The value of extracts of *Ficus lutea* (Moraceae) in the management of Type II diabetes in a mouse obesity model

Oyinlola Oluwunmi Olaokun
(B.Sc, M.Sc Biochemistry)

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Supervisor: Prof V. Naidoo
Co-supervisors: Prof J.N. Eloff
Dr L.J. McGaw

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Declaration

I declare that the thesis hereby submitted to the University of Pretoria for the degree of doctor of philosophy has not been previously submitted by me for a degree at this or any other university, that it is my own work in design and in execution, and that all material contained herein has been duly acknowledged.

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Mrs O.O. Olaokun

Dedication

This work is dedicated to the memory of my father (Late Mr Olatunji Korede) and to a colleague and friend (Late Olukemi Ore Udom who started her PhD in the Phytomedicine Programme but passed away beforecompleting). May their souls rest in perfect peace

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Conference presentations

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Paper: O.O. Olaokun, L.J. McGaw, J.N. Eloff and V. Naidoo. Antioxidant activity, total polyphenolic content and in vitro inhibition of activity of digestive enzymes significant to diabetes by leaf extracts of ten South African *Ficus* species presented at the 38th Annual Conference of the South African Association of Botanists (SAAB), 15th – 18th January 2012, University of Pretoria, Hatfield.

Paper: O.O. Olaokun, V. Naidoo and J.N. Eloff. Alpha glucosidase inhibitory activity of five South African *Ficus* species presented at the 38th Annual conference of Physiology Society of Southern Africa, 27th - 29th September, 2010, East London, Eastern Cape.

Paper: O.O. Olaokun, V. Naidoo and J.N. Eloff. Alpha amylase inhibitory activity of five South African *Ficus* species presented at the Greengold Symposium, 14th – 16th September 2009 at The Farm Inn Conference Centre, Pretoria.

Articles prepared from this thesis for publication

O.O. Olaokun, L.J. McGaw, J.N. Eloff and V. Naidoo. Evaluation of the inhibition of carbohydrate hydrolysing enzymes, antioxidant activity and polyphenolic content of extracts of ten African *Ficus* species (Moraceae) used traditionally to treat diabetes. Manuscript submitted to BMC Complementary and Alternative Medicine.

O.O. Olaokun, L.J. McGaw, J.N. Eloff and V. Naidoo. Cytotoxicity activity and the effect of ten South African *Ficus* species on stimulation of glucose uptake and insulin release in relevant cell lines (Manuscript).

O.O. Olaokun, L.J. McGaw, J.N. Eloff and V. Naidoo.The *in vitro* antidiabetic potential of the fractions of the extract of *F. lutea* (Manuscript).

Abstract

Diabetes mellitus is a chronic disease characterised by prolonged hyperglycaemia,especially post-prandial, in association with the consumption of diets that promote obesity. While different types of the disease have been identified, Type II diabetes also known as insulin dependent diabetes is most prevalent. Treatment for patients with this disease is usually a combination of exercise, low caloric diet and specific medical intervention through the use of allopathic medicines or surgery. While the number of treatment option is large, unfortunately, treatment is usually associated with complication such as drug adverse reactions and failure to halt disease progression. As a result new therapies are required. Herbal medicines such as those derived from the *Ficus* species, which have been used traditionally in the treatment of diabetes, may serve as new source of drug therapies. The aim of this study was to evaluate the effectiveness of selected South African *Ficus* species for their potential ability to manage Type II diabetes using *in vitro* and *in vivo* screening models. Dried and ground leaves of ten *Ficus* species were extracted separately with acetone, chloroform and hexane for determination of its phytochemical constituents. Since acetone extracted more variety of compounds, the extracts was used for determination of total polyphenol content, antioxidant activity, α-amylase and α-glucosidase inhibitory activity, cytotoxicity, glucose uptake in primary cell cultures and established cell lines, and insulin release in pancreatic cell lines. The most active extract (*F. lutea*) was subjected to solvent-solvent fractionation and the six fraction subsequently evaluated by the same assays. The most active fraction (ethyl acetate) was hereafter subjected to fractionation for the isolation of bioactive compound(s) or direct evaluation in a mouse obesity model.

