

**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

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 before completing). May their souls rest in perfect peace

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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 μ g/ml. Although the extract of *F. lutea* had the highest sucrase ($64.31 \pm 3.57\%$) inhibitory activity at concentration of 0.5 mg/ml, the EC_{50} of *F. sycomorus* (217 ± 69 μ g/ml) was the best followed by *F. lutea* (289 ± 111 μ g/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 μ g/ml) and more toxic (126.0 ± 6.8 μ g/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 μ g/ml) induced greater glucose uptake of $10.8 \pm 1.8\%$ for muscle and of $32.0 \pm 8.4\%$ 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 μ g/ml) increase glucose uptake by $14.9 \pm 2.3\%$ and $19.3 \pm 0.6\%$ 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 μ M) 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 $120.8 \pm 11.1\%$ at the highest concentration (500 μ g/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 μ g/ml), while the n-butanol fraction had the highest α -amylase inhibitory activity (26.5 ± 1.3 μ g/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 LD₅₀ against the Vero cells (LD₅₀ = 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

DECLARATION-----	II
DEDICATION-----	III
ACKNOWLEDGEMENT -----	IV
CONFERENCE PRESENTATIONS-----	V
ARTICLES PREPARED FROM THIS THESIS FOR PUBLICATION-----	V
ABSTRACT -----	VI
TABLE OF CONTENTS -----	VIII
LIST OF FIGURES -----	XIII
LIST OF TABLE-----	XVIII
LIST OF ABBREVIATIONS -----	XIX
CHAPTER 1 -----	1
1.1 Introduction-----	1
1.2 Hypothesis -----	2
1.3 Aim -----	2
1.4 Objectives-----	2
CHAPTER 2 -----	4
2 LITERATURE REVIEW-----	4
2.1 Introduction-----	4
2.2 Glucose Metabolism-----	6
2.2.1 Digestion -----	6
2.2.2 Absorption and transport of glucose-----	8
2.2.3 Entry of Glucose into cells-----	9
2.3 Insulin -----	18
2.3.1 Structure and biochemistry -----	18
2.3.2 Mechanism of insulin secretion -----	19
2.3.3 Mechanism of insulin action-----	20
2.3.4 Physiology of normal insulin secretion -----	20

2.4	Diseases of improper glucose metabolism -----	21
2.4.1	Type I diabetes mellitus -----	21
2.4.2	Type II diabetes mellitus -----	23
2.4.3	Other forms of diabetes-----	29
2.4.4	Complications of diabetes-----	29
2.5	Management and treatment of diabetes-----	31
2.5.1	Therapy for weight loss -----	31
2.5.2	Pharmacological therapy of insulin response or release -----	34
2.6	Medicinal plants and diabetes-----	37
2.6.1	Polyphenols -----	37
2.6.2	Polyphenols and diabetes -----	38
2.6.3	Polyphenols and obesity-----	39
2.6.4	Polyphenols and antioxidant defence-----	40
2.6.5	Potential risk associated with polyphenols -----	40
2.7	Selection of plant species -----	41
2.7.1	Ethnomedical uses of <i>Ficus</i> species -----	41
2.8	Conclusion-----	42
CHAPTER 3 -----		43
3	MATERIALS AND METHODS -----	43
3.1	Materials -----	43
3.1.1	Reagents and chemicals -----	43
3.1.2	Cell lines and primary cell cultures-----	43
3.1.3	Plant material -----	44
3.1.4	The selected <i>Ficus</i> species -----	44
3.2	Methods -----	51
3.2.1	Preparation of leaf extracts-----	51
3.2.2	Phytochemical analysis of extracts -----	52
3.2.3	Total polyphenolic content-----	52

