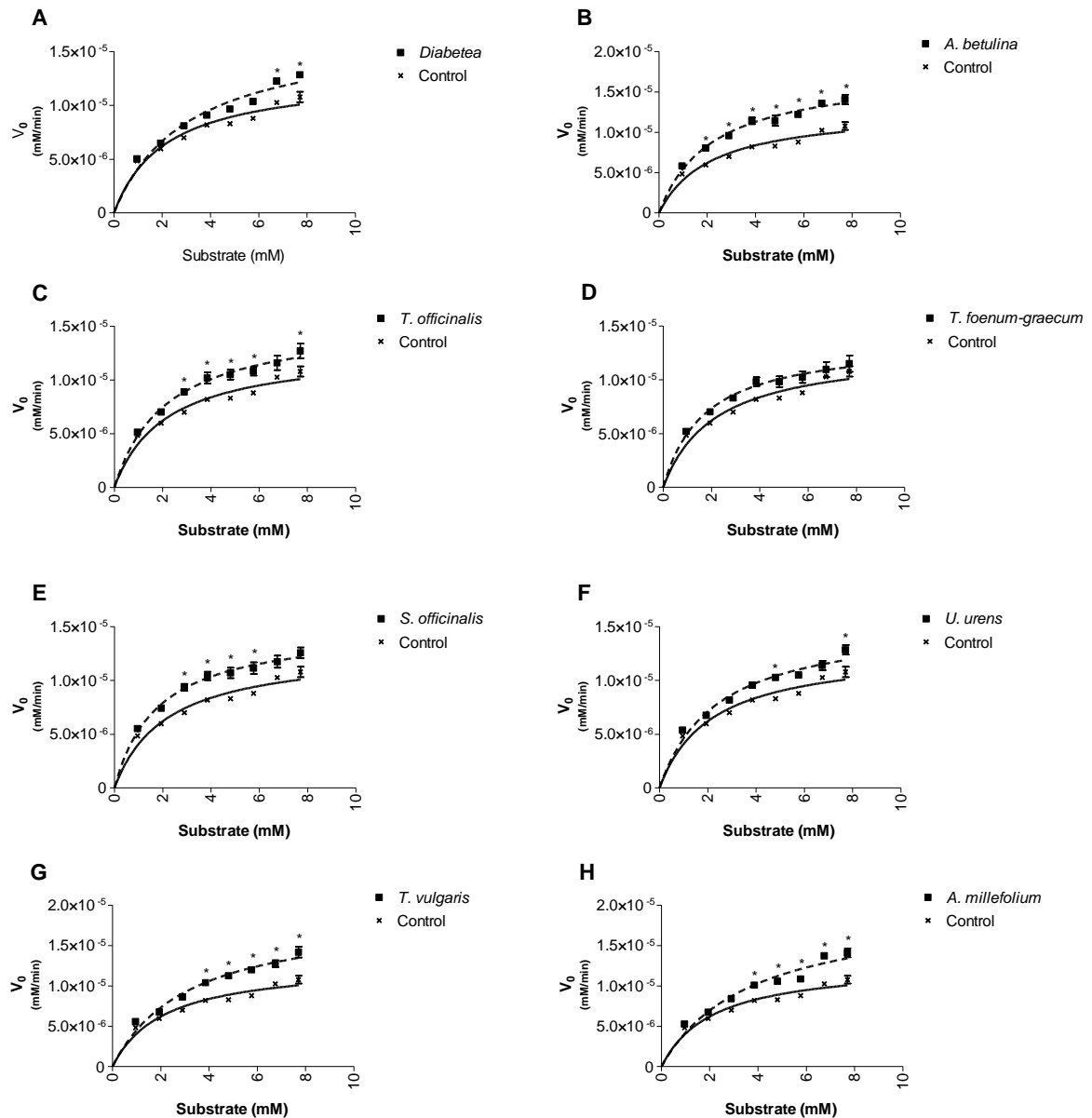


Figure 57: Michaelis-Menten graphs indicating the effect of each hot water extract on the activity of α -amylase at different substrate (starch) concentrations (mM). Significance (*) was considered at $p < 0.05$. The solid line represents the control and dashed line that of the respective extract.

