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

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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.

______________________________
Mrs O.O. Olaokun
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 before completing). May their souls rest in perfect peace
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Articles prepared from this thesis for publication


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 options is large, unfortunately, treatment is usually associated with complications 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 sources 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 were 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 fractions subsequently evaluated by the same assays. The most active fraction (ethyl acetate) was thereafter 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...
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<td>2, 2–Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
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<td>AMP</td>
<td>Adenosine monophosphate</td>
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<td>Analysis of variance</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>AUCC</td>
<td>Animal Use and Care Committee</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>Baso</td>
<td>Basophiles</td>
</tr>
<tr>
<td>BEA</td>
<td>Benzene: ethanol: ammonium hydroxide</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>brs</td>
<td>Broad singlet</td>
</tr>
<tr>
<td>brd</td>
<td>Broad Doublet</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Carbon</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CEF</td>
<td>Chloroform: ethyl acetate: formic acid</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CoASH</td>
<td>Coenzyme A not attached to an acyl group</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlated Spectroscopy</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>δ</td>
<td>Delta</td>
</tr>
<tr>
<td>dd</td>
<td>Doublet of Doublets</td>
</tr>
<tr>
<td>DEPT</td>
<td>Distortionless Enhancement by Polarisation Transfer</td>
</tr>
<tr>
<td>DHAP</td>
<td>Dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s minimal essential medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
</tbody>
</table>
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
EC50 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
H2SO4 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$_2$PO$_4$  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
MgSO$_4$ 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$_2$PO$_4$ Sodium hydrogen phosphate
NaOH   Sodium hydroxide
Neut   Neutrophils
NFkB   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
Pi     Inorganic phosphate
PKC    Protein Kinase C
Plt C  Platelets count
PPAR\(\gamma\)  Peroxisome proliferators activated receptor \(\gamma\)
\(R^2\)  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
TNF\(\alpha\)  Tumour necrosis factor \(\alpha\)
UCP-2  Uncoupling protein 2
UPBRC  University of Pretoria Biomedical Research Centre
UV  Ultraviolet
WBC/WCC  White blood cell count
WHO  World Health Organisation