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 beforecompleting). 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 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

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 *Ficus* 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 *Ficus* 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 *In vivo* 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 *Ficus* species ---------- 71

4.1.6 α-Glucosidase inhibitory activity of extracts of *Ficus* species ---------- 73

4.1.7 The enzyme kinetics of α- amylase and α- glucosidase inhibition by extract of *F. lutea* -- 75

4.1.8 Cytotoxicity of the acetone extracts of the ten *Ficus* 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 *F. lutea* ---------- 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 *F. lutea* ----- 91

4.2.5 Inhibition of α-glucosidase activity by the fractions of acetone extract of *F. lutea* --------- 93

4.2.6 Cytotoxicity of the fractions from acetone extract of *F. lutea* ---------- 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 F. lutea ---------- 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 *In vivo* 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 *Ficus* species-- 119

5.3 Isolation of the active components from the acetone extract of *F. lutea* --------------------------- 124

5.4 The isolated compounds from the ethyl acetate fraction of *F. lutea* -------------------------------- 125

5.5 *In vivo* 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 *et al.*, 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 *Ficus capreifolia* (van Noort and Rasplus, 2004).. 44

Figure 3-2 *Ficus cordata* (van Noort and Rasplus, 2004)... 45

Figure 3-3 *Ficus craterostoma* (van Noort and Rasplus, 2004)... 46

Figure 3-4 *Ficus glumosa* (van Noort and Rasplus, 2004).. 46

Figure 3-5 *Ficus lutea* (van Noort and Rasplus, 2004) 47

Figure 3-6 *Ficus natalensis* (van Noort and Rasplus, 2004) 48

Figure 3-7 *Ficus polita* (van Noort and Rasplus, 2004)... 49

Figure 3-8 *Ficus religiosa*(Warrier *et al.*, 2995) 49

Figure 3-9 *Ficus sycomorus* (van Noort and Rasplus, 2004) 50

Figure 3-10 *Ficus thonningii* (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 *Ficus* 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 *F. capreifolia* (1), *F. cordata* (2), *F. craterostoma* (3), *F. glumosa* (4), *F. lutea* (5), *F. natalensis* (6), *F. polita* (7), *F. religiosa* (8), *F. sycomorus* (9), and *F. thonningii* (10). 69

Figure 4-3 Antioxidant TLC chromatograms of acetone, chloroform and hexane extracts of different *Ficus* 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 *F. capreifolia* (1), *F. cordata* (2), *F. craterostoma* (3), *F. glumosa* (4), *F. lutea* (5), *F. natalensis* (6), *F. polita* (7), *F. religiosa* (8), *F. sycomorus* (9), and *F. thonningii* (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 *Ficus* 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 *Ficus* 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 *Ficus* 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 *Ficus* 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 ± standard error of mean, n=9) exposed to the acetone extracts of the ten *Ficus* 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 ± standard error of mean, n=9) exposed to the acetone extracts of the ten *Ficus* species and insulin at 1 mM glucose concentration. 80

Figure 4-11 Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells± standard error of mean, n=9) exposed to the acetone extracts of the ten *Ficus* species and insulin. 81

Figure 4-12 Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells ± standard error of mean, n=9) exposed to the acetone extract of *F. lutea* 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 ± standard error of mean, n=9 for) exposed to the acetone extracts of the ten *Ficus* species, metformin and insulin. 84

Figure 4-14 Glucose uptake in H-4-11-E liver cells (as percentage of untreated control cells ± standard error of mean, n=9) exposed to the different concentrations of the acetone extract (represented by the different colours) of *F. lutea* 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 ± standard error of mean, n=9) exposed to the acetone extracts of the ten *Ficus* species and insulin...... 86

Figure 4-16 Insulin secreted in RIN-m5F pancreatic cell (as percentage of untreated control cells ± standard error of mean, n=6) exposed to the acetone extract of *F. lutea* and glibenclamide (positive control) in glucose free medium. 87

Figure 4-17 Effect of the extract of *F. lutea* on RIN-m5F pancreatic cell viability (as percentage of untreated control cells ± standard error of mean, n=6) after stimulating insulin secretion 88

Figure 4-18 The correlation between percentage cell viabilty of RIN-m5F pancreatic β-cells and percentage insulin secretion by the acetone extract of *F. lutea*. 88

Figure 4-19 Percentage yield of fractions was calculated as (dry fraction weight/dry extract weight) × 100. 89

Figure 4-20 TLC chromatograms of fractions from the crude acetone extract of *F. lutea* 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 *F. lutea* (0.5 mg/ml). Results are expressed as mean ± 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 *F. lutea.* 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 *F. lutea.* 94

Figure 4-24 Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells ± standard error of mean, n=9) exposed to the fractions of acetone extract of *F. lutea.* 95

Figure 4-25 Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells ± 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 ± standard error of mean, n=9 for) exposed to the fractions of acetone extract of *F. lutea*. 97

Figure 4-27 Glucose uptake in H-4-11-E rat liver cells (as percentage of untreated control cells ± 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 ± standard error of mean, n=6) exposed to the ethyl acetate fraction from crude acetone extract of *F. lutea.* 99

Figure 4-29 Effect of the ethyl acetate fraction from the extract of *F. lutea* on RIN-m5F pancreatic cell viability (as percentage of untreated control cells ± standard error of mean, n=6) after stimulating insulin secretion. 99

Figure 4-30 The correlation between percentage cell viabilty of RIN-m5F pancreatic β-cells and percentage insulin secretion by the ethyl acetate fraction of the extract of *F. lutea*. 100

Figure 4-31 The structure of AFL1 (lupeol) isolated from the leaves of *F. lutea*. 100

Figure 4-32 The structure of compound AFL2 (stigmasterol) isolated from the leaves of *F. lutea*. ... 101

Figure 4-33 The structure of compound AFL3 (α-amyrin acetate) isolated from the leaves of *F. lutea*. 102

Figure 4-34 The structure of AFL4 (epicatechin) isolated from the leaves of *F. lutea*. 103

Figure 4-35 The structure of AFL5 (epiafzelechin) isolated from the leaves of *F. lutea*. 103

Figure 4-36 Glucose uptake in C2C12 muscle cells (as percentage of untreated cells control cells ± standard error of mean, n=9) exposed to the fractions of acetone extract of *F. lutea*. 105

Figure 4-37 Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells ± 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 ± standard error of mean, n=9 for) exposed to the fractions of acetone extract of *F. lutea*. 107

Figure 4-39 Glucose uptake in H-4-II-E rat liver cells (as percentage of untreated control cells ± 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 ± 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± standard error of mean, n=6) after stimulating insulin secretion. 109

Figure 4-42 The correlation between percentage cell viabilty 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 *F. lutea*) on body weight of CD1 mice (mean ± 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 *F. lutea*) on food intake of CD1 mice ((mean ± 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 *F. lutea*) on faecal output ((mean ± 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 ± 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 ± 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 *Ficus* 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 *Ficus* 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 *Ficus* species 74

Table 4-4 Cytotoxicity activity of acetone extracts of the *Ficus* species (LC_{50} in µg/ml ± SE) 77

Table 4-5 The total polyphenol content of fractions of the crude acetone extract of *F. lutea*.............. 91

Table 4-6 The EC_{50} of α-amylase and α-glucosidase activity of the fractions of the acetone extract of *F. lutea* 92

Table 4-7 Cytotoxicity activity of fractions from extract of *F. lutea* (LC_{50} in µg/ml ± SE) 94

Table 4-8 The EC_{50} sucrase activity of compounds from ethyl acetate fraction of *F. lutea* 104

Table 4-9 The effect of diets (with or without ethyl acetate fraction of *F. lutea*) on haematological parameters of mice 117

Table 4-10 The effect of diets (with or without ethyl acetate fraction of *F. lutea*) 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
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

CHAPTER 1

1.1 Introduction

In many developing countries, herbal medicines are of vital importance in primary health care (Kamboj, 2000). This is supported by literature in behavioural and pharmacological sciences with animals and people using a number of different plants for the control of disease symptoms and related illnesses in their environment (Hart, 2004; Cousins and Huffman, 2002). One of such disorder is diabetes mellitus, which is a chronic disease characterised by prolonged hyperglycaemia,especially post-prandial,in association with the consumption of diets that promote obesity.

The World Health Organisation (WHO) estimated that 171 million people were diagnosed with diabetes mellitus in 2000 and predicts that the prevalence of the disease will increase to 366 million in 2030 (a staggering increase of 144% over 30 years)if no action is taken (Wild *et al.*, 2004). As a result, diabetes is one of the most rapidly growing non-communicable diseases and the fourth/fifth leading cause of death globally (Amos *et al.*, 1997). While for numerous years, diabetes was considered a disease of the affluent in first world countries, the disease is also on the increase in Africa where the number of people with diabetes is predicted to increase from 13.3 million in 2000 to 33.3 million in 2030 (an increase of 148%)(Wild *et al.*, 2004). In South Africa, the number of people suffering from diabetes is also high and estimated to have a prevalence of 3.4% in the 24 million South Africans between the ages of 20 to 79 (Rheeder, 2006). Type II diabetes is the most common form of diabetes which goes undiagnosed at the early stage because there are no physical clinical manifestation (Alberti and Zimmet, 1998). However, when hyperglycaemia is severe, it usually presents with symptoms of diabetes (e.g. excessive thirst, frequent urination and weight loss) or a complication related to diabetes (retinopathy, nephropathy) or as a chance finding in an asymptomatic individual (Albertiand Zimmet, 1998; Baynes, 2006). Although numerous hypoglycaemic therapies are available for the management of Type II diabetes they all have a number of limitations, such as adverse effects and high rates of secondary failure (Nathan *et al.*, 2006; Virally *et al.*, 2007), with the main reason for their failure being tolerance. From current evidence it appears that the latter results from a decreased sensitivity to insulin.

As an alternate to the use of drugs that mimic the insulin effect or enhances insulin release, it has been suggested that weight modulation may be a better treatment modality,as weight loss is known to improve insulin sensitivity, overall glycaemic control and decrease mortality rates (Norris *et al.*, 2004). The latter is so well accepted that most doctors will unequivocally prescribe a change in diet in addition to pharmaceutical management in their diabetic patient (Anderson *et al.*, 2003). Unfortunately diet changes only result in moderate weight loss as it is difficult to achieve long term weight loss control,as weight lost is slowly regained due to resumption of poor eating habits and/or the physiological abnormalities induced by the obesity (Norris *et al.*, 2004).

It has been proposed that weight controlling medication may be a better or adjunct means of controlling diabetes than the current pharmaceutical agents (Lee and Aronne, 2007). The polyphenols present in plants may possibly be this alternate source. As a class they are naturally occurring phytochemicals of which some, such as catechins, anthocyanins, have been shown to regulate pathways that are involved in energy metabolism, adiposity and obesity (Meydani and Hasan, 2010), in addition to exerting an antidiabetic effect (Mai *et al.*, 2007). Several species of the genus *Ficus* are used traditionally in a wide variety of ethnomedical remedies all over the world (Hansson *et al.*, 2005; Koné *et al.*, 2004). They have long been used in folk medicinein the treatment of different ailments including diabetes (Watt and Breyer-Brandwijk, 1962; Trivedi *et al.*, 1969). From historical studies, these plants are known to have several phytochemical constituents including polyphenols which are among the naturally occurring antidiabetic agents (Ramadan *et al.*, 2009; Andrade-Cetto *et al.*, 2008). The antidiabetic potential of these species though has been confirmed *in vivo* but the mechanism of activity remains speculative.

1.2 Hypothesis

Plants within the *Ficus* species possess *in vitro* antidiabetic activity and *in vivo* weight reducing activity.

1.3 Aim

To evaluate the effectiveness of selected South African*Ficus* species in the management of Type II diabetes using either *in vitro* or *in vivo* models of efficacy.

1.4 Objectives

1. To evaluate the antioxidant activity of crude plant extracts of selected *Ficus* species in relation to their inherent total polyphenolic concentration.
2. To evaluate the *in vitro* α- amylase and α- glucosidase inhibitory activity of crude plant extracts of selected *Ficus* species.
3. To evaluate the efficacy of selected crude plant extracts of selected *Ficus* species for their ability to stimulate glucose uptake in established fat (3T3-L1), muscle (C2C12) and liver (H-4-II-E) cells.
4. To evaluate the efficacy of selected crude plant extracts of selected *Ficus* species in stimulating insulin release in an established insulin producing pancreatic cell line (RIN-m5F)
5. To evaluate the efficacy of selected crude plant extracts of selected *Ficus* species for their ability to stimulate glucose uptake in primary rat muscle and fat cell cultures.
6. To further evaluate the most active extracts from the above mentioned assays in the same assays through fractionation and the isolation of active compounds.

7. To evaluate the *in vitro* cytotoxicity of crude plant extracts and selected fractions using Vero monkey kidney and C3A human liver hepatoma cell lines.
8. To evaluate the most active and non-toxic fraction from the above mentioned assays for its ability to stimulate weight loss in a model of mouse obesity.

CHAPTER 2

2 Literature review

2.1 Introduction

Every living organism is made up of membrane bound cells which are the structural and functional unit of life. Mammals are multicellular organism containing different types of cells which vary in size, shape and specialised functions, to such an extent that cells that are similar in structure and functions aggregate to form tissues while different tissues with similar functions aggregate to form organs (Nelson and Cox, 2005). Nonetheless despite their specialised functions, these different cells are all characterised by a similar biological structure, which is classified as eukaryotes. The unique characteristic of the eukaryotic cells is the presence of an organised nucleus and specialised organelles which function synergistically (**Error! Reference source not found.**):

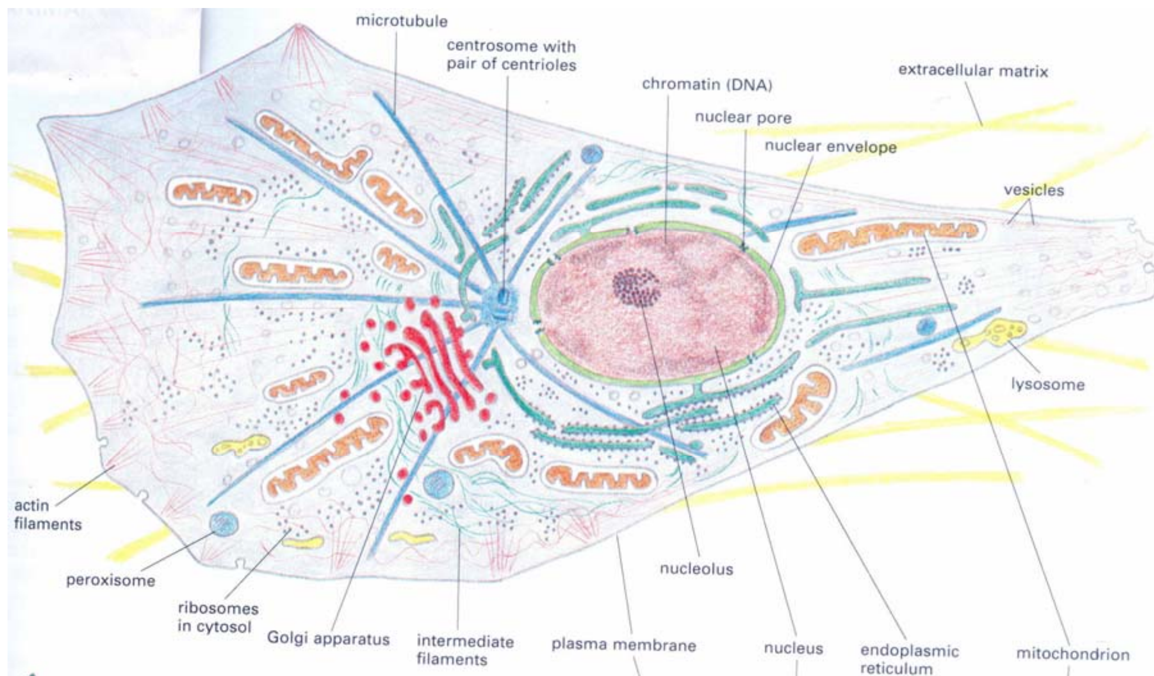

Figure 2-1:The features of eukaryotic animal cell structure (Alberts *et al.*, 1998).

- Cell membrane. – Each cell is surrounded by a phospholipid bilayer in which ion channels, receptors and other transporters are present. In addition to keeping the cytoplasm and organelles contained within the cell, the cell membrane is extremely important in controlling how substances enter via diffusion, facilitated diffusion, active transport or pinocytosis in addition to containing specific signalling proteins known as receptors.

- Cytoplasm. – The cytoplasm is enclosed by the plasma membrane and it is the content of the eukaryotic cell excluding the organelles. The cytoplasm is the site for many metabolic reactions that are fundamental to the cell's existence e.g. protein biosynthesis, glycolysis and pentose phosphate pathway (Alberts *et al.*, 1998).

- Nucleus. – The nucleus is the most prominent organelle in animal cell enclosed by double membrane called the nuclear envelope. The nucleus contains the cell's genetic material (where they are stored and replicated), extremely long linear DNA (deoxyribonucleic acid) molecules which when complex with proteins such as histones form chromosomes. The nucleus is the control centre of the cell. The nucleus contains sub-nuclear bodies such the nucleoli which are involved in assembly of ribosomes exported to the cytoplasm where they translate mRNA (messenger ribonucleic acid) into proteins (Alberts *et al.*, 1998).

- Mitochondria. –These are organelles containing their own DNA and RNA (ribonucleic acid) and a complete transcription and translation system including ribosomes enabling them to synthesize their own protein. The main function of the mitochondria is to synthesize energy in form of adenosine triphosphate (ATP) which is the main energy source of the cell (Hüttemann *et al.*, 2007).

- Endoplasmic reticulum.–Endoplasmic reticulum (ER) is an irregular maze of space (cisternal space or lumen) enclosed by a membrane. The ER is involved in the synthesis of secretory and membrane proteins;biosynthesis of phospholipids, cholesterol and steroids; the degradation of glycogen; detoxification reaction; and the regulation of intracellular calcium (Baumann and Walz, 2001).

- Golgi apparatus. – This comprises stacks of flattened membrane cisternae surrounded by small vesicles (Nebenfuhr and Staehelin, 2001). The Golgi apparatus is the site for the processing, modification and attachment of carbohydrate moleculesto proteins and lipids through the secretory pathway. It also serves as a station for sorting and transport of proteins which it receives from the ER and delivers to the plasma membrane or other intracellular sites (Altan-Bonnet at al., 2004).

- Ribosomes. – These are small particles in the cell which are composed of protein and RNA molecules called ribonucleoprotein complexes (Byrne, 2009). Ribosomes can exist as either free or membrane bound ribosomes with the free ribosomes synthesizing protein that are released in the cytoplasm where they are used while the membrane bound ribosomes are

attached to the ER (called rough ER) synthesize protein for export to other organellesor excretion (Alberts *et al.*, 1998).

- Centrosome. –Centrosomes are small structures (1-2 μm^3) on one side of the nucleus when the cell in not in mitosis. The centrosome plays an important role in organising both the interphase cytoskeleton and the bipolar mitotic spindle, ensuring correct segregation of chromosomes to prevent aneuploidy (Loffler *et al.*, 2006).

- Cytoskeleton. – The cytoskeleton is responsible for the cell's ability to organise its intracellular components including the organelles, to adopt a variety of shapes and to carry out coordinated movements such as transport of organelles from one place to another, the segregation of chromosomes into two daughter cells in mitosis, and the pinching apart of animal cells at cell division (Nelson and Cox, 2005).

- Lysosomes. – These are membranous sacs of hydrolytic enzymes that degrade proteins, phospholipids, nucleic acids and oligosaccharides (Alberts *et al.*, 1998).

- Vacuoles. – These are enclosed compartments that are filled with water which contains inorganic molecules, organic molecules and enzymes. Sometimes they may contain solids which have been engulfed (Alberts *et al.*, 1998).

While each organelle has a specific function within the cell, their functionality is dependent onthe availability of adenosine triphosphate (ATP) as an energy source. At the subcellularlevel this ATP is formed by the mitochondria from glucose. The glucose involved in ATP generation is derived from dietary sources in a process known as glucose metabolism (Tiwari and Rao, 2002).

2.2 Glucose Metabolism

2.2.1 Digestion

Digestion is the mechanical (chewing) and chemical (enzymatic action) breakdown of food into smaller components that are more easily absorbed into the blood stream (Reed and Wickham, 2009). While the digestion of all classes of food begins in the mouth with the mechanical process of mastication, for certain carbohydrate such as starches and dextrins, chemical digestion also begins in the mouth (Pedersen *et al.*, 2002). Here the food mixes with saliva containing the α-amylase enzyme called ptyalin (Pedersen *et al.*, 2002),which begins the conversion of starch and dextrins into short chain polysaccharide fragments, maltotriose (the di- and trisaccharides of α-D-(1→4) glucose), oligosaccharides called limit dextrins, fragments of amylopectin containing α-D-(1→6) branch

pointsor disaccharide maltose, all through the hydrolysis of the internal glycosidic linkages of the carbohydratemolecule (Nelson and Cox, 2005)(Figure 2-2). However, because food remains in the mouth for only a short time, only about 5% of the starches eaten get hydrolysed by the time ofswallowing and about 20% by the time of reaching the stomach (Guyton and Hall, 2000).

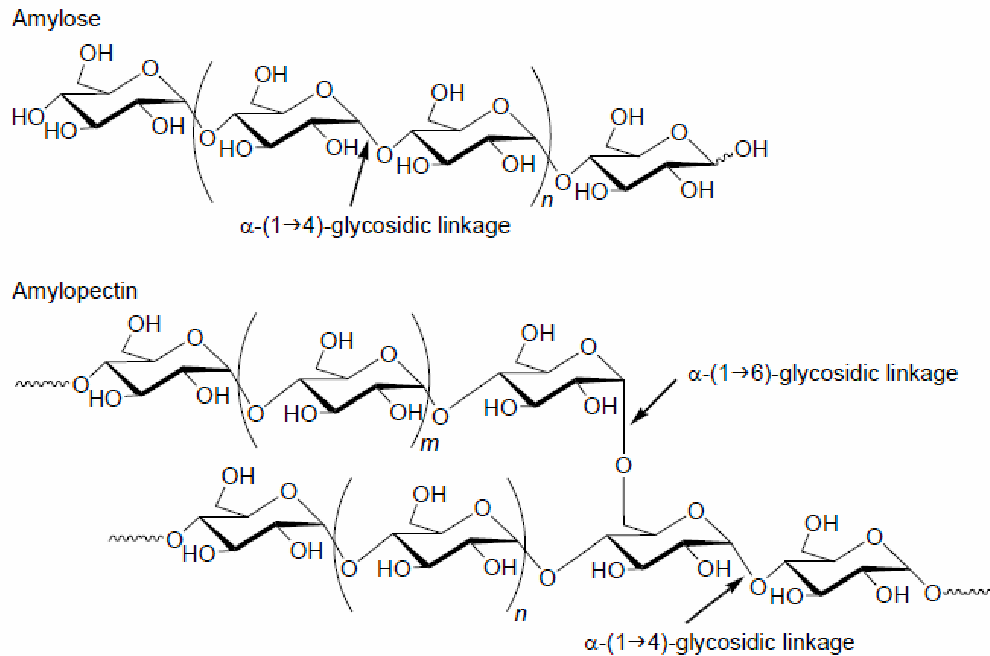

Figure 2-2:Illustration of the point of hydrolysis of the internal glycosidic linkages of starch. α-Amylase from saliva and the pancreas hydrolyse the α-D-(1→4) bond of amylose and; α-D-(1→6) and α-D-(1→4) bonds of amylopectin yielding short chain polysaccharides fragments anddisaccharides (Kadokawa, 2012).

After deglutition carbohydrate metabolism is temporarily terminated by the acidic environment of the stomach, and only reinitiates once the chyme enters the duodenum, where it is once again exposed to α-amylase enzyme this time produced by the pancreas. In comparison, the pancreatic α-amylase is more active than the salivary α-amylase with more efficient hydrolyses of the carbohydrates into maltose, sucrose and lactose (Hanhineva *et al.*, 2010).

Hereafter the formed maltose, sucrose and other disaccharides are exposed to a family of brush border enzymes collectively known as the glucosidases. These specific enzymes are specific to a disaccharideand are responsible for the splitting of disaccharides to their constituent monosaccharides e.g. lactose is metabolised by lactase into a molecule of glucose and a molecule of galactose, maltose by maltase into two molecules of glucose, sucrose by sucrase into a molecule of glucose and a molecule of fructose,and α-dextrin by α-dextrinase to several molecules of glucose (Figure 2-3). Of all the formed monosaccharides, glucose is the major product which is absorbed forfurther utilisation (Nelson and Cox 2005).

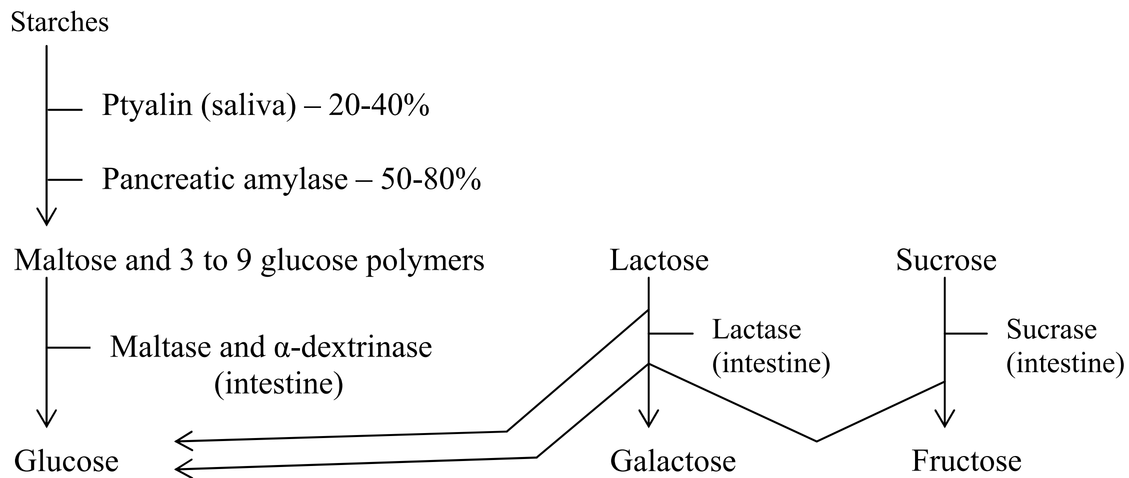

Figure 2-3:Illustrations of digestion of starch in the human gut by enzymes (α-amylase and α-glucosidases) to yield monosaccharide as the final product(Guyton and Hall, 2000).

2.2.2 Absorption and transport of glucose

The newly formed glucose is rapidly absorbed across the wall of the small intestine with absorption being complete before reaching the terminal ileum. Due to the water soluble nature of glucose, its absorption is reliant on the presence of a specific co-transport protein that is driven by the facilitated absorption of sodium (symport dependent on sodium transport) i.e. in the absence of sodium no glucose absorptionresults (Nelson and Cox, 2005). The transporter has been named the Sodium - Glucose symporter (SGLUT). This driving force for the movement of sodium into the cell is created by the active sodium-potassium pump (Na^{+}/K^{+}ATPase) which depletes the cytoplasm of sodium by moving three sodium molecules out of the cytoplasm and two potassium molecules therein in order to maintain electrical neutrality (Figure 2-4). The glucose then leaves the cell via the basal cell membrane through the passive and specific glucose transporter GLUT2(Nelson and Cox, 2005) and enters into the blood capillaries.

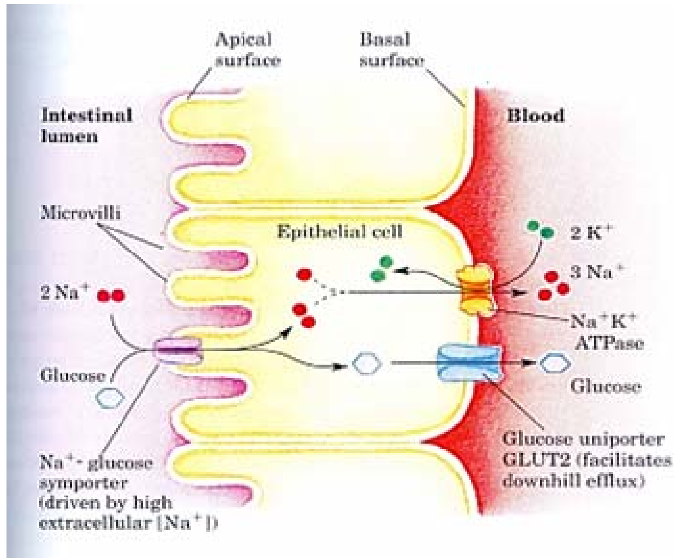

Figure 2-4: Illustration of the transport of glucose across the the intestinal epithelium.
Entry of glucose into epithelial cells is mediated by SGLUT located in the apical surface. Glucose is co-transported with sodium ion (Na^+) across the apical plasma membrane into the epithelial cell. It moves through the cell to the basal surface, where it passes into the blood via GLUT2, a passive glucose transporter. The Na^+/k^+ ATPase continues to pump Na+ outward to maintain the Na^+ gradient that drives glucose uptake (Nelson and Cox, 2005).

2.2.3 Entry of Glucose into cells

Glucose has to be transported from the blood across the cell membrane into the cellular cytoplasm before it can be used by the body's tissue cells (Guyton and Hall, 2000). However, with the membrane of cells being impermeable to glucose, the cell is reliant on transmembrane proteins transporters fordiffusion into the cell. The glucose transporters in non-epithelial mammalian tissues are part of the family of glucose transporters known as the GLUT receptors. These receptors may or may not be insulin dependent (requires insulin for function)(For more details please refer to section 2.3.3):

- GLUT1 transportersare present in the cell membrane of almost all cell types (highest expression is in the red blood and brain microvessels). Under basal physiological conditions the GLUT1 transporter continuously allows for glucose entry. These receptors are non-insulin dependent.

- GLUT2 transporters, which are also non-insulin dependent are found in the pancreatic β-cell, liver, kidney and small intestine (basolateral membrane). In these tissues, the uptake of glucose is dependent on plasma glucose concentrations i.e. facilitated diffusion (Scheepers *et al.*, 2004).

- GLUT3 transporters are present in the neurons of the brain and foetal muscle. In these tissues, the uptake of glucose is dependent on plasma glucose concentrations i.e. facilitated diffusion (Scheepers *et al.*, 2004).

- GLUT4 transporters are present in muscle and fat cells. GLUT4 transportersare normally sequestered in intracellular organelles where they remain inactive (Scheepers *et al.*, 2004; Saltiel and Kahn, 2001). However, on binding of insulin to the insulin cell surface receptor,the GLUT4 transporter translocates from its intracellular location to the cell surface. Once in its active position the GLUT4 immediately and rapidly allows for the transport of glucose (for as long as insulin is bound to its receptor) intracellular at a rate of 10 times or more than in its absence (Scheepers *et al.*, 2004).

Oncewithin the cellglucose gets phosphorylated by glucokinase (in the liver) and hexokinase (in other cells) into glucose-6-phosphate which can no longer leave the cell. The phosphorylation step is almost completely irreversible (except in certain cells e.g. hepatocytes) and serves to ensure that glucose is retained by cells for either energy generation or storage (Guyton and Hall, 2000).

2.2.3.1 Energy Utilisation

While glucose is the main fuel source of the body, cells do not have the capacity to make use of glucose directly but only in the form of ATP, which is generated under aerobic oranaerobic conditions.

2.2.3.1.1 Glycolysis (Energy generation under anaerobic conditions)

Glycolysis is a tensteps, two stages, sequential enzymatic breakdown of a molecule of glucose into two molecules of pyruvate, and takes place within the cytoplasm (Figure 2-5). The first stage requires energy in the form of ATP to start the process. In this pathway, glucose is first phosphorylated to glucose-6-phosphate, then isomerised to fructose-6-phosphate and further phosphorylated to fructose-1, 6-bisphosphate. At the end of the first phase, two molecules of glyceraldehyde-3-phosphate are produced with two molecules of ATP being utilised.

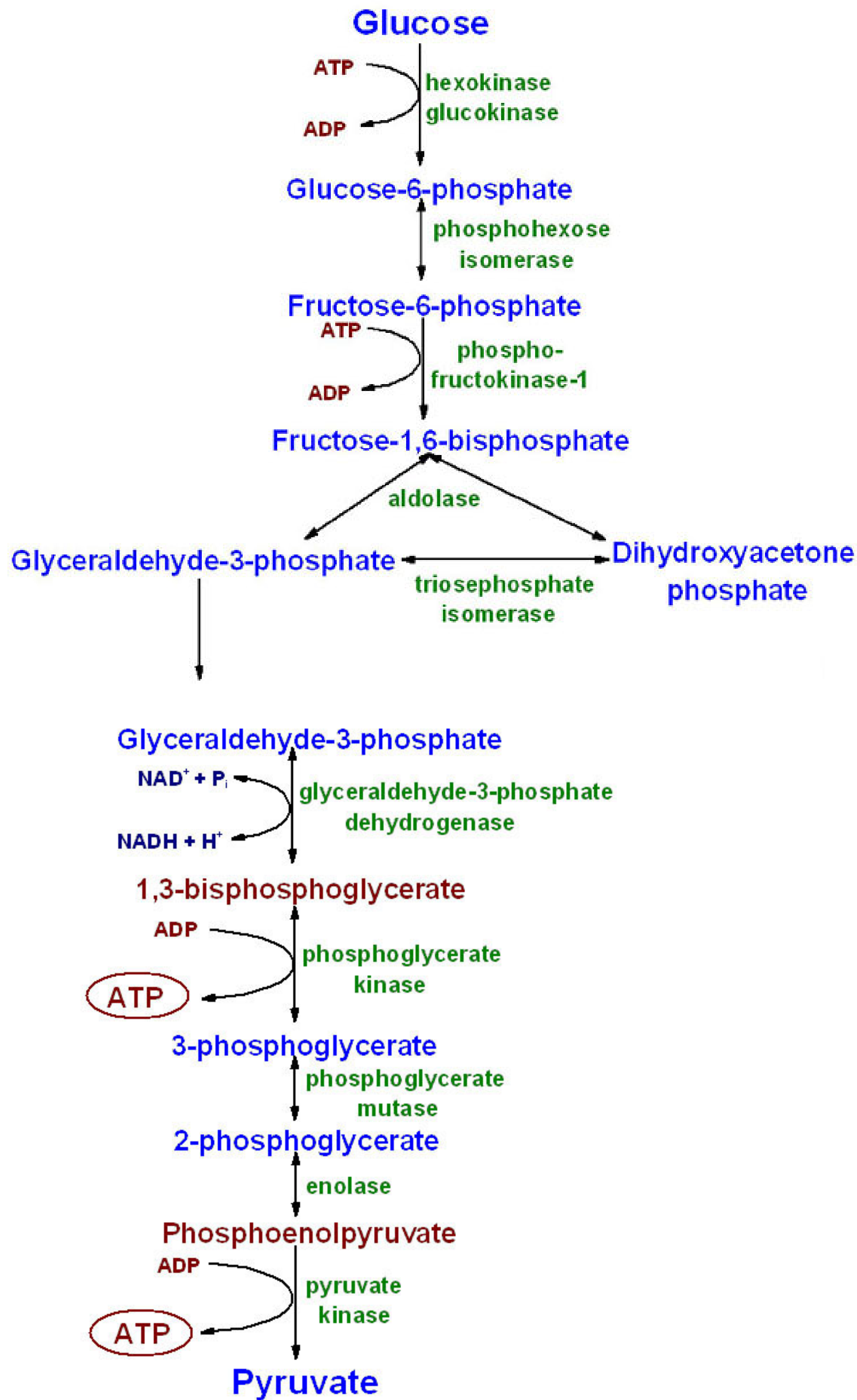

Figure 2-5: Illustration of the ten steps which make up glycolysis (King, 1996).
Glucose is phosphorylated to glucose-6-phosphate, isomerased to fructose-6-phosphate, phosporylated to fructose-1,6-bisphosphate and then split to glyceraldehyde-3-phosphate (GLAP) and dihydroxyacetone phosphate (DHAP). DHAP is isomerase to GLAP. After 5 steps enzyme reactions pyruvate is produced. For each glucose molecule that passes through the first phase, two glyceraldehyde 3-phosphate are formed. Both pass through the second phase and 2 pyruvate, 2 ATP and 2 NADH molecules are the end product (Nelson and Cox, 2005).

The second stage is the energy generation phase. The two molecules of glyceraldehyde-3-phosphate produced in the first stage undergo enzymatic oxidation and two molecules of pyruvate are produced

with four molecules of ATP and two molecules of NADH (reduced nicotinamide adenine dinucleotide) generated as energy. With two molecules of ATP being utilised in the first phase, this process yields two molecules of ATP and two molecules of NADH as energy. The pyruvate molecules formed eventually get reduced to lactic acid under anaerobic condition by accepting electrons from NADH, while the NADH itself is oxidised to NAD^+ (nicotinamide adenine dinucleotide), regenerating the NAD^+ that is required to maintain the glycolytic process (Nelson and Cox, 2005).

2.2.3.1.2 ATP generation under aerobic conditions

Pyruvate is the principal mitochondrial substrate and is produced by glycolysis as mentioned above. The pyruvate moleculesformed in the cytoplasm undergo carrier mediated transport across the inner mitochondrial membrane into the mitochondrial matrix where they undergo oxidative decarboxylation with a loss of the carboxyl group as a carbon dioxide (waste product) and generation of one molecule of NADH (Maechler and Wollheim, 2001). A total of two molecules of carbon dioxide and two molecules of NADH areproduced from the two pyruvate molecules per one glucose molecule.The remaining two carbon atoms become the acetyl group of acetyl coenzyme A (acetyl CoA), a crucial intermediate metabolite for the citric acid cycle in the mitochondrial matrix (Figure 2-6).