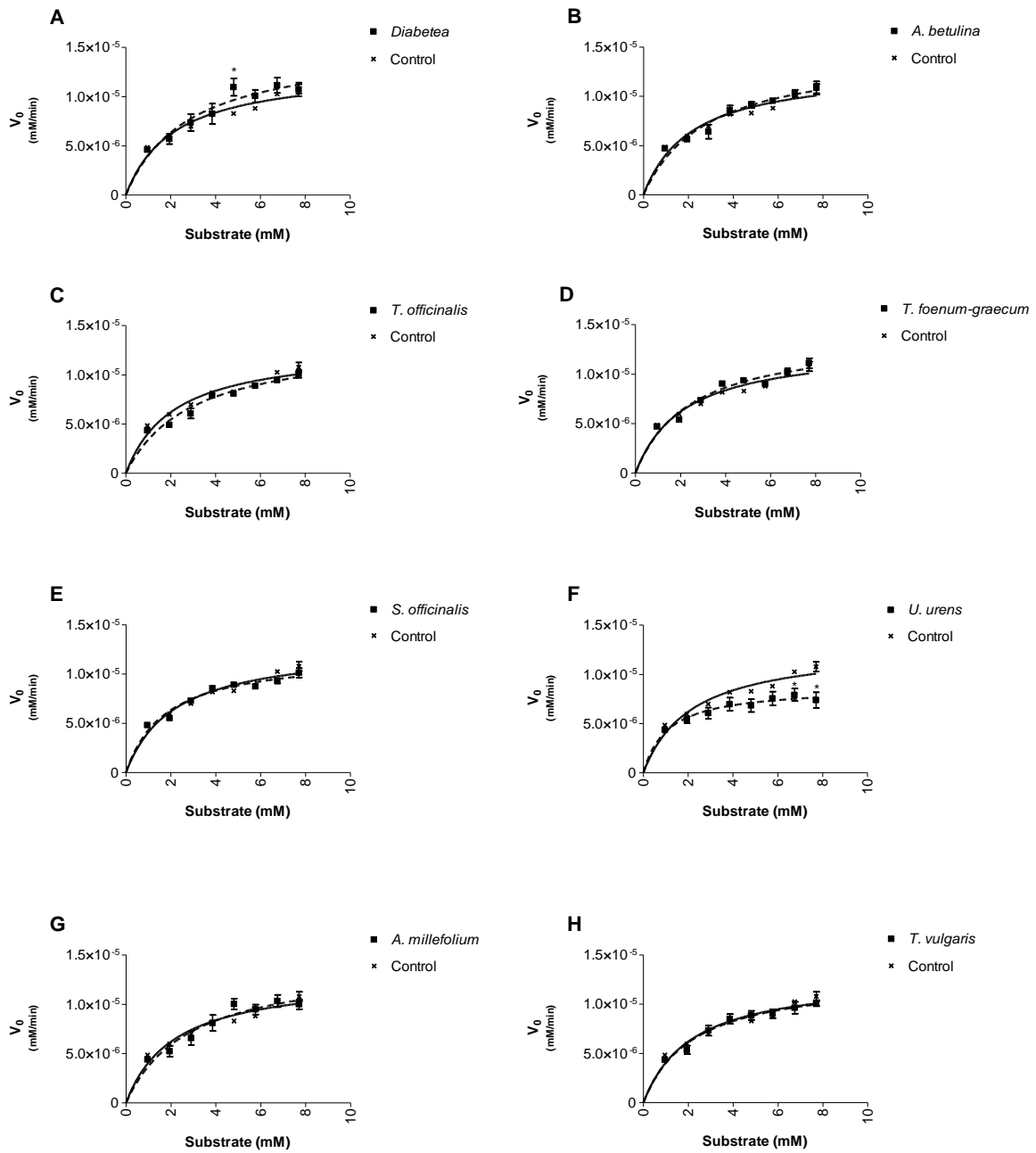


Figure 58: Michaelis-Menten graphs indicating the effect of each dichloromethane extract on the activity of α -amylase at different substrate (starch) concentrations (mM). Significance (*) was considered at $p < 0.05$. The solid line represents the control and dashed line that of the extract.