3.2.4	Trolox equivalent antioxidant capacity (TEAC)-----	52
3.2.5	α -Amylase inhibition assay -----	53
3.2.6	α - Glucosidase inhibition assay -----	54
3.2.7	Kinetics of inhibition against α - amylase and α - glucosidase activities -----	55
3.2.8	Calculation of EC ₅₀ -----	55
3.2.9	Cytotoxicity assay -----	55
3.2.10	Glucose uptake in primary cell cultures -----	56
3.2.11	Glucose uptake in established cell lines -----	58
3.2.12	Insulin secretion assay-----	60
3.2.13	Solvent-solvent fractionation and isolation of compounds-----	60
3.2.14	Isolation of compounds -----	61
3.2.15	General experimental procedures -----	61
3.2.16	<i>In vivo</i> assay -----	61
3.2.17	Statistical analyses -----	66
CHAPTER 4 -----		67
4	RESULTS-----	67
4.1	Crude extracts -----	67
4.1.1	Extraction of plants -----	67
4.1.2	Phytochemical analysis-----	68
4.1.3	Antioxidant activity -----	68
4.1.4	Total polyphenol content and antioxidant activity-----	70
4.1.5	α -Amylase inhibitory activity of extracts of <i>Ficus</i> species -----	71
4.1.6	α -Glucosidase inhibitory activity of extracts of <i>Ficus</i> species -----	73
4.1.7	The enzyme kinetics of α - amylase and α - glucosidase inhibition by extract of <i>F. lutea</i> --	75
4.1.8	Cytotoxicity of the acetone extracts of the ten <i>Ficus</i> species -----	76
4.1.9	Glucose uptake activity in primary rat abdominal muscle culture-----	77
4.1.10	Glucose uptake in primary rat epididymal fat cells -----	78
4.1.11	Glucose uptake activity in C2C12 muscle cells -----	78

4.1.12	Glucose uptake activity in H-4-11-E liver cells -----	82
4.1.13	Glucose uptake in 3T3-L1 pre-adipocytes-----	85
4.1.14	Insulin secretion in RIN-m5F pancreatic cells-----	87
4.2	Solvent-solvent fractionation of extract of <i>F. lutea</i> -----	89
4.2.1	Percentage yield of fractions-----	89
4.2.2	Antioxidant activity-----	89
4.2.3	Total polyphenol content-----	90
4.2.4	Inhibition of α -amylase activity by the fractions from the acetone extract of <i>F. lutea</i> -----	91
4.2.5	Inhibition of α -glucosidase activity by the fractions of acetone extract of <i>F. lutea</i> -----	93
4.2.6	Cytotoxicity of the fractions from acetone extract of <i>F. lutea</i> -----	94
4.2.7	Glucose uptake in C2C12 muscle cells-----	95
4.2.8	Glucose uptake activity in H-4-11-E liver cells-----	96
4.2.9	Insulin secretion in RIN-m5F pancreatic cells -----	98
4.3	Structure Elucidation of Compounds from the ethyl acetate fraction of <i>F. lutea</i> -----	100
4.3.1	Structure Elucidation of AFL1 or Lupeol (1) -----	100
4.3.2	Structure Elucidation of AFL2 or Stigmasterol (2)-----	101
4.3.3	Structure Elucidation of AFL3 or α -Amyrin Acetate (3) -----	101
4.3.4	Structure elucidation of AFL4 or Epicatechin (4) -----	102
4.3.5	Structure elucidation of AFL5 or Epiafzelechin (5)-----	103
4.3.6	Inhibition of α -glucosidase activity by the compounds -----	104
4.3.7	Glucose uptake in C2C12 muscle cells-----	104
4.3.8	Glucose uptake in H-4-11-E liver cells-----	106
4.3.9	Insulin secretion in RIN-m5F pancreatic cells -----	108
4.4	<i>In vivo</i> study -----	110
4.4.1	The effect of different diets on body weight-----	110
4.4.2	The effect of different diets on food intake -----	111
4.4.3	The effect of different diets on faecal weight -----	112
4.4.4	The effect of high calorie diet on blood glucose concentration -----	113

4.4.5	The effect of different diets on full blood count parameters -----	115
4.4.6	The effect of different diets on serum chemistry parameters-----	115
4.4.7	The effect of diets on gross pathological changes in CD1 mice. -----	115
CHAPTER 5 -----		119
5	DISCUSSION-----	119
5.1	Selection of solvent for extraction of plants -----	119
5.2	Efficacy of crude extracts of the ten <i>Ficus</i> species-----	119
5.3	Isolation of the active components from the acetone extract of <i>F. lutea</i> -----	124
5.4	The isolated compounds from the ethyl acetate fraction of <i>F. lutea</i> -----	125
5.5	<i>In vivo</i> assay -----	126
CHAPTER 6 -----		130
6	CONCLUSION -----	130
6.1	Future Work -----	131
REFERENCES -----		132
APPENDIX -----		150