Immediately acetyl coenzyme A enters the citric acid cycle, where it donates its acetyl group to a four carbon compound oxaloacetate, to commence the eight step enzymatic oxidation reaction and at the end of the cycle, oxaloacetate is regenerated which starts a new cycle. For each one acetyl group that enters the citric acid cycle, two molecules of carbon dioxide are released as waste products.One molecule of GTP (guanosine triphosphate) a close relative of ATP, three molecules of NADH and one molecule of $FADH_2$ (reduced flavin adenine dinucleotide) are generated as energy. At the end of glycolysis and the citric acid cycle, one molecule of glucose would have generatesfour molecules of ATP andtwenty-four high energy electrons in the form of hydride ions which are carried by NADH and $FADH_2$ for entry into the electron transport chain (Nelson and Cox, 2005).

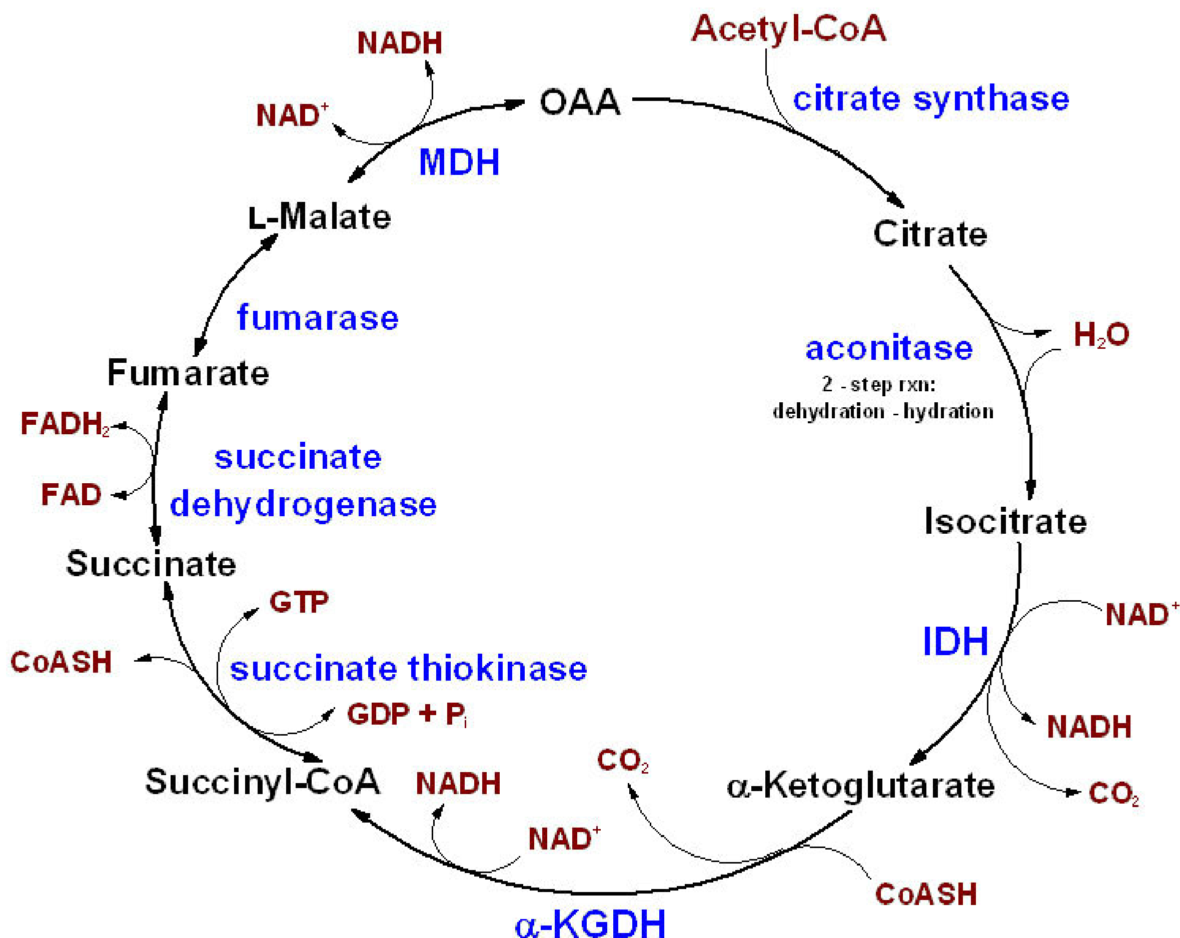

Figure 2-6:Illustration of the reactions which make up the citric acid cycle (King, 1996).
The acetate group of acetyl Co-A condenses with (OAA) oxaloacetate to form citric acid. Energy is conserved by electrons transferred to FAD and NAD^+, forming $FADH_2$ and $NADH+H^+$. At each turn of the cycle, three molecules of NADH, one molecules of $FADH_2$, one molecule of GTP (ATP) are generated. (Nelson and Cox, 2005). IDH = isocitrate dehydrogenase, α-KGDH = α-ketoglutarate dehydrogenase (2-oxoglutarate dehydrogenase), MDH = malate dehydrogenase and CoASH = coenzyme A not attached to an acyl group.

The electron transport chain is responsible for oxidative phosphorylation and is embedded within the inner mitochondrial membrane. Oxidative phosphorylation is a two stage enzymatic generation of ATP. The first stage is the oxidation of hydrogen by the splitting ofhydrogen atoms(derived from NADH and $FADH_2$) into hydrogen ions(protons) and electrons, and the second stage is the utilisation of the energy generated during the oxidative reactions to phosphorylate ADP to ATP (Guyton and Hall, 2000). The electron transport chain consists of electron carriers, which are large enzyme complexes namely complex I, II, III, IV and V (Figure 2-7) arranged in order of increasing redox potential (Alberts *et al.*, 1998). Oxidative phosphorylation begins with the entry of electrons into the respiratory chain at complex I (NADH) and II ($FADH_2$). Complex II, succinate dehydrogenase, is also an integral part of the citric acid cycle. The electrons from both complexes I and II move to complex III.

The flow of electrons along the respiratory chain drives the expulsion of protons from the mitochondrial matrix, which establishes a steep electrochemical gradient across the inner mitochondrial membrane i.e. the mitochondrial matrix becomes negative. More importantly since the inner membrane is impermeable to protons their re-entry into the matrix, to re-establish electrical neutrality, is dependent on entry through the dedicated transmembrane carrier ATP synthase, which is located in the inner mitochondrial membrane projecting into the matrix. The flow of the protons down the electrochemical gradient through a pore in the ATP synthase causes the ATP synthase to spin thereby providing energy to drive the synthesis of ATP from ADP and inorganic phosphate.

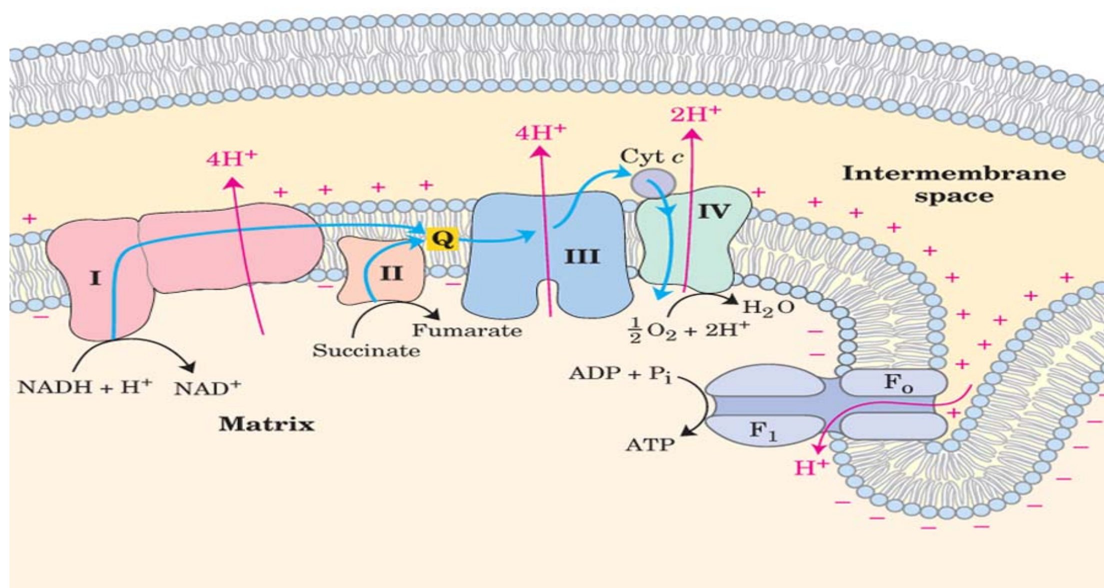

Figure 2-7:Illustration of the mechanism of oxidative phosphorylation (Nelson and Cox, 2005).
As a high energy electron is passed along the electron transport chain starting with the electron donated by NADH, some energy is released to drive the respiratory enzyme complexes that pump H^+ out of the matrix space. The resulting electrochemical proton gradient across the inner membrane drives H^+ back through ATP synthetase, a transmembrane protein complex that uses the energy of the H+ to synthesise ATP from ADP and Pi in the matrix (Alberts *et al.*, 1998).

The ATP produced is subsequently transferred into the cytoplasm in exchange for ADP by the enzyme adenine nucleotide translocator (Maechler and Wollheim, 2001). For every two electrons that pass through the electron transport chain (representing hydrogen atoms carried each by NADH and $FADH_2$), about three molecules of ATP are synthesized. The breakdown of glucose by glycolysis and critic acid cycle produce a total of twenty-four hydrogen atoms and twenty of these hydrogen atoms are undergo oxidative phosphorylation in the mitochondrial matrix. At the end of oxidative phosphorylation, additional 30 ATP molecules are metabolised accounting for almost 90% of the total energy generated (Guyton and Hall, 2000).

2.2.3.2 Storage of glucose

After entry into the cells, glucose can either be immediately converted to ATP for use by the cell (see section 2.2.3.1 above)or be stored in the form of glycogen (low osmotic active polymer of glucose) or fat. Glycogenesis is the process of glycogen formation from glucose. The glucose-6-phosphate formed (Figure 2-5) is converted to glucose-1-phosphate, then to uridine diphosphate glucose and finally converted into glycogen (Figure 2-8). All cells are capable of storing glycogen in large cytosolic granulesto various extents, with cells like the hepatocytesbeing capable of storing larger amount of glycogen (5% to 8% of their weight), and muscle cells to a lesser extent (1%-3% of their weight)(Guyton and Hall, 2000). The elementary particle of glycogen, the β-particle about 21 nm in diameter, consists of up to 55,000 glucose residues with about 2,000 non-reducing ends(Nelson and Cox, 2005).

The body does however only have limited capacity for the storage of glycogen(Guyton and Hall, 2000). Once the glycogen stores are saturated, the additional glucose is converted into triglycerides by the liver cells and transported as very low density lipoprotein for storage in the adipose tissue all over the body. The first step in the synthesis of triglycerides is the conversion of glucose to acetyl-CoA. Malonyl CoA and NADPH are the principal intermediates in the polymerisation process. The second step is the esterification of glycerol and the synthesised fatty acid with chains between 14 and 18 carbon atoms to form triglycerides. A major difference between glycogen and fat, is that glycogen is an immediately available store of glucose while fat is less readily available because in mammals fat cannot be metabolisedanaerobically (Guyton and Hall, 2000).

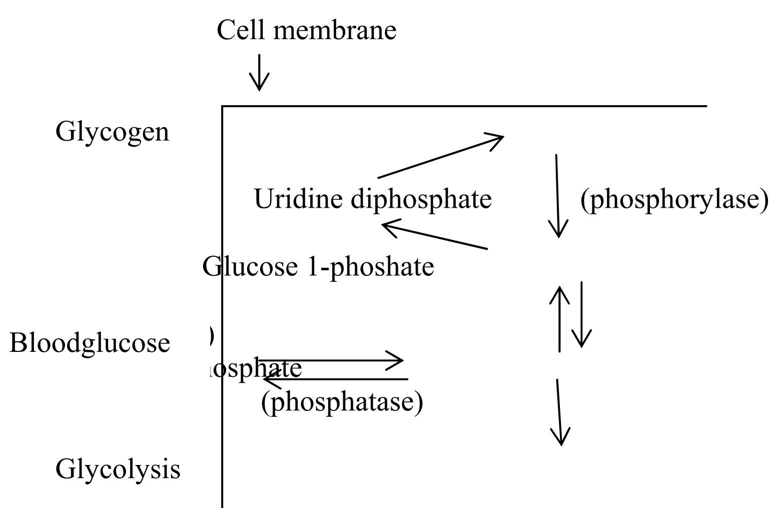

Figure 2-8:Illustration of the chemical reactions of glycogenesis and glycogenolysis.
The mechanism of inter-conversions between glycogen into glucose 6-phosphate (in all tissues) and liver glycogen into blood glucose. The latter is specific to the liver as the phosphatase enzyme required for release of glucose from within cell is present only in the hepatocytes (Guyton and Hall, 2000).

2.2.3.3 Regulation of glucose

Blood glucose in mammals is maintained within very tight limits (about 80 to 110 mg/dl), despite the large fluctuations that result following food intake (Tiwari and Rao, 2002, Guyton and Hall, 2000).The reasons beingto supply the brain, retina and the germinal epithelium of the gonads with sufficient glucose to meet their energy requirement as entry of glucose into these tissues in non-insulin dependent and based purely on a passive concentration (see section2.2.3). In addition, this specific concentration is strictly maintained to prevent intra- and extra-cellular dehydration, as glucose is highly osmotically active.

After the ingestion of a carbohydrate rich diet, increases in the blood glucose to concentration of about 140 mg/dl can be expected. This increase in blood glucose concentration stimulates the release of insulin, by the pancreatic β-cells, which subsequently decrease the concentration of blood glucose to the above mentioned "normal limit". Insulin reduces the blood glucose concentration by increasing the rate of glucose uptake in the muscle and fat cells and inhibiting glucose production (gluconeogenesis) by the hepatocytes. Conversely during the period of fasting, after overnight fast or in between meals, insulin levels are usually low and blood glucose concentrations are maintained by the supply of glucose from the liver through gluconeogenesis and glycogenolysis under the control of glucagon also produced by the β-cells (Nelson and Cox, 2005; Guyton and Hall, 2000).

- Glycogenolysis: This is the breakdown of the muscle and liver's store of glycogen to produce glucose 6-phosphate in the cells which is subsequently used to provide energy. Glycogenolysis does not occur in reversal of the same pathway that formed glycogen (Figure 2-8); rather each succeeding glucose unit of the outer branches of the glycogen enters the glycolytic pathway through the action of three enzymes: glycogen phosphorylase, glycogen debranching enzyme and phosphoglucomutase (Nelson and Cox, 2005; Guyton and Hall, 2000).

  When there is demand for glucose, two hormones; epinephrine (which acts on muscle and liver) and glucagon (which acts only on liver) are released into the blood and cause the formation of cyclic AMP within the muscle and liver cells. The formation of this substance initiates a cascade of reactions that activates glycogen phosphorylase which catalysis the splitting (phosphorylation) of α-(1-4) glycolytic linkage between two glucose residues at a non-reducing end of glycogen removing the terminal glucose residue as α-D-glucose-1-phosphate. The glycogen phosphorylase continues to split the glucose unit until it reaches four

glucose units away from anα-(1-6) branch point and its action stops. Further degradation by this enzyme can occur only after the debranching enzyme catalysed two successive reactions that transfers branches and hydrolysed the glucosyl residue at C-6. Glucose-1-phosphate the end product of glycogen phosphorylase reaction is converted to glucose-6-phosphate by phosphoglucomutase (Nelson and Cox, 2005; Guyton and Hall, 2000).

The glucose-6-phosphate formed from muscle glycogen can hereafter enter the glycolysis pathway.In the liver, however, glycogen breakdown serves to replenish glucose concentrations in the blood. A key enzyme glucose-6-phosphatase that is present as a transmembrane protein on the ER of the liver and kidney is responsible for the hydrolysis of glucose-6-phosphate. This glucose-6-phosphate is transported from the cytosol to the active site on the luminal side of the ER where the glucose-6-phospate is hydrolysed to glucose and phosphate. The glucose formed is transported to the cytosol and out of the liver through GLUT2. Because muscle and the adipose tissues lack glucose-6-phospatase, they cannot convert the glucose-6-phosphate formed by glycogen breakdown to glucose and therefore do not contribute glucose to the blood (Nelson and Cox, 2005).

- Gluconeogenesis: As mentioned above (see section2.2.3.2 above) energy can also be stored in fat tissue (adipose tissue) as droplets of triacylglycerol (fat) within the adipocytes. Triacylglycerol molecules are formed from esterification of fatty acids and glycerol by enzymes found within the adipocytes. When fuel demand rises, especially during fasting, glucagon is released into the blood and activates hormone-sensitive lipases in the adipocytes which hydrolyse stored triacylglycerol with resultant release of large quantities of fatty acids and glycerol into the blood. The free fatty acids hereafter become the primary source of energy for the muscle and liver. However, because some cells like the brain cells mainly use glucose for energy, the liver degrades some non-essential amino acids by transamination and deamination and uses their carbon skeleton in addition to glycerol derived from the adipose tissue as substrates to produce glucose via gluconeogenesis.

  After a few days of fasting, the acetyl CoA produced by fatty acid oxidation start to accumulate due to depletion of the critic acid cycle intermediates required for its conversion to glucose. As a result, the accumulating acetyl CoA molecules condensate to form acetoacetic acid, β-hydroxybutyrate or acetone, collectively known as the ketone bodies, which can be used as an energy source by the heart, skeletal muscle and brain during prolonged fasting and starvation. The ketone bodies like glucose can cross the blood brain

barrier and can be used by the brain cells with about two-third of the its energy being derived from β-hydroxybutyrate (Nelson and Cox, 2005; Guyton and Hall, 2000).

Insulin and glucagon therefore function as important feedback control systems for maintaining a normal blood glucose concentration. The liver also play an important role in glucose regulation by removing glucose from the blood when present in excess or returning it to the blood when low (Guyton and Hall, 2000). While glucagon and insulin are the main control hormones regulating glucose concentrations within the plasma, other hormones may also play a role in its control. The growth hormone (somathotropin), thyroxine and cortisol also increase plasma glucose concentration in conditions characterised by growth/production, increased activity or stress (Nelson and Cox, 2005; Burén and Eriksson, 2005).

When fat reserves are depleted, the degradation of essential proteins begins, protein synthesis is stopped and large quantities of proteins are released into the blood. The blood amino acids concentration rise considerable, and most of the excess amino acids can used either directly for energy (Nelson and Cox, 2005; Guyton and Hall, 2000) via gluconeogenesispathway.

2.3 Insulin

2.3.1 Structure and biochemistry

Insulin is a small protein with 51 amino acids composed of two chains (A and B) linked by disulphide bonds with molecular weight of 5802 (Wilcox, 2005). Insulin synthesis begins in the pancreatic β-cells in the form of an inactive single chain 110 amino acids precursor, preproinsulin(molecular weight of about 11,500) with an amino acid terminal called signal sequence (Figure 2-9). This terminal signal sequence with 24 amino acids directs the synthesis of preproinsulin into the lumen of the rough ER where it is converted to proinsulin by the cleavage of the terminal signal sequence and folded to acquire its characteristic 3-dimensional structure. Secretory vesicles transferthe proinsulin from the rough ER to the Golgi apparatus, where proinsulin is processed to form hexamers(Wilcox, 2009).The proinsulin hexamers are stored in secretory granule within the β-cell until they are released. While within the secretory granule, the C (connecting) chain (35 amino acids) that links the A and B chains together is cleaved to form the insulin in a process known as maturation. When insulin is released, C peptide and proinsulin are also secreted into circulation by exocytosis (Reiter and Gardner, 2003), although the proinsulin is virtually inactive while the C peptide is devoid of activity.

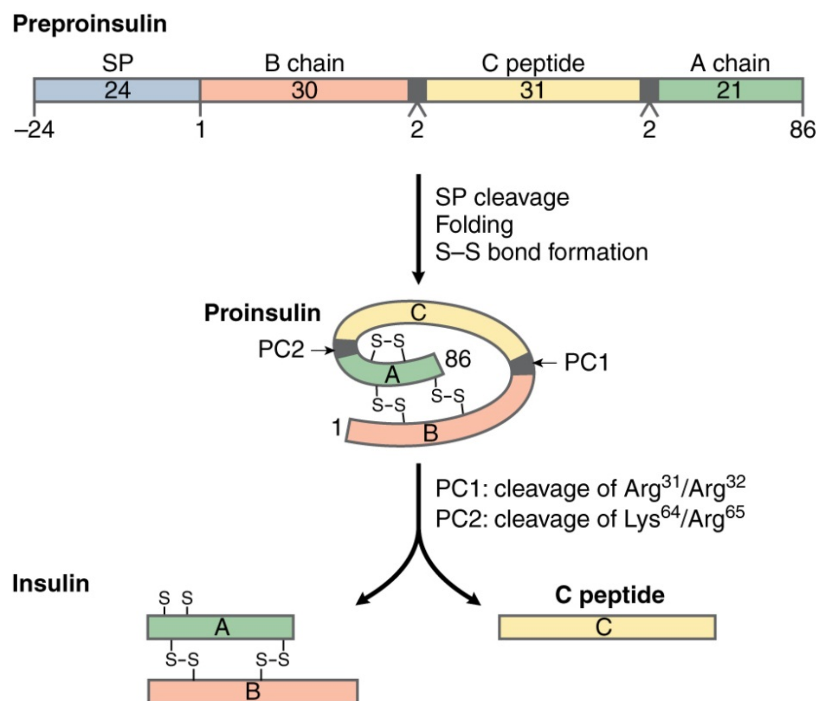

Figure 2-9: Illustration of the process of maturation of insulin from the larger precursor preproinsulin molecule via a proteolytic process (Brunton, 2011).
Removal of a 23 amino acid segment at the terminus of preproinsulin and formation of three disulphide bonds produces proinsilin. Further prolteolytic cuts remove the C peptide from the proinsulin to produce matured insulin composed of A and B chains (Nelson and Cox, 2005).

2.3.2 Mechanism of insulin secretion

As mentioned above, increases in blood glucose stimulates the release of insulin in concentration dependent manner from the pancreas. The absorbed glucose reaches the pancreas via the hepatic portal vein and enters the β- cells by facilitated transport, mediated by the GLUT2 transporter (see section 2.2.2 and 2.2.3 above). Once within the cell, the glucose is phosphorylated by the hexokinase IVenzyme initiating the process of glycolysis (Maechler and Wollheim, 2001). After the complete oxidation of glucose in the pancreatic β-cell mitochondria, the produced ATP is exported to the cytoplasm wherebythe increasing concentration induce the closing of the ATP- gated efflux K^+channels, with subsequent depolarization of β-cells, and opening of voltage-sensitive Ca^{2+} channels in the cell membrane. This resultant influx of Ca^{2+}induces further mobilization of Ca^{2+} from the sarcoplasmic reticulum, microfilament contraction and the exocytosis of insulin from its storage granules (Figure 2-10) (Nelson and Cox, 2005; Saltiel and Kahn, 2001).

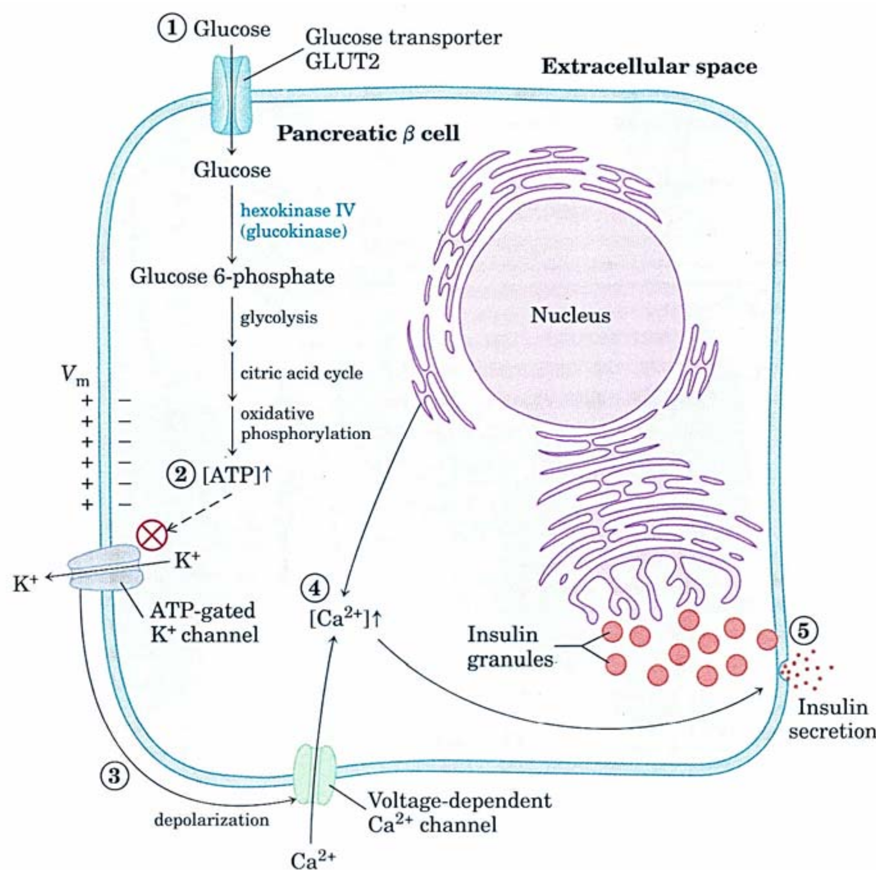

Figure 2-10: Illustration of glucose induced regulation of insulin secretion by pancreatic β-cell.
When the blood glucose levels is high, active metabolism of glucose in the β-cell raises intracellular ATP which leads to the closing of the K^+ channels in the plasma membrane, depolarising the membrane. In response to the change in plasma potential, voltage-gated Ca^{2+} channels in the plasma membrane open, allowing Ca^{2+} to flow into the cell; this raises the cytosolic Ca^{2+} enough to trigger insulin release by exocytosis (Nelson and Cox, 2005).

2.3.3 Mechanism of insulin action

Insulin released into the bloodstream gets carried to the liver, muscle and fat cells (Figure 2-10) (Saltiel and Kahn, 2001) where it binds reversibly to cell surface transmembrane receptors. This insulin receptor is a ligand- activated tyrosine kinase receptor. The binding of insulin to the receptor induces conformational changes in the insulin receptor molecule in the β-subunit with subsequent increase in its tyrosine kinase activity leading to the autophosphorylationof multiple tyrosine residues within its intracellular region (Saltiel and Kahn, 2001).This phosphorylation finally stimulates the translocation of the glucose transporter GLUT4 from the intracellular location up to the plasma membrane in muscle and adipocytes, allowing glucose to enter the cells across the concentration gradient (Bryant *et al.*, 2002).

2.3.4 Physiology of normal insulin secretion

During periods of preprandial, blood glucose level is maintained at 80 to 90 mg/dl while basal insulin is secreted at 25 ng/min/kg of body weight(a level that has only a slight physiological activity (see

section 2.3.2 above). Immediately postprandial, blood glucose concentration is increased to two or three times the normal range. This stimulates the release of insulin in two stages (biphasic pattern) (Guyton and Hall, 2000);

1. Insulin concentrations increase rapidly initially to almost tenfold basal levels within 3 to 5 minutes of the elevation of blood glucose, and resultsfrom the immediate release of insulin from the matured secretory granules (Pratley and Weyer, 2001). However, this initial high secretion of insulin is not sustained, and the insulin concentration decreases back to the basal levels after a further 5 to 10 minutes (Pratley and Weyer, 2001).
2. Continued high blood glucose levels leads to a slower second phase of insulin secretion after approximately 15 minutes and subsequently a much higher concentration than the initial phase, with peaks being achieved within 2 to 3 hours or as long as the high concentration of glucose persist. This second phase insulin secretion is derived from stored secretory granules and from activation of the enzyme system that synthesizes (see section 2.3.2above) and releases new insulin from the cells (Pratley and Weyer, 2001).

The early phase insulin secretion is pivotal in the transition from fasting state to fed state, as it serves to supress hepatic glucose production, lipolysis in addition to preparing target cells for the action of insulin. The second phase is responsible for the overall decrease in postprandial hyperglycaemia (Virally at al., 2007).

2.4 Diseases of improper glucose metabolism

Diabetes mellitus is a metabolic disorder of multiple aetiologies characterised clinically as a disease of disturbances in carbohydrate, fat and protein metabolism brought about through defects in insulin secretion (i.e. β-cell dysfunction), insulin action (i.e. peripheral insulin resistance in muscle and fat cells), or both. Type I and Type II diabetes mellitus are the most predominant in the world (Sherwin, 2004).

2.4.1 Type I diabetes mellitus

2.4.1.1 Epidemiology

Type I diabetes, also known as insulin dependent diabetes mellitus (IDDM), is generally diagnosed in children or young adults with the most common acute symptoms being polyuria, polydipsia, tiredness, blurred vision and weight loss (Roche *et al.*, 2005).

While data on sub-Saharan Africa are scarce (Mbanya *et al.*, 2010), this form of the disease in Europe had an incidence of 94, 000 in under age 15 years in 2005 and predicted to rise to 160, 000 in 2030 (Patterson *et al.*, 2009).In a study by Amos *et al.*(1997), the incidence of Type I diabetes in sub-Saharan Africa was estimated at 85 000 individuals for 1995. This study further speculated that the number of affected persons would increase to 219 000 by 2010. In contrast to the global population, an unusual finding amongst the African population is that the local population appears to develop the disease at an average age of 22 to 29 years (Motala, 2002).

2.4.1.2 Pathophysiology

Type I diabetes is a complicated disease and is usually a combination of a number of different factors, of which immunological factors are most important:

- **Immunological**: Type I diabetes is often the result of an autoimmune disease that causes the selective destruction of the insulin-producing β-cells of the pancreas (Morran *et al.*, 2008). Numerous autoantibodies have been isolated from people with Type I diabetes, and include insulin autoantibodies (IAA) (which neutralises the produced insulin), islet cell antibodies (ICA) (which target and destroys the β-cells), and autoantibodies that target other pancreatic β-cell associated components such as glutamic acid decarboxylase (GAD) and tyrosine phosphatase-related IA-2 molecules (IA-2A) (all proteins found in pancreatic β-cells) (Morran *et al.*, 2008; Knip and Siljander, 2008), and a pancreatic β-cell specific zinc transporter (ZnT8-Slc30A8) (Wenzlau *et al.*, 2007).

- **Genetic:** Current evidence suggests the presence of a genetic predisposition to developing Type I diabetes. In Caucasians, the general incidence for the occurrence of Type I diabetes is only 0.4% for the entire population but substantially higher at 5 to 6% if one has an affected relative (Kelly *et al.*, 2001). Genome-wide screens for susceptibility genes have identified more than ten chromosomal loci potentially associated with the disease (Cox *et al.*, 2001; Van Belle *et al.*, 2011), of which the two most important ones are the human leukocyte antigen (HLA) locus and the insulin gene locus, contributing approximately 42% and 10% respectively to familial inheritance (Morran *et al.*, 2008). For the HLA locus, it has been speculated that mutations ona region located on chromosome 6may result in abnormalities which subsequently leads to the autoimmune responseaccounting for more than 50% of genetic susceptibility to Type I diabetes (Reimann *et al.*, 2009). While the gene encoding insulin, located on chromosome 11 is another susceptible factor contributing 10% to genetic susceptibility (Reimann *et al.*, 2009).

- **Environmental factors**:While speculative, a number of environmental factors have been tentatively linked to the induction of Type I diabetes(Reimann *et al.*, 2009). Factors blamed include viral exposure (such as enterovirus, adenovirus, Coxsackie B virus, cytomegalovirus, hepatitis C Virus, mumps virus, rotavirus and rubella virus)(Morran *et al.*, 2008), exposure to food contaminantsbafilomycin A1 (bafA1, a macrolide antibiotic) or streptozotocin (Zimmet *et al.*, 2001) or other pathogens early in life that prime the immune system (Reimann *et al.*, 2009).Short term breastfeeding (3months) with early weaning onto cow milk (increased numbers of antibodies towards cow milk detected in children with newly diagnosed Type I diabetes) (Virtanen and Knip, 2003) and wheat-based diet with gluten (Van Belle *et al.*, 2011) may also induce immune intolerance.

Irrespective of the cause, the pathophysiology of Type I diabetes is linked to the absence of insulin production by the beta cells. With the absence of insulin, glucose is unable to enter into the cells, with resultant accumulation of glucose in the plasma leading to an initial hyperglycaemia, intracellular dehydration and osmotic diuresis (Guyton and Hall, 2000). As a result of inability to use an essential food source, the body shift energy utilisation to the fat stores. Unfortunately the use of fatty acids as an energy source is also eventually exhausted due to the depletion of the citric acid cycle intermediates, with subsequent accumulation and overproduction of the ketone bodies. In the absence of insulin, virtually all protein synthesis is also stopped while catabolism of protein increases. This degradation of amino acids leads to enhanced urea excretion in the urine, with resulting protein wasting and extreme weakness (Guyton and Hall, 2000). Finally the ketone bodies accumulate to such an extent that the body succumbs for keto-acidosis.

2.4.1.3 Clinical presentation

Patients with Type I diabetes, typicallypresent with severe weight loss, polyuria and polydipsia while in severe cases patients may collapsed due to ketoacidosis (ketoacidosis is a potentially life-threatening condition caused by the build-up of ketone bodies with resultant drop of the physiological pH to 6.9)(Nelson and Cox, 2005; Guyton and Hall, 2000).

2.4.2 Type II diabetes mellitus

2.4.2.1 Epidemiology

Type II diabetes mellitus, also known as non-insulin dependent diabetes mellitus (NIDDM) or adult onset, is a slower form of the disease with milder symptoms (Nelson and Cox, 2005). Type II diabetes is associated with predominant insulin resistance with a relative insulin deficiency; or a predominant insulin secretory defect with or without insulin resistance (Zimmet *et al.*, 2001).Type IIis estimated to

account for approximately 90% of all diabetes mellitus cases globally (Goldstein, 2007). Most importantly this form of the disease is becoming more prevalent in children, teenagers and adolescents due to an increasing sedentary lifestyle and associated obesity (Pinhas-Haniel and Zeltler, 2005). In 2007, it was estimated that approximately 197 million people worldwide had impaired glucose tolerance (Hossain *et al.*, 2007).

2.4.2.2 Pathophysiology of Type II diabetes

2.4.2.2.1 Factors Predisposing to Diabetes

- **Genetic predisposition**:Type II diabetes is foremost a disease that occurs within family groups which confers a 2.4 fold increased risk for the disease (Stumvoll *et al.*, 2005). Children with one parent with Type II diabetes have a 38% risk of developing the disease while those with both parents having the disease have a 60% chance of developing the disease (Stumvoll *et al.*, 2005).

- **Obesity**: About 90% of Type II diabetes is attributable to excess body weight, as a result of the interaction of fat and insulin function (Hossain *et al.*, 2007).While excess fat in any region of the body increases the risk of developing Type II diabetes, abdominal fat ('central' obesity) appears to be a bigger risk factor (Montague and O'Rahily, 2000). From a medical standpoint, a person is considered obese when they have a body mass index (BMI) greater than 30 kg/m^2 as opposed to BMI not greater than 25kg/m^2 in healthy individuals (BMI is calculated as body weight in kilograms divided by the height in metres squared [kg/m^2]) (Dixon *et al.*, 2011).

  The levels of plasma free fatty acid are usually elevated in the obese and are most likely due to the free fatty acid release associated with excess visceral fat mass (Boden and Shulman, 2002). Studies have speculated that elevated circulating free fatty acid may contribute to the underlying pathophysiology of Type II diabetes, in particular, the development of insulin resistance both in muscle and the liver (Shulman, 2000; Boden and Shulman, 2002), due to pronounced lipolysis that directly blocks insulin signalling pathways (Milnar *et al.*, 2007). The numerous hormones and inflammatory factors released by the fat tissue are also indicative of a link between obesity and diabetes (Reimann *et al.*, 2009).

- **Urbanization**:Diabetes is a disease of urbanization with several studies associating the disease with higher prevalence in urban than in the rural areas within the same country (Jain and Saraf, 2010). While speculative, it has been suggested that urbanization is accompanied by a shift from labour intensive occupational activities to more sedentary service-based

occupations that require less energy due to computerization and mechanization and improved transport (Abubakari *et al.*, 2009; Zimmet *et al.*, 2001).

- **Low birth weight and thrifty phenotype**:Foetal under-nutrition and small birth weight for gestational age has been proposed as risk factors for Type II diabetes among adults and youth, aspoor foetal growth appears to correlate with a reduced numbers of pancreatic β-cells and a concurrent diminished capacity to make insulin (Hussain *et al.*, 2007). According to the thrifty phenotype hypothesis (Hussain *et al.*, 2007; Hales and Barker, 2001), the combination of being undernourished in utero followed by a nutritionally overabundant environment later in life may unmask certain foetally programmed predispositions such as central adiposity, decreased pancreas β-cell growth, sub-normal insulin secretory response and insulin receptors functions (Hussain *et al.*, 2007). These abnormalities in turn, may increase susceptibility to insulin resistance and Type II diabetes (Hussain *et al.*, 2007; Eriksson *et al.*, 2001). While the pathophysiology is not clear, it appears that the undernourished foetus may have less stimulus to produce insulin due to lower overall nutritional load within the body i.e. foetal β-cell development is stimulated by the level of foetal nutrition. As a result of the lower number β-cells, the foetus, when exposed to better nutrition post-natally, is more susceptible to obesity and subsequent diabetes later in life. It has also been speculated that people born thin at birth tend to be insulin resistant in adulthood as their metabolism is geared towards fuel conservation (Phillips, 1996).