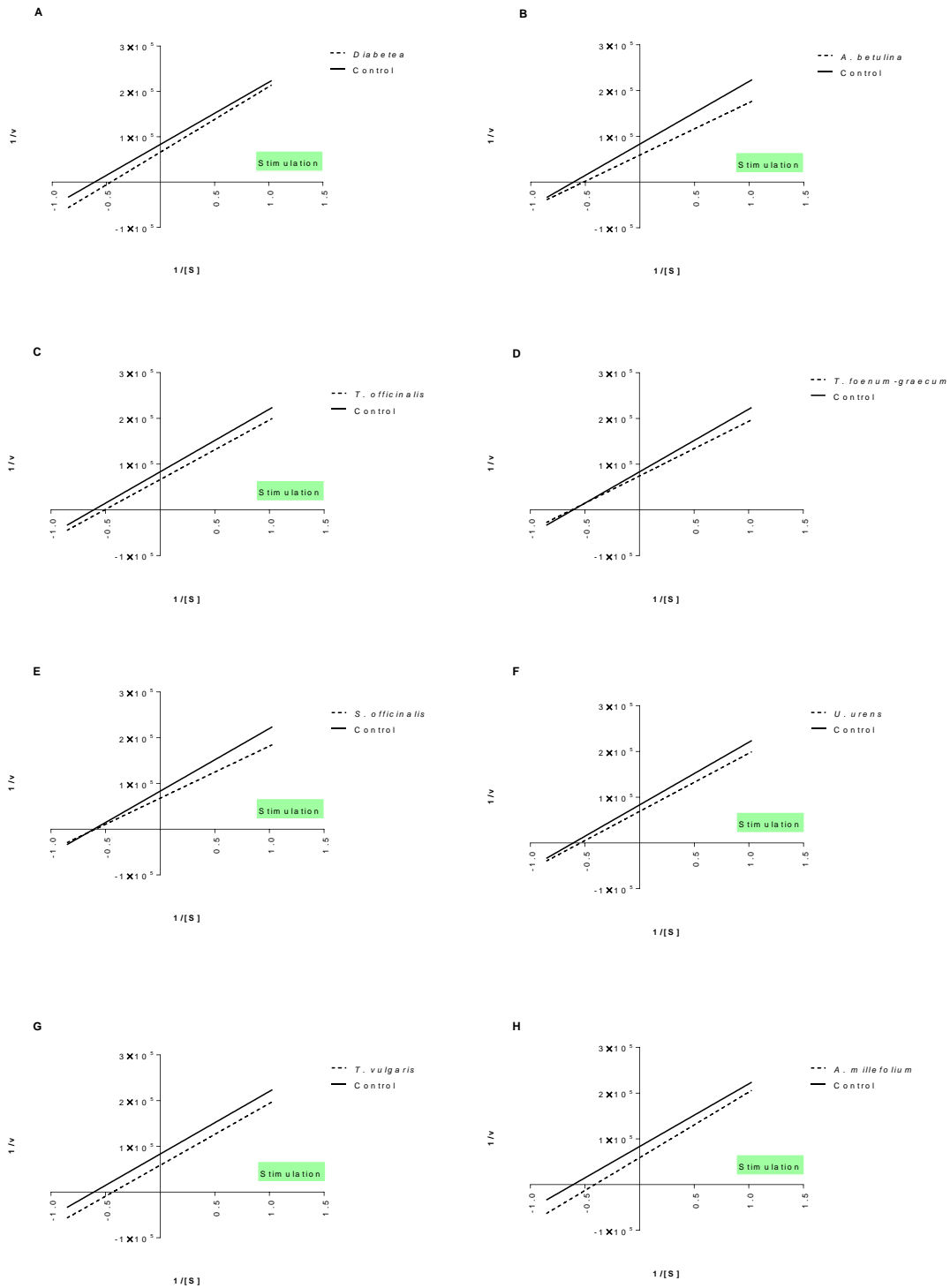


Figure 59: Lineweaver-Burk plots indicating the modes of inhibition exerted by the hot water extracts against α -amylase. The green box indicates significant ($p < 0.05$) stimulation at any one substrate concentration. V – reaction velocity, S – substrate concentration. The solid line represents the control and dashed line that of the extract.