List of Figures

Figure 2-1: The features of eukaryotic animal cell structure (Alberts <i>et al.</i> , 1998).	4
Figure 2-2: Illustration of the point of hydrolysis of the internal glycosidic linkages of starch.	7
Figure 2-3: Illustrations of digestion of starch in the human gut by enzymes (α -amylase and α -glucosidases) to yield monosaccharide as the final product (Guyton and Hall, 2000).....	8
Figure 2-4: Illustration of the transport of glucose across the the intestinal epithelium.	9
Figure 2-5: Illustration of the ten steps which make up glycolysis (King, 1996).	11
Figure 2-6: Illustration of the reactions which make up the citric acid cycle (King, 1996).....	13
Figure 2-7: Illustration of the mechanism of oxidative phosphorylation (Nelson and Cox, 2005).....	14
Figure 2-8: Illustration of the chemical reactions of glycogenesis and glycogenolysis.....	15
Figure 2-9: Illustration of the process of maturation of insulin from the larger precursor preproinsulin molecule via a proteolytic process (Brunton, 2011).....	19
Figure 2-10: Illustration of glucose induced regulation of insulin secretion by pancreatic β -cell.	20
Figure 2-11: Illustration of Pancreatic β -cell dysfunction Type II diabetes.....	27
Figure 2-12: Superoxide production by the mitochondrial electron transport chain.....	28
Figure 2-13: Speculated pathways by which glucose metabolism can lead to the production of reactive oxygen species (ROS) (Robertson, 2004).	30
Figure 3-1 <i>Ficus capreifolia</i> (van Noort and Rasplus, 2004).....	44
Figure 3-2 <i>Ficus cordata</i> (van Noort and Rasplus, 2004).....	45
Figure 3-3 <i>Ficus craterostoma</i> (van Noort and Rasplus, 2004).....	46
Figure 3-4 <i>Ficus glumosa</i> (van Noort and Rasplus, 2004).....	46
Figure 3-5 <i>Ficus lutea</i> (van Noort and Rasplus, 2004)	47
Figure 3-6 <i>Ficus natalensis</i> (van Noort and Rasplus, 2004)	48
Figure 3-7 <i>Ficus polita</i> (van Noort and Rasplus, 2004).....	49
Figure 3-8 <i>Ficus religiosa</i> (Warrier <i>et al.</i> , 2995)	49
Figure 3-9 <i>Ficus sycomorus</i> (van Noort and Rasplus, 2004)	50
Figure 3-10 <i>Ficus thonningii</i> (van Noort and Rasplus, 2004).....	51