- **Physical inactivity**:Physical inactivity independent of body size has been identified as a risk factor for Type II diabetes, with regular physical activity reducing the risk of Type II diabetes by 20-60% (Hussain *et al.*, 2007). The WHO estimates that 41% of the global population are inactive, and up to 60% fail to achieve the recommended 30 minutes of moderate activity for most days of the week (Abubakari *et al.*, 2009). Amongst those already afflicted by Type II diabetics, less fit individuals (people with less than 30 minutes activity per week) appear to have twice the risk of death in comparison to the fit (Wei *et al.*, 2000). Physical activity of moderate and vigorously intensity and duration is associated with decreased risk of conversion of impaired glucose tolerance to diabetes, even in the absence of significant weight loss (Hussain *et al.*, 2007). Exercise, or being physically active, enhances glucose uptake in skeletal muscle cells by increasing insulin dependent translocation of the glucose transporter GLUT4 from intracellular vesicles to the plasma membrane, improves insulin mediated muscle blood flow, increases non-oxidative glycolysis and enhances glucose storage as muscle glycogen. More importantly, acute exercise increases glucose uptake into muscle by a mechanism that is distinct from the insulin stimulated phosphoinositol-3-kinase pathway (Hussain *et al.*, 2007).

2.4.2.2.2 Factors resulting in TypeII diabetes

Insulin resistance: Insulin resistance is considered to be present when the biologically normal concentrations or supra-normal concentrations of insulin are inadequate to stimulate both glucose (≥140 mg/dl) uptake into the skeletal muscle and the suppression of endogenous gluconeogenesis within the liver, which results in high circulating blood glucose levels (Bryant *et al.*, 2002). As for the decreased insulin production, decreased insulin response results in hyperglycaemia for the same reasons. The insulin resistance in muscle and liver does not result from a decrease in insulin receptor number but from a defect in the insulin signalling pathway (Virally *et al.*, 2007). Insulin resistance in the muscle is characterised by defects at many levels including decreases in kinase activity, the concentration and phosphorylation of insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2), glucose transporter (GLUT4) translocation and the activity of intracellular enzymes (Virally *et al.*, 2007). Insulin resistance in the liver is also characterised by defects at many levels including increases in glucagon concentration, glucose-6 phosphate and phosphoenolpyruvate carboxykinase (PEPCK) activity (enzymes of gluconeogenesis), concentration of plasma free fatty acids, hepatic fatty acid oxidation and acetyl-CoA (Virally *et al.*, 2007).

Pancreatic β-cell dysfunction (impaired insulin secretion): In non-diabetic patients the β-cell adapts its secretory rate to the level determined by insulin sensitivity of the target tissues and blood glucose concentrations (Virally *et al.*, 2007).Similarly in insulin-resistant states, the normal pancreatic β-cell responds to chronic blood glucose levels by increasing insulin secretion to maintain normoglycaemia in a process termed β-cell compensation. This compensation involves the expansion of β-cell mass, enhanced insulin biosynthesis, and enhanced β-cell function (Figure 2-11)(Prentki and Nolan, 2006). Type II diabetes results when the β-cell compensation can no longer be sustained at the level required to maintain normoglycaemia (Prentki and Nolan, 2006). While it is unknown if this dysfunction is genetic and/or acquired (Virally *et al.*, 2007), β-cell dysfunction appears to be present earlyin the pathophysiology of the diseasei.e. even before changes in blood glucose levels can be determined via clinical measurements (Prentki and Nolan, 2006).

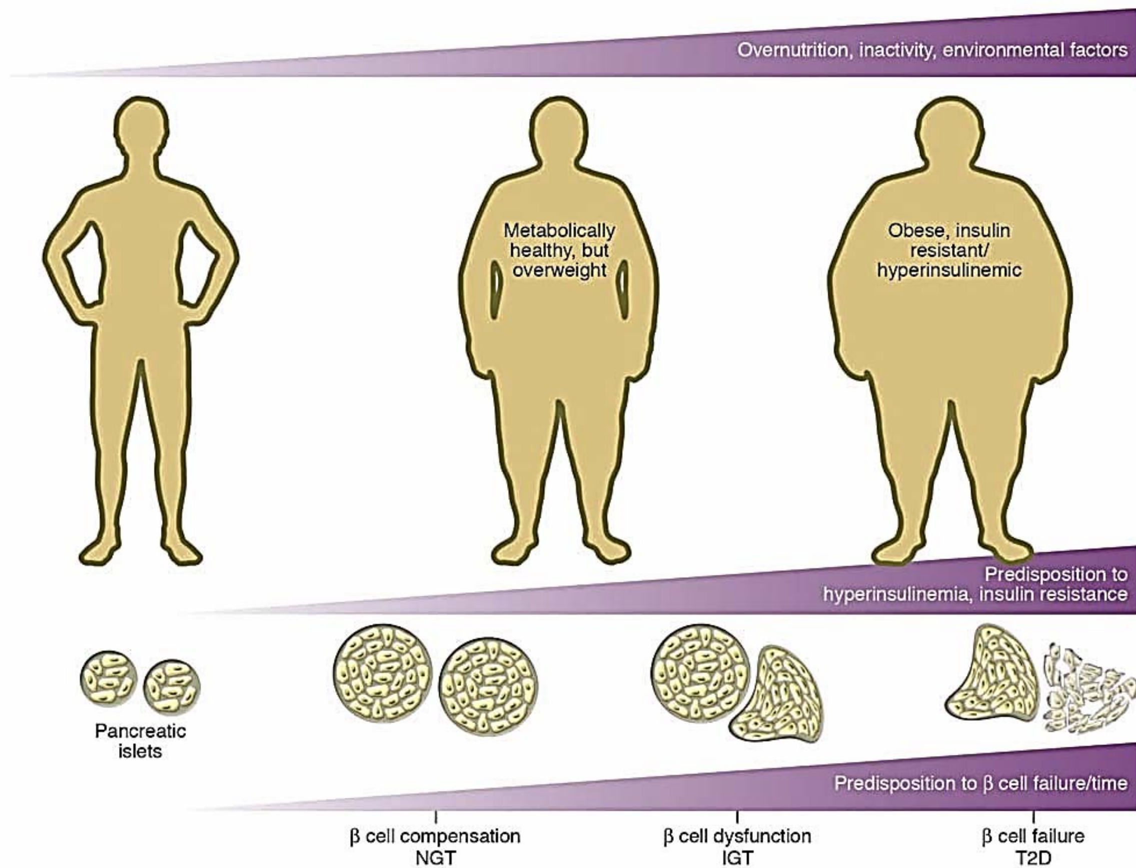

Figure 2-11:Illustration of Pancreatic β-cell dysfunction Type II diabetes.
Type II diabetes develops in response to excessive calorie intake and physical inactivity in people that have underlying genetic and/or acquired predisposition to both insulin resistance and /or hyperinsulinaemia and β-cell dysfunction. Over time, pancreatic β-cell compensation fails, resulting in a gradual decline in β-cell function. Consequently, there is a degeration from normal glucose tolerance (NGT) to impaired glucose tolerance (IGT) and finally to established Type II diabetes (T2D) (Prentki and Nolan, 2006).

2.4.2.2.3 Cellular mechanisms involved in β-cell dysfunction

- **The β-cell exhaustion and ER stress:** The compensating β-cell's prolonged and chronic stimulation by the high blood glucose levels leads to the physical depletion of insulin stores so that further release of insulin is no longer possible (known as tachyphylaxis). The β-cell function does return to normal, once sufficient times is allowed for the re-synthesis and storage of insulin (Robertson *et al.*, 2003). However, until such time that insulin stores are replenished, the circulation of glucose goes uncontrolled by the body. ER stress has been proposed as the potential cellular mechanism for β-cell exhaustion resulting from defect in proinsulin synthesis due to the high demand placed on the ER for the synthesis of proinsulin by the compensating islet of pancreatic β-cell (Prentki and Nolan, 2006).

- **Glucose toxicity:** The speculation that hyperglycaemia itself can desensitize β-cell and increase apoptosis led to the concept of glucose toxicity (Marchetti *et al.*, 2008). It has been speculated that the high glucose concentration produces deleterious effects through several

mechanisms including increased oxidative phosphorylation, over activation of the hexosamine pathway and enhanced activity of protein kinase C (Marchetti *et al.*, 2008). All these pathways lead to increased production of reactive oxygen species and consequently oxidative stress, changes in the expression of free radical scavenging enzymes and alteration of mitochondria (Marchetti *et al.*, 2008). The major manifestation of glucose toxicity occurs in the β-cell evident as a defective insulin gene expression (defective pancreas duodenum homeobox-1 mRNA [PDX-1 mRNA]), diminished insulin content and/or defective insulin secretion (Robertson and Harmon, 2006).

- **Mitochondrial dysfunction with production of reactive oxygen species (ROS):** Prolonged hyperglycaemia in the obese promotes increased metabolism of glucose and free fatty acids through mitochondrial oxidation with resultant increased mitochondrial potential and superoxide production (Lowell and Shulman, 2005; Brownlee, 2003). Increased superoxide production causes increased exposure of the β-cell to ROS and activation of uncoupling protein-2 (UCP-2) (protein which uncouples oxidative phosphorylation) (Krauss *et al.*, 2003) (**Error! Reference source not found.**). Chronically elevated levels of free fatty acids and glucose induce the UCP-2 genes in the β-cell. Increased UCP-2 helps to safely dissipate the elevated mitochondrial membrane potential, ROS and promote fuel detoxification (oxidation of these fuels becomes increasingly coupled to heat production rather than ATP production) at the expense of ATP synthesis and insulin secretion (**Error! Reference source not found.**)(Krauss *et al.*, 2003; Prentki and Nolan, 2006).

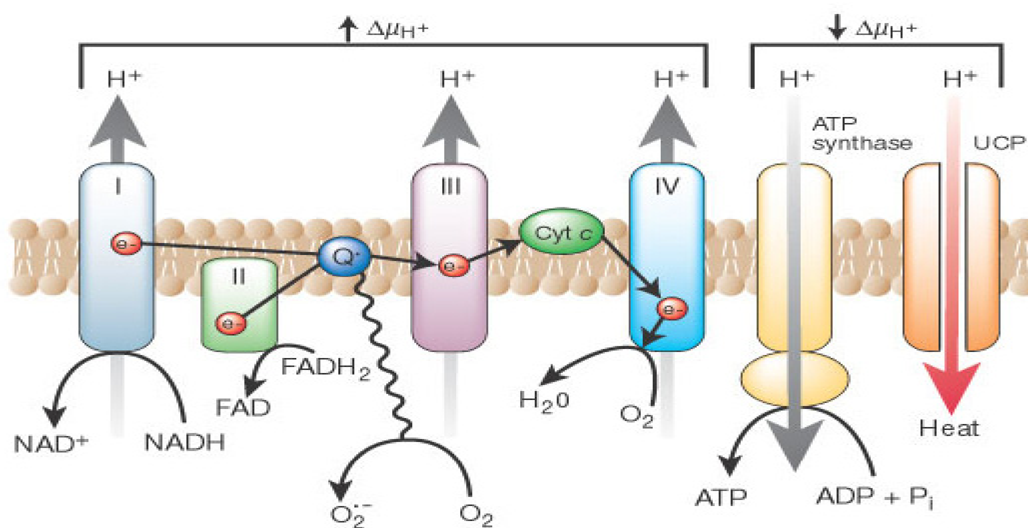

Figure 2-12: Superoxide production by the mitochondrial electron transport chain.
Hyperglycaemia increases the levels of electron donors (NADH and $FADH_2$). This generate a high mitochondrial membrane potential by pumping protons across the mitochondrial inner membrane and consequently inhibits electron transport at complex III, increasing the half-life of free radical intermediates of coenzyme Q which reduces O_2 to superoxide (Brownlee, 2001).

- **Islet amyloid:** Amyloid deposits are found in most but not all individuals with TypeII diabetes (Knowles *et al.*, 2002). This islets amyloid consists of deposits of amyloid polypeptide, also known as amylin that is normally co-secreted with insulin at a tenfold lower rate. While the physiological role of islet amyloid polypeptide is unclear, it is postulated that the initial demands of insulin secretion lead to increase in production and subsequent aggregates which appear to be cytotoxic.

2.4.3 Other forms of diabetes

Gestational diabetes is another form of diabetes, defined as a state of glucose intolerance during pregnancy that usually subsides after delivery but has a major implication for subsequent risk of Type IIdiabetes, as pregnancy serves as an 'environmental' stressor that reveals a genetic predisposition (Permutt *et al.*, 2005). Other less common forms of diabetes include the rare genetically determined disease maturity onset diabetes of the young (MODY), diabetes resulting from surgery, and other illnesses that constitute only 1-5% of cases (Permutt *et al.*, 2005).

2.4.4 Complications of diabetes

The following are some of the most common complications of diabetes

- Retinopathy: This is the most common complication of diabetes that damages the eyes. It is a disorder that can affect the peripheral retina, the macular, or both and is a leading cause of visual disability and blindness in people with diabetes (Cade, 2008). Total or partial loss of vision is characterised vitreous haemorrhage or retinal detachment while central loss of vision is through retinal vessel leakage and macular edema (Cade, 2008).

- Nephropathy: This is a complication of diabetes that damages the kidneys. The kidney is made up of hundreds of thousands of small units called nephrons. These nephrons filter blood and remove waste from the body. However in people with diabetes, the nephrons are modified by the thickening of the basement membrane and result in reduced filtration, albuminuria and renal failure (Ahmed, 2005).

- Neuropathy: This is a complication of diabetes that damages the nerves. It is characterised by segmental demyelination and axonal degeneration of peripheral neurons thus resulting in reduced nerve conduction and blood flow. It may present clinically as pain or numbness or limbs or as impotence in men (Ahmed, 2005).

- Wound healing: Normal wound healing occurs in a complex co-ordinated sequence of events, cells migrate into the wound, inflammatory response, the proliferation of different cell types, formation of matrix components, remodelling and closure of the wound. Impaired wound healing is common in diabetic patients and it is the leading cause of amputations (Ahmed, 2005).

2.4.4.1 Oxidative stress and complication of diabetes

Oxidative stress is the excessive generation of highly reactive molecules (free radicals) such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), or insufficient removal of them (Johansen *et al.*, 2005). Studies have shown that prolonged chronic hyperglycaemia can lead to oxidative stress provoked by hyperglycaemia-induced generation of free radicals (**Error! Reference source not found.**) or by the impairment of endogenous antioxidant defense, and has been implicated in the development and progression of diabetic complication (Johansen *et al.*, 2005).

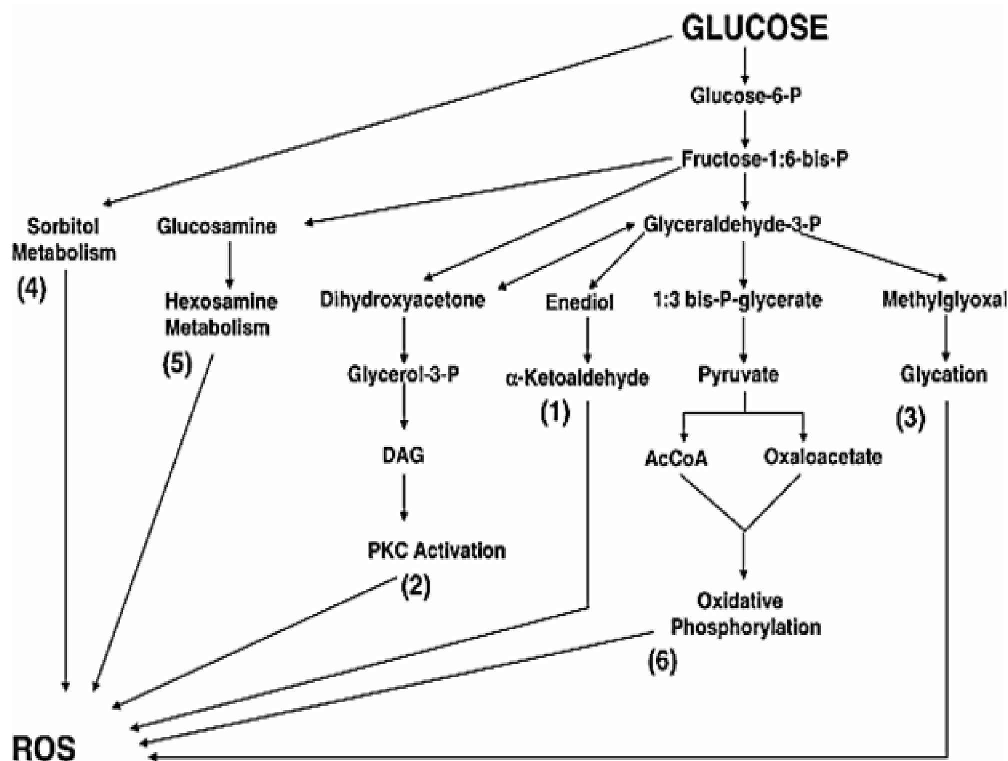

Figure 2-13: Speculated pathways by which glucose metabolism can lead to the production of reactive oxygen species (ROS) (Robertson, 2004).
Under physiological conditions, glucose primarily undergoes glycolysis and oxidative phosphorylation (6). Under pathological conditions of hyperglycaemia, high glucose levels can overwhelm the glycolytic process and inhibit glyceraldehyde catabolism, which causes glucose, fructose 1,6-bis phosphate and glyceraldehyde 3-phosphate to be shunted to other pathways: (1) enolization and α-ketoaldehyde formation, (2) PKC activation, (3) dicarbonyl formation and glycation, (4) sorbitol metabolism, and (5) hexosamine metabolism.

2.4.4.2 The mechanisms involved in the complications of diabetes

The following are two of the speculated mechanisms through which hyperglycaemia-associated free radical can lead to diabetic complications in insulin independent tissues.

- Increased intracellular advanced glycation endproducts (AGEs) formation: With hyperglycaemia, intracellular glucose concentration rises and glucose reacts non-enzymatically with proteins to form advanced glycation endproducts (AGEs). AGEs bind to structural components of the connective tissue matrix or basement membrane, such as collagen, resulting in sclerosis of the renal glomeruli, thickening of the capillary basement and atherosclerosis (Lapolla *et al.*, 2005). These AGEs can also bind to specific receptors (RAGEs) in smooth muscles, monocytes, macrophages and endothelial cells leading to the an increase in the production of NFκB, a transcription factor involved in vascular function, inflammation and pathology (Giacco and Brownlee, 2010).

- Increased polyol pathway flux: The polyol pathway is a sub-pathway for glycolysis (Mizuno *et al.*, 1999) catalysed by aldose reductase that reduces glucose by NADPH to sorbitol (polyol). Aldose reductase is found in tissues such as nerves, retina, lens, glomerulus and vascular cells (Giacco and Brownlee, 2010). In these tissues that are insulin independent, hyperglycaemia activates the aldose reductase, increases polyol pathway flux and damages the cells through increase in redox stress (Giacco and Brownlee, 2010). In addition the polyol pathway also oxidises sorbitol to fructose by sorbitol dehydrogenase. The formation of both sorbitol and fructose deplete NADPH in cells causing redox imbalance (Edwards *et al.*, 2008). The activation of the sorbitol pathway has been implicated in the lens cataract formation, peripheral neuropathy and nephropathy (Maritim *et al.*, 2003).

2.5 Management and treatment of diabetes

The aim of therapy in Type II diabetes is to reach normal glycaemia levels. With insulin resistance being fundamental in the pathogenesis of Type II diabetes, intervention is initially aimed towards improvement in tissue sensitivity/responsiveness. This often involves lifestyle intervention, with moderate exercise and weight loss, and pharmacological management (Tuomilehto *et al.*, 2001).

2.5.1 Therapy for weight loss

Lifestyle intervention through a low calorie diet and regular exercise is the best approach for patients with obesity and Type II diabetes.However, the physiological abnormalities of diabetes may make

weight loss more difficult and harder to maintain, therefore therapy (weight reducing drugs and surgery) promoting weight loss can be an effective adjunct when appropriate (Lee and Aronne, 2007).

2.5.1.1 Pharmacologic agents for weight loss

Numerous pharmacotherapies for weight loss in overweight or obese patients are available and have different mechanisms including:

2.5.1.1.1 Appetite suppressants

Most appetite suppressants work primarily by increasing the availability of anorexogenic neurotransmitters which are norepinephrine, serotonin, dopamine, or some combination of these neurotransmitters in the central nervous system (Yanovski and Yanovski, 2002). Noradrenergic agents include phendimetrazine and benzphetamine. Serotonergic agents act by increasing the release of serotonin, inhibiting its reuptake or both. The mixed noradrenergic-serotonergic agents e.g. sibutramine, is an inhibitor of both norepinephrine reuptake and serotonin reuptake and also weakly inhibits dopamine reuptake (Yanovski and Yanovski, 2002). Tachycardia, dry mouth, constipation, nervousness, insomnia and high blood pressure are some of the associated side effects (Moyers 2005). For safety reasons fenfluramine, dexfenfluramine and silbutramine have been withdrawn for sale in numerous countries (Moyers, 2005).

2.5.1.1.2 Decreased absorption of fats

Gastric and pancreatic lipases are required to hydrolyse triglycerides from dietary fat into absorbable free fatty acids and monoglycerides (DeWald *et al.*, 2006). Through the inhibition of these lipases, the digestion and absorption of ingested dietary fat and fat soluble vitamins is reduced (DeWald *et al.*, 2006). Tetrahydrolipstatin (Orlistat) inhibits gastric, pancreatic and pancreatic carboxylester lipase locally in the lumen of the stomach and small intestine with an estimated reduction of fat absorption by about 30% (Leung *et al.*, 2003). The side effects associated with use of orlistat include reduction in absorption of fat soluble vitamins, flatus with discharge, increased defaecation and oily spotting (Moyers, 2005).

2.5.1.1.3 Increased metabolism/thermogenesis

Ephedrine and caffeine are thermogenic agents that promote weight loss by enhancing energy expenditure. Thermogenesis may be achieved through central activation of the sympathetic nervous system or peripherally by stimulation of atypical adrenoreceptors (DeWald *et al.*, 2006). The side

effects from this combination include tremor, tachycardia, increased systolic blood pressure, insomnia and dizziness (DeWald *et al*., 2006).

2.5.1.2 Surgical therapy

There is evidence that significant weight loss achieved using lifestyle and pharmacological methods by obese, particularly severely obese people is modest and rarely sustained because of the constant upward pressure of counter-regulatory mechanisms of body weight (Lee and Aronne, 2007) making surgery an option (Dixon *et al*., 2011).

Bariatric surgery for weight loss can be classified into 2 groups; intestinal malabsorption and gastric restriction (DeWald *et al*., 2006).

2.5.1.2.1 Malabsorptive procedures

The aim of the malabsorptive procedures is to limit the surface area available for absorption in the small intestines. The procedures that have been used are:

- **Jejunoileal bypass**: In this procedure the proximal jejunum is anastomosed to the terminal ileum. The complications of this procedure include liver failure, cirrhosis, oxalate kidney stone, protein malnutrition, metabolic bone disease and renal dysfunction (DeWald *et al*., 2006).

- **Biliopancreatic diversion**: This involves diverting biliary and pancreatic secretions to distal 50 cm of ileum(DeWald *et al*., 2006). The complications associated with this procedure include protein-calorie malnutrition, metabolic bone disease, deficiencies in fat soluble vitamins, iron, calcium and vitamin B_{12}.

- **Duodenal switch**: The duodenal-switch procedure connects the jejunum rather than the ileum to the proximal duodenum (DeWald *et al*., 2006).

2.5.1.2.2 Restrictive procedure

In contrast to limiting absorption, the restrictive procedures limit the total amount of food a person may consume:

- **Gastroplasty**: this restricts the storage capacity of the stomach to decrease the ability to consume solid foods. Because the procedure uses surgical stapling devices, it is also called a gastric stapling. This procedure consists of construction of a small pouch of the upper stomach

with a restricted outlet along the lesser curvature. The stomach is partitioned with staples and a window is created in the stomach to create the outlet(Ellison and Ellison, 2008). Complications include staple line disruption, pouch dilatation, anaemia, outlet obstruction, incisional hernia and gastroesophageal reflux disease (Ellison and Ellison, 2008).

- **Gastric banding**: this procedure involves an adjustable hollow silastic band placed around the proximal stomach, creating a restrictive pouch. A tube and port are extended from the band to the anterior rectus sheath. The band is secured with gastrogastric sutures to prevent slippage. The port can be accessed through the skin to adjust the band size based on weight loss results (Ellison and Ellison, 2008). The absence of staple line and the associated risk of staple-line dehiscence are theoretical advantage of this technique (DeWald *et al.*, 2006). Complications include gastric slippage, gastric stoma obstruction, band erosion, gastric injury and necrosis, esophageal and gastric pouch dilatation (Ellison and Ellison, 2008).

- **Gastric bypass**: this procedure consists of construction of a proximal gastric pouch with a Y-shaped outlet of jejunum of varying length. The stomach is partitioned by a staple line bypassing the lower stomach. This procedure is both a restrictive and malabsorptive procedure with the degree of malabsorption determined by the length of the jejunum attached at the stomach outlet (Ellison and Ellison, 2008). Complications include wound infections, leaks from the staple/suture anastomosis line, acute gastrointestinal obstruction, gastrointestinal hemorrhage, stomal/marginal ulcer, vitamins and mineral deficiencies and cholesterol stone (Ellison and Ellison, 2008).

Although bariatric surgical procedures have been shown to effectively achieve weight loss in many obese Type II diabetes patients as they decrease food consumption (Rubino, 2006), there are a number of associated complications.

2.5.2 Pharmacological therapy of insulin response or release

2.5.2.1 Reduction of absorption of carbohydrate

Restriction of carbohydrate-containing foods is a method of reducing alimentary absorption of carbohydrate (Virally *et al.*, 2007). Starting from the very beginning of carbohydrate metabolism, release of glucose and transport across the intestinal brush border membrane down to the blood stream, have been targets to reduce the post-prandial hyperglycaemic peak(s). This has been achieved by retarding the absorption of glucose through the inhibition of the carbohydrate hydrolysing enzymes, α-amylase and α-glucosidase, or manipulating the glucose transporter (SGLUT)in the

digestive tract. Acarbose, miglitol and voglibose are competitive inhibitors of the α-glucosidases, the enzymes located in the brush border of enterocytes responsible for cleavage of oligosaccharides. This inhibitory effects delays glucose absorption and consequently decreases the postprandial glucose peak and insulin response to a meal (Virally *et al.*, 2007).Flatulence, abdominal distension and diarrhoea resulting from the fermentation of unabsorbed carbohydrate in the bowel and occasionally increased liver enzymes are some of the adverse side effects resulting in treatment cessation or poor compliance (Virally *et al.*, 2007).

2.5.2.2 Increased pancreatic insulin secretion

The sulfonylurea class of drugs (e.g. glibenclamide, tolbutamide, talazamide, glyburide, glipizide) increase in insulin secretion by binding to a specific sulfonylurea receptor (SUR-1) of the K^+-channel of the pancreatic β-cell (Virally *et al.*, 2007), thereby stimulating the closing of the ATP-sensitive potassium channel (K_{ATP}). The decrease in potassium results in depolarization of the β-cell membrane, calcium flux and exocytosis of insulin (Virally *et al.*, 2007). The efficacy of sulfonylurea drugs is therefore limited to the number of functional β- cells, which due to progressive loss of β-cells over time renders them ineffective after a period of time. Hypoglycaemia, weight gain, hyperinsulinaemia and hepatic and renal impairment are some of the adverse effects of sulfonylureas (Stumvoll *et al.*, 2005).

2.5.2.3 Nonsulfonylurea insulin secretagogue

Glitinides (e.g. repaglinide, netaglinitde) are non-sulfonylurea insulin secretagogues that require the presence of glucose for action and work by closing an ATP-sensitive potassium channel. This class of drug use at least two receptor binding sites on the pancreatic β-cell, and one of these sites is the sulfonylurea receptor (SUR-1) (Virally *et al.*, 2007).These agents have a shorter action than the sulfonylurea drugs and are associated with lower risk of hypoglycaemia (Rendell, 2004). Hypoglycaemia, weight gain and hyperinsulinaemia are some of the associated adverse side effects (Virally *et al.*, 2007).

2.5.2.4 Reduced hepatic glucose output

Thebiguanides (e.g. metformin) reduce of hepatic glucose production (gluconeogenesis and glycogenolysis) and to a lesser extent improves peripheral insulin sensitivity (Hundal and Inzucchi, 2003). The mechanism of action of metformin while speculative has been attributed to the stimulation of adenosine monophosphate activated protein kinase (AMPK) (Hundal and Inzucchi, 2003). Metallic taste, abdominal discomfort, anorexia and diarrhoea are adverse side effects and sometimes lead to drug discontinuation by patients (Virally *et al.*, 2007).

2.5.2.5 Increased insulin sensitivity

The thiazolidinedione (e.g. pioglitazone, rosiglitazone) primarily activate PPARγ (peroxisome proliferators activated receptor γ) receptors in adipose tissue and subsequently alter adipose metabolism and distribution(Nolan *et al.*, 2000).The thiazolidinediones also reduce circulating concentrations of pro-inflammatory cytokines that promote insulin resistance (e.g. TNF-α and interleukin 6) and at the same time increase concentrations of adiponectin, which has insulin-sensitising and anti-inflammatory properties. The multiple effects of thiazolidinediones on adipose tissue metabolism and cross-talk of these signals with liver and skeletal muscle, as well as pancreatic β-cells, may account for the enhancement of insulin action and improvement in insulin secretion (Stumvoll *et al.*, 2007). Unlike metformin, thiazolidinediones act mainly at peripheral level and to a lesser degree on hepatic glucose production (Virally *et al.*, 2007). Fluid retention, weight gain, congestive heart failure, oedema and anaemia are some of the associated adverse side effects.

2.5.2.6 Exogenous insulin

Exogenous insulin is the only available treatment for patients with the absolute insulin deficiency (TypeI diabetes)while inType II,because of the progressive nature of the disease diabetes, most patients will eventually require insulin (Fonseca and Kulkarni, 2008). However in contrast to physiological insulin which is under fine endogenous control, the administration of exogenous insulin is intermittent with resultant poorer responses. This can result in hypoglycaemia (sometimes fatal) from mismatch occurring between carbohydrate intake and circulating insulin concentrations. Insulin analogues with different pharmacokinetic profiles that are less likely to cause hypoglycaemia when used appropriately have been developed; however, cost currently restricts their use in developing countries (Bolli *et al.*, 1999; Sachs and Bonner, 2000). Weight gain and hypoglycaemia are some of the risk factors of insulin therapy.

2.5.2.7 Limitation of conventional therapy

While these therapies can control many aspects of diabetes, none has so far convincingly demonstrated that they can significantly alter the progressive loss of pancreatic insulin secretion that would eventually require exogenous insulin supplementation. Furthermore despite the availability of therapeutic agents, the pathological complications associated with the disease and the mortality continues to increase. In addition the conventional treatments available for the management of Type II diabetes have their own inherent side effects. As a result, newer and more effective drugs/compounds need to be developed.

One of the suggested methods to overcome this problem would be the use of herbal remedies, and these herbal drugs have been prescribed widely because of their perceived effectiveness, fewer side effects and relatively low cost (Venkatesh *et al.*, 2004). Proof for this comes from the extraction of metformin, from *Galega officinalis* (Goat's Rue, French Lilac) which was used as a treatment for diabetes in Medieval Europe (Bailey and Day, 2004).

2.6 Medicinal plants and diabetes

Traditional medicine, derived mainly from plants, play a major role in the management of diabetes mellitus in developing countries where most people have limited resources and do not have access to modern treatment (Ali *et al.*, 2006). Furthermore, about 80% of the world's population makes use (wholly or partly) of plant-based drugs (Kamboj, 2000). There is also an increase in demand in industrially-developed countries to use alternative approaches to treat diabetes, such as plant-based medicines, due to the side effects associated with the use of insulin and oral hypoglycaemic agents (Ali *et al.*, 2006).

In South Africa, like many African countries, medicinal plants and herbal formulations play an important role in the daily health care of the people (Anonymous, 2002). The country is rich in floral biodiversity which has provided health practitioners and traditional healers with an immense pool of "natural pharmacy" from which ingredients are selected for the preparation of herbal medicine (Mahomed and Ojewole, 2006). It is estimated that more than 4000 plant taxa are used in herbal formulations countrywide (Mulholland, 2005). Katerere and Eloff (2006) identified many African species that have been used traditionally to treat diabetes.

At present more than 200 pure compounds isolated from plant sources have been reported to show blood glucose lowering activity *in vivo* (Broadhurst *et al.*, 2000). Amongst these compounds are polyphenols, alkaloids, glycosides, saponins, galactomannan gum, polysaccharides, peptidoglycans, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions (Mukherjee *et al.*, 2006). Of these, the polyphenols have appeared to be the most promising agents.

2.6.1 Polyphenols

Polyphenols are a large and heterogeneous group of phytochemicals of plant based food such as tea, coffee, wine, cereal grains, vegetables, legumes and fruits (Hanhineva *et al.*, 2010). Polyphenols are plant secondary metabolitessynthesized as protective agents from biological and environmental stresses (Pandey and Rizvi, 2009). The structural diversity of the polyphenols extends from simple

one-phenol hydroxybenzoic and hydroxycinnamic acids to large polymeric molecules like proanthocyanidins and ellagitannins (Hanhineva *et al.*, 2010). The two main types of polyphenols are flavonoids and phenolic acids with flavonoids distributed among various classes of flavones, flavonols, flavanols, flavanones, isoflavones, proanthocyanidins and anthocyanins (Scalbert *et al.*, 2005). Some of the common flavonoids are quercetin, a flavanol in onion, tea and apple; catechin, a flavanol found in tea and several fruits; hesperetin, a flavanone in citrus fruits; cyanidin, an anthocyanin responsible for the colour of many red fruits; daidzein, isoflavone in soybean; and proanthocyanidin responsible for the astringency or bitterness of grape or cocoa (Scalbert *et al.*, 2005). Phenolic acid such as caffeic acid is present in many fruits and vegetables, and ferulic acid is present in cereals (Scalbert *et al.*, 2005).

2.6.2 Polyphenols and diabetes

Numerous mechanisms have been evaluated for the ability of the polyphenols to reduce blood glucose:

- **Precipitation of proteins**: One of the best-known properties of polyphenolic compounds, with the exception of the low molecular weight phenols, is their capacity to bind and precipitate protein(Bravo, 1998) with the highly polymerized tannins beingthe most effective. With the gastrointestinal tract being rich in digestive enzymes, polyphenols may be able to inhibit these enzymes with subsequent reduction in dietary carbohydrate hydrolysis and the postprandial glycaemic response (Bravo, 1998).A variety of polyphenols have been shown to inhibit α-amylase and α-glucosidase activities *in vitro* (Hanhineva *et al.*, 2010). Matsui *et al.* (2001a) demonstrated that a polyphenolic rich extracts from the sweet potato (*Ipomoea batatas* L.) roots and morning glory flowers (*Pharbitis nil* cv. Scarlett O'Hara) enriched in anthocyanins were effective inhibitors of rat intestinal α-glucosidase andhuman α-amylase. Subsequently, they demonstrated that diacylated anthocyanins from these sources were most effective against α-glucosidase (Matsui *et al.*, 2001b) and were capable of inducing an anti-hyperglycaemic effect in rats (Matsui *et al.*, 2002). Specific isolated anthocyanins that inhibited α-glucosidase activity *in vit*ro are Cyanidin-3-α-O-rhamnoside and pelargonidin-3-α-O-rhamnoside (Cazarolli *et al.*, 2008). Other polyphenols such as (+) catechin, (-) epicatechin, (-) epigallocatecin and epicatechin gallate from tea, isoflavones from soybeans, phenolic compounds, tannic acids and chlorogenic acid have also been shown to be potent inhibitors of α-glucosidase (Tiwari and Rao, 2002).

- **Competition with glucose**: Flavonoids are speculated to be absorbed in the intestine and in some cases compete with glucose in unknown absorption mechanisms (Cazarolli *et al.*, 2008). A soybean extract containing genistein and daidzedin isoflavonoids decreased *in vitro* glucose

absorption into the brush border membrane vesicles of rabbits while naringenin reduced glucose uptake in the intestinal brush border vesicles of diabetic rats (Cazarolli *et al.*, 2008).

- **Glucose transport**: The influence of polyphenols on glucose transporters had been studies *in vitro* and several flavonoids and phenolic acids have been associated with inhibition of glucose transport. (-) Epicatechin gallate (-) epigallocatechin gallate and (-) epigallocatechin markedly reduced glucose absorption through the competitive inhibition of sodium-dependent glucose transpoter-1 (SGLUT1) (Cazarolli *et al.*, 2008). The sodium-dependent SGLUT1 mediated glucose transport was also inhibited by chlorogenic , ferrulic, caffeic and tannic acids, quercetin monoglucosides, tea catechins and naringenin while glucose transporter GLUT2 was inhibited by quercetin, myricetin, apigenin and tea catechins (Hanhineva *et al.*, 2010).