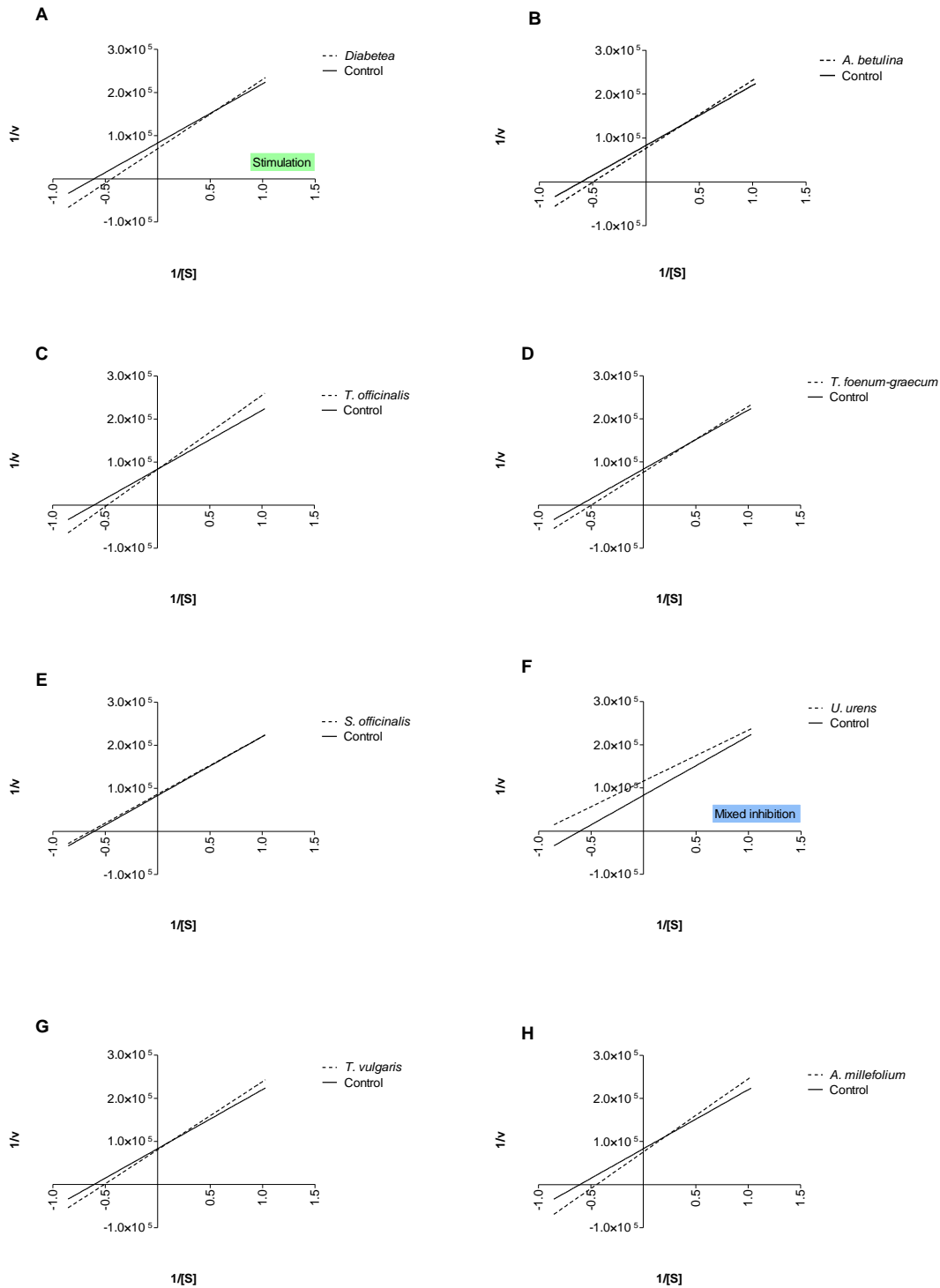


Figure 60: Lineweaver-Burk plots representing the modes of inhibition exerted by the dichloromethane extracts against α -amylase. The green and blue boxes indicate significant ($p < 0.05$) stimulation or inhibition of α -amylase activity respectively. V – reaction velocity, S – substrate concentration. The solid line represents the control and dashed line that of the extract.