Figure 4-1 Percentage extract yield (W/V) calculated as (dry extract weight/dry starting material weight) x 100. Different solvents; acetone, chloroform and hexane were used separately to extract plant material.....	67
Figure 4-2 TLC chromatograms of acetone, chloroform and hexane extracts of ten <i>Ficus</i> species, developed with BEA, CEF, EMW and FAWE (for acetone extracts only) sprayed with acidified vanillin to show compounds. Lanes from left to right are <i>F. capreifolia</i> (1), <i>F. cordata</i> (2), <i>F. craterostoma</i> (3), <i>F. glumosa</i> (4), <i>F. lutea</i> (5), <i>F. natalensis</i> (6), <i>F. polita</i> (7), <i>F. religiosa</i> (8), <i>F. sycomorus</i> (9), and <i>F. thonningii</i> (10).....	69
Figure 4-3 Antioxidant TLC chromatograms of acetone, chloroform and hexane extracts of different <i>Ficus</i> species separated by CEF, EMW and FAWE (for acetone extracts only) sprayed with 0.2% DPPH. Clear zones indicate antioxidant activity. Lanes from left to right are <i>F. capreifolia</i> (1), <i>F. cordata</i> (2), <i>F. craterostoma</i> (3), <i>F. glumosa</i> (4), <i>F. lutea</i> (5), <i>F. natalensis</i> (6), <i>F. polita</i> (7), <i>F. religiosa</i> (8), <i>F. sycomorus</i> (9), and <i>F. thonningii</i> (10).....	70
Figure 4-4 The correlation between log of percentage α -amylase inhibitory activity and log of total polyphenolic contents (mg gallic acid equivalent/g dry weight of sample) of acetone extracts from the ten South African <i>Ficus</i> species.	73
Figure 4-5 The correlation between log of percentage α -amylase inhibitory activity and the log of TEAC of acetone extracts from the ten South African <i>Ficus</i> species.	73
Figure 4-6 The correlation between log of percentage α -glucosidase inhibitory activity and log of total polyphenolic contents (mg gallic acid equivalent/g dry weight of sample) of acetone extracts from ten South African <i>Ficus</i> species.	75
Figure 4-7 The correlation between log of percentage α -glucosidase inhibitory activity and the log of TEAC of acetone extracts from the ten South African <i>Ficus</i> species.	75
Figure 4-8 Lineweaver-Burk double reciprocal plots for kinetic analysis analysis of enzyme reactions.	76
Figure 4-9 Glucose uptake in rat abdominal primary muscle culture (as percentage of untreated control cells \pm standard error of mean, n=9) exposed to the acetone extracts of the ten <i>Ficus</i> species and insulin at 1 mM glucose concentration.....	79
Figure 4-10 Glucose uptake in rat epididymal primary fat cell culture (as percentage of untreated control cells \pm standard error of mean, n=9) exposed to the acetone extracts of the ten <i>Ficus</i> species and insulin at 1 mM glucose concentration.....	80
Figure 4-11 Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells \pm standard error of mean, n=9) exposed to the acetone extracts of the ten <i>Ficus</i> species and insulin.....	81

Figure 4-12 Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells \pm standard error of mean, n=9) exposed to the acetone extract of <i>F. lutea</i> in medium containing different concentrations of insulin (represented by the different colours) in the presence of different concentrations of insulin (x-axis).....	82
Figure 4-13 Glucose uptake in H-4-11-E rat liver cells (as percentage of untreated control cells \pm standard error of mean, n=9 for) exposed to the acetone extracts of the ten <i>Ficus</i> species, metformin and insulin.	84
Figure 4-14 Glucose uptake in H-4-11-E liver cells (as percentage of untreated control cells \pm standard error of mean, n=9) exposed to the different concentrations of the acetone extract (represented by the different colours) of <i>F. lutea</i> in medium containing different concentrations of insulin (x-axis).....	85
Figure 4-15 Glucose uptake in 3T3-L1 pre-adipocytes (as percentage of untreated control cells \pm standard error of mean, n=9) exposed to the acetone extracts of the ten <i>Ficus</i> species and insulin.....	86
Figure 4-16 Insulin secreted in RIN-m5F pancreatic cell (as percentage of untreated control cells \pm standard error of mean, n=6) exposed to the acetone extract of <i>F. lutea</i> and glibenclamide (positive control) in glucose free medium.....	87
Figure 4-17 Effect of the extract of <i>F. lutea</i> on RIN-m5F pancreatic cell viability (as percentage of untreated control cells \pm standard error of mean, n=6) after stimulating insulin secretion.....	88
Figure 4-18 The correlation between percentage cell viability of RIN-m5F pancreatic β -cells and percentage insulin secretion by the acetone extract of <i>F. lutea</i>	88
Figure 4-19 Percentage yield of fractions was calculated as (dry fraction weight/dry extract weight) \times 100.....	89
Figure 4-20 TLC chromatograms of fractions from the crude acetone extract of <i>F. lutea</i> developed with BEA, CEF, EMW and FAWE sprayed with (A) acidified vanillin to show compounds and (B) 0.2% DPPH with clear zone indicating antioxidant activity. Lanes from left to right are fractions of hexane (1), chloroform (2), dichloromethane (3), ethyl acetate (4), n-butanol (5) and water (6).	90
Figure 4-21 The percentage inhibition of α -amylase and α -glucosidase (sucrase) activity by the six fractions of the crude acetone extract of <i>F. lutea</i> (0.5 mg/ml). Results are expressed as mean \pm SEM (n=9).....	92
Figure 4-22 The correlation between α -amylase inhibitory activities (%) and total polyphenolic contents (mg gallic acid equivalent/g dry weight of sample) of the fractions of the acetone extract of <i>F. lutea</i>	93