The acetone extract of *F. lutea* had the highest polyphenolic content (56.85 ± 1.82 mg GAE/g dry weight), the strongest antioxidant activity (4.80 ± 0.90 TEAC) and the highest α-amylase inhibitory activity with an EC_{50} value of 9.42 ± 2.01 µg/ml. Although the extract of *F. lutea* had the highest sucrase (64.31 ± 3.57%) inhibitory activity at concentration of 0.5 mg/ml, the EC_{50} of *F. sycomorus* (217 ± 69 µg/ml) was the best followed by *F. lutea* (289 ± 111 µg/ml). Based on the correlation coefficient between polyphenol and alpha amylase inhibition (0.80) and alpha glucosidase (sucrase) inhibition (0.84), and the partial non-competitive manner by which the acetone extract of *F. lutea* inhibited the α-amylase and α-glucosidase enzymes, the polyphenols appear to be in part responsible for the evident activity. All ten *Ficus* species were less toxic than doxorubicin (positive control) but contained compounds that are generally relatively more toxic to the Vero kidney cells than to the C3A liver cells. The extract of *F. craterostoma* was the least toxic to the C3A and Vero cells, while the LC_{50} for the extract of *F. lutea* extract were relatively non-toxic to the Vero cells (214.8 ± 5.0 µg/ml) and more toxic (126.0 ± 6.8 µg/ml) to the C3A cell line.

In the glucose uptake assays using primary rat abdominal muscle or epididymal fat cells, *F. lutea* acetone extracts (200 µg/ml) induced greater glucose uptake of 10.8 ± 1.8% for muscle and of 32.0 ± 8.4% for fat respectively, in comparison to the DMSO control wells.A similar response was seen with the established C2C12 muscle and H-4-II-E liver cell lines, where *F. lutea* in a dose related manner increased glucose uptake and at the highest concentration (500 µg/ml) increase glucose uptake by 14.9 ± 2.3% and 19.3 ± 0.6% respectively. In contrast no result was quantifiable in the established 3T3-L1 pre-adipocytes cell line, most likely due to a flaw in the methodology. The concurrent insulin addition, (1 and 10 µM) also potentiated the glucose utilisation in the *F. lutea* treated C2C12 and H-4-II-E cells. On addition of extracts to the RIN-m5F pancreatic β-cells, the extract of *F. lutea* stimulated a dose related increase in insulin release with insulin secretion of 120.8 ± 11.1% at the highest concentration (500 µg/ml) and concurrent dose related decrease in cell viability in comparison to the untreated control. As a result it would appear that *F. lutea* acetone extracts have a dual mechanism behind its ability to reduce glucose concentrations.

The extract of *Ficus lutea,* was further subjected to solvent-solvent fractionation in hexane, chloroform, dichloromethane, ethyl acetate, n-butanol and water due to its superior response. The ethyl acetate fraction had the highest polyphenolic content (100.5 ± 1.6 mg GEA/g dried extract) and the highest sucrase inhibitory activity (126.8 ±30.6 µg/ml), while the n-butanol fraction had the highest α-amylase inhibitory activity (26.5 ± 1.3 µg/ml). Nonetheless the inhibition of the α-amylase

enzyme activity by the various fractions was in all cases lower than that for the crude extract. In the cytotoxic assay using Vero monkey kidney and C3A liver cell line, the hexane fraction was the least toxic while the ethyl acetate fraction was relatively non-toxic, it had the lowest LD_{50} against the Vero cells (LD_{50} = 126.9 ± 1.5 µg/ml). In the glucose uptake assays, the ethyl acetate fraction stimulated the greatest glucose uptake into the C2C12 muscle and H-4-II-E liver cells in dose responsive manner, with no added benefits being achieved through the concurrent addition of insulin. The ethyl acetate fraction also enhanced insulin secretion in RIN-m5F pancreatic β-cells, albeit to a lower extent than the crude extract with dose related decrease in cell viability. With the ethyl acetate fraction being the most active fraction with moderately toxicity, further isolation was attempted. Five compounds were isolated, namely lupeol, stigmasterol, α-amyrin acetate, epicatechin and epiafzelechin, with all of the compounds except epiafzelechin previously known to possess antidiabetic activity. The ethyl-acetate fraction was also evaluated for its weight reducing potential in obese mouse model. Unfortunately no in vivo activity was discernible. In conclusion, this study is the first to report on the *in vitro* antidiabetic activity of the extract of *F. lutea*.