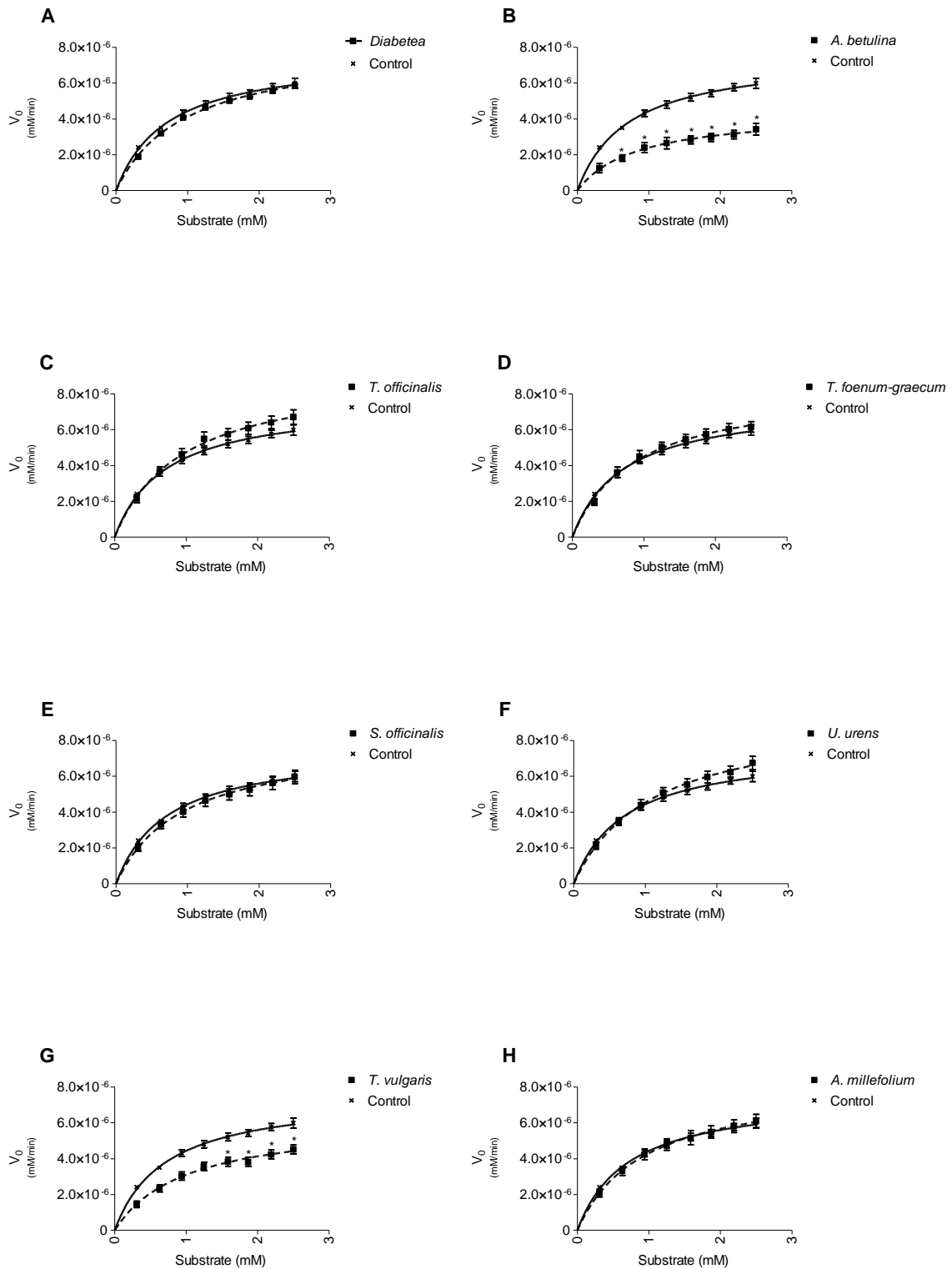


Figure 61: Michaelis-Menten graphs indicating the effect of each hot water (HW) extract on the activity of α -glucosidase at different substrate concentrations (mM). Significance (*) was considered at $p < 0.05$. The solid line represents the control and dashed line that of the extract.