Figure 4-23 The correlation between α -glucosidase inhibitory activities (%) and total polyphenolic contents (mg gallic acid equivalent/g dry weight of sample) of the fractions of the acetone extract of <i>F. lutea</i> .	94
Figure 4-24 Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells \pm standard error of mean, n=9) exposed to the fractions of acetone extract of <i>F. lutea</i> .	95
Figure 4-25 Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells \pm standard error of mean, n=9) exposed to difference concentrations of the ethyl acetate fraction (represented by the different colours) in medium containing different concentrations of insulin (x-axis).	96
Figure 4-26 Glucose uptake in H-4-11-E rat liver cells (as percentage of untreated control cells \pm standard error of mean, n=9 for) exposed to the fractions of acetone extract of <i>F. lutea</i> .	97
Figure 4-27 Glucose uptake in H-4-11-E rat liver cells (as percentage of untreated control cells \pm standard error of mean, n=9) exposed to different concentration of the ethyl acetate fraction (different colours) in medium containing different concentrations of insulin (x-axis).	98
Figure 4-28 Insulin secreted in RIN-m5F pancreatic cells (as percentage of untreated control cells \pm standard error of mean, n=6) exposed to the ethyl acetate fraction from crude acetone extract of <i>F. lutea</i> .	99
Figure 4-29 Effect of the ethyl acetate fraction from the extract of <i>F. lutea</i> on RIN-m5F pancreatic cell viability (as percentage of untreated control cells \pm standard error of mean, n=6) after stimulating insulin secretion.	99
Figure 4-30 The correlation between percentage cell viability of RIN-m5F pancreatic β -cells and percentage insulin secretion by the ethyl acetate fraction of the extract of <i>F. lutea</i> .	100
Figure 4-31 The structure of AFL1 (lupeol) isolated from the leaves of <i>F. lutea</i> .	100
Figure 4-32 The structure of compound AFL2 (stigmasterol) isolated from the leaves of <i>F. lutea</i> .	101
Figure 4-33 The structure of compound AFL3 (α -amyrin acetate) isolated from the leaves of <i>F. lutea</i> .	102
Figure 4-34 The structure of AFL4 (epicatechin) isolated from the leaves of <i>F. lutea</i> .	103
Figure 4-35 The structure of AFL5 (epiafzelechin) isolated from the leaves of <i>F. lutea</i> .	103
Figure 4-36 Glucose uptake in C2C12 muscle cells (as percentage of untreated cells control cells \pm standard error of mean, n=9) exposed to the fractions of acetone extract of <i>F. lutea</i> .	105
Figure 4-37 Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells \pm standard error of mean, n=9) exposed to the epiafzelechin at different concentrations (represented by the different colours) in medium containing different concentrations of insulin (on the x-axis).	106