- **Insulin utilisation**: Polyphenolic fractions from plants may also alter glucose utilization in mammals through an insulin-like effect (Broadhurst *et al.*, 2000). The supplementation of genistein and rutin in diabetic rats increased plasma insulin concentrations (Cazarolli *et al.*, 2008). Within the anthocyanins and anthocyanidins group, cyanidin-3-glycoside and delphinidin-3-glycoside potent insulin secretagogues while pelargonidin-3-glycoside and its aglycon, pelargonidin, have also been able to significant increase in insulin secretion (Cazarolli *et al.*, 2008).

- **Regeneration of pancreatic beta cells**: Polyphenols from some plants reportedly also have the ability to stimulate pancreatic β-cells regeneration. Intraperitoneal injection of (-) epicatechin in alloxan treated mice demonstrated β-cell regenerative capacity; similarly soybean isoflavoniods, genistein and daidzedin preserved the insulin production by β-cells (Hanhineva *et al.*, 2010), while quercetin increased the number of pancreatic islets in both normoglycaemic and diabetic rats (Cazarolli *et al.*, 2008).

2.6.3 Polyphenols and obesity

Some dietary polyphenols have been shown to modulate physiological and molecular pathways that are involved in energy metabolism, adiposity and obesity, which may facilitate weight loss and prevent weight gain (Meydani and Hassan, 2010). Several studies have demonstrated that consumption of green and white teas containing catechins, in particular epigallocatechin gallate (EGCG), fruits such as blueberries with anthocyanins, red grapes and wine with resveratrol and spices like turmeric which contain curcumins may stimulate weight loss (Meydani and Hassan, 2010). The mechanisms through which these polyphenols induce weight loss was speculated to include

suppression of fat absorption from the gut, uptake of glucose by skeletal muscles, suppression of anabolic pathways, stimulation of apoptosis of matured adipocytes, and reduction of chronic inflammation associated with adiposity (Meydani and Hassan, 2010).

2.6.4 Polyphenols and antioxidant defence

Polyphenols with catechol groups (aromatic rings with two hydroxyl groups in the ortho position) have greater antioxidant potency than those with simple phenol groups (aromatic rings with a single hydroxyl group). Quercetin and epigallocatechin, for example, have been shown to be excellent traps for $ABTS^+$ and are almost five times more active than trolox, a water-soluble vitamin E analogue, at equivalent molar concentrations (Scalbert *et al.*, 2005). It has been shown in several *in vitro* studies that the relatively polar polyphenols regenerate or spare lipophilic antioxidants, such as vitamin E while antioxidant effects of polyphenols may be more easily observed when the status in other antioxidants is low (Scalbert *et al.*, 2005). The antioxidant efficiency of polyphenols may depend on the extent of absorption and metabolism of these compounds, as well as the activity of methoxylated and conjugated forms circulating in plasma (Bravo, 1998).

It has been demonstrated that for some plant extracts atleast, a positive correlation exists between antidiabetic activity and flavonoid content (Sezik *et al.*, 2005; Aslan *et al.*, 2007). The phenolic groups in the polyphenols can accept an electron to form relatively stable phenoxyl radicals, thereby disrupting chain oxidation reactions in cellular components (Scalbert at al., 2005). As antioxidants, these compounds may prevent the progressive impairment of pancreatic beta-cell function due to oxidative stress (Song *et al.*, 2005).

2.6.5 Potential risk associated with polyphenols

While several studies have focused mainly on the beneficial effects of polyphenols, there are likely to be side effects with increased intake of polyphenols, particularly beyond common levels of exposure associated with the diet. Many of the proposed side effects are speculative. Flavonoids may influence the thyroid function and have goitrogenic effects. A reduction in thyroid peroxidase activity was observed in rats fed a diet supplemented with genistein, and these effects of genistein and other related compounds (such as daidzein, kaempferol, quercetin or naringenin) on thyroid function are more pronounced when iodine is deficient (Scalbert *et al.*, 2005). High intake of catechins or green tea extracts containing very high concentration of EGCG causes hepatotoxicity, inflammation of and necrotic damage to the liver (Meydani Hasan, 2010). The catechol group of polyphenols (coffee, tea, wine) directly chelate iron from the gut thus inhibiting non-heme iron absorption especially when polyphenols are consumed together with the source of iron, though the impact of dietary polyphenols

on iron status is not as firmly established (Scalbert *et al.*, 2005). There are reports of animals consuming plants rich in tannins showing signs of acute toxicity while no such acute toxicity has been reported in human consumption of dietary polyphenols (Scalbert *et al.*, 2005).

2.7 Selection of plant species

The use of *Ficus* species as food or pharmacological agents to improve human health has a history of about eleven thousand years in Ayurvedic and Traditional Chinese Medicine (TCM) (Lansky *et al.*, 2008).The genus *Ficus*, commonly referred to as fig, is a genus of about 800 species of woody trees, shrubs and vines in the family Moraceae found in all tropical habitat types with about 100 species in Africa and the surrounding islands (Serrato *et al.*, 2004). Many of these species possess auxiliary aerial root systems extending to the ground from their branches or trunks. All *Ficus* species possess latex-like material within their vasculature, affording protection and self-healing from physical assaults (Lansky *et al.*, 2008).

2.7.1 Ethnomedical uses of *Ficus* species

In folk medicine, *Ficus* species are reported to have hypotensive, antidiabetic, mild laxative, antirheumatic, antibacterial and digestive activities. They are also used to treat cough, chest conditions and as an anthelmintic against intestinal parasites. They have been used as anti-inflammatory agents in urinary tract infections, sore throats, ulcerated noses, to reduce fever, and to cure tuberculosis and piles. Externally, they have been used to treat eczema, to cure tinea and leprosy, to treat cracks in the soles of the feet, and as a dressing for boils (Watt and Breyer-Brandwijk, 1962; Ramadan *et al.*, 2009).

From what is known about the chemistry of the different species and from the genetic commonalities between different *Ficus* species, there seem to be some overlap, and similarities between different *Ficus* species do occur (Lansky and Paavilainen, 2010). Therefore, if a particular activity has been demonstrated to occur in one *Ficus* species, another *Ficus* species could be an alternative and possibly better source of the same activity and may be more easily obtainable (Lansky and Paavilainen, 2010). Phytochemical investigations of some *Ficus* species revealed that polyphenolic compounds constitute the major components of stembark (Li *et al.*, 2006; Sandabe *et al.*, 2006). Also present are triterpenes of different types, sterols, flavonoids, coumarins, alkaloids and other miscellaneous compounds (Ramadan *et al.*, 2009). Some other studies reported the presence of antioxidant activity of some *Ficus* species which attributed the antioxidant activity to the polyphenolic content (Abdel-Hameed, 2009).

A survey of the literature indicates that about 12*Ficus* species have *in vivo* antidiabetic activity from laboratory animal diabetic studies. These are *Ficus banghalensis* L. (Elder, 2004), *Ficus carica* L. (Perez *et al.*, 1996), *Ficus racemosa* L (Rao *et al.*, 2002), *Ficus hispida* L. f. (Ghosh *et al.*, 2004),

Ficus deltoidea (Adam *et al.*, 2012), *Ficus microcarpa* L.f. (Kumar *et al.*, 2007),*Ficus arnottiana* Miq. (Mazunder *et al.*, 2009), *Ficus religiosa* L. (Pandit *et al.*, 2010), *Ficus thonningii* Blume (Musabayane *et al.*, 2007), *Ficus glumosa* Del. (Madubunyi *et al.*, 2012), *Ficus glomerata* Roxb.(Kar *et al.*, 2003) and *Ficus sycomorus* L. (Adoun *et al.*, 2011). However, of the 23 *Ficus* species growing in South Africa (Jordaan, 2000), very little research has been done on their antidiabetic activity.

From a search of the available literature, there was only one report by Musabayane *et al.* (2007) on the effect of *Ficus thonningii* stembark ethanolic extract on blood glucose. Based on the scant ethnobotanical and ethnomedical information available for the South African species, ten South African *Ficus* species were selected for antidiabetic investigation. These plants are: *Ficus capreifolia, Ficus cordata, Ficus craterostoma, Ficus glumosa, Ficus lutea, Ficus natalensis, Ficus poita, Ficus religiosa, Ficus sycomorus* and *Ficus thonningii*.

2.8 Conclusion

Diabetes mellitus is a state of persistent and chronic hyperglycaemia which once thought to be an elite disease is now one of the most prevalent non-communicable diseases in recent times with severe clinical signs. Weight loss through increased physical activity in addition to low calorie food has been long suggested to improve glucose tolerance. However, since it is not easy for people to change habits formed over years, drugs and bariatric surgery are alternative weight loss therapy suggested for obese Type II diabetic. Unfortunately due to severe side effects associated with the use of these commercially available therapies, failure of the current agents to alter the natural progress of the disease newer therapies are needed. Herbal medicines, used traditionally for the treatment of many diseases including diabetes may serve as a source of these alternates. For this study 10 *Ficus* species will be explored as alternative therapies for diabetes.The description of the species used is provided in chapter 3.

Chapter 3

3 Materials and Methods

3.1 Materials

3.1.1 Reagents and chemicals

Folin-Ciocalteu reagent, gallic acid, sodium carbonate (Na_2CO_3), potassium persulphate, ABTS [2, 2–azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)], potato starch, porcine pancreatic α- amylase enzyme (type VI), sodium chloride (NaCl), sodium phosphate, 3, 5-dinitrosalicylic acid (DNS), sodium potassium tartrate, sodium hydroxide (NaOH), acarbose, rat intestinal acetone powder, potassium phosphate, sucrose, TRIS (hydroxymethyl) aminomethane, the glucose oxidase kit (GAGO 20) and trolox (a synthetic water soluble vitamin E analogue), bovine serum albumin (BSA), minimal essential medium (MEM), Dulbecco's MEM (DMEM), Roswell Park Memorial Institute medium 1640 (RPM1640), foetal calf serum (FCS), glutamine, trypsin-EDTA, berberine chloride, phosphate buffered saline (PBS), sodium pyruvate, Hanks balanced salt solution (HBSS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid (HEPES), collagenase (type 11), penicillin/streptomycin, glucose oxidase kits and D-glucose were purchased from Sigma (South Africa). Gentamicin (Virbac), insulin (Sanof; Aventis), Trypan blue (Fluka), doxorubicin (Pfizer) and Insulin (Rat) ELISA kit (DRG Instruments GmbH, Germany Frauenbergst). Sodium hydrogen carbonate ($NaHCO_3$), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulphate ($MgSO_4$), potassium hydrogen phosphate (KH_2PO_4), dimethyl sulphoxide (DMSO), acetone, methanol, hydrogen chloride (HCl), sulphuric acid (H_2SO_4) and Whatman No. 1 filter paper were purchased from Merck (South Africa). Corn oil was purchase from the University of Pretoria, Faculty of Veterinary Sciences pharmacy, condensed milk (clover) was bought from a local supermarket and the standard rodent chow was sourced from Epol foods.The absorbance measurements were read using a microtitre plate reader (VERSAmax, Molecular Devices Labotec).

3.1.2 Cell lines and primary cell cultures

C2C12 mouse muscle myoblast (CRL-1772), 3T3-L1 mouse pre-adipocytes fibroblast (CL-173), H411E rat hepatoma (CRL-1548), RIN-m5F rat insulinoma (CRL-11065), C3A human liver cells (CRL-10741) were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and Vero African green monkey kidney cells were obtained from the Department of Veterinary Tropical Diseases (Faculty of Veterinary Sciences, University of Pretoria)(The cultures have been maintained for over ten years and are not deposited at the ATCC). The tissues/organs (abdominal muscle and epididymal fat pad) used in this study for isolation of cells were collected as waste by-products from

other animal studies. Ethics approval was received from the Animal Use and Care Committee (AUCC), University of Pretoria, for animal use with approval number V069-09.

3.1.3 Plant material

The leaves of ten *Ficus* species were collected at the Manie van der Schijff Botanical Garden (University of Pretoria), South Africa in February 2009, and voucher specimens were conserved in the HGWJ Schweikerdt Herbarium of the University of Pretoria. The names of plant species and voucher numbers are as follow: *Ficus capreifolia* Delile - PRU 33124; *Ficus cordata* Thunb - PRU 35501; *Ficus craterostoma* Mildbr. & Burret.- PRU 48293; *Ficus glumosa* Delile - PRU 38554, *Ficus lutea* Vahl.- PRU 074568; *Ficus natalensis* Hochst - PRU 078082; *Ficus polita* Vahl - PRU 35945; *Ficus religiosa* L. - PRU 115415; *Ficus sycomorus* L. - PRU 066173; and *Ficus thonningii* Blume - PRU 57036. The leaves were separated from the stems and dried at room temperature. The dried plant materials were milled to a fine powder in a Macsalab mill (Model 200 LAB, Eriez® Bramley) and stored at room temperature in closed glass bottles in the dark until extracted (Eloff, 1998a).

3.1.4 The selected *Ficus* species

3.1.4.1 *Ficus capreifolia* Delile (sandpaper fig)

It is a shrub, but may become a small, slender tree up to 7 m; occurring in swamps, and frequently forming tangled thickets along river banks and on sandy islands in the larger rivers. The bark is greenish grey to whitish and smooth, and the branches are often long and whip-like. The leaves are alternate or opposite (unusual in *Ficus*); elliptic with the surface very rough and scabrid resembling sandpaper. Figs are borne singly on fairly long slender stalks in the axils of the leaves, and are pear-shaped, and green to yellow in colour (Coates-Palgrave, 2002).

Figure 3-1*Ficus capreifolia* (van Noort and Rasplus, 2004)

Traditional use includes treatment of infections (Duke, 1975). In Tanzania the leaf sap and a decoction of the root are taken for the treatment of schistosomiasis, and the leaf sap or the powdered root is sprinkled on syphilitic ulcers (Brink, 2010) (Figure 3-1).

3.1.4.2 *Ficus cordata* Thunberg (Namaqua Rock fig)

It is usually a medium-sized tree, about 10 m in height and rather slender, but old species can be up to 20 m, with a wide spread, characteristically associated with rocks where its pale roots flattened against the rock-faces are distinctive; common in dry, stony deserts. The bark is pale grey and rather smooth. The leaves are ovate, or heart-shaped, dark green and hairless. Figs are paired in the axils of the leaves, without stalks, finely velvety and brown to yellowish in colour (Coates-Palgrave, 2002) (Figure 3-2).

The stembark of *Ficus cordata*is used traditionally in Cameroon, in the treatment of diarrhoeal infections, tuberculosis and as an anthelmintic (Kuete *et al.*, 2008).

Figure 3-2*Ficus cordata* (van Noort and Rasplus, 2004)

3.1.4.3 *Ficus craterostoma* Warb. Ex Mildbraed & Burret (Forest fig)

It is usually a small to medium-sized tree reaching 12 m in height, occurring in evergreen forest margins, swamp forest and in deep, and heavily wooded mountain ravines or on rocky outcrops in the open; it can be a strangler. The bark is grey and, relatively smooth. The leaves are spirally arranged to alternate and even sub-opposite, thinly leathery and hairless. Figs occur in the axils of the leaves, without stalks, and are not hairy, slightly warty and yellow to reddish in colour (Coates-Palgrave, 2002) (Figure 3-3).

In South Africa, the Xhosa people boil the fresh leaves of *Ficus craterostoma* and take the decoction orally for stomach-ache (Bhat and Jacobs, 1995).

Figure 3-3*Ficus craterostoma* (van Noort and Rasplus, 2004)

3.1.4.4 *Ficus glumosa* Delile (Hairy rock fig)

This is a small to medium-sized tree 5-10 m tall or sometimes a large tree reaching 13 m, occurring on rocky outcrops when it can become a rock-splitter, along dry watercourses, or in open country frequently in valleys where it reaches its greatest size. The bark is pale grey to yellowish grey, smooth to slightly rough, flaking off. Branchlets are finely hairy and may be marked with large leaf scars. Leaves are oval to oblong, roundish, green or greyish green, matured leaves are usually hairless above, and the under-surface is finely hairy, sometimes with scattered long hairs. Figs are in pairs in the leaf axils and often clustered at the end of the branches (Coates-Palgrave, 2002) (Figure 3-4).

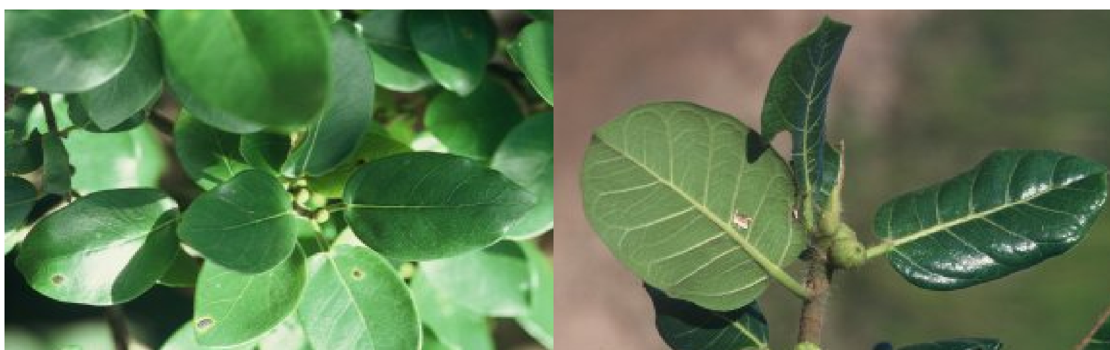

Figure 3-4*Ficus glumosa* (van Noort and Rasplus, 2004)

The traditional medicinal uses are numerous. In Cote d'Ivoire the latex is applied to alleviate pain from sprains while the latex diluted in water in the Central African Republic is used to treat diarrhoea (Jansen and Cardon, 2005). In Zimbabwe the latex is dropped into the eyes for the treatment of sore

eyes (Jansen and Cardon, 2005). Powdered bark mixed with latex is used in Nigeria to plug carious teeth while in the Central African Republic a decoction of the bark is used as a mouthwash against toothache (Jansen and Cardon, 2005). A bark macerate is applied on the eyes of new born babies to prevent conjunctivitis while the pounded bark is applied topically against headache. In addition pounded bark, soaked in water, is drunk against stomach disorders while a decoction of pounded bark is used to wash sores daily until recovery (Jansen and Cardon, 2005). It is also used in the treatment of skin diseases (Hutchings *et al.*, 1996) and diabetes (Madubunyi *et al.*, 2010).

3.1.4.5 *Ficus lutea* Vahl (Giant leaved fig)

This species is a large, spreading tree reaching 25 m occurring in evergreen and riverine fringe forest. The bark is dark grey; the bole usually short and often buttressed, with the branches widely spreading. Leaves are very large, crowded at the end of the branches, ovate to elliptic or oblong and shiny green. Figs occur in ones or twos per leaf axil, and are tightly crowded at the tips of the branchlets or just below the leaves, and furry or smooth and yellow in colour (Coates-Palgrave, 2002) (Figure 3-5).

The leaves of *Ficus lutea* are used traditionally in the treatment of sores and boils (Marwah *et al.*, 2006). In combination with other plants, macerations are taken orally for the treatment of rabies, madness and anaplasmosis. A decoction of the ground leaves is used to massage the abdomen to treat placenta retention (Chifundera, 1998).

Figure 3-5*Ficus lutea* (van Noort and Rasplus, 2004)

3.1.4.6 *Ficus natalensis* Hochstetter (Coastal strangler fig)

This species is very variable in size and habit, from a small to medium-sized tree up to almost 20 m in height and densely leafy, often a strangler; occurring in a variety of habitats. The bark is grey and smooth, often with thin aerial roots hanging down the branches. Leaves are spirally arranged but

tending to be alternate or even sub-opposite, very variable, oblong to narrowly obovate, generally rather small, and dark green, thinly leathery and hairless. Figs are in pairs in the axils of or just below the leaves, hairless and reddish orange (Coates-Palgrave, 2002) (Figure 3-6).

The leaves are used as compresses and poultices for wounds, boils, warts and other growths (Hutchings *et al.*, 1996). Roots are used for colic and when added to another plant, as a snake bite remedy while the bark is used for influenza (Watt and Breyer-Brandwijk, 1962). The fruits and leaves are also used for treatment of pain and venereal diseases (Cousins and Huffman, 2002).

Figure 3-6*Ficus natalensis* (van Noort and Rasplus, 2004)

3.1.4.7 *Ficus polita* Vahl (Heart- leaved fig)

This is a large tree, 10-16 m tall, occurs in evergreen forest, coastal forest and dune forest. The bark is light brown to grey-brown, flaking in small sections, the stem is inclined to the buttressed and the main branches with warty protuberances bear the figs. The leaves are broadly ovate, dark green and glossy above. Figs are in clusters, borne on dwarf knobby branchlets either on the main branches or on the trunk, without hairs, and are yellowish-green to purplish-green in colour when mature (Coates-Palgrave, 2002) (Figure 3-7).

The plant is used for treatment of dyspepsia, infectious diseases, abdominal pains and diarrhoea (Kuete *et al.*, 2011), respiratory disorders and certain skin diseases (Hutchings *et al.*, 1996).

Figure 3-7*Ficus polita* (van Noort and Rasplus, 2004)

3.1.4.8 *Ficus religiosa* L (bo tree or sacred fig)

This is a large or medium sized, evergreen or deciduous tree, 20 m tall and 1.5-2 m diameter, irregularly-shaped, with wide-spreading branches and without aerial roots from the branches. The trunk is regularly shaped, often with low buttresses. The bark is grey with brownish specks, smooth, exfoliating in irregular rounded flakes. Leaves are alternate, spirally arranged and broadly ovate, glossy, coriaceous (leathery), dark green leaves. Figs occur in pairs, and are rounded, flat-topped green, smooth, and ripen to purple with red dots (Warrier *et al.*, 1995; Singh *et al.*, 2011; Makhija *et al.*, 2010) (Figure 3-8).

Ficus religiosa is reported to have numerous therapeutic uses in folk medicine. The leaf juice has been used for the treatment of asthma, cough, sexual disorders, diarrhoea, haematuria, ear-ache and toothache, migraine, eye troubles, gastric problems and scabies (Sirisha *et al.*, 2010). The leaf decoction has been used as an analgesic for toothache; fruits for the treatment of asthma, other respiratory disorders and scabies; stembark is used in gonorrhoea, bleeding, paralysis, diabetes, diarrhoea, bone fracture, and as an antiseptic and astringent (Sirisha *et al.*, 2010).

Figure 3-8*Ficus religiosa*(Warrier *et al.*, 2995)

3.1.4.9 *Ficus sycomorus* L (sycamore fig)

This is a tree of about 5-25 m in height, fine and spreading; frequently occurs along river banks, forming a distinctive part of the riverine thicket, and also in mixed woodland often on termite mounds. The bark is distinctive yellow to greenish yellow, occasionally creamy brown, smooth, powdery or flaking; the stem may develop buttresses in the very large specimens. Leaves are spirally arranged, sometimes almost alternate, large, ovate to elliptic or almost circular, dark green, rough and harsh to the touch. Figs are quite large, up to 3 cm in diameter, and yellowish to reddish in colour (Coates-Palgrave, 2002) (Figure 3-9).

The powdered stembark of *Ficus sycomorus* is soaked in water and the aqueous solution is used in both human and animal medicine. In human it is used for the treatment of ailments such as diabetes (Etuk *et al.*, 2010), mental illness, wound dressing, diarrhoea (Sandabe and Kwari, 2000). It is used to stimulate milk production in cows (Venter and Venter, 1996). A decoction of the bark and latex is used for chest conditions, coughing and scrofula (Venter and Venter, 1996). Leaves are used against jaundice and as an antidote for snake bite while roots are used as an anthelmintic (Venter and Venter, 1996). Inflamed areas are treated with the milky latex and ringworm is treated with the bark and milky latex (Venter and Venter, 1996).

Figure 3-9*Ficus sycomorus* (van Noort and Rasplus, 2004)

3.1.4.10 *Ficus thonningii* Blume (bark-cloth fig)

This species is an evergreen tree about 6-21 m in height, with a rounded to spreading and dense crown. It is sometimes epiphytic, often a strangler; and the trunk is fluted or multi-stemmed. The bark on the young branches is hairy, but smooth and grey on older branches and stems, often with aerial roots hanging down from branches. Leaves are simple, glossy, dark green, thin and papery or slightly leathery. Figs are in leaf axils, sometimes below the leaves and are yellow or red in colour (Coates-Palgrave, 2002) (Figure 3-10).

The bark is important in local medicine, and an infusion of the bark is used in treating diabetes (Etuk *et al.*, 2010), colds, sore throat, dysentery, wounds, constipation, nose-bleed and to stimulate lactation (Venter and Venter, 1996). The latex is used for wound fever, while an infusion of the root and fibre is taken orally to help prevent abortion (Venter and Venter, 1996). The powdered root is taken in porridge to stop nosebleed; and the milky latex is dropped into the eye to treat cataracts. The fruits and leaves are used to treat bronchitis and urinary tract infections (Venter and Venter, 1996).

Figure 3-10*Ficus thonningii* (van Noort and Rasplus, 2004)

3.2 Methods

3.2.1 Preparation of leaf extracts

The powdered plant material (2 g) was individually extracted with 20 ml of acetone, hexane or chloroform (technical grade - Merck) in polyester centrifuge tubes using a platform shaker (Labotec model 20.2 shaking machine) at room temperature for 30 min (Suffness and Douros, 1979; Eloff, 1998c). The extracts were centrifuged at 500 x g for 5 min in a Rotofix 32 A Hettich centrifuge and the supernatant filtered through Whatman No. 1 filter paper. This procedure was repeated three times on the same plant material with fresh solvent to ensure that all possible compounds were exhaustively extracted. The filtered extracts of each species were combined into pre-weighed glass vials and the solvent was left to evaporate at room temperature under a stream of cold air. The percentage yield was expressed in terms of air-dried mass of the leaves. The extracts obtained were used for the subsequent assays.

3.2.2 Phytochemical analysis of extracts

Ten microlitres of extracts, reconstituted to 10 mg/ml in acetone, were loaded onto the TLC plates (Merck, silica gel 60 F_{254}) and elutedwith one of the following solvents (Kotze and Eloff, 2002):

1. Polar/neutral = ethyl acetate: methanol: water (EMW) (10:1.35:1, v/v/v)
2. Intermediate polarity/acidic = chloroform: ethyl acetate: formic acid (CEF) (10:8:2, v/v/v)
3. Non-polar/basic = benzene: ethanol: ammonium hydroxide (BEA) (18:2:0.2, v/v/v)
4. Polar = ethyl acetate: water: formic acid: acetic acid (FAWE) (70:20:3:2 v/v/v) (adapted from Phytomedicine Programme protocol)

Following elution, the developed plates were sprayed with vanillin-sulphuric acid [0.1 g vanillin (Sigma) dissolved in 28 ml methanol and 1 ml sulphuric acid added] and heated for approximately 3 min at 110ºC for optimal colour development. A second set of plates were also sprayed with 0.2% 1, 1-diphenyl-2-picryl-hydrazyl (Sigma) (DPPH) in methanol as an indicator of antioxidant activity (Deby and Margotteaux, 1970).

3.2.3 Total polyphenolic content

The amount of total polyphenolic compounds was determined using the method described by Djeridane *et al.* (2006). This method depends on the reduction of Folin-Ciocalteu reagent (a 2N phosphomolybdic-phosphotungstic reagent) by phenols to a mixture of blue oxides. The commercial reagent was diluted 10-fold with double-distilled water. A standard curve was developed using gallic acid as the standard. Different concentrations of gallic acid were prepared in 80% methanol in a 96-well microtitre plate, and their absorbance was immediately recorded at 760 nm. The crude acetone extracts were dissolved in 80% methanol to a final concentration of 1 mg/ml. The plant extract (100 µl of the 1 mg/ml) was dissolved in 500 µl (1/10 dilution) of the Folin-Ciocalteu reagent. After the addition of 1000 µl of distilled water the mixture was allowed to stand for 1 min at room temperature, where after 1500 µl of a 20% Na_2CO_3 solution was added. The final mixture was shaken, followed by an incubation period of 1h in the dark at room temperature. The absorbance of all samples was measured at 760 nm. All analyses were performed in triplicate and repeated on three different occasions. The results are expressed in mg of gallic acid equivalent (GAE) per gram of dry weight of plant material.

3.2.4 Trolox equivalent antioxidant capacity (TEAC)

The total antioxidant activity was estimated using the trolox equivalent antioxidant capacity (TEAC) test of Re *et al.* (1999). In this assay, the relative capacity of antioxidant to scavenge the $ABTS^{+}$ [2, 2–

azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical compared to the antioxidant potency of trolox (6–hydroxyl–2,5,7,8-tetramethlchromane-2-carboxylic acid), a synthetic water soluble vitamin E analogue, was measured.The $ABTS^+$ radical was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulphate in the dark for 12 - 16 h at room temperature. Prior to assay, the pre-formed $ABTS^+$ radical was diluted in methanol (about 1:89 v/v) and equilibrated to give an initial absorbance (A_i) of 0.700 (±0.02) at 734 nm. The crude acetone extracts were made up in methanol to a concentration of 0.0625 mg/ml to 1 mg/ml and the trolox was made up to a concentration of 0.0625 mg/ml to 0.5 mg/ml in methanol. The crude acetone extract or trolox standard (20 µl) was added to the $ABTS^+$ radical (180 µl) and the mixture allowed to react for 6 minutes, and the absorbance (A) read at 734 nm in a spectrophotometer at one-minute intervals. Appropriate solvent blanks were included in each assay. The percentage change in absorbance was calculated for each concentration of extracts and trolox. To calculate the TEAC, the gradient of the plot of the percentage inhibition of absorbance versus concentration plot for the extract under investigation is divided by the gradient of the plot for trolox (Re *et al.*, 1999). As a result, the TEAC of a substance is a ratio value and has no unit. All assays were carried out in triplicate and repeated three times. The percentage change in absorbance (A) was measured as follows;

$$\%\ \text{change in A} = \left(\frac{A_i\ ABTS^+(734nm) - \text{New mean A}\ ABTS^+}{A_i ABTS^+(734nm)}\right) \times 100$$

3.2.5 α-Amylase inhibition assay

An adapted α-amylase inhibition assay as described by Ali *et al.* (2006) was utilised. The dried crude acetone extracts were re-dissolved in undilutedDMSO to a concentration of 20 mg/ml and used for the α-amylase inhibition assay. Ice cold porcine pancreatic α-amylase solution (200 µl) at 4 unit/ml (type VI) was pre-incubated with 40 µl of crude acetone extracts and 160 µl of distilled water, and mixed in a screw-top plastic tube. The reaction was started by the addition of 400 µl of potato starch (0.5%, w/v) in 20 mM phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride, and thereafter incubated at 25°C for 3 min. Final concentrations in the incubation mixture were plant extract (1 mg/ml), 0.25% (w/v) starch and 1 unit/ml enzyme. An aliquot of the mixture (200 µl) was removed and placed into a separate tube containing 100 µl DNS colour reagent solution (96 mM 3, 5-dinitrosalicylic acid, 5.31M sodium potassium tartrate in 2M NaOH) and placed into an 85°C water bath. After 15 min, this mixture was removed from the water bath, cooled and diluted with 900 µl distilled water. Alpha-amylase activity was ascertained indirectly by measuring the concentration of the non-metabolised starch in the mixture at 540 nm. Control incubations, representing 100% enzyme activity were conducted by replacing the plant extract with undilutedDMSO (40 µl). For the blanks

(negative controls) the enzyme solution was replaced with distilled water and the same procedure was carried out as above. Acarbose was used as positive control (acarbose 1 mg/ml: α-amylase 1 unit/ml). The α-amylase inhibition activity was expressed as;

$$\% \text{ Inhibition} = 100 \times \left(\frac{\Delta A_{Control} - \Delta A_{Sample}}{\Delta A_{Control}}\right)$$

$$\Delta A_{Control} = A_{Test} - A_{Blank}$$

$$\Delta A_{Sample} = A_{Test} - A_{Blank}$$

3.2.6 α- Glucosidase inhibition assay

A commercial rat intestine acetone powderwas purchased and used to prepare enzyme solution according to the method of Oki *et al.* (1999) with slight modifications. In brief, rat intestinal acetone powder (25 mg/ml) was homogenized in ice-cold 50 mM phosphate buffer. After centrifugation at 6 000 x g for 15 min, the clear supernatant obtained was used as the enzyme solution for further analysis.The rat intestinal sucrase inhibitory activity was determined using the method of Bhandari *et al.* (2008), with a slight modification. Sucrose (200 µl of a 56 mM solution) in 0.1 M potassium phosphate buffer (pH 7) was mixed with 100 µl of crude acetone extracts (2.5 mg/ml) in 50% aqueous dimethyl sulphoxide (DMSO) in a test tube. After pre-incubation at 37°C for 5 min, 200 µl of rat intestinal α-glucosidase solution prepared as described above was added. Whereas 100 µl DMSO (50% in water) was used in place of the plant extract to represent 100% enzyme activity, acarbose (0.1 mg/ml) was used as the positive control and for the blanks (negative controls) the enzyme solution was replaced with distilled water and the same procedure was carried out as above.Final concentrations in the incubation mixture for plant extracts were 0.5 mg/ml and 0.02 mg/ml for acarbose.

After thoroughly mixing, the test sample, solvent and positive control test tubes were incubated at 37°C for 20 min and the reaction stopped by adding 750 µl of 2 M Tris–HCl buffer (pH 6.9). The amount of liberated glucose was determined by the glucose oxidase method using a commercial test kit (Sigma GAGO 20) according to the manufacturer's instructions. The absorbance of the wells was measured at 540 nm and the inhibitory activity was calculated using the following formula:

$$\% \text{ Inhibition} = 100 \times \left(\frac{\Delta A_{Control} - \Delta A_{Sample}}{\Delta A_{Control}}\right)$$

$$\Delta A_{Control} = A_{Test} - A_{Blank}$$

$$\Delta A_{Sample} = A_{Test} - A_{Blank}$$

3.2.7 Kinetics of inhibition against α- amylase and α- glucosidase activities

The kinetics of inhibition of the crude acetone extract of *F. lutea* against α- amylase and α-glucosidase activities were measured by increasing substrate concentrations of starch (0.016% - 0.5%) and sucrose (7 mM – 56 mM) respectively in the absence and presence of extract of *F. lutea* at concentrations of 25 - 100 µg/ml for α-amylase inhibitory assay and 62.5 – 250 µg/ml for α-glucosidase inhibitory assay. The type of inhibition was determined by Lineweaver-Burk double reciprocal plot analysis of the data, which was calculated according to Michaelis-Menten equation $\left(v = \frac{V_{max}[S]}{K_m + [S]}\right)$ (Kim *et al.*, 2005; Goutelle *et al.*, 2008). Where v = initial velocity of reaction, V_{max} = the maximum rate of reaction, [S] = substrate concentration and K_m = the substrate concentration at which the rate of the enzymatic reaction is half V_{max} (Goutelle *et al.*, 2008).

3.2.8 Calculation of EC_{50}

The concentration of crude/fractions/compounds that inhibited 50% of the enzyme activity (EC_{50}) was determined for α- amylase and α-glucosidase inhibitory assay in the programme Kinetica 5 (Thermo). The concentration versus response relationship was plotted and fitted to a Hill's model (Goutelle *et al.*, 2008). The model was described by the following equation; $E = E_0 \frac{E_{max} C^{\alpha}}{EC_{50}^{\alpha} + C^{\alpha}}$ where: *E* = predicted effect of the drug, E_0 = baseline response (effect at a drug concentration of 0), E_{max} = maximum drug induced effect, *C* = drug concentration at time t, EC_{50} = plasma drug concentration at which 50% maximal effect is obtained, and α = sigmoidicity factor (Hill exponent). Concentration *C* and EC_{50} are expressed either as µg/ml/mg/ml while α has no unit (Goutelle *et al.*, 2008).

3.2.9 Cytotoxicity assay

3.2.9.1 Vero monkey kidney cells

The acetone extracts of the ten *Ficus* species were screened for cytotoxic activity in human liver C3A and Vero African green monkey kidney cell lines using the MTT assay (Mosmann, 1983; McGaw *et al.*, 2007). Cells were maintained in MEM supplemented with 0.1% gentamicin and 5% FCS. Previous established confluent monolayer were trypsinized for 20 min, and re-suspended in fresh MEM for final seeding at a density of 5.0×10^4 cells/ml into each well (200 µl) of 96-well microtitre plate for the toxicity assays. Wellsof columns 1 and 12 were only filled with growth media (200 µl) to minimise the 'edge effect' and maintain humidity. Following overnight incubation at 37ºC in a 5% CO_2, the cells in the microtitre plates were used for cytotoxicity assay. The media was removed and replaced with 200 µl of fresh media containing plant extracts. Prior to fresh medium with plant extracts, stock solutions of dried crude plant extracts were prepared by reconstitution with undiluted

DMSO to a concentration of 100 mg/ml. This was further diluted with growth mediato tenconcentrations (1 to 1000 µg/ml)and used for the assays. Since DMSO was used as the reconstitution solvent, it was used as solvent (negative) control at concurrent concentrations as for the plant extracts with 1% DMSO in growth media as the highest concentration, while doxorubicin was used as the positive control.The cells were thereafter incubated for 2 days. All extracts were tested in quadruplicate and repeated at a later occasion.