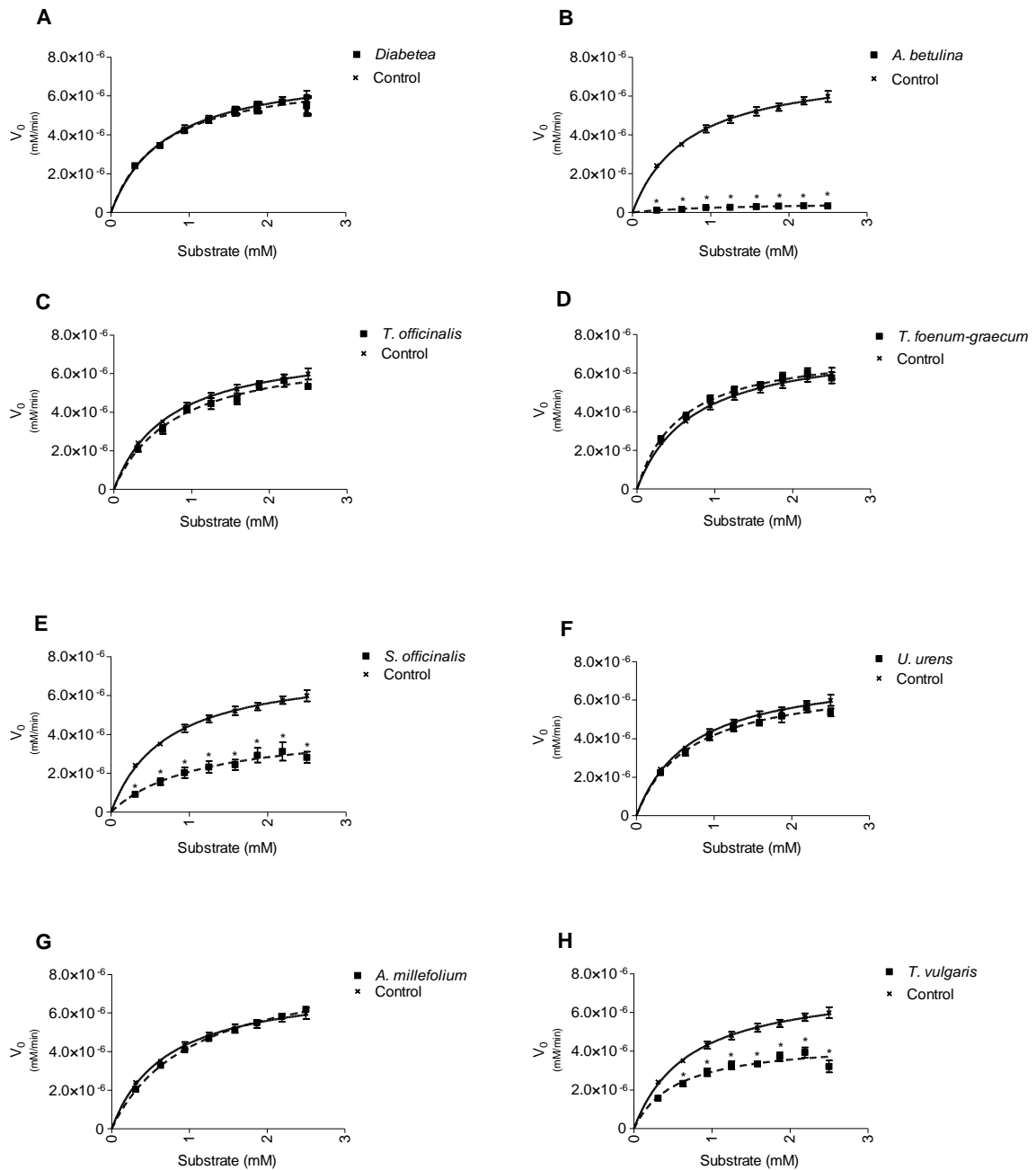


Figure 62: Michaelis-Menten graphs indicating the effect of each dichloromethane herb extract on the activity of α -glucosidase at different substrate concentrations (mM). Significance (*) is considered as $p < 0.05$. The solid line represents the control and dashed line that of the extract.