Figure 4-38 Glucose uptake in H-4-11-E rat liver cells (as percentage of untreated control cells \pm standard error of mean, n=9 for) exposed to the fractions of acetone extract of <i>F. lutea</i>	107
Figure 4-39 Glucose uptake in H-4-II-E rat liver cells (as percentage of untreated control cells \pm standard error of mean, n=9) exposed to the compound epiafzelechin in medium containing different concentrations of insulin.	107
Figure 4-40 Insulin secreted in RIN-m5F pancreatic cells (as percentage of untreated control cells \pm standard error of mean, n=6) exposed to the isolated compound (epiafzelechin).	108
Figure 4-41 Effect of epiafzelechin on RIN-m5F pancreatic cell viability (as percentage of untreated control cells \pm standard error of mean, n=6) after stimulating insulin secretion.	109
Figure 4-42 The correlation between percentage cell viability of RIN-m5F pancreatic β -cells and percentage insulin secretion by epiafzelechin.	109
Figure 4-43 The effect of high calorie and normal diet with and without treatment (the ethyl acetate fraction of <i>F. lutea</i>) on body weight of CD1 mice (mean \pm S.E.M.). The initial body weight at period 0 was when obesity state was attained by mice prior to commencement of treatment for about 7 weeks.	111
Figure 4-44 The effect of high calorie and normal diet with and without treatment (the ethyl acetate fraction of <i>F. lutea</i>) on food intake of CD1 mice ((mean \pm S.E.M.). Food intake at period 0 was when obesity state was attained by mice prior to commencement of treatment for about 7 weeks.	112
Figure 4-45 The effect of high calorie and normal diet with and without treatment (the ethyl acetate fraction of <i>F. lutea</i>) on faecal output ((mean \pm S.E.M.). Faecal output at period 0 was when obesity state was attained by mice prior to commencement of treatment for about 7 weeks.	113
Figure 4-46 The effect of high calorie diet on blood glucose concentrations of CD1 mice ((mean \pm S.E.M.). Fasting blood glucose concentrations and glucose tolerance tests (GTT) at period 0 when obesity state was attained by CD1 mice prior to commencement of treatment.	114
Figure 4-47 The effect of different diets on blood glucose concentrations of CD 1 mice ((mean \pm S.E.M.). Fasting blood glucose concentrations and glucose tolerance tests (GTT) of CD1 mice after 6 weeks of treatment.	115

List of Table

Table 3-1 Nutritional contents of food (g) to induce obesity and after inducement of obesity.....	63
Table 3-2 Weight prediction chart for the CD1 mice.....	63
Table 3-3 Chart for placement of CD1 mice into treatment group once obesity was induced.....	64
Table 3-4 Codes assigned to the treatments group and the numbers of animals.....	64
Table 4-1 Percentage yield, total polyphenol content and antioxidant activity of crude acetone extracts of leaves of ten <i>Ficus</i> species	71
Table 4-2 The percentage inhibition of α -amylase activity (1 ml/mg) and concentration leading to 50% inhibition (EC_{50}) of crude acetone extracts of the ten <i>Ficus</i> species.....	72
Table 4-3 The percentage inhibition of α - glucosidase activity (0.5 ml/mg) and concentration leading to 50% inhibition (EC_{50}) of crude acetone extracts of ten <i>Ficus</i> species	74
Table 4-4 Cytotoxicity activity of acetone extracts of the <i>Ficus</i> species (LC_{50} in $\mu\text{g/ml} \pm \text{SE}$)	77
Table 4-5 The total polyphenol content of fractions of the crude acetone extract of <i>F. lutea</i>	91
Table 4-6 The EC_{50} of α -amylase and α -glucosidase activity of the fractions of the acetone extract of <i>F. lutea</i>	92
Table 4-7 Cytotoxicity activity of fractions from extract of <i>F. lutea</i> (LC_{50} in $\mu\text{g/ml} \pm \text{SE}$)	94
Table 4-8 The EC_{50} sucrase activity of compounds from ethyl acetate fraction of <i>F. lutea</i>	104
Table 4-9 The effect of diets (with or without ethyl acetate fraction of <i>F. lutea</i>) on haematological parameters of mice	117
Table 4-10 The effect of diets (with or without ethyl acetate fraction of <i>F. lutea</i>) on serum chemistry parameters of mice	118

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
<i>brs</i>	Broad singlet
<i>brd</i>	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
<i>dd</i>	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
FADH ₂	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 ₂ SO 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
<i>m</i>	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_2PO_4	Sodium hydrogen phosphate
NaOH	Sodium hydroxide
Neut	Neutrophils
NF κ B	Nuclear factor κ B
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
<i>Pi</i>	Inorganic phosphate
PKC	Protein Kinase C

Plt C	Platelets count
PPAR γ	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
<i>t</i>	Triplet
TCM	Traditional Chinese Medicine
TEAC	Trolox equivalent antioxidant capacity
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TNF α	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