After the above stated incubation period, the medium was removed by aspiration and the wells rinsed with PBS (200 µl) to remove traces of the plant extract, prior to fresh media together with 30 µl of MTT (5 mg/ml in PBS) being placed into each well. After a further incubation period of 4h, the medium was carefully aspirated without disturbing the MTT crystals at the bottom of the wells and replaced with 50 µl of undilutedDMSO. The concentration of MTT reduced was measured at 570 nm after gentle shaking using a microtitre plate reader with a 1cm corrected pathlength (VERSAmax). The wells containing only medium and MTT were used to blank the reader. The percentage cell viability was calculated as the percentage change in MTT concentrations in comparison to the untreated cells. The LC_{50} values were calculated from a plot of log of concentration versus average absorbance represents the concentration of plant extract that resulted in a 50% reduction of absorbance in comparison to the untreated cells.

3.2.9.2 C3A liver cell line

The C3A liver cells are an establishedhuman liver cell lines that have retained some of the drug metabolising enzymes and are used as relevant model for evaluating the cytotoxic effects of products of metabolic alteration of chemicals (Flynn and Ferguson, 2008). The C3A cells were cultured in MEM supplemented with 10% FCS in the absence of antibiotics. Previous established 70-80% confluent monolayer were trypsinized for 20 min, and re-suspended in fresh MEM at a density of 5.0×10^4 cells/ml into a 96-well microtitre plate (200 µl). Hereafter the same method as for the Vero monkey kidney cells was used.

3.2.10 Glucose uptake in primary cell cultures

3.2.10.1 Preparation of rat abdominal skeletal muscle

Rat abdominal muscle was isolated and prepared by modification of the method described by Gray and Flatt (1997). Briefly, 8 week old non-fasting male Sprague-Dawley rats (n=12) (170-250g) supplied by the University of Pretoria Biomedical Research Centre (UPBRC) were euthanized by anisoflurane overdose. Working under sterile conditions, abdominal muscle was excised and placed

into a sterile beaker containing PBS. Muscle tissue was washed twice in 10 ml PBS to remove blood, sectioned into pieces of approximately 10-20 mg and transferred into 20 ml KRB-BSA in 50 ml Schott bottles for gentle agitation at 37°C for 20 min in 5% carbon dioxide in oxygen (carbogen).

3.2.10.1.1 Glucose uptake assay

Each muscle square (20mg) was dispensed into a well of a 48-well microtitre plate containing 450 µl KRB-BSA supplemented with 2mM-sodium pyruvate at glucose concentration of 1 mM. Plant extracts (125 – 2000 µg/ml), insulin (1, 10, 100, 1000 µM; positive control), 10% DMSO in media (solvent control), or medium only (50 µl) was added into wells and incubated at 30°C for 45 min. The final concentrations in the incubation mixture were plant extracts (12.5- 200 µg/ml), insulin (0.1, 1, 10, 100 µM) and 1% DMSO. After the incubation period, the glucose concentration in the medium was determined by the glucose oxidase method using a commercial test kit (Sigma GAGO 20) according to the manual instructions. The absorbance of the wells was measured at 540 nm in a microplate reader (VERSAmax) and the glucose uptake was calculated using the following formula:

$$\%\ \text{Glucose utilised} = 100 \times \left(\frac{\Delta A_{\text{Control (untreated cells)}} - \Delta A_{\text{Sample (treated cells)}}}{\Delta A_{\text{Control (untreated cells)}}}\right).$$

All experiments were carried out in triplicate on each of three different occasions.

3.2.10.2 Isolated rat (epididymal) adipose cells

Isolated adipocytes were obtained under sterile conditions using a method modified from Rodball (1964) and maintained in culture as described by Martz *et al.* (1986) with modification. Briefly, the epididymal fat pads removed under sterile conditions from Sprague-Dawley rats (170-250g) euthanized by anisoflurane overdose, were weighed and rinsed in phosphate buffered saline. Isolated cells were obtained by shaking finely minced tissue in sterile polypropylene containers in an incubator at 37°C for 1 h in DMEM containing 25mM HEPES, collagenase type 11 (1 mg/ml), and albumin (40 mg/ml). After the shaking, cells were filtered through nylon mesh (1000 µm) and centrifuged at 400 x g for 1 min for layered separation. The bottom layer was carefully aspirated from the container to leave only the cells in the top layer. The isolated cells were washed three times in HEPES buffered Kreb-Ringer (KRH) solution, pH 7.0, consisting of 20 mM HEPES, 120 mM NaCl, 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 2.5 mM KCl, 1 mM NaH_2PO_4, 1 mM sodium pyruvate and 1% bovine serum albumin by always removing the bottom layer by aspiration. Penicillin (20 units/ml) and streptomycin (20 mg/ml) were added to all buffers. The cells were finally re-suspended in polypropylene tubes containing the same buffer (4 ml of buffer per 1 g of the original weight) and allowed 40 min to

calibrate at 37°C without shaking. Adipocyte number and viability were determined by trypan blue exclusion.

3.2.10.2.1 Glucose uptake assay

To prepare the primary adipocytes for the glucose uptake assay, the cells were suspended in medium supplemented with 1 mM D-glucose in sterile polypropylene containers and placed in a shaking water bath and incubated at 37°C for 20 min. The cell suspension (200 µl) was plated at a density of 1.0×10^3 cells/well of a 96-well microtitre plate and 50 µl of plant extracts (62.5 – 1000 µg/ml), insulin (0.5, 5, 50, 500 µM; positive control), 5% DMSO in media (solvent control), or media only was added. Final concentrations in the incubation mixture were plant extracts (12.5 – 200 µg/ml), insulin (0.1, 1, 10, 100 µM) and 1% DMSO. The microtitre plates were incubated at 37ºC in a 5% CO_2 for 1 h. After the incubation, the glucose concentration in the medium was determined by the glucose oxidase described above. Each experiment was repeated three times.

3.2.11 Glucose uptake in established cell lines

3.2.11.1 Complete growth medium

The C2C12 myocytes, 3T3-L1 pre-adipocytes and H-4-II-E hepatoma cell lines are used for glucose uptake because they have different glucose transporters. While undifferentiated C2C12 myocytes and 3T3-L1 pre-adipocytes predominantly express GLUT1 glucose transporter that is strictly involved in the regulation of basal glucose transport, the differentiated C2C12 myocytes and the adipocytes express GLUT4 insulin-responsive glucose transporter (Ariga *et al.*, 2008). Thus, differentiation changes the number and composition of GLUT proteins, especially increasing the cellular GLUT4 content, contributing to the developing the insulin-responsive glucose transport system in myotubes and adipocytes (Ariga *et al.*, 2008). The H-4-II-E hepatoma cells predominantly express GLUT2 glucose transporter (Scheepers *et al.*, 2004).

The C2C12 myocytes were maintained in DMEM culture medium supplemented with10% foetal bovine serum (FBS), 4 mM glutamine. The 3T3-L1 pre-adipocytes were maintained in DMEM culture medium supplemented 10% bovine calf serum, 4 mM glutamine. The H-4-11-E hepatoma cells were maintained in MEM culture medium supplemented with 10% FBS, 2 mM glutamine, while the RIN-m5F insulinoma cell were maintained in RPMI-1640 with 2 mM glutamine supplemented with 10% FBS, 10 mM HEPES and 1 mM sodium pyruvate.

3.2.11.2 Culturing of cells

Frozen vials were revived according to ATCC guideline. Each cell lines were revived on different days. The vial containing cells was rapidly thawed under gentle agitation in a 37^oC water bath. The content of vial was transferred to a centrifuge tube containing 9 ml of complete growth medium for each cell type (pre-incubated for 15 min at 37^oC under humidity at 5% CO_2 to avoid excessive alkalinity) and centrifuged at 125 x g for 5-7 min. The supernatant was discarded while the pellet was re-suspended in fresh growth medium (2 ml) of which 1 ml (3.0 x 10^3 - 5.0 x 10^3 cells/ml) was dispensed into T25 flasks containing 9 ml growth medium for incubation at 37^oC in a humidified atmosphere at 5% CO_2. The following day the medium was replaced with fresh medium. Medium was changed every 2-3 days, until the monolayers required trypsinisation for sub-culturing or experiments at approximately 80% confluence. Trypsinisation made use of trypsin-EDTA (3 ml) for 5-10 min, centrifuged at 1500 for 2 min, prior to suspension in fresh growth medium (2ml) for counting.

3.2.11.3 Glucose uptake assay

The amount of glucose uptake was determined by the methods of Yin *et al.* (2008) and Deutschlander *et al.* (2009). The C2C12 muscle myocytes (25,000 cells/ml), the 3T3-L1 pre-adipocytes (30,000 cells/ml) and the H-4-11-E hepatoma cells (30,000 cells/ml) suspended in DMEM supplemented with 0.25% BSA were seeded (200 µl) into wells of 96-well microtitre plates. Only media (200 µl) was added into wells of column 1 and 12 to minimise the "edge effect". After incubation at 37^oC in a 5% CO_2 incubator for 4 days (C2C12 and 3T3-L1) and 2 days (H-4-11-E), the cells in the microtitre plates were used for glucose uptake assay.

Prior to glucose uptake assay, plant extracts were prepared by reconstitution in undiluted DMSO to a concentration of 100 mg/ml which was further diluted with appropriate growth medium before using them for assay. The medium in each of the test wells were removed by aspiration and immediately replaced with 100 µl of growth medium containing plant extracts at concentrations of 15, 31, 63, 125, 250 and 500 µg/ml. The cells were incubated at 37^oC in a 5% CO_2 incubator for 1 h (C2C12), 1½ h (3T3-L1) and 3 h (H-4-11-E) with the various treatments. Insulin (0.1, 1, 10, 100 µM) served as the positive control for the C2C12 and 3T3-L1 cells and was incubated for 1 h and 1½ h respectively while metformin and insulin (0.1, 1, 10, 100 µM) werethe positive control for H-4-11-E cells. Cells treated with metformin were incubated for 24 h. Since DMSO was the solvent of reconstitution, it served as the solvent control in all cases. The final highest DMSO content of plant extracts was 1% and this served as the highest concentration of DMSO used in solvent controls. After incubation, the glucose concentration in the medium was determined by the glucose oxidase method using a commercial test kit (Sigma GAGO 20) as mentioned above.

3.2.12 Insulin secretion assay

The amount of Insulin secretion by RIN-m5F was determined by the method of Persaud *et al.* (1999). The RIN-m5F insulinoma cells (100,000 cells/ml) were suspended in fresh growth medium (RPMI-1640 supplemented with 10% FBS, 10 mM HEPES and 1 mM sodium pyruvate) seeded (200 µl) into 96-well microtitre plate. Only media (200 µl) was added into wells of column 1 and 12 to minimise the "edge effect". Cells were left in the incubator at 37°C in a 5% CO_2 to adhere for 48 h. After 48 h incubation, the medium was removed by aspiration and cells were pre-incubated in a glucose free Kreb-Ringer bicarbonate buffer (135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$ and 1.5 mM $CaCl_2$) pH 7.4 supplemented with 1mg/ml BSA and 10 mM HEPES for 2 h. The medium in test wells of column 3-11 were removed and replaced with 100 µl of glucose free Kreb-Ringer bicarbonate buffer containing plant extract at 4 concentrations (62.5, 125, 250 and 500 µg/ml). Glibenclamide (0.1, 1, 10 µM) was used as positive control and test wells of column 2 were replaced with plain medium (untreated cells control). The cells were incubated at 37°C in a 5% CO_2 incubator for 1 h. The insulin content of supernatants was determined using DRG diagnostic Insulin (Rat) ELISA kit according to the manufacturer's instructions. The assays were run in duplicate and repeated three times.

$$\%\ \text{Insulin secreted} = \left[\left(\frac{\Delta A_{\text{Sample (treated cells)}}}{\Delta A_{\text{Control (untreated cells)}}}\right) \times 100\right] - 100$$

The toxicity of the extract and pure compound to the RIN-m5F pancreatic β-cells after the 2h of exposure was concurrently determinedusing the MTT assay as described for the Vero monkey kidney cells. The percentage cell viability was calculated as the percentage change in MTT concentrations in comparison to the untreated cells.

3.2.13 Solvent-solvent fractionation and isolation of compounds

One and half kilograms of the dried powdered leaves of *F. lutea*were extracted with acetone and concentrated using a rotary evaporator (Büchi R-114). The partially dried extract was allowed to dry at room temperature under a stream of cold air. The weighed dried crude acetone extract was re-dissolved in 50% acetone in water, and successively and exhaustively partitioned (by liquid-liquid extraction) with hexane, chloroform/ dichloromethane, ethyl acetate and n-butanol (in order of increasing polarity). To get an indication of the chemical composition, the fractions were analysed by thin layer chromatography (TLC) using aluminium-backed TLC plates using the following elution system BEA, CEF, EMW and FAWE sprayed with vanillin-sulphuric acid. A second set of TLC were

sprayed with 0.2% 1, 1-diphenyl-2-picryl-hydrazyl (Sigma) (DPPH) to evaluate for antioxidant activity (Deby and Margotteaux, 1970).

3.2.14 Isolation of compounds

Due to highest activity, the ethyl acetate fraction was subjected to a silica gel column chromatography and eluted with an increasing polarity of n-hexane (Hex), ethyl acetate (EtOAc) and methanol (MeOH) to afford 115 fractions of 500 mL each. Fraction 18 eluted with n-hex : EtOAc (85:15) crystallized in the same solvent system to yield compound 1 (23 mg) while a combined fractions 16-19 [n-hex : EtOAc (90:10, 85:15)] and 20-25 [n-hex : EtOAc (80:20)] crystallized also in n-hex:EtOAc (85:15) to afford compound 1 (10 mg) and compound 2 (25 mg), respectively. Fractions 10-30 (1g) eluted with n-hex:EtOAc (95:5) was further subjected to a purification silica gel column chromatography using n-hexane and EtOAc (0 - 100%) to afford 139 fractions of 50 ml each and sub-fraction 76 (23 mg) yield compound 3 (17.4 mg) after preparative TLC (Merck, silica gel 60 F254). Fractions 46-52 eluted with n-hex:EtOAc (70:30) was also subjected to similar silica gel column chromatography as fractions 10-30 followed by preparative TLC to afford compound 4 (15 mg) and compound 5 (21 mg).

3.2.15 General experimental procedures

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with a Bruker spectrometer at 500 MHz and Variant spectrometer at 400 MHz. Chemical shifts (δ) are quoted in parts per million (ppm) from internal standard tetramethylsilane (TMS). Column chromatography was performed on MN silica gel 60 (0.063-0.2 mm / 70-230) mesh. Preparative TLC was performed using high-purity grade powder silica gel (60 A, 2-25 µm) Sigma-Aldrich, Germany. Precolated plates of TLC silica gel 60 F_{254} (Merck, Germany) were used for monitoring fractions and spots were detected with UV light (254 and 365 nm) and then sprayed with 30 % H_2SO_4 followed by heating up to 110 °C.

3.2.16 *In vivo* assay

3.2.16.1 Maintenance of animals

Ethics approval was received from the Animal Use and Care Committee (AUCC) of the University of Pretoria (V060) in compliance with the South African National Standard on the housing and care of laboratory animals. Healthy male CD1 male mice (n = 40) of age 5 weeks old were purchased from Onderstepoort Veterinary Institute (OVI), Pretoria and housed at the University of Pretoria Biomedical Research Centre (UPBRC) in conventional Eurostandard type II cages (Techniplast) at the

temperature of 22°C (± 2°C), controlled relative humidity (50%-60%) in a light/dark cycle of 12 hours. Environmental enrichment, e.g. suspended bells, cardboards and sticks, were provided to keep the mice busy. Animals were housed on cloth bedding for the duration of the study for easy collection of faecal samples. Animals were initially housed in pairs but later single housed due to fighting. The mice had free access to water and standard rodent chow (radiated) for one week during the one week acclimatisation period prior to the introduction of the experimental diets.

3.2.16.2 Experimental design

After acclimatisation, all mice were fed a high caloric diet until obesity was induced (on or before 13 weeks) prior to treatment. The high caloric diet was the standard rodent chow to which fat (42%) and sugar(36%) was added (Table 3-1). Obesity was defined as the animal being 5 g higher than the weight for the species at the specific age (Table 3-2). However, since the mice failed to attain an obese state at the same time, the animals were treated only when they were individually considered obese according to a growth chart (Table 3-2). Hereafter, the mice were randomly assigned (Table 3-3) into one of the four treatment groups below (as coded in Table 3-4) and kept on the diet for the particular treatment plan (Table 3-1) for the next 7 weeks. The treatment groups were based on two treatment plans and their associated controls, and are as follows:

- Treatment Plan 1: High caloric diet intake with ethyl acetate fraction. The following treatment aimed to show that weight loss was possible in obese animals maintained on a high-caloric diet in the presence (Group 2) of ethyl acetate fraction of *F. lutea* extract (1 g/kg) in comparison to the high-caloric diet alone (Group 1). This treatment option was to stimulate the practice of failing to reduce caloric intake in a weight loss programme.

- Treatment Plan 2: Normal caloric diet intake with ethyl acetate fraction. The following treatment aimed to show that weight loss was possible in obese animals maintained on a normal-caloric diet in the presence (Group 4) of the ethyl acetate fraction of *F. lutea* extract in comparison to the normal-caloric diet alone. This treatment option was essentially to stimulate the standard practice of decreasing caloric intake in a weight loss programme.

Table 3-1Nutritional contents of food (g) to induce obesity and after inducement of obesity

Ingredient	High calorie diet (g)	High calorie diet with ethyl acetate fraction (g)	Normal diet (g)	Normal diet with ethyl acetate fraction (g)
Standard rodent chow	60	59.79	210	209.79
Condensed milk (clover)[a]	100	100	-	-
Corn oil	55	55	-	-
Ethyl acetate fraction	-	0.21	-	0.21

[a]The condensed milk contained 8.2g total fat and 57g sugar per 100g milk

3.2.16.3 Preparation of diets

The standard rodent chow was crushed with mortar and pestle, milled to powderin a Kenwood blender, packaged in plastic bag and stored in dry place at 4°C. The food was prepared for each treatment separately. The high calorie diet was prepared by adding the entire ingredient (Table 3-1 and Table 3-5) into a Kenwood mixer and mixing at the lowest speed. The resulting dough was cut into small pieces and moulded into pellets. The normal diet was prepared in the same manner in the absence of the added fat. For fortification, the treatment was prepared by mixing 1 g of ethyl acetate fraction of crude extract of *F. lutea* per kilogram of either high fat or normal diet in a Kenwood mixer prior to moulding. The prepared diets were oven dried at 70°C for 24 hour, allowed to cool, and packaged into labelled plastic bag. The diets were prepared every two days and animal food was changed at the interval of 3 days (every Mondays, Wednesdays and Fridays) to prevent oxidation of the fats and deterioration of the ethyl acetate fraction of *F. lutea* extract.

Table 3-2Weight prediction chart for the CD1 mice

Age (weeks)	Weight	Treatment weight
6	32.9	37.9
7	35.7	40.7
8	37.6	42.6
9	38.3	43.3
10	40.3	45.3
11	41.2	46.2
12	41.8	46.8

Source: Anonymous,2011

Table 3-3Chart for placement of CD1 mice into treatment group once obesity was induced

Order	Code for treatment	Order	Code for treatment	Order	Code for treatment
1	4	13	1	26	2
2	2	14	3	27	1
3	3	15	4	28	1
4	4	16	4	29	4
5	3	17	1	30	2
6	2	18	3	31	4
7	2	19	3	32	2
8	3	20	3	33	2
9	4	21	4	34	2
10	2	22	2	35	4
11	3	23	3	36	4
12	3	25	1	37	1

Table 3-4Codes assigned to the treatments group and the numbers of animals

Code	Treatment group	Total number of animals
1	High caloric diet	6
2	High caloric diet + ethyl acetate fraction	10
3	Normal Diet	10
4	Normal Diet + ethyl acetate fraction	10

3.2.16.4 Body weight, food intake and faecal weight

Body weight, food intake and faecal weight for each mouse were measured every Mondays, Wednesdays and Fridays using a rodentscale (Jadever Snug II precision balance). Body weight was reported as measured on the scale. Food eaten was determined by the difference between place and remaining food. For correctness, any food found in the bottom of the cage on the cloth bedding was collected and weight along with the remaining food. Faecal weight was reported as the amount of faeces on the cloth bedding as measured with the scale.

3.2.16.5 Baseline blood glucose and glucose tolerance test (GTT).

After the induction of obesity and prior to commencement of treatments, aglucose tolerance test was performed per mouse using a single drop of blood collected by needle prick from the tail. Mice were starved for 6 hours prior to the determination of baseline blood glucose levels. After the evaluation ofthe baseline sample, each mouse was treated with a sterile solution (SABAX 50% dextrose) of 2 g of glucose/kg body weight intraperitoneally (i.p). Blood glucose concentrations were sequentially sampled from each animal at 5, 10, 15, 30, 60 and 90 minutes. Blood glucose levels were measured by dropping blood on Contour TS glucose test strips (Bayer) and the blood glucose values displayed on the screen of the glucometer (Bayer, Contour TS) was immediately recorded. The entire process was repeated after 6 weeks of treatment. The area under the plasma glucose versus time graph was estimated using the linear trapezoidal rule and used for comparisons.

3.2.16.6 Clinical pathology

The animals were terminally bled under isoflurane anaesthesia after the 7 weeks of feeding. The haematological parameters evaluated were red blood cell count (RBC or RCC), red cell distribution (RCD), white blood cell count (WBC or WCC), haemoglobin (Hb), haematocrit (ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), neutrophils (neut), eosinophil (eos), basophiles (baso), lymphocytes (lymph), monocytes (mono), platelets count (Plt C) and mean platelet volume (MPV) using which machine. Serum chemistry parameters evaluated were alkaline phosphatase activity (ALP), alanine aminotransferase (ALT), urea, plasma glucose, creatinine, total protein, cholesterol, globulin, albumin and triglycerides using which machines. All parameters were performed using standard laboratory procedures at the clinical pathology laboratory of the Department of Companion Animal Studies, Faculty of Veterinary Science of the University of Pretoria.

3.2.16.7 Pathology

All test mice were submitted for necropsy. All gross pathological changes were recorded for each animal and subsequently microscopy examination of the organs showing evidence of gross pathology in animals was conducted. In addition, the liver, kidneys, pancreas, heart and blood vessel to the legs were collected for histological pathological examination. The selected organs were collected in 10% buffered formalin. Tissue fixation was for 48 hours, thereafter; organs were cut and processed in an automated histological tissue processor according to standard operating procedure of the Section of Pathology, Faculty of Veterinary Science of the University of Pretoria. After tissue processing the wax blocks were sectioned at 6 μm and stained with Haematoxylin and Eosin in an automated histological

stainer and then mounted before histological evaluation was conducted. Histopathological evaluations were undertaken by Dr Botha of IDEXX VetPath.

3.2.17 Statistical analyses

All *in vitro*experiments were performed in triplicate and repeated on three different occasions to yield nine dose-response curves. Statistical analyses of antioxidant activity, total polyphenols content, α-amylase and α-glucosidase inhibitory activities, glucose uptake assays, insulin secretion,body weight, food intake, faecal output as well as haematological and serum chemistry parametersfor high calorie diet, high calorie diet with treatment and normal diet and normal diet with treatment were evaluated by one-way analysis of variance (ANOVA) and considered to be significantly different at p 0.05. Non normal data were log-transformed prior to statistical testing. When significance was found, location of significance was determined by Bonferroni and Tukey HSD multiple comparison*post hoc* tests. The correlation coefficients ($R^{2)}$ between antioxidant, polyphenols, α-amylase and α-glucosidase inhibitory activities, and between viabilityRIN-m5F pancreatic β-cells and insulin secretion were also determined. All analyses were undertaken in SPPS 20 (IBM). Data are presented as the mean ± standard error of mean (S.E.M.). For the glucose tolerance test differences before and after treatment were ascertained using a paired t-test.

Chapter 4

4 Results

4.1 Crude extracts

4.1.1 Extraction of plants

Hexane extracted the lowest mass of material from the leaves of the ten *Ficus* species, while the highest mass was obtained with acetone as the extractant except for *F. cordata*, *F. natalensis* and *F. polita* for which chloroform produced the highest yield (Figure 4-1). In comparison acetone and chloroform had the same yield of extraction. The percentage yield ranged from 2.3% to 3.7% (acetone extracts), 1.6% to 3.5% (chloroform extracts), and 1.1% to 2.3% (hexane extracts). The acetone extract of *F. lutea* had the highest yield (3.7%), followed by *F. polita* (3.2%), while *F. capreifolia* had the lowest yield (2.3%). The chloroform extract of *F. polita* had the highest yield (3.5%), followed by *F. natalensis* (3.0%), while *F. capreifolia* had the lowest yield (1.6%). The hexane extract of *F. polita* had the highest yield (2.3%), followed by *F. natalensis* (2.2%), while *F. capreifolia* resulted in the lowest yield (1.1%).

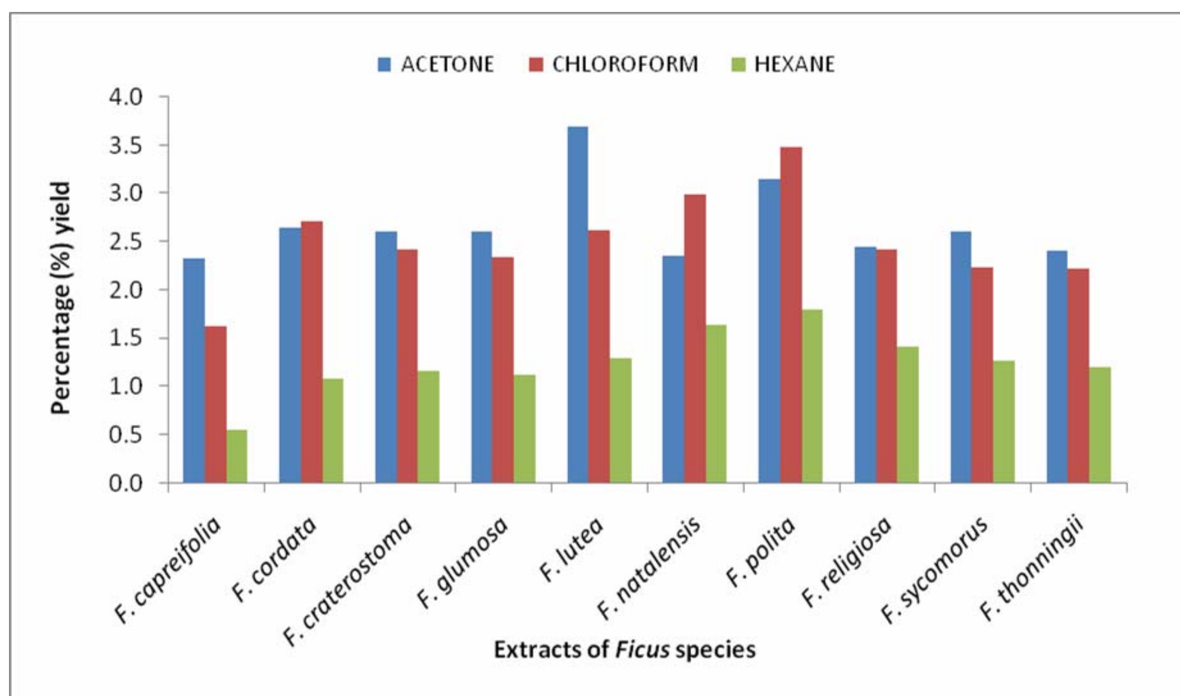

Figure 4-1Percentage extract yield (W/V) calculated as (dry extract weight/dry starting material weight) x 100. Different solvents; acetone, chloroform and hexane were used separately to extract plant material.

4.1.2 Phytochemical analysis

The TLC chromatogram of the ten *Ficus* species sprayed with vanillin-sulphuric acid produced a number of bands (Figure 4-2). The TLC chromatographic profile of the ten *Ficus* species showed many similar compounds within a solvent extractant. A comparison of the chemical profile in the form of bands for the extracts of acetone, chloroform and hexane showed that hexane had the least number of visible bands separated by the different TLC elution systems (BEA, CEF and EMW). Only the acetone extracts were developed in the FAWE eluent system as acetone is known to extract compounds which are poorly separated by the other three eluent systems used.

Acetone extracted compounds with different colours including compounds which turned red when plates were sprayed with acidified vanillin spray reagent. The BEA solvent system gave a better resolution of the compounds present in the crude extracts than CEF, EMW or FAWE. However, some compounds at the base of the plates (red in colour) which the elution systems of BEA and CEF could not resolve, probably because they are highly polar compounds, were resolved with the EMW and FAWE elution systems, with the latter being the superior system. Based on the colour reaction and the polar nature of the compounds it was suspected that these compounds were most likely polyphenolic compounds.

4.1.3 Antioxidant activity

The TLC chromatograms of the ten *Ficus* species were sprayed with 0.2% 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) in methanol as a qualitative indicator of antioxidant activity (Figure 4-3). The antioxidant TLC chromatograms of the ten *Ficus* species detected no activity in the chloroform and hexane extracts in all the elution systems (Figure 4-3). Antioxidant activity was detected in the acetone extracts with all the elution systems. For the BEA and CEF eluents, the compounds exhibiting antioxidant activity were situated at the base of the TLC plate while these compounds were better resolved with the EMW and FAWE elution systems. Antioxidant activity was detected in the acetone extracts of *F. craterostoma*, *F. glumosa*, *F.lutea*, *F. polita* and *F. sycomorus*.

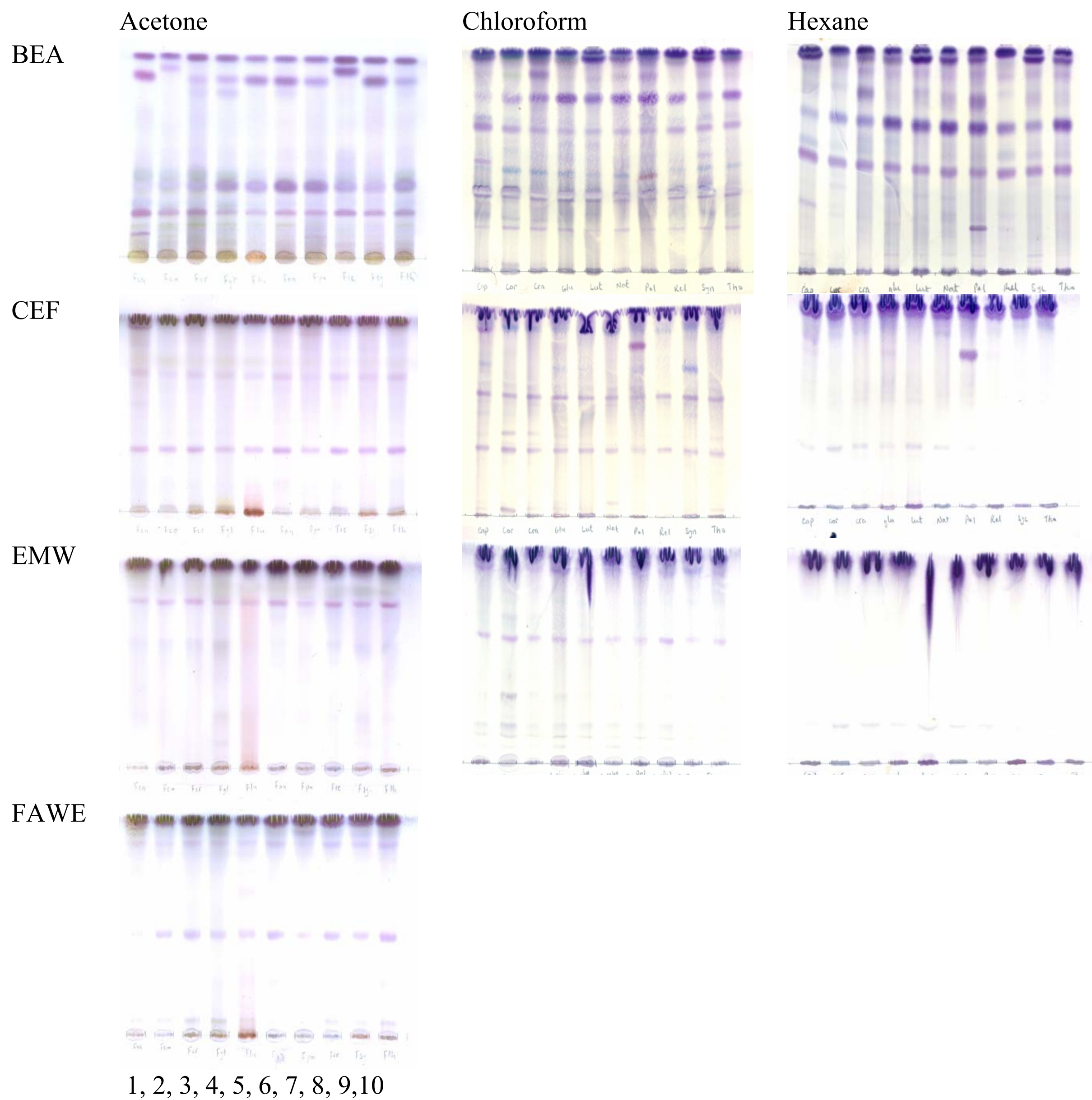

Figure 4-2TLC chromatograms of acetone, chloroform and hexane extracts of ten *Ficus* species, developed with BEA, CEF, EMW and FAWE (for acetone extracts only) sprayed with acidified vanillin to show compounds. Lanes from left to right are *F. capreifolia* (1), *F. cordata* (2), *F. craterostoma* (3), *F. glumosa* (4), *F. lutea* (5), *F. natalensis* (6), *F. polita* (7), *F. religiosa* (8), *F. sycomorus* (9), and *F. thonningii* (10).

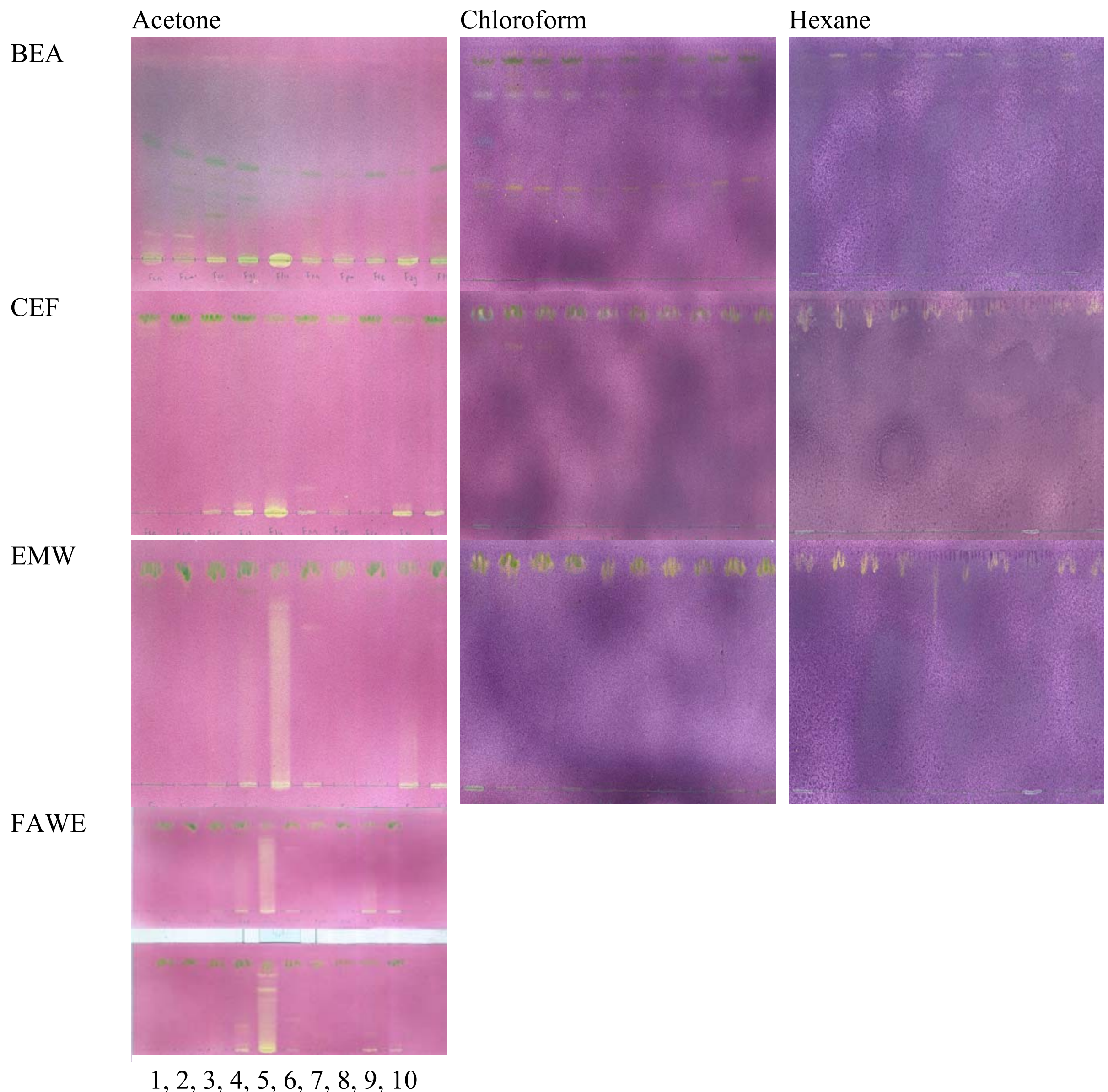

Figure 4-3Antioxidant TLC chromatograms of acetone, chloroform and hexane extracts of different *Ficus* species separated by CEF, EMW and FAWE (for acetone extracts only) sprayed with 0.2% DPPH. Clear zones indicate antioxidant activity. Lanes from left to right are *F. capreifolia* (1), *F. cordata* (2), *F. craterostoma* (3), *F. glumosa* (4), *F. lutea* (5), *F. natalensis* (6), *F. polita* (7), *F. religiosa* (8), *F. sycomorus* (9), and *F. thonningii* (10).