Table of Contents

List of Figures

List of Table

List of Abbreviations

ABTS	2, 2–Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate protein kinase
ATP	Adenosine triphosphate
AGEs	Advanced glycation endproducts
ALP	Alkaline phosphatase activity
ALT	Alanine aminotransferase
α	Alpha
α-KGDH	α-Ketoglutarate dehydrogenase
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
AUCC	Animal Use and Care Committee
β	Beta
Baso	Basophiles
BEA	Benzene: ethanol: ammonium hydroxide
BMI	Body mass index
brs	Broad singlet
brd	Broad Doublet
BSA	Bovine serum albumin
C	Carbon
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CEF	Chloroform: ethyl acetate: formic acid
CO_2	Carbon dioxide
CoA	Coenzyme A
CoASH	Coenzyme A not attached to an acyl group
COSY	Correlated Spectroscopy
DAG	Diacylglycerol
δ	Delta
dd	Doublet of Doublets
DEPT	Distortionless Enhancement by Polarisation Transfer
DHAP	Dihydroxyacetone phosphate
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethyl sulphoxide

DNA	Deoxyribonucleic acid
DNS	3, 5-Dinitrosalicylic acid
DPPH	1, 1-Diphenyl-2-picryl-hydrazyl
EGCG	Epigallocatechin gallate
ER	Endoplasmic reticulum
EMW	Ethyl acetate: methanol: water
EC_{50}	Effective concentration that will produce 50% inhibition
Eos	Eosinophil
EtOAc	Ethyl acetate
FAD	Flavin adenine dinucleotide
FADH2	Reduced flavin adenine dinucleotide
FAWE	Ethyl acetate: water: formic acid: acetic acid
FBS	Foetal bovine serum
GAD	Glutamic acid decarboxylase
GAE	Gallic acid equivalent
GLAP	Glyceraldehyde-3-phosphate
GLUT	Glucose transporter
GTP	Guanosine triphosphate
GTT	Glucose tolerance test
H^+	Hydrogen ion (proton)
HCl	Hydrogen chloride
H_2SO_4	Hydrogen sulphate
Hb	Haemoglobin
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid
Hex	n-Hexane
HLA	Human leukocyte antigen
HMBC	Heteronuclear Multiple Bond Connectivity
HSQC	Heteronuclear Single Quantum Coherence
Ht	Haematocrit
IAA	Insulin autoantibodies
ICA	Islet cell antibodies
IDDM	Insulin dependent diabetes mellitus
IDH	Isocitrate dehydrogenase
IRS	Insulin receptor substrate
i.p.	Intraperitoneally
K^+	Potassium ion
KCl	Potassium chloride

KH_2PO_4	Potassium hydrogen phosphate
KRB	Kreb-Ringer biocarbonate
KRH	HEPES buffered Kreb-Ringer
LC_{50}	Lethal concentration that will kill 50% cells
Lymph	Lymphocytes
m	Multiplet
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MDH	Malate dehydrogenase
MEM	Modified essential medium
MgSO4	Magnesium sulphate
$MgCl_2$.	Magnesium chloride
MODY	Maturity onset diabetes of the young
Mono	Monocytes
MPV	Mean platelet volume
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaCl	Sodium chloride
NAD^+	Oxidised nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
Na^+/K^+ATPase	Sodium-potassium pump
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium hydrogen carbonate
NaH_2PO_4	Sodium hydrogen phosphate
NaOH	Sodium hydroxide
Neut	Neutrophils
NFκB	Nuclear factor κB
NIDDM	Non-insulin dependent diabetes mellitus
NMR	Nuclear magnetic resonance
OAA	Oxaloacetate
OVI	Onderstepoort Veterinary Institute
PBS	Phosphate buffered saline
PDX-1	Pancreas duodenum homeobox-1
PEPCK	Phosphoenolpyruvate carboxykinase
P*i*	Inorganic phosphate
PKC	Protein Kinase C

Plt C	Platelets count
PPARγ	Peroxisome proliferators activated receptor γ
R^2	Correlation coefficient
RBC/RCC	Red blood cell
RCD	Red cell distribution
RNA	Ribonucleic acid
RAGEs	Receptors for advanced glycation endproducts
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute medium 1640
RNS	Reactive nitrogen species
s	Singlet
SEM	Standard error of mean
SGLUT	Sodium - Glucose symporter (sodium dependent glucose transporter)
SUR-1	Sulfonylurea receptor-1
t	Triplet
TCM	Traditional Chinese Medicine
TEAC	Trolox equivalent antioxidant capacity
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TNFα	Tumour necrosis factor α
UCP-2	Uncoupling protein 2
UPBRC	University of Pretoria Biomedical Research Centre
UV	Ultraviolet
WBC/WCC	White blood cell count
WHO	World Health Organisation