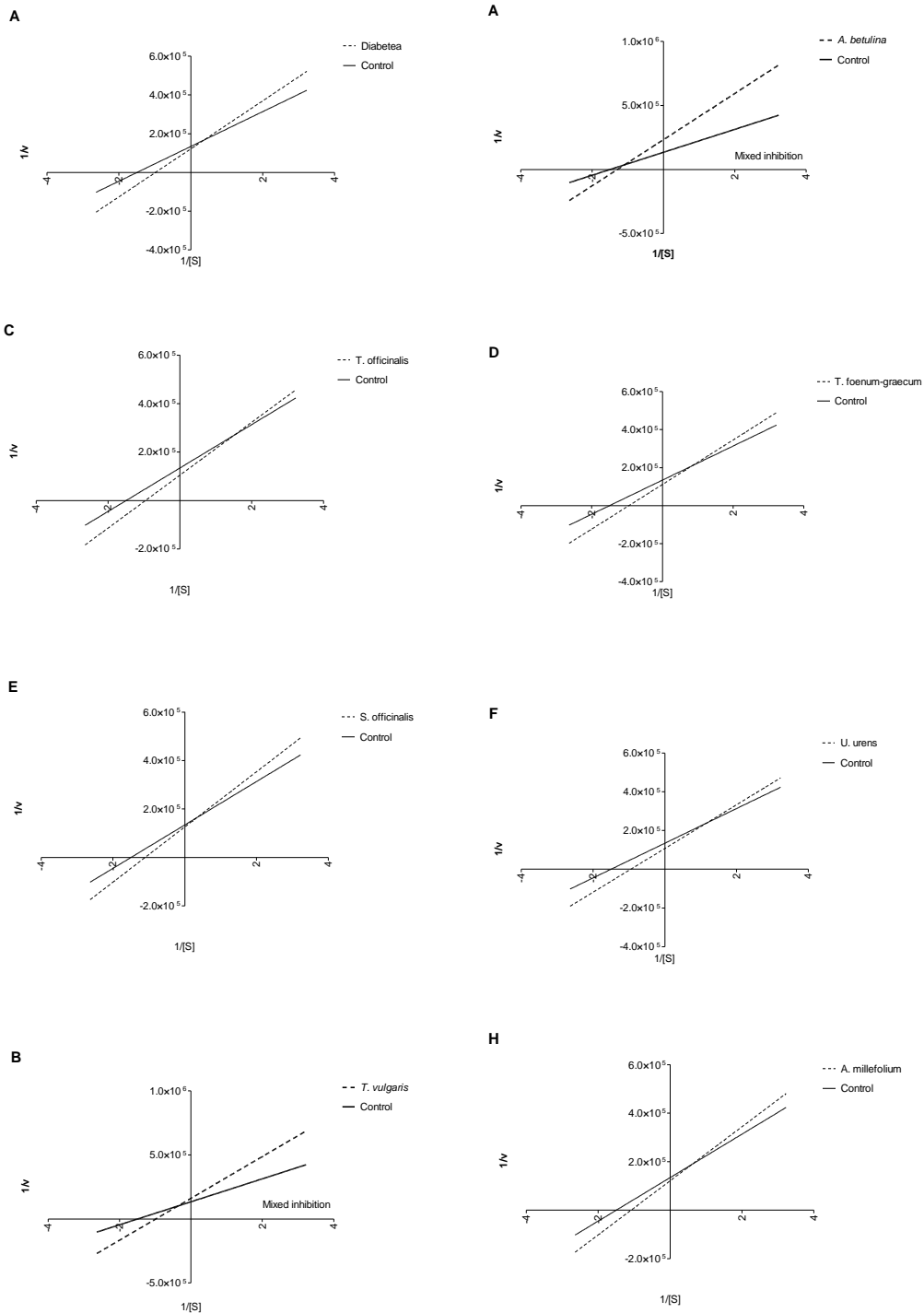


Figure 63: Lineweaver-Burk plots indicating the modes of inhibition exerted by the hot water herb extracts against glucosidase. The blue boxes indicate significant inhibition of α -glucosidase activity. V – reaction velocity, S – substrate concentration. The solid line represents the control and dashed line that of the extract.