4.1.4 Total polyphenol content and antioxidant activity

The total polyphenol content of the crude acetone extracts of the ten *Ficus* species varied widely (Table 4-1). The total polyphenol content ranged from 4.64 to 56.85 mg GAE/g dry weight of plant. When the total polyphenol content of each extract was compared, the extract of *F. lutea* (56.85 ± 1.82 mg/g) was found to have a significantlyhigher content (*p* 0.001) followed in decreasing order by extracts of *F. glumosa* and *F. sycomorus* with total polyphenol content of 19.24 ± 0.79 and 12.33 ± 0.26 mg GEA/g dry weight of plant respectively. The extracts with the lowest values in decreasing

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order were *F. natalensis, F. capreifolia* and *F. thonningii* with total polyphenolic content of 4.75 ± 0.92, 4.73 ± 0.26 and 4.64 ± 0.28 mg GAE/g dry weight of plant respectively.

Table 4-1Percentage yield, total polyphenol content and antioxidant activityof crude acetone extracts of leaves of ten *Ficus* species

Plants extract	[a]Total polyphenol (mg GAE/g dry weight)	[ab]Antioxidant activity TEAC
Ficus capreifolia	4.73 ± 0.26[c]	0.34 ± 0.05[c]
Ficus cordata	8.23 ± 1.00[d]	0.27 ± 0.03[c]
Ficus craterostoma	9.80 ± 0.93[d]	0.66 ± 0.06[d]
Ficus glumosa	19.24 ± 0.79[e]	1.29 ± 0.30[e]
Ficus lutea	56.85 ± 1.82[f]	4.80 ± 0.90[f]
Ficus natalensis	4.75 ± 0.92[c]	0.69 ± 0.08[d]
Ficus polita	8.04 ± 0.52[d]	0.31 ± 0.06[c]
Ficus religiosa	5.40 ± 0.35[c]	0.59 ± 0.18[c]
Ficus sycomorus	12.33 ± 0.26[e]	1.91 ± 0.19[e]
Ficus thonningii	4.64 ± 0.48[c]	0.77 ± 0.06[d]

[a]Values are means (n=9) ± standard error;
[b]Antioxidant activity (Trolox equivalent antioxidant capacity); [c,d,e,f]No significant difference between extracts with same value, but significant difference p 0.05 between different values.

The antioxidant activities of the extracts, expressed as trolox equivalent antioxidant capacity (TEAC) are presented in Table 4-1. The crude acetone extracts of the ten *Ficus* species had different antioxidant capacities (Table 4-1). The total antioxidant activity for the ten *Ficus* species varied widely from 4.80 ± 0.90 to 0.27 ± 0.03 TEAC under the assay conditions. The antioxidant activity of the extract of *F. lutea* (4.80 ± 0.90 TEAC) was significantly different (p 0.001) when compared with that of the other extracts, followed by *F. sycomorus* and *F. glumosa* in decreasing order with antioxidant activity of 1.91 ± 0.18 and 1.29 ± 0.30 TEAC respectively. The extracts with the lowest TEAC in decreasing order were *F. capreifolia*, *F. polita* and *F. cordata* with 0.34 ± 0.05, 0.31 ± 0.06 and 0.27 ± 0.03 TEAC respectively.

4.1.5 α-Amylase inhibitory activity of extracts of *Ficus* species

The crude acetone extracts of the ten *Ficus* species all demonstrated a potential to inhibit the α-amylase enzyme system (Table 4-2). The extract of *F. lutea* had the highest inhibitory potential with 95.4 ± 1.2% inhibition at concentration of 1 mg/ml. This was followed by the extract of *F. glumosa*

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with65.1 ± 3.0% inhibition at the same concentration while acarbose, the positive control, had 96.7 ± 0.3% inhibition at a concentration of 0.04 mg/ml. The extracts of all the other *Ficus* species moderately inhibited α-amylase activity between 40% and 45%, except for the extracts of *F. religiosa* and *F. thonningii* with low inhibitory activities of 35.3 ± 2.8% and 37.6 ± 2.7% respectively.

Table 4-2The percentage inhibition of α-amylase activity (1 ml/mg) and concentration leading to 50% inhibition (EC_{50}) of crude acetone extracts of the ten *Ficus* species

plant	(%) α- amylase inhibition[a]	EC_{50} (µg/ml)
Ficus capreifolia	43.8 ± 3.3[b]	100
Ficus cordata	45.9 ± 5.2[b]	100
Ficus craterostoma	48.3 ± 1.6[b]	11.41 ± 4.68[b]
Ficus glumosa	65.1 ± 3.0[c]	100
Ficus lutea	95.4 ± 1.2[d]	9.42 ± 2.01[b]
Ficus natalensis	43.7 ± 1.8[b]	17.85 ± 4.42[b]
Ficus polita	40.2 ± 2.6[b]	100
Ficus religiosa	35.3 ± 2.8[b]	100
Ficus sycomorus	40.0 ± 2.8[b]	100
Ficus thonningii	37.6 ± 2.7[b]	100
Acarbose	96.7 ± 0.3	0.04 ± 0.03

[a]% α-amylase inhibitory activity of crude acetone extracts of ten South African *Ficus* species (1 mg/ml) and control (acarbose) (0.04 mg/ml). Results expressed as % mean ± SEM (n=9).[b,c,d]No significant difference between extracts with same value, but significant difference *p* 0.05 between different values

The concentration of crude acetone extracts of the ten *Ficus* species and acarbose leading to inhibition of 50% of α-amylase activity (EC_{50}) is presented in Table 4-2. The extract of *F. lutea* was the most potent inhibitor with an EC_{50} of 9.42 ± 2.01 µg/ml followed by *F. craterostoma* (EC_{50} = 11.41 ± 4.68 µg/ml) and *F. natalensis* (EC_{50} = 17.85 ± 4.42 µg/ml) with no significant difference (*p* 0.05) among them, and acarbose with a value of 0.04 ± 0.03 µg/ml. The EC_{50} values for the extracts of the other *Ficus* species were above 100 µg/ml and therefore had limited anti α-amylase activity.

The correlation coefficient between total polyphenolic content and inhibition of α-amylase activity by the crude acetone extracts of the ten *Ficus* species R^2 was 0.81 (Figure 4-4). This indicates that the inhibition of α-amylase activity by the crude acetone extracts of the ten *Ficus* species may be due to the total polyphenolic content. Conversely, the correlation coefficient between antioxidant activity (TEAC) and inhibition of α-amylase activity by the crude acetone extracts of the ten *Ficus* species R^2

was 0.46 (Figure4-5). This indicates that the compound(s) responsible for inhibition of α-amylase activity by the crude acetone extracts of the ten *Ficus* species is most likely not be responsible for the antioxidant activity although there may be some of overlap.

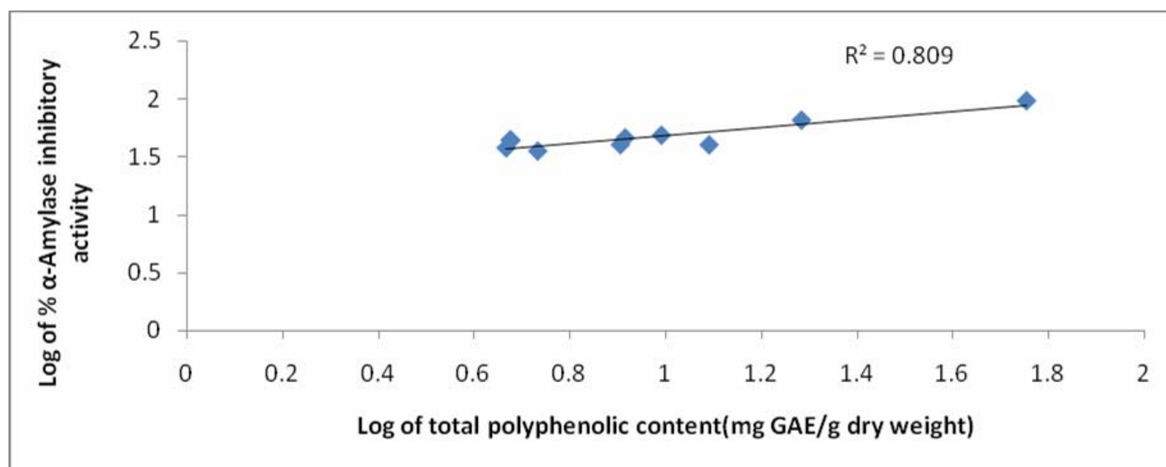


Figure 4-4The correlation between log of percentage α-amylase inhibitory activity and log of total polyphenolic contents (mg gallic acid equivalent/g dry weight of sample) of acetone extracts from the ten South African *Ficus* species.

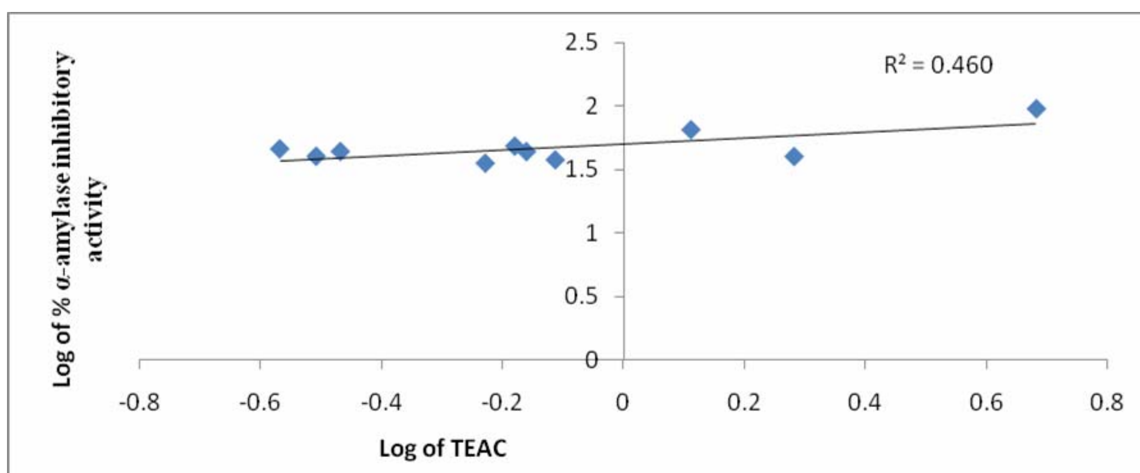


Figure4-5The correlation between log of percentage α-amylase inhibitory activity and the log of TEAC of acetone extracts from the ten South African *Ficus* species.

4.1.6 α-Glucosidase inhibitory activity of extracts of *Ficus* species

The crude acetone extracts of the ten *Ficus* species were generally weak inhibitors of α-glucosidase (Table 4-3). All the extracts of the ten *Ficus* species, except for the extract of *F. lutea*, inhibited α-glucosidasewith inhibitory activity below 40% under the assay conditions. The extract of *F. lutea* had the highest inhibitory activity of 64.3 ± 3.6% at a concentration of 0.5 mg/ml and was significantly

different (p 0.05) from the other extracts. The extracts of *F. glumosa* and *F. sycomorus* inhibited α-glucosidase activity by 38.7 ± 6.5% and 35.7 ± 5.4% respectively. The extract of *F. religiosa* inhibited α-glucosidase activitythe least (17.6 ± 8.0%). Acarbose (positive control) potently inhibited α-glucosidase activity (84.8 ± 1.7%) at a concentration of 0.02 mg/ml.

The concentration of extracts of the ten *Ficus* species and acarbose leading to inhibition of 50% of α-glucosidase activity (EC_{50}) is presented in Table 4-3. The extract of *F. sycomorus* was more potent than all the other extracts with an EC_{50} of 217 ± 69 µg/ml, followed closely by the extract of *F. lutea* (290 ± 111 µg/ml), with no significant difference between them (p 0.05). The EC_{50} of acarbose was 3.4 ± 0.5 µg/ml.

Table 4-3The percentage inhibition of α- glucosidase activity (0.5 ml/mg) and concentration leading to 50% inhibition (EC_{50}) of crude acetone extracts of ten *Ficus* species

plant	(%) α- glucosidase inhibition[a]	EC_{50} (µg/ml)
Ficus capreifolia	24.3 ± 1.7[b]	1000
Ficus cordata	22.0 ± 3.6[b]	1000
Ficus craterostoma	28.2 ± 7.0[b]	1000
Ficus glumosa	38.7 ± 6.5[b]	1000
Ficus lutea	64.3 ± 3.6[c]	290 ± 111[b]
Ficus natalensis	23.6 ± 8.1[b]	1000
Ficus polita	29.2 ± 5.8[b]	1000
Ficus religiosa	17.6 ± 8.0[b]	1000
Ficus sycomorus	35.7 ± 5.4[b]	217 ± 69[b]
Ficus thonningii	25.3 ± 5.0[b]	1000
Acarbose	84.8 ± 1.7	3.4 ± 0.5

[a]% α-glucosidase inhibitory activity of crude acetone extracts of ten South African *Ficus* species (0.5 mg/ml) and control (acarbose) (0.02 mg/ml). Results expressed as % mean ± SEM (n=9);
[b,c] No significant difference between extracts with same value, but significant difference p 0.05 between different values

The correlation coefficient between total polyphenolic content and inhibition of α-glucosidase activity by the crude acetone extracts of the ten *Ficus* species R^2 was 0.85 (Figure 4-6). This indicates that the inhibition of α-glucosidase activity by the crude acetone extracts of the ten *Ficus* species may be ascribed in large part to the total polyphenolic content.In addition, the correlation coefficient between antioxidant activity (TEAC) and inhibition of α-glucosidase activity by the crude acetone extracts of the ten *Ficus* species R^2 was 0.67 (Figure 4-7). This indicates that the compound(s) responsible for the

inhibition of α-glucosidase activity by the crude acetone extracts of the ten *Ficus* species may possible be responsible for the antioxidant activity.

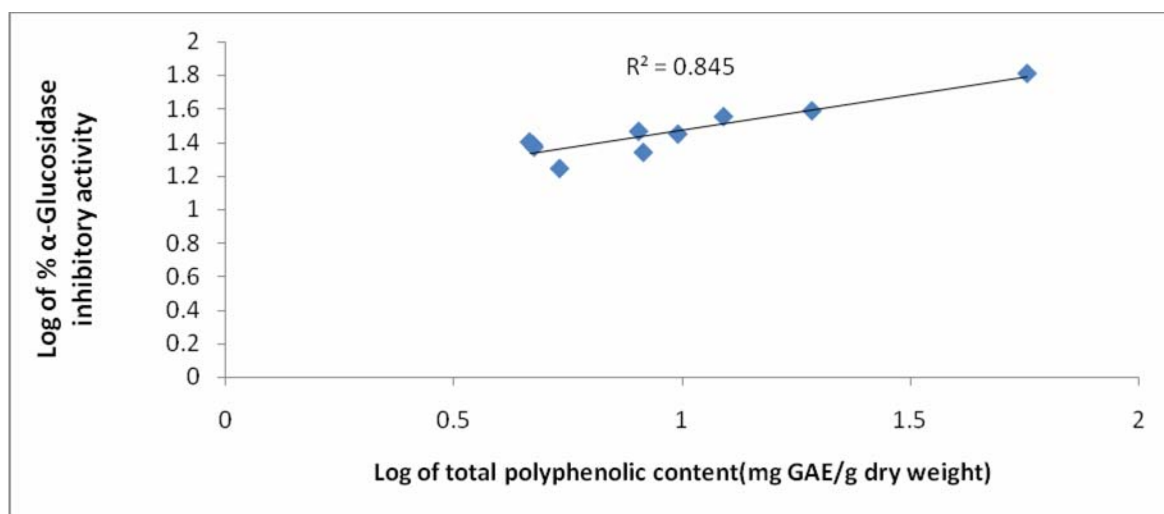


Figure 4-6The correlation between log of percentage α-glucosidase inhibitory activity and log of total polyphenolic contents (mg gallic acid equivalent/g dry weight of sample) of acetone extracts from ten South African *Ficus* species.

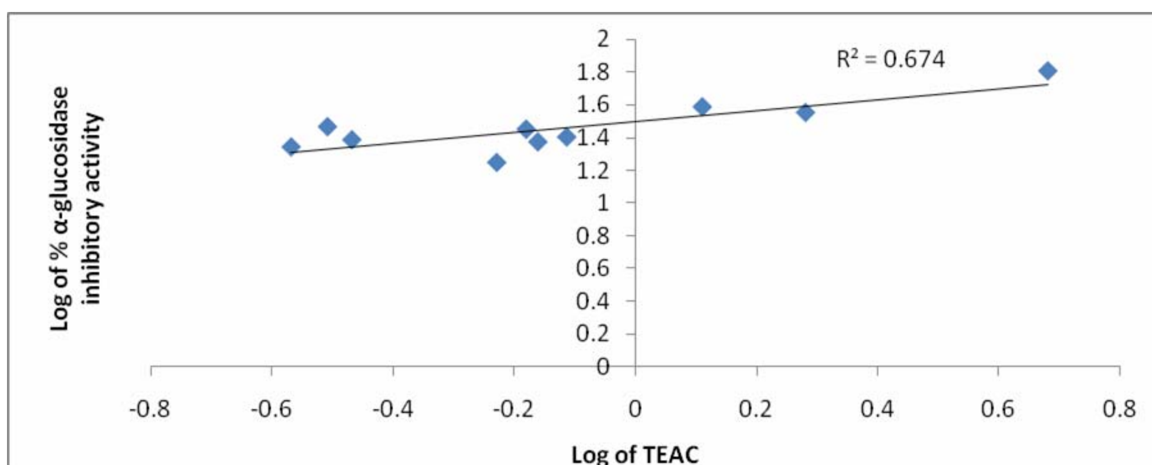


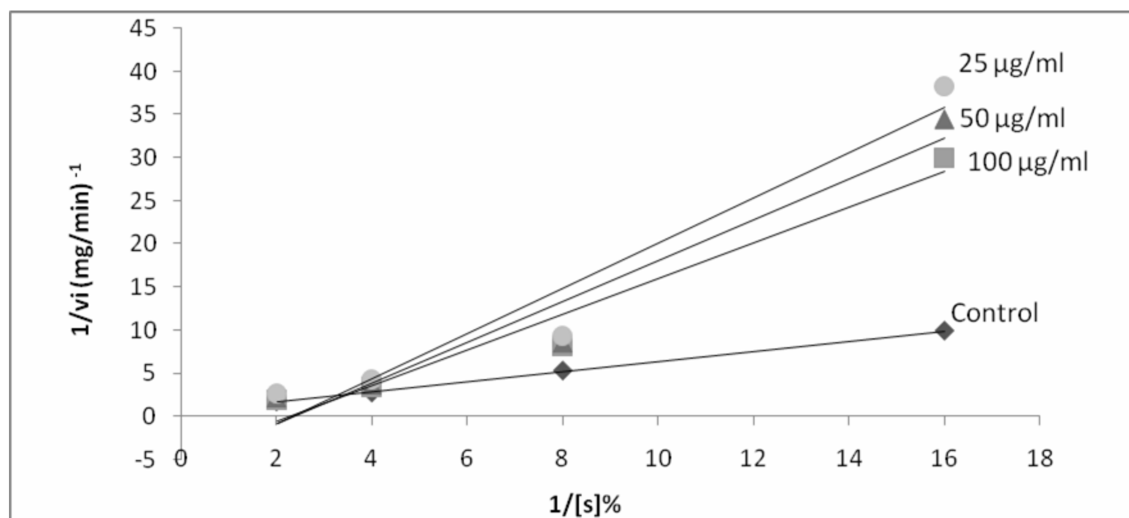
Figure 4-7The correlation between log of percentage α-glucosidase inhibitory activity and the log of TEAC of acetone extracts from the ten South African *Ficus* species.

4.1.7 The enzyme kinetics of α- amylase and α- glucosidase inhibition by extract of *F. lutea*

Analysis of the α-amylase and α-glucosidase kinetics by the crude acetone extract of *F. lutea* is shown in Figure 4-8. For the α-amylase (Figure 4-8A) and α-glucosidase (Figure 4-8B) inhibition by *F. lutea*, the intersection of the double reciprocal plot was seated at a point above the +1/[s] axis, indicating that *F. lutea* acts as a partially non-competitive-type inhibitor of α-amylase and α-glucosidase, indicating

that the active chemical(s) within the plant function was due the chemical inactivation of the enzyme, most likely via protein precipitation.

(A)



(B)

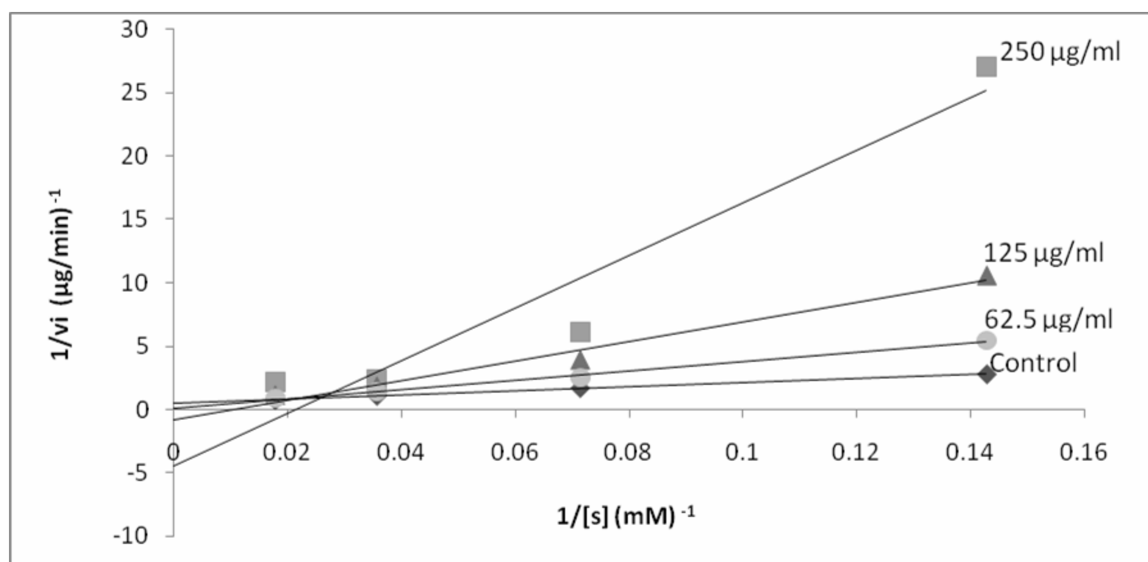


Figure 4-8Lineweaver-Burk double reciprocal plots for kinetic analysis analysis of enzyme reactions.

(A) The inhibition of porcine pancreatic α-amylase. The reciprocal of initial velocity (1/vi) of substrate (starch) versus reciprocal of different substrate concentration (%) (1/ [S]).(B) Inhibition of rat intestinal α-glucosidase.The reciprocal of initial of initial velocity (1/vi) of substrate (sucrose) versus reciprocal of different substrate concentration (mM^{-1}) (1/ [S]) in the absence and presence of different concentrations of extract of *Ficus lutea.*

4.1.8 Cytotoxicity of the acetone extracts of the ten *Ficus* species

The cytotoxic effect of the acetone extracts of the ten *Ficus* species against the Vero monkey kidney and C3A human liver cell lines are presented in Table 4-4. The extracts were less cytotoxic against the

Vero monkey kidney and C3A cell lines when compared to doxorubicin, the positive control. For the Vero kidney cells, the extract of *F. craterostoma* (356.2 ± 9.6 µg/ml), *F. lutea* (214.8 ± 5.0 µg/ml) and *F. religiosa* (110.9 ± 8.2 µg/ml) werethe least toxic based on their LC_{50}in parenthesis while the extract of *F. thonningii* (68.0 ± 1.0 µg/ml) and *F. natalensis* (69.2 ± 8.0 µg/ml) was the most toxic based on their LC_{50}in parenthesis. For the C3A liver cells, the LC_{50} for the extract of *F. craterostoma* (> 1000 µg/ml) and *F. religiosa* (922.9 ± 4.7 µg/ml) are the least toxic in decreasing order while the LC_{50} for the extract of *F. polita* (44.8 ± 1.8 µg/ml) was the most toxic. In all assays, the extract of *F. polita* was relatively toxic, while the extract of *F. craterostoma* was the least toxic. All the *Ficus* species, except extracts of *F. lutea* and *F. polita,* were more toxic to the Vero monkey kidney cell line than the C3A cell line.

Table 4-4Cytotoxicity activity of acetone extracts of the *Ficus* species (LC_{50} in µg/ml ± SE)

Plant	Vero kidney cells	C3A liver cells
F. capreifolia	85.3 ± 2.0	108.4 ± 0.8
F. cordata	76.7 ± 1.4	166.3 ± 1.9
F. craterostoma	356.2 ± 9.6	1000
F. glumosa	72.7 ± 9.2	127.6 ± 2.6
F. lutea	214.8 ± 5.0	126.0 ± 6.8
F. natalensis	69.2 ± 8.0	113.8 ± 7.4
F. polita	90.9 ± 1.4	44.8 ± 1.8
F. religiosa	110.9 ± 8.2	922.9 ± 4.7
F. sycomorus	101.8 ± 1.1	151.6 ± 4.3
F. thonningii	68.0 ± 1.0	491.4 ± 9.9
Doxorubicin	17.0 ± 0.1	6.7 ±0.6

Values are means of triplicate determinations done three times (n=9) ± standard error.

4.1.9 Glucose uptake activity in primary rat abdominal muscle culture

The effect of the acetone extracts of the ten *Ficus* species on glucose uptake in primary rat abdominal primary muscle culture at 1 mM glucose concentration is presented in Figure 4-9. The extract of *F. lutea* significantly (p 0.001) induceda dose related glucose uptake in muscle culture with the highest glucose uptake of 10.8 ± 1.8% at a concentration of 200 µg/ml. This was followed by extracts of *F. thonningii* (8.1 ± 0.7%), *F. natalensis* (7.0 ± 7.7%) and *F. glumosa* (5.9 ± 2.7%) at the same concentration. The extracts of the other *Ficus* species did not enhanced glucose uptake in muscle cell culture in that the extracts had values lower than that of the solvent control (DMSO) (5.7 ± 1.4%). Insulin, the positive control significantly (p 0.001) enhanced glucose uptake (35.7 ± 1.0%) in muscle

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culture at the highest concentration of 100 µM. Only the extract of *F. lutea* and insulin enhanced concentration dependent glucose uptake in the primary muscle cells.

4.1.10 Glucose uptake in primary rat epididymal fat cells

The effect of the acetone extracts of the ten *Ficus* species on glucose uptake in rat epididymal fat cells at 1 mM glucose concentration is presented in Figure 4-10. The extracts of *F. lutea* and *F. glumosa*significantly (*p* 0.001) induced a dose related glucose uptake in the primary fat cell culture with the highest glucose uptake of 32.0 ± 8.4% and 31.6 ± 5.7% respectively for the extracts of *F. lutea* and *F. glumosa*at the highest concentration of 200 µg/ml. The extracts of the other *Ficus* species did not enhance glucose uptake in the primary fat cell culture in that the extracts had values lower than that of the solvent control (DMSO) (20.6 ± 4.2%). The extract of *F. thonningii* did not enhance glucose uptake in the primary fat cell culture at all concentrations tested. Insulin, the positive control significantly (*p* 0.001) enhanced dose related increase in glucose uptake with the highest uptake of 82.2 ± 2.0% resulting at a concentration of 100 µM.

4.1.11 Glucose uptake activity in C2C12 muscle cells

The effect of the acetone extracts of the ten *Ficus* species at different concentrations (15 µg/ml – 500 µg/ml) on glucose uptake in C2C12 muscle cells is presented in Figure 4-11. Only the extract of *F. lutea*, in a dose related manner, significantly (*p* 0.001) enhanced glucose uptake in C2C12 muscle cells with the highest glucose uptake of 14.9 ± 2.3% at the highest concentration (500 µg/ml). The extract of *F. lutea* did not, however, enhance glucose uptake in C2C12 muscle cells at concentrations below 63 µg/ml. Although the extracts of the other species failed to enhance glucose uptake at 500 µg/ml in that they had values lower than DMSO, the positive control (1.8 ± 0.3%), some species did show some activity at the lower concentrations. The extract of *F. glumosa* enhanced glucose uptake of 4.5 ± 2.6% at the concentration of 250 µg/ml and the extract of *F. thonningii* enhanced glucose uptake of 3.3 ± 4.1% at the concentration of 125 µg/ml. The extract of *F. sycomorus*did not enhance glucose uptake in C2C12 muscle cells at all of concentrations tested. Insulin, the positive control significantly (*p* 0.001) enhanced glucose uptake in C2C12 muscle cells with the highest uptake of 19.1 ± 3.7% at the concentration of 10 µM.

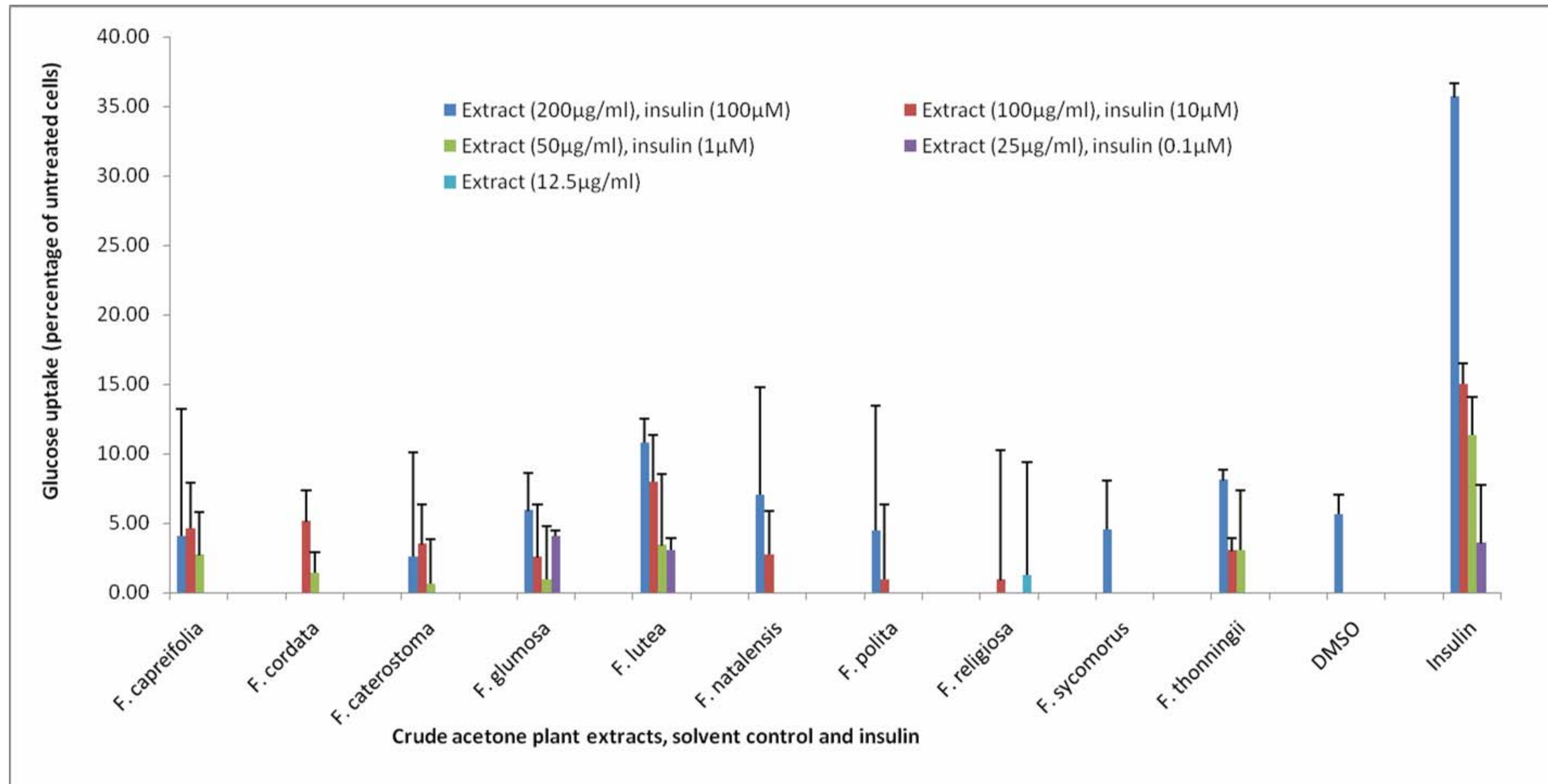

Figure 4-9Glucose uptake in rat abdominal primary muscle culture (as percentage of untreated control cells ± standard error of mean, n=9) exposed to the acetone extracts of the ten *Ficus* species and insulin at 1 mM glucose concentration.

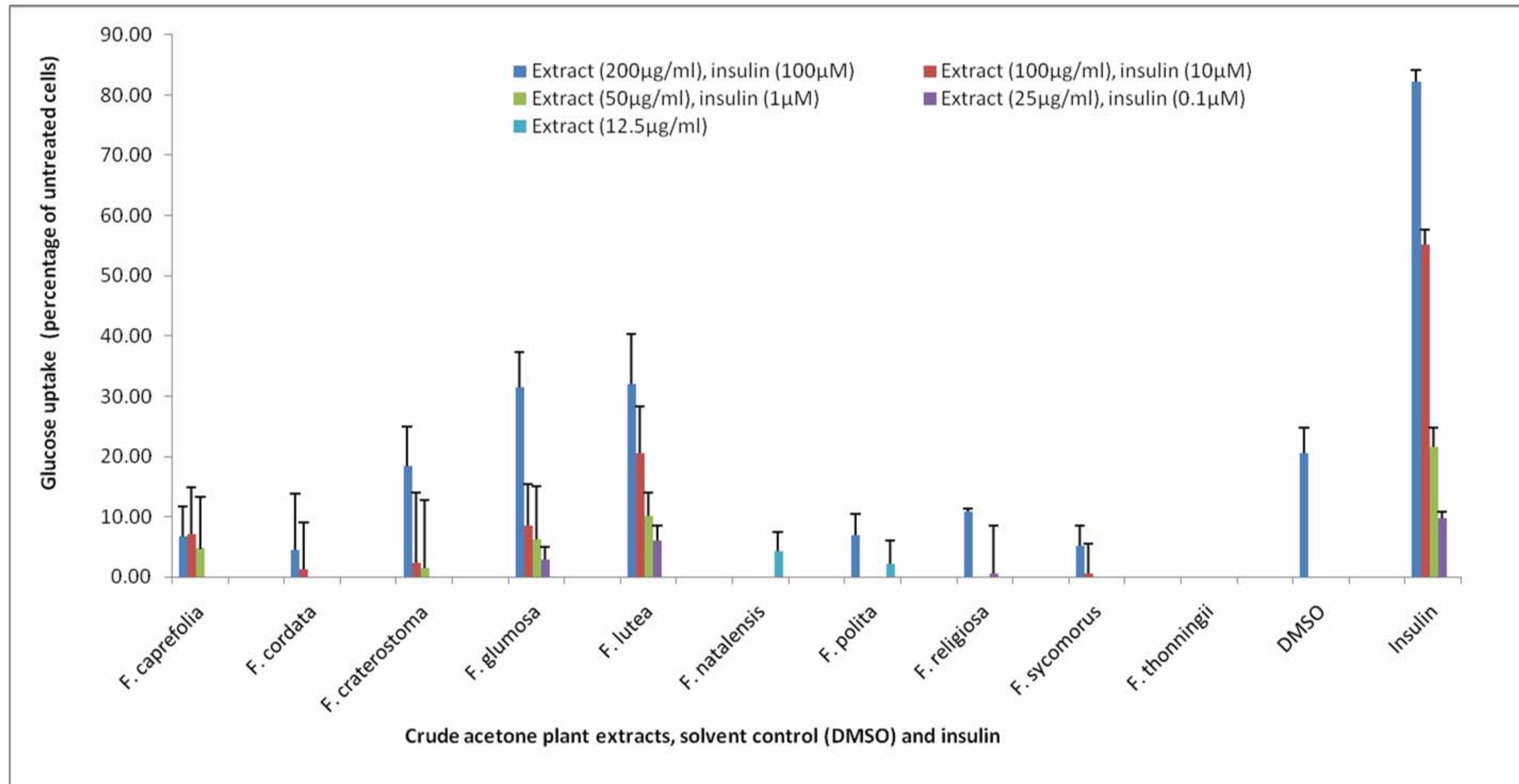

Figure 4-10Glucose uptake in rat epididymal primary fat cell culture (as percentage of untreated control cells ± standard error of mean, n=9) exposed to the acetone extracts of the ten *Ficus* species and insulin at 1 mM glucose concentration.

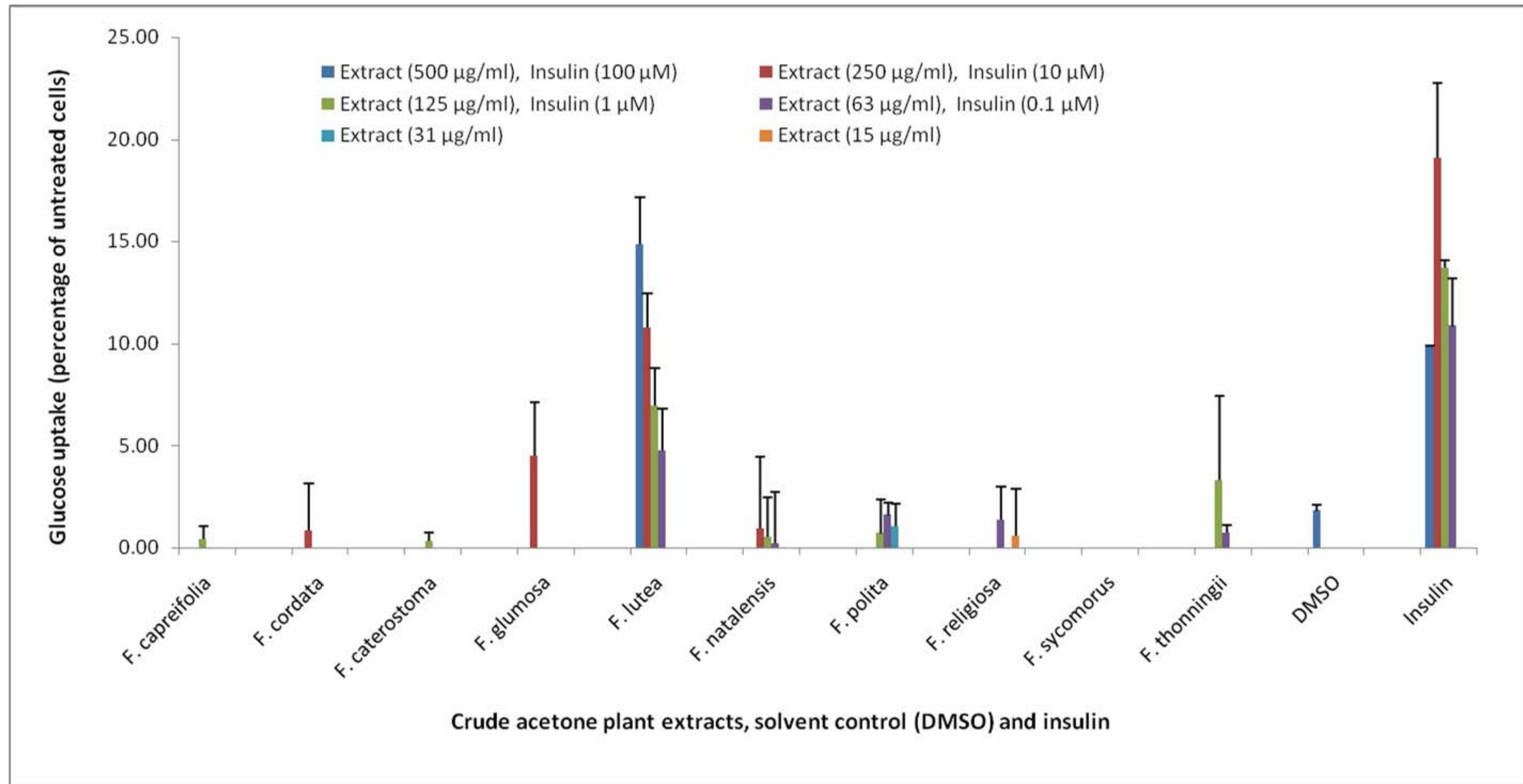

Figure 4-11Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells± standard error of mean, n=9) exposed to the acetone extracts of the ten *Ficus* species and insulin.

Since the extract of *F. lutea* enhanced the highest uptake of glucose in C2C12 muscle cells, the effect of the extract on *F. lutea*(at different concentrations; 15 µg/ml – 500 µg/ml) on glucose uptake in C2C12 muscle cells in the presence of insulin at two different concentrations (1 µM and 10 µM) was investigated. The result of insulin-mediated glucose uptake in C2C12 muscle is presented in Figure 4-12. Insulin at the two concentrations tested significantly (*p* 0.001) influenced the glucose uptake in the C2C12 muscle cells. The medium containing the extract of *F. lutea* in the presence 1 µM and 10 µM insulin significantly (*p* 0.05) increased the glucose uptake in the C2C12 muscle cells (19.5 ± 0.7% and 20.8 ± 1.6% respectively) at the highest concentration of 500 µg/ml in comparison to the insulin control (14.9 ± 2.3%). For the uptake of glucose at the other concentrations of the *F. lutea* extract, the results were mixed.

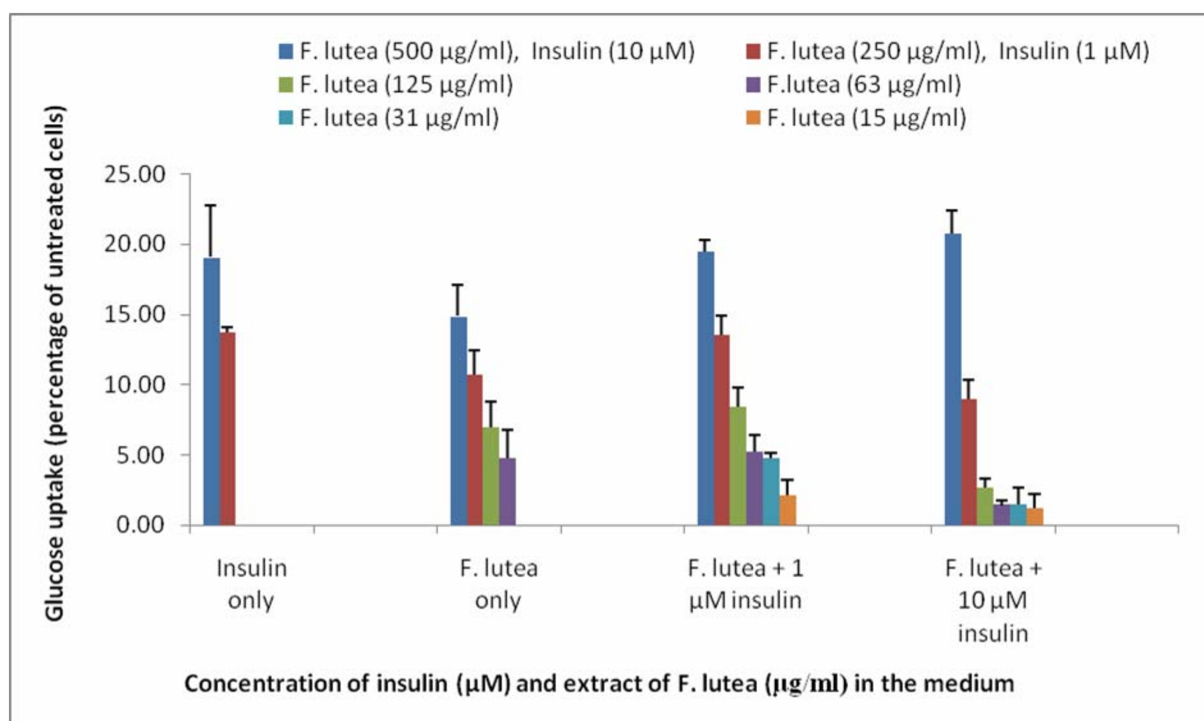

Figure 4-12Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells ± standard error of mean, n=9) exposed to the acetone extract of *F. lutea* in medium containing different concentrations of insulin (represented by the different colours) in the presence of different concentrations of insulin (x-axis).

4.1.12 Glucose uptake activity in H-4-11-E liver cells

The effect of the acetone extracts of the ten *Ficus* species at different concentrations (15 µg/ml – 500 µg/ml) on glucose uptake in H-4-II-E liver cells is presented in Figure 4-13. The extract of *F. lutea* did not significantly enhanced glucose uptake in H-4-II-E liver cells with the highest glucose uptake of glucose being only 19.3 ± 0.6% at the concentration of 500 µg/ml. None of the other concentrations enhanced glucose uptake in that their values were lower than for DMSO, the solvent control (3.4 ±

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1.8%). The extracts of *F. cordata* and *F. thonningii* enhanced glucose release rather than uptake in the H-4-II-E liver cells (values below 0%) at all the concentrations tested. Metformin and insulin were used as positive controls. Metformin had a maximum glucose uptake of 18.1 ± 0.6% at the highest concentration of 100 µM while insulin significantly (p 0.001) enhanced glucose uptake in the liver cells with maximum glucose uptake of 17.7 ± 2.1% at the concentration of 10 µM.

Since the extract of *F. lutea* enhanced the highest glucose uptake in H-4-II-E liver cells, the effect of the extract on *F. lutea*(at different concentrations; 15 µg/ml – 500 µg/ml) on glucose uptake in H-4-II-E liver cells in the presence of insulin at two different concentrations (1 µM and 10 µM) was investigated. The result of insulin-mediated glucose uptake in H-4-II-E liver cells is presented inFigure 4-14. Insulin at the two concentrations tested significantly (p 0.001) influenced the glucose uptake in the H-4-II-E liver cells exposed to the extract of *F. lutea*. The medium containing insulin at 1 µM (p 0.05) and 10 µM (p 0.001) significantly enhanced glucose uptake in H-4-11-E liver cells (21.8 ± 1.6% and 24.6 ± 1.7% respectively) in the presence of the extract of *F. lutea* at 500 µg/ml in comparison to the *F. lutea* alone (19.3 ± 0.6%). The effect for the other doses was variable with no describable dose response interaction being present.The medium containing 10 µM insulin also increased glucose uptake in H-4-11-E liver cells exposed to the extract of *F. lutea* at the concentrations of 31 µg/ml and 15 µg/ml (3.2 ± 2.5% and 2.3 ± 2.3% respectively).

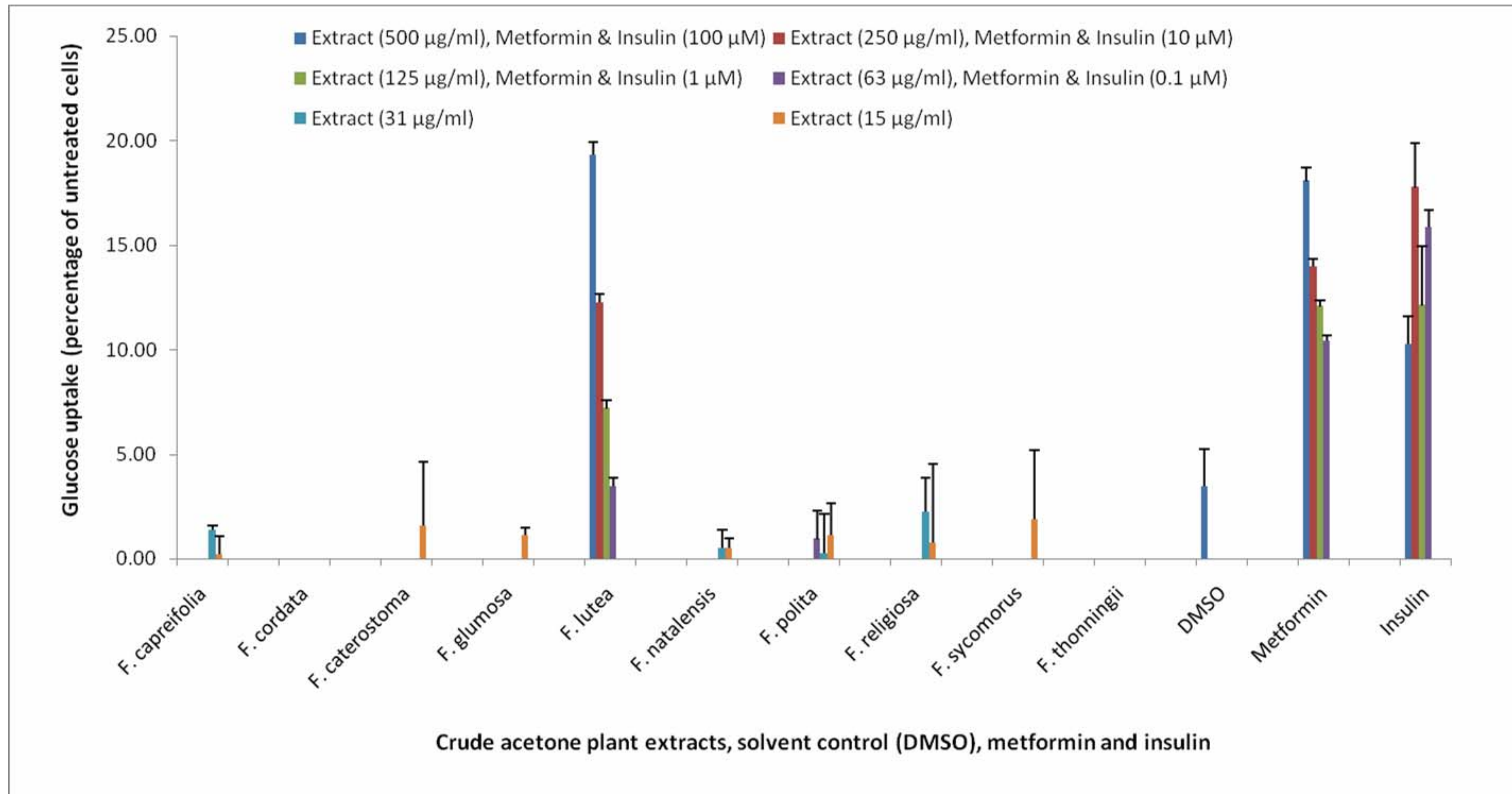

Figure 4-13Glucose uptake in H-4-11-E rat liver cells (as percentage of untreated control cells ± standard error of mean, n=9 for) exposed to the acetone extracts of the ten *Ficus* species, metformin and insulin.

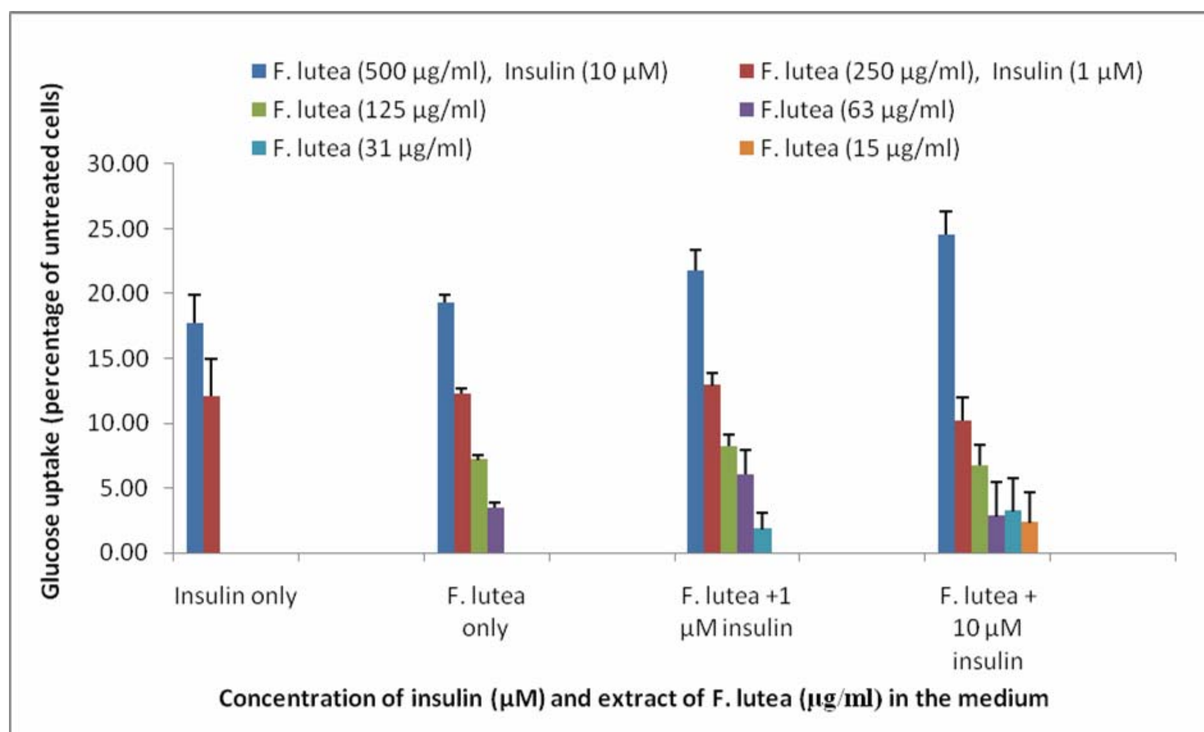

Figure 4-14Glucose uptake in H-4-11-E liver cells (as percentage of untreated control cells ± standard error of mean, n=9) exposed to the different concentrations of the acetone extract (represented by the different colours) of *F. lutea* in medium containing different concentrations of insulin (x-axis).

4.1.13 Glucose uptake in 3T3-L1 pre-adipocytes

The effect of acetone extracts of the ten *Ficus* species at different concentrations (15 µg/ml – 500 µg/ml) on glucose uptake in 3T3-L1 pre-adipocytes is presented in Figure 4-15. None of the extracts of the *Ficus* species enhanced glucose uptake in the 3T3-L1 pre-adipocytes at the highest concentration of 500 µg/ml in that they had values lower than DMSO the solvent control.Only the extract of *F. polita* enhanced glucose uptake(7.2 ± 3.7%) in 3T3-L1 pre-adipocytes at the lowest concentration (15 µg/ml). Insulin, the positive control significantly (*p* 0.001) enhanced glucose uptake in 3T3-L1 pre-adipocytes with the highest glucose uptake of 23.7 ± 2.1% at the concentration 10 µM while the uptake in the DMSO treated cells were 6.5 ± 1.1%.

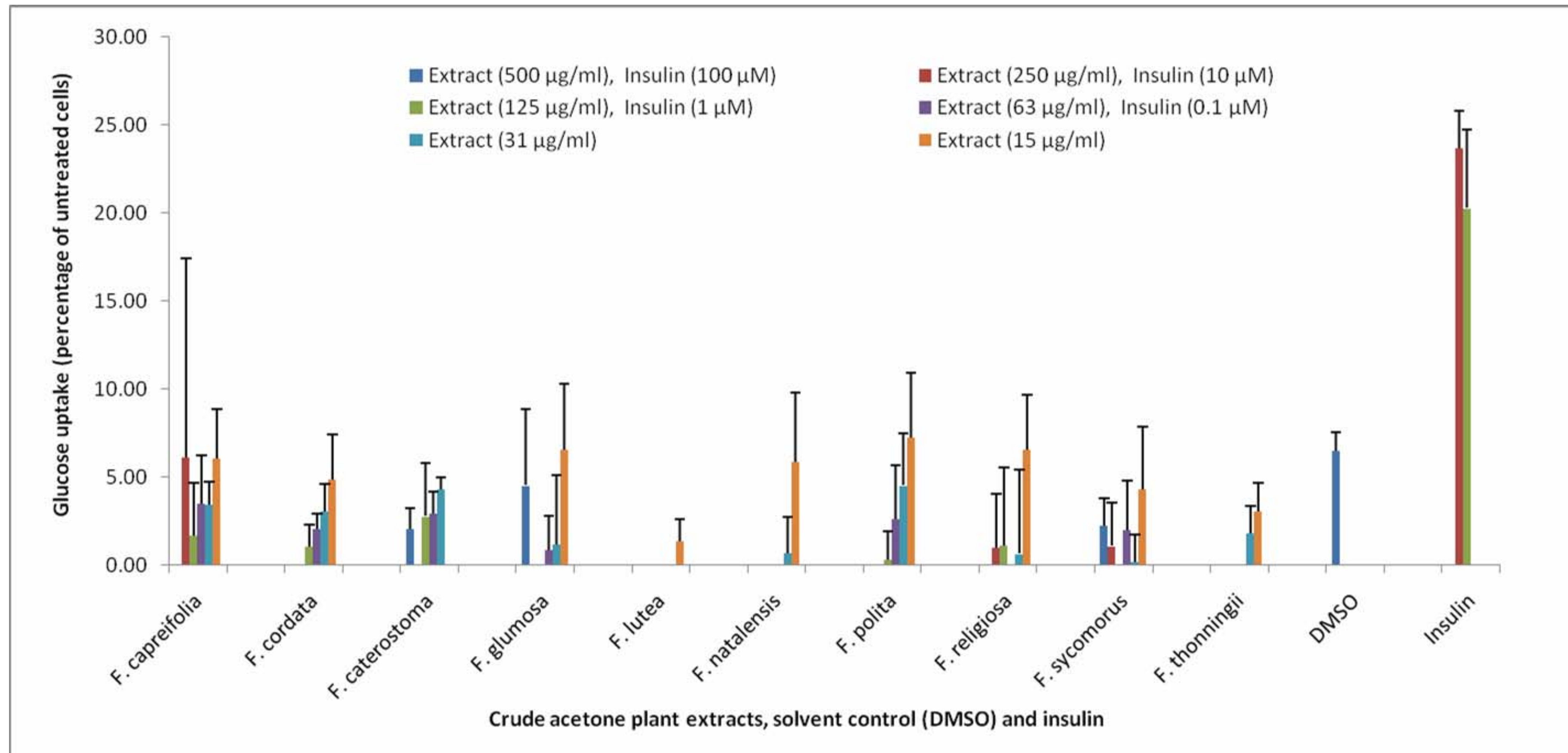

Figure 4-15Glucose uptake in 3T3-L1 pre-adipocytes (as percentage of untreated control cells ± standard error of mean, n=9) exposed to the acetone extracts of the ten *Ficus* species and insulin.

4.1.14 Insulin secretion in RIN-m5F pancreatic cells

Since the cells (muscle and liver cells) exposed to the extract of *F. lutea* utilised more glucose than cells exposed to the extracts of the nine other *Ficus* species, the effect of extract of *F. lutea* at different concentrations (62.5 µg/ml – 500 µg/ml) on insulin secretion in RIN-m5F pancreatic β-cells incubated in medium free of glucose (non-stimulatory condition) was evaluated and was compared with the untreated control cells. The RIN-m5F pancreatic β-cells exposed to the extract of *F. lutea* resulted in a dose related increase in insulin secretion (Figure 4-16). The insulin secreted significantly (*p* 0.001) increased from 26.4 ± 69.7% to 120.8 ± 11.1% as the concentration of the extract of *F. lutea* was increased from 62.5 µg/ml to 500 µg/ml. Likewise the RIN-m5F pancreatic β-cells exposed to glibenclamide (positive control) significantly (*p* 0.001) secreted insulin in a dose dependent manner with the highest insulin secretion (142.7 ± 13.9%) at the concentration of 100 µM.

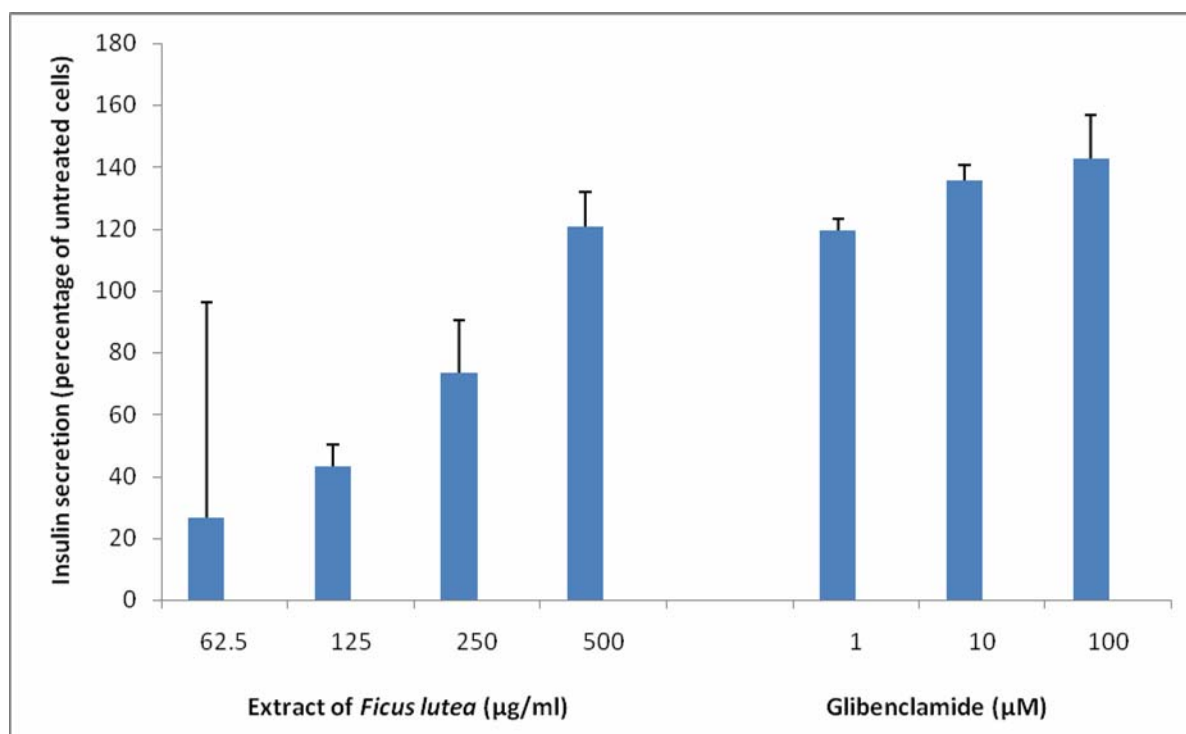

Figure 4-16Insulin secreted in RIN-m5F pancreatic cell (as percentage of untreated control cells ± standard error of mean, n=6) exposed to the acetone extract of *F. lutea* and glibenclamide (positive control) in glucose free medium.

The effect of extract of *F. lutea* on the viability of RIN-m5F pancreatic β-cells was investigatedsubsequent to the insulin secretion assay. The result showed that RIN-m5F cells exposed to the extract of *F. lutea* had a mild dose related decrease in cell viability from 98.8 ± 9.9% to 81.0 ± 1.3% as concentration was increasedfrom 62.5 µg/ml to 500 µg/ml (Figure 4-17). The correlation coefficient between the viability of RIN-m5F pancreatic β-cells and insulin secretion by the extract of *F. lutea* $R^2$was 0.72 (Figure 4-18). This indicates that the stimulation of RIN-m5F pancreatic β-cells

by the extract of *F. lutea* to secrete insulin may in part be attributed to the disruption of cell membrane.

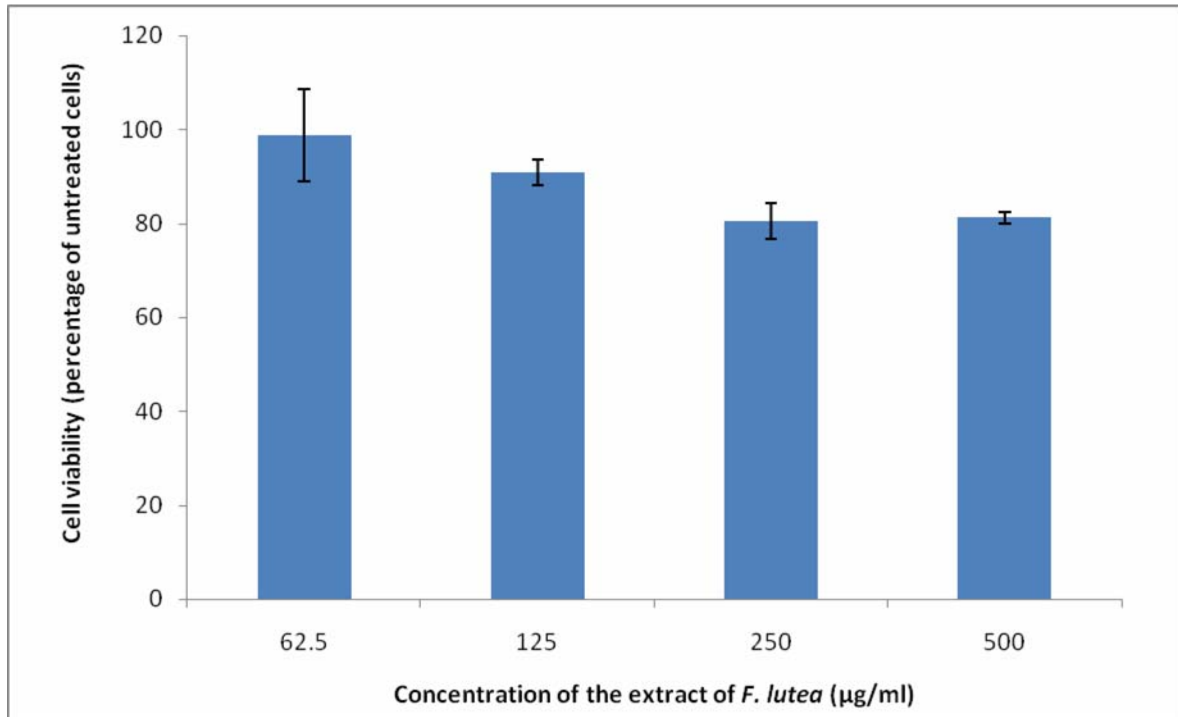

Figure 4-17Effect of the extract of *F. lutea* on RIN-m5F pancreatic cell viability (as percentage of untreated control cells ± standard error of mean, n=6) after stimulating insulin secretion

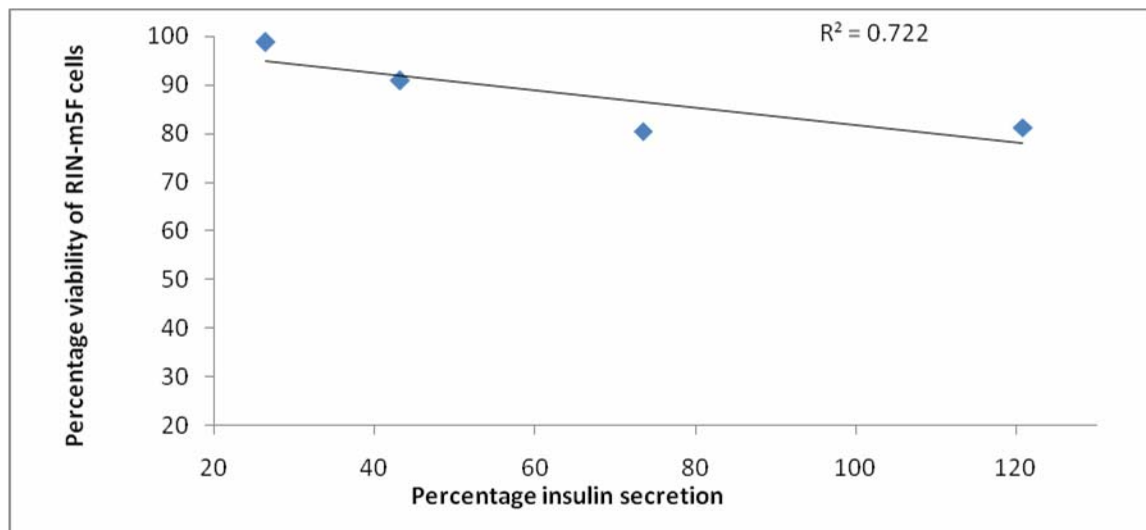

Figure 4-18The correlation between percentage cell viabilty of RIN-m5F pancreatic β-cells and percentage insulin secretion by the acetone extract of *F. lutea*.

4.2 Solvent-solvent fractionation of extract of *F. lutea*

4.2.1 Percentage yield of fractions

The powdered leaf of *F. lutea* (3g) each was weighed into two beakersand was extracted with acetone to produce about 112.5 mg of dried crude extract which was subjected to solvent-solvent fractionation. The percentage yield of the solvent-solvent fractionation of the crude acetone extract of *F. lutea* is presented (Figure 4-19). The percentage yield of ranged from 13.7% - 42.4% (15.4 mg – 47.7 mg) with hexane extracting the lowest mass 15.4 mg (13.7%) and ethyl acetate extracting the highest mass 47.7 mg (42.4%) followed by n-butanol 41.0 mg (36.4%).

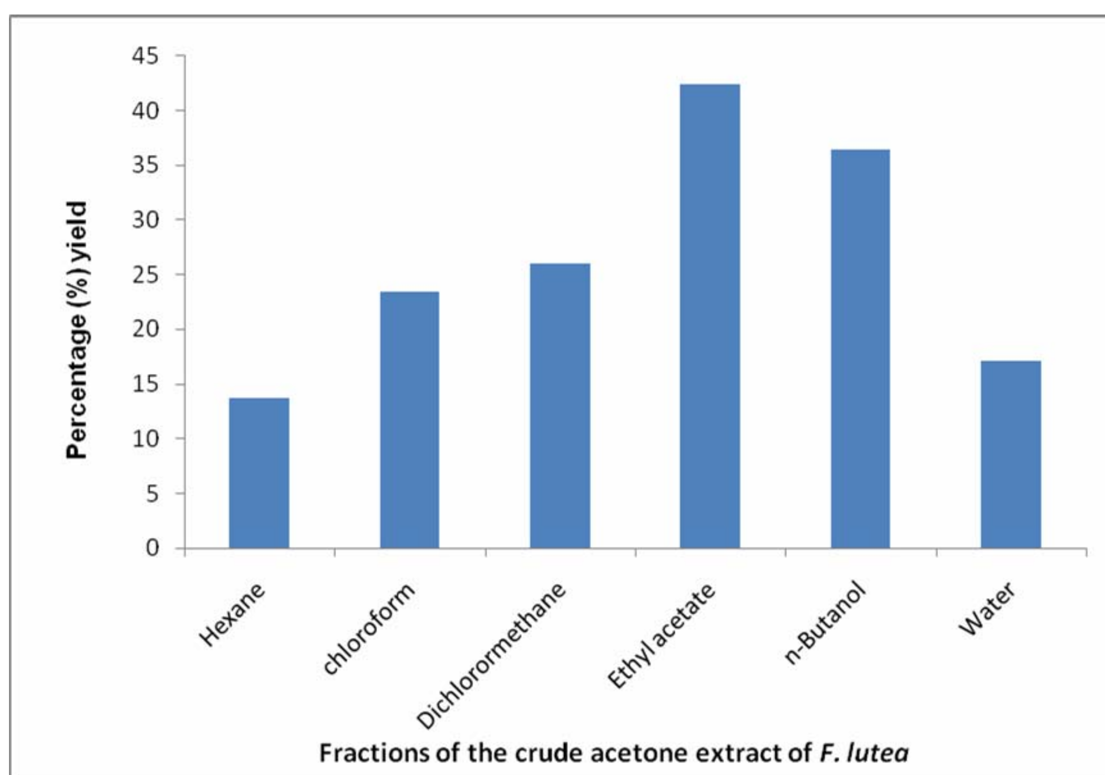

Figure 4-19Percentage yield of fractions was calculated as (dry fraction weight/dry extract weight) × 100.

4.2.2 Antioxidant activity

The TLC chromatograms of the six fractions from the acetone extract of *F. lutea* sprayed with vanillin and 0.2% 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) are presented in Figure 4-20B. For BEA and CEF elution systems, the compounds exhibiting antioxidant activity are seated at the base of the TLC plate while those compounds are better resolved with the EMW and FAWE elution system. The antioxidant TLC chromatograms of fractions did not detect significant antioxidant activity in the hexane,

chloroform and dichloromethane fractions. However, antioxidant activity was detected in the ethyl acetate, n-butanol and water fractions.

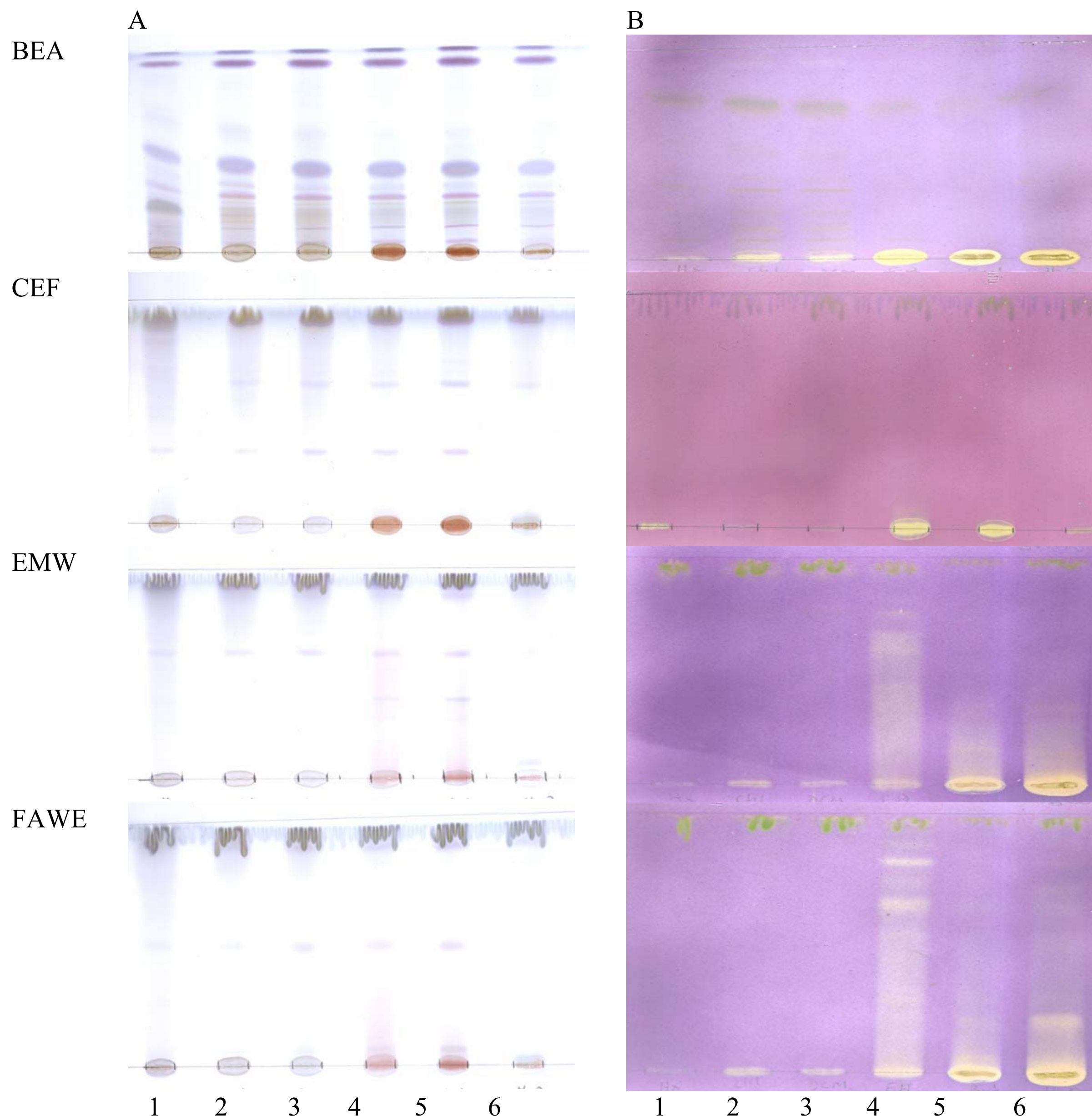

Figure 4-20TLC chromatograms of fractions from the crude acetone extract of *F. lutea* developed with BEA, CEF, EMW and FAWE sprayed with (A) acidified vanillin to show compounds and (B) 0.2% DPPH with clear zone indicating antioxidant activity. Lanes from left to right are fractions of hexane (1), chloroform (2), dichloromethane (3), ethyl acetate (4), n-butanol (5) and water (6).