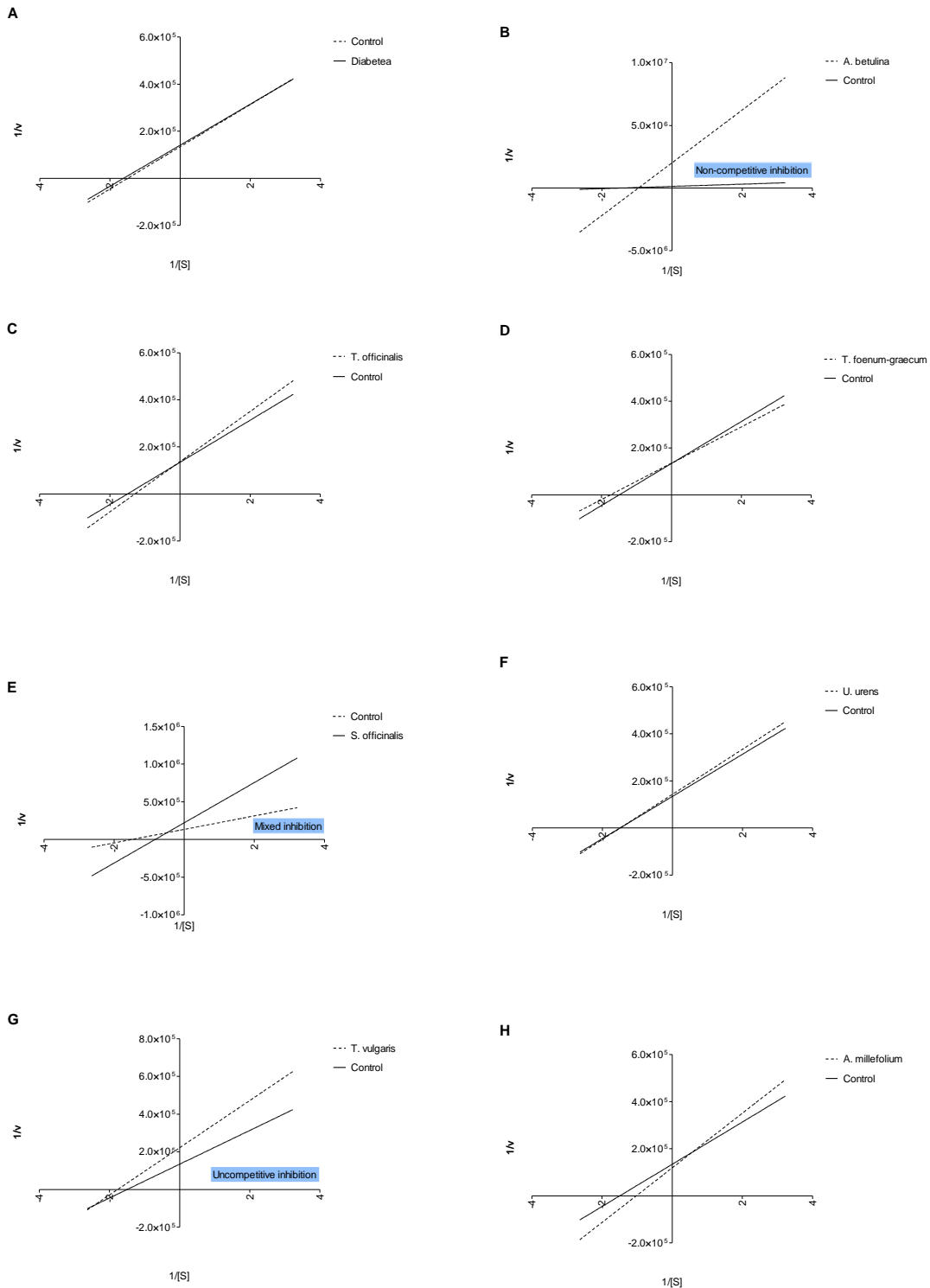


Figure 64: Lineweaver-Burk plots indicating the modes of inhibition exerted by the dichloromethane herb extracts against α -glucosidase. The blue boxes indicate significant inhibition of α -glucosidase activity. V – reaction velocity, S – substrate concentration. The solid line represents the control and dashed line that of the extract.

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