4.2.3 Total polyphenol content

The total polyphenol content of the six fractions from the acetone extract of *F. lutea* varied widely (Table 4-5), ranging from 10.32 ± 0.82 to 100.51 ± 1.60 mg GAE/g dry weight of extract. When the total polyphenol content of each fractions was compared, the ethyl acetate fraction (100.51 ± 1.60

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mg/g dry weight of extract) was found to have significantly higher content (p 0.001) followed by n-butanol fraction (79.58 ± 0.50 mg/g dry weight of extract).

Table 4-5The total polyphenol contentof fractions of the crude acetone extract of *F. lutea*

Fractions	[ab]Total polyphenol (mg GEA/g dry weight extract)
Hexane	14.86 ± 1.43[c]
Chloroform	10.32 ± 0.82[c]
Dichloromethane	11.83 ± 2.32[c]
Ethyl acetate	100.51 ± 1.60[d]
n-butanol	79.58 ± 0.50[e]
water	13.34 ± 0.85[c]

[a]Values are means of triplicate determinations done three times (n=9) ± standard error;
[b]Total polyphenolic contents (mg gallic equivalent/g dry weight of extract) of crude acetone extract of *F. lutea.*
[c,d,e]No significant difference between extracts with same value, but significant difference p 0.05 between different values

4.2.4 Inhibition of α-amylase activity by the fractions from the acetone extract of *F. lutea*

The fractions of the crude acetone extract of *F. lutea* had potential to inhibit α-amylase activity (Figure-4-21). The n-butanol fraction had the highest inhibitory potential (88.3 ± 0.8%), followed by the ethyl acetate fraction with 78.3 ± 2.5% inhibition, both at 0.5 mg/ml. The dichloromethane and water fractions inhibited α-amylase with 52.3 ± 0.5% and 34.4 ± 4.5% inhibition respectively, while hexane and chloroform inhibited α-amylase activity at between 40% and 48%.

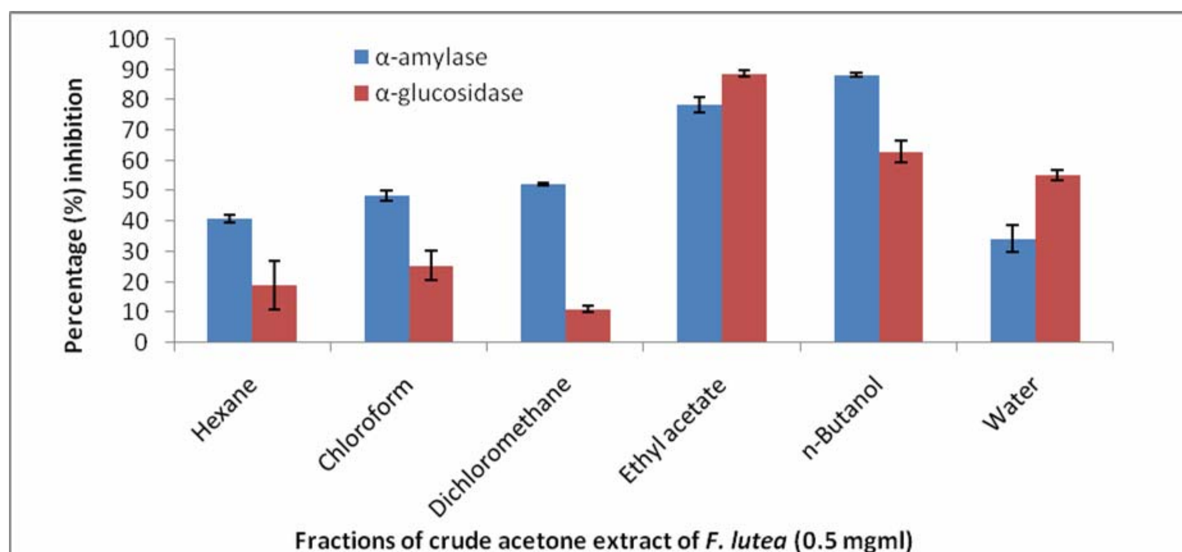

Figure 4-21The percentage inhibition of α-amylase and α-glucosidase (sucrase) activity by the six fractions of the crude acetone extract of *F. lutea* (0.5 mg/ml). Results are expressed as mean ± SEM (n=9).

The concentration of the six fractions of the extract of *F. lutea* leading to inhibition of 50% of α-amylase activity (EC_{50}) is presented in Table 4-6. The n-butanol fraction was the most potent inhibitor with an EC_{50} value of 26.50 ± 1.22 µg/ml followed by the ethyl acetate fraction (39.53 ± 7.10 µg/ml) with a significant difference between them (*p* 0.05*)*.

Table 4-6The EC_{50} of α-amylase and α-glucosidase activity of the fractions of the acetone extract of *F. lutea*

Fractions	α-Amylase inhibition (EC_{50}) µg/ml	α-Glucosidase inhibition (EC_{50}) µg/ml
Hexane	1000	1000
Chloroform	1000	1000
Dichloromethane	1000	854.51 ± 56.92[a]
Ethyl acetate	39.53 ± 7.10[a]	126.78 ± 30.62[b]
n-Butanol	26.50 ± 1.22[b]	195.17 ± 63.60[b]
Water	1000	558.40 ± 51.67[a]

[a,b,c]No significant difference between extracts with same value, but significant difference *p* 0.05 between different values

The correlation coefficient between total polyphenolic content and the percentage inhibition of α-amylase activity by the fractions of the acetone extract of *F. lutea* R^2 was 0.81 (Figure 4-22). Most of the points of the graph are at the lower concentration as the four fractions of *F. lutea* contained low amount of polyphenolic compounds and weak α-amylase activity inhibitor while the two fractions

contained higher amount of polyphenolic compounds and are potent inhibitors of activity of α-amylase. This indicates that the inhibition of α-amylase activity by the fractions of extract of *F. lutea* may be due to thepolyphenolic compounds therein.

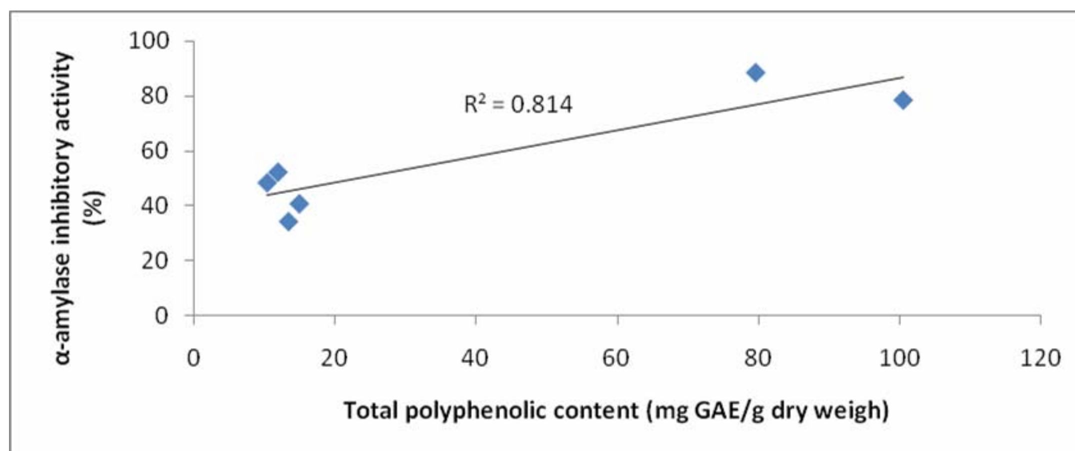

Figure 4-22The correlation between α-amylase inhibitory activities (%) and total polyphenolic contents (mg gallic acid equivalent/g dry weight of sample) of the fractions of the acetone extract of *F. lutea.*

4.2.5 Inhibition of α-glucosidase activity by the fractions of acetone extract of *F. lutea*

The fractions of the crude acetone extract of *F. lutea* inhibited α-glucosidase (sucrase) activity (Figure 4-21). The ethyl acetate fraction had the highest inhibitory potential (88.6 ± 1.0%) followed by the n-butanolic and water fractions with 63.0 ± 3.7% and 55.3 ± 1.7% inhibition respectively all at the concentration of 0.5 mg/ml. The other three fractions weakly inhibited the activity of α-glucosidase with percentage inhibition less than 30%.

The concentration of the six fractions of the extract of *F. lutea* leading to inhibition of 50% of α-glucosidase activity (EC_{50}) is presented in Table 4-6. The ethyl acetate fraction was the most potent inhibitor with an EC_{50} value of 126.78 ± 30.62 µg/ml followed by the n-butanol fraction (195.17 ± 63.60 µg/ml) with no significant difference between them (p 0.05). The correlation coefficient between total polyphenolic content and the percentage inhibition of α-glucosidase activity by the fractions of the acetone extract of *F. lutea* R^2 was 0.74 (Figure 4-23). As above, most of the points of the graph are at the lower concentration as four fractions of *F. lutea* contained low amount of polyphenolic compounds and weak α-glucosidase activity inhibitor while the other two fractions contained higher amount of polyphenolic compounds and are potent inhibitors of activity of α-glucosidase. This indicates that the inhibition of α-glucosidase activity by the fractions of extract of *F. lutea* may be due to the total polyphenolic content.

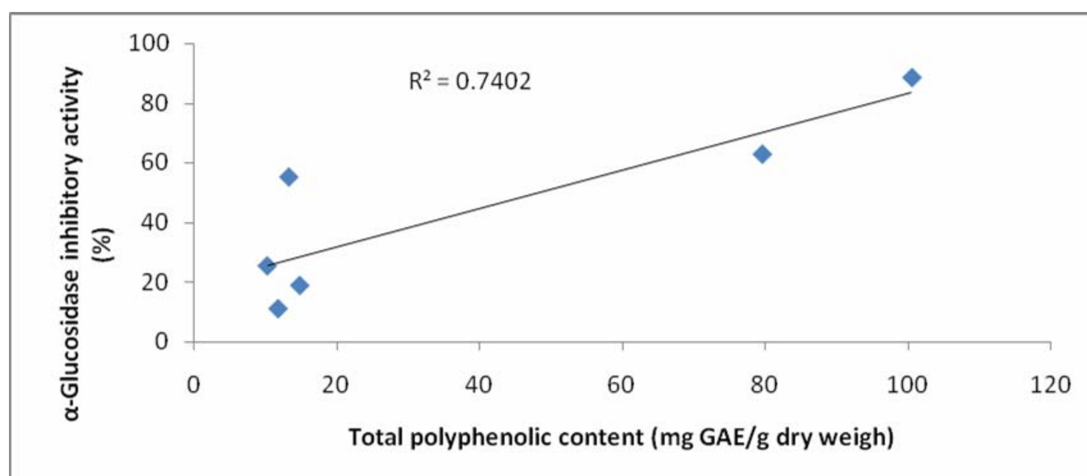

Figure 4-23The correlation between α-glucosidase inhibitory activities (%) and total polyphenolic contents (mg gallic acid equivalent/g dry weight of sample) of the fractions of the acetone extract of *F. lutea.*

4.2.6 Cytotoxicity of the fractions from acetone extract of *F. lutea*

The cytotoxic activities of the fractions against the Vero monkey kidney and the human C3A liver cell lines are presented in Table 4-7. In all assays, the hexane fraction was the least toxic (LD_{50} value >1000 µg/ml). The fractions were relatively more cytotoxic to the Vero kidney cells when compared to the C3A liver cells. The ethyl acetate fraction was relatively non-toxic although it had the lowest LD_{50} against the Vero cells (LD_{50} = 126.9 ± 1.5 µg/ml), while the n-butanol fraction was the most cytotoxic to the C3A liver cells with LC_{50} of 76.8 ± 0.4 µg/ml.

Table 4-7Cytotoxicity activity of fractions from extract of *F. lutea* (LC_{50} in µg/ml ± SE)

Fractions	Vero kidney cells	C3A liver cells
Hexane	1000	1000
chloroform	389.6 ± 1.8	615.7 ± 3.9
Dichloromethane	302.3 ± 2.1	1000
Ethyl acetate	126.9 ± 1.5	1000
n-Butanol	216.1 ± 2.9	76.8 ± 0.4
Water	ND	ND

Values are means of triplicate determinations done three times (n=9) ± standard error;

ND: not determined

4.2.7 Glucose uptake in C2C12 muscle cells

The effect of fractions of the acetone extract of *F. lutea* at different concentrations (15 µg/ml – 500 µg/ml) on glucose uptake in C2C12 muscle cells is presented in Figure 4-24. Of all the fractions, only the ethyl acetate and n-butanol fractions significantly (p 0.001) increased glucose uptake in the cells in a dose responsive manner with the ethyl acetate fraction enhancing the highest glucose uptake of 31.2 ± 1.5% at the highest concentration (500 µg/ml) followed by the n-butanol fraction enhancing glucose uptake of 25.9 ± 1.2% as the same concentration. The hexane, chloroform and dichloromethane fractions enhanced very low glucose uptake at only the lowest concentration (15 µg/ml).

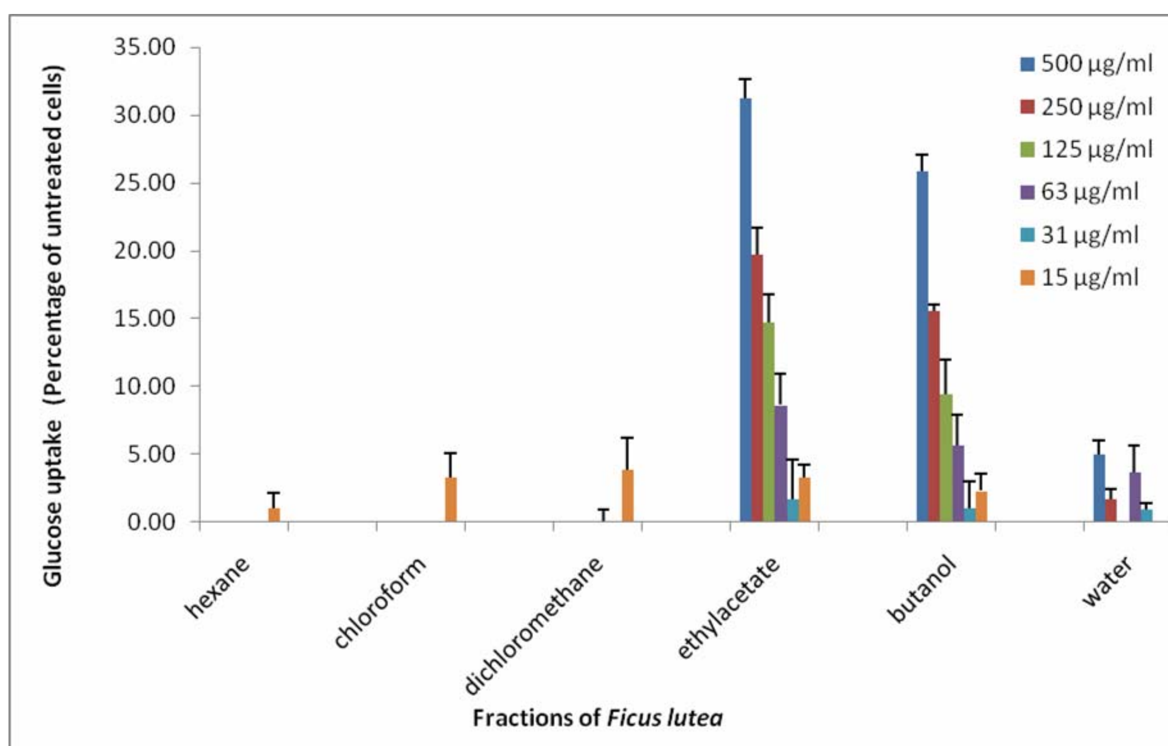

Figure 4-24Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells ± standard error of mean, n=9) exposed to the fractions of acetone extract of *F. lutea.*

With the ethyl acetate fraction significantly enhancing glucose uptake, the effect of concurrent insulin on glucose uptake in the presence of ethyl acetate fraction was investigated. The uptake of glucose by C2C12 cells treated with ethyl acetate fraction at different concentrations (15 µg/ml – 500 µg/ml) in medium containing two different concentrations of insulin (1 µM and 10 µM) is presented in Figure 4-25. The insulin-mediated glucose uptake of C2C12 exposed to the ethyl acetate fraction at different insulin concentrations of 1 µM and 10 µM was 26.8 ± 0.8% and 28.8 ± 0.7% respectively compared to ethyl acetate alone (31.2 ± 1.5%) at the highest ethyl acetate fraction concentration (500 µg/ml). Insulin at the two concentrations significantly (p 0.001) inhibited cell exposed to ethyl acetate from

increasing glucose uptake in comparison to ethyl acetate fraction in the absence of insulin. However since the difference was minor, the effect was not considered to be biologically significantly different.

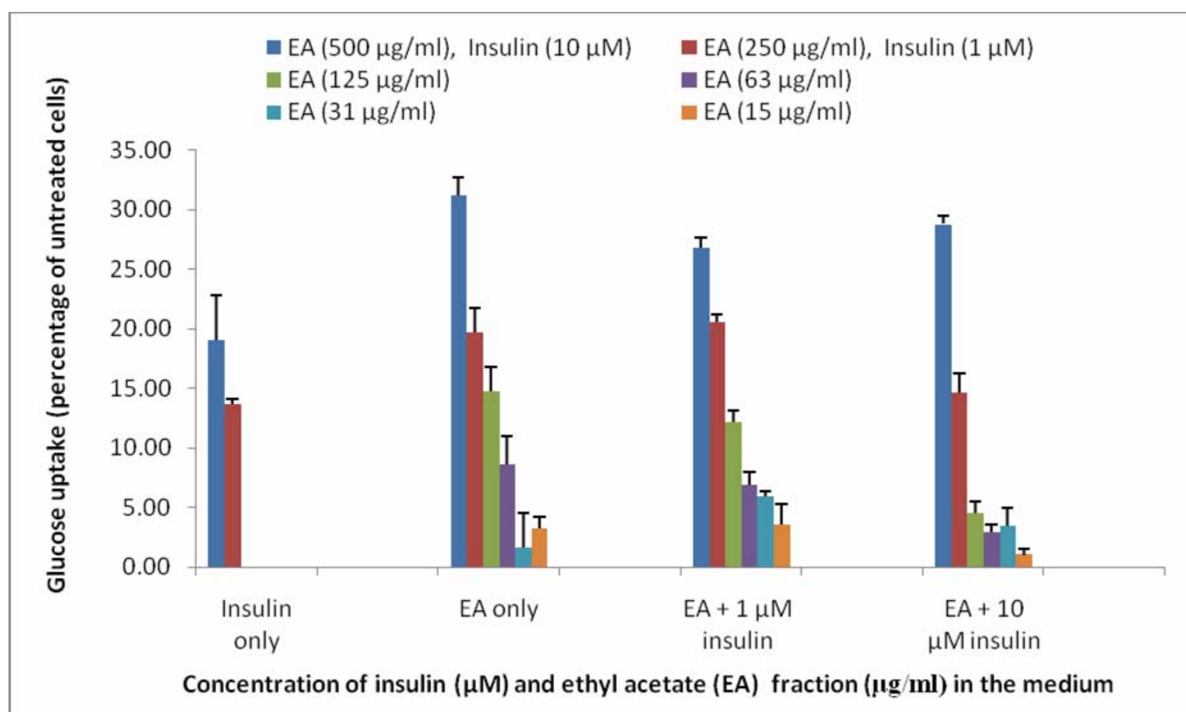

Figure 4-25Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells ± standard error of mean, n=9) exposed to difference concentrations of the ethyl acetate fraction (represented by the different colours) in medium containing different concentrations of insulin (x-axis).

4.2.8 Glucose uptake activity in H-4-11-E liver cells

The effect of fractions at different concentrations (15 µg/ml – 500 µg/ml) on glucose uptake activity in H-4-11-E liver cells is presented in Figure 4-26. All fractions minimally enhanced glucose utilisation activity in H-4-11-E liver cells at the lowest concentration (15 µg/ml). Only the ethyl acetate and n-butanol significantly (*p* 0.001) increased glucose uptake in a dose responsive manner. The ethyl acetate fraction enhanced the highest (40.0 ± 2.8%) glucose uptake at the concentration of 500 µg/ml and this was followed by the n-butanol fraction (25.9 ± 1.4%) at the same concentration.

With the ethyl acetate fraction significantly enhancing glucose uptake activity, the effect of concurrent insulin on ethyl acetate mediated glucose uptake was evaluated. The effect of ethyl acetate fraction at different concentrations (15 µg/ml – 500 µg/ml) in medium containing two different concentrations of insulin (1 µM and 10 µM) on glucose uptake is presented in Figure 4-27. The insulin-mediated glucose uptake in H-4-II-E liver cells exposed to ethyl acetate fraction at different insulin

concentrations of 1 µM and 10 µM was 27.0 ± 0.8% and 29.5 ± 0.7% respectively when compared to ethyl acetate fraction alone (40.0 ± 2.8%) at the highest concentration (500 µg/ml).

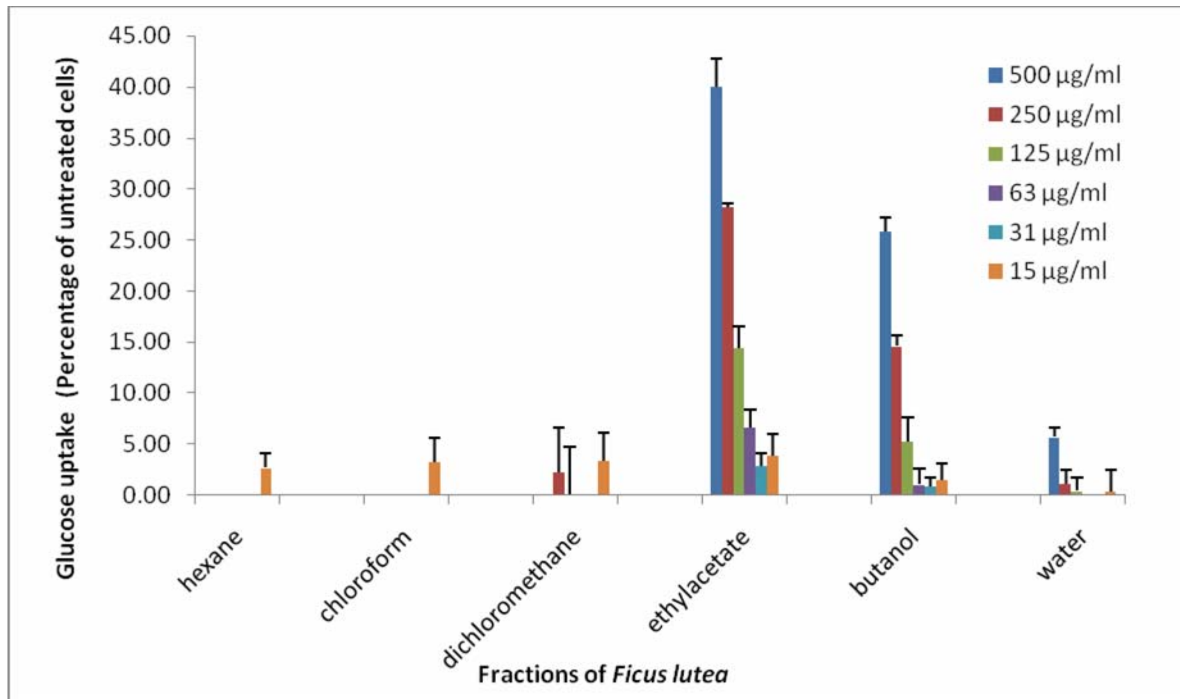

Figure 4-26Glucose uptake in H-4-11-E rat liver cells (as percentage of untreated control cells ± standard error of mean, n=9 for) exposed to the fractions of acetone extract of *F. lutea*.

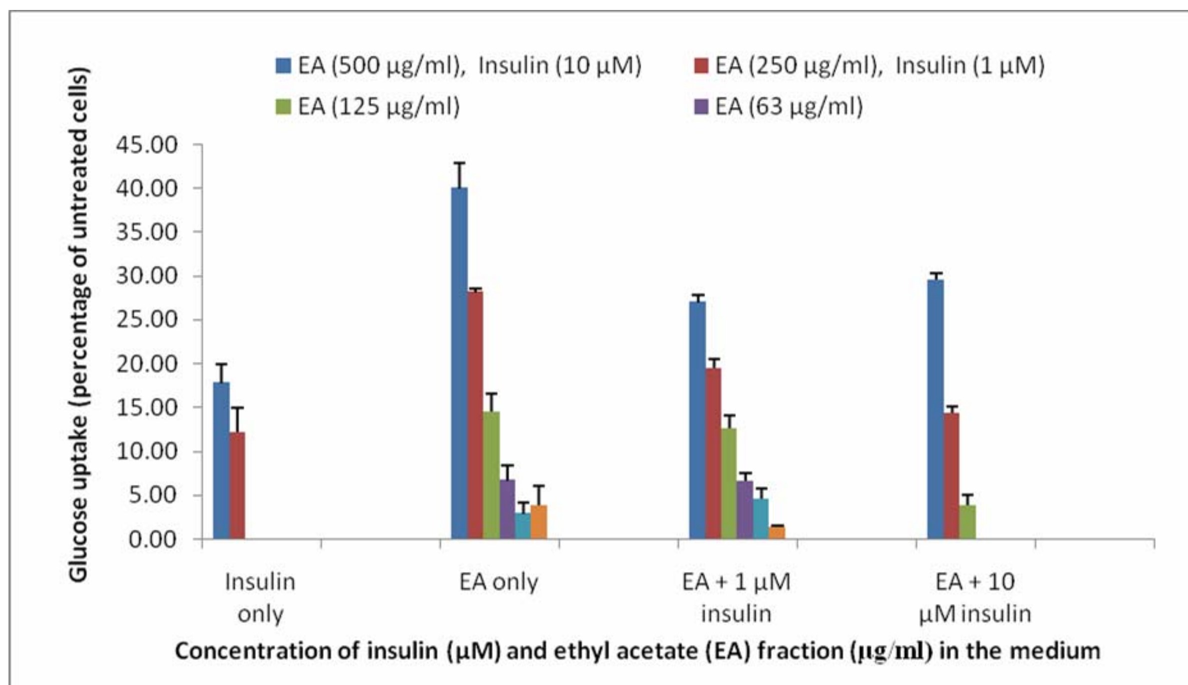

Figure 4-27Glucose uptake in H-4-11-E rat liver cells (as percentage of untreated control cells ± standard error of mean, n=9) exposed to different concentration of the ethyl acetate fraction (different colours) in medium containing different concentrations of insulin (x-axis).

4.2.9 Insulin secretion in RIN-m5F pancreatic cells

With the ethyl acetate fraction of *F. lutea* being the most active fraction in stimulating glucose uptake in the treated C2C12 muscle and H-4-II-E cells, the effect of this fraction at different concentrations (62.5 µg/ml – 500 µg/ml) on insulin secretion was investigated in RIN-m5F pancreatic β-cells and was compared with the untreated control cells. The RIN-m5F pancreatic cells exposed to the ethyl acetate fraction resulted in a dose related increase in insulin secretion (Figure 4-28). The insulin releasedsignificantly (*p* 0.001) increased from 33.0 ± 7.3% at the concentration of 62.5 µg/ml to 115.0 ± 4.4% at the concentration of 250 µg/ml. The 500 µg/ml dose has no superior response to the 250 µg/ml dose, indicating that the maximum effect has plateaued.

The effect of the ethyl acetate fraction of *F. lutea* on the viability of RIN-m5F pancreatic β-cells after insulin secretion assay was investigated (Figure 4-29). The result showed that the RIN-m5F cells exposed to the ethyl acetate fraction had dose related decrease in cell viability from 91.9 ± 8.6% to 50.2 ± 2.8% as concentration was increased from 62.5 µg/ml to 500 µg/ml. The correlation coefficient between the viability of RIN-m5F pancreatic β-cells and insulin secretion by the ethyl acetate fraction of the extract of *F. lutea* R^2 was 0.76 (Figure 4-30). This indicates that the stimulation of RIN-m5F pancreatic β-cells by the ethyl acetate fraction of the extract of *F. lutea* to secrete insulin may in part be attributed to the disruption of cell membrane.

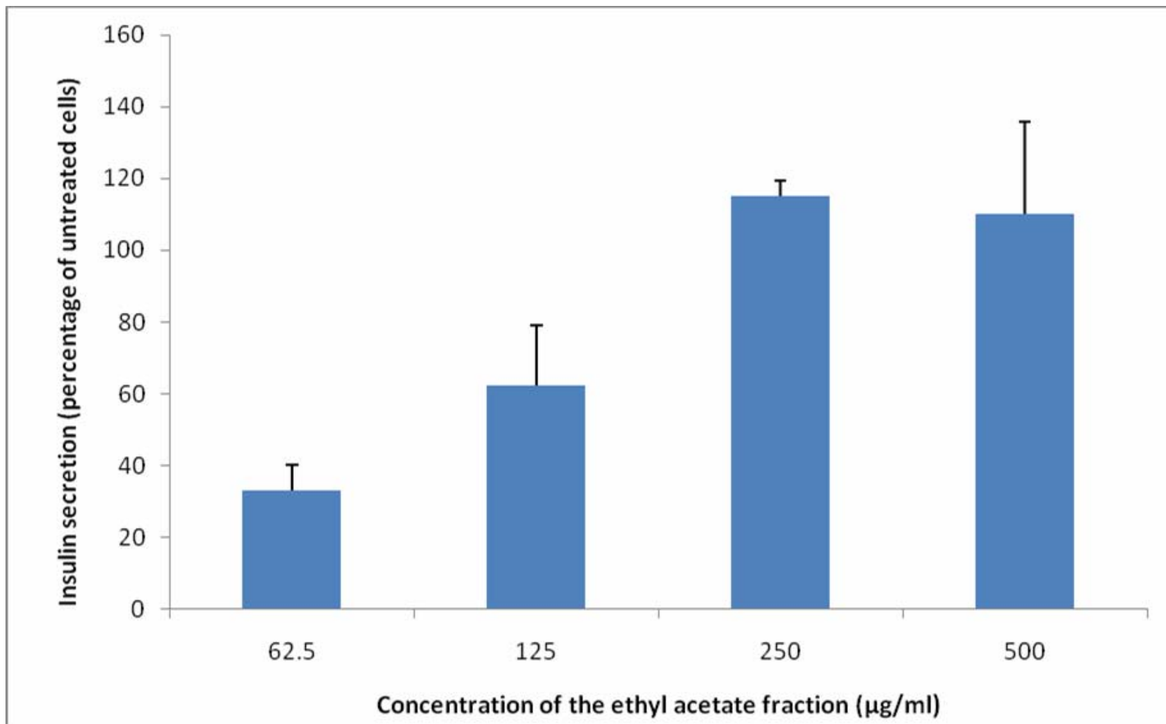

Figure 4-28Insulin secreted in RIN-m5F pancreatic cells (as percentage of untreated control cells ± standard error of mean, n=6) exposed to the ethyl acetate fraction from crude acetone extract of *F. lutea.*

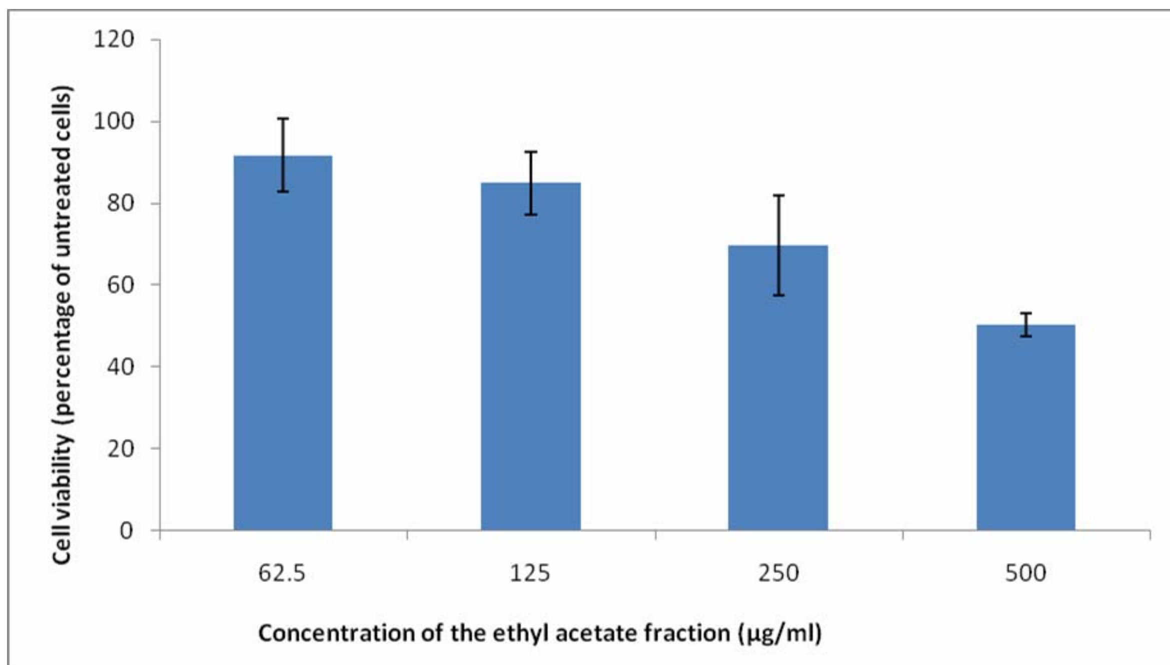

Figure 4-29Effect of the ethyl acetate fraction from the extract of *F. lutea* on RIN-m5F pancreatic cell viability (as percentage of untreated control cells ± standard error of mean, n=6) after stimulating insulin secretion.

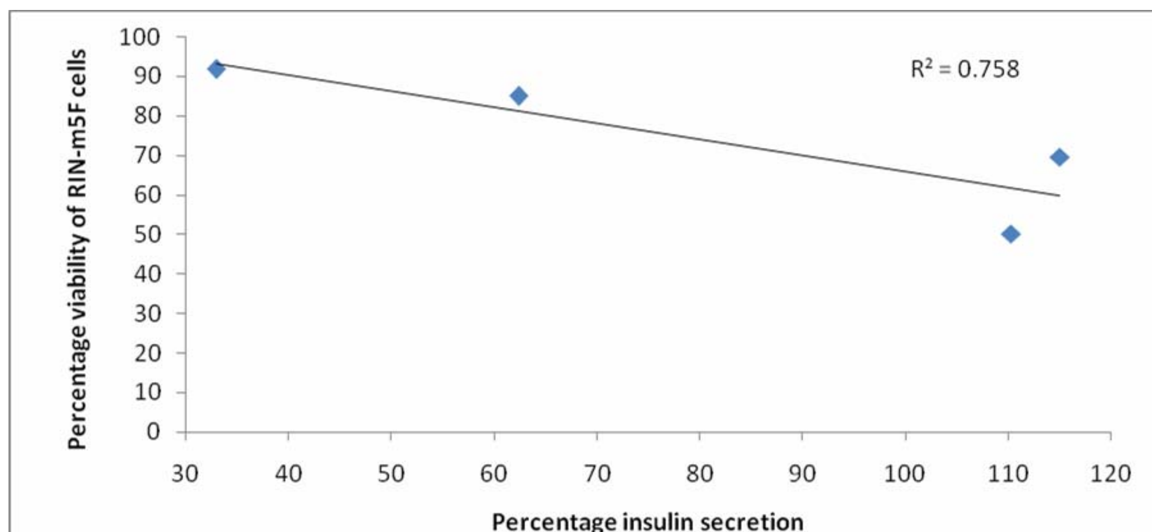

Figure 4-30 The correlation between percentage cell viabilty of RIN-m5F pancreatic β-cells and percentage insulin secretion by the ethyl acetate fraction of the extract of *F. lutea*.

4.3 Structure Elucidation of Compounds from the ethyl acetate fraction of F. lutea

4.3.1 Structure Elucidation of AFL1 or Lupeol (1)

AFL1 was obtained as white powder. Its ^{1}H and ^{13}C NMR spectra (Figure 1 and 2 in Appendix) exhibited signals at δ 4.68 (*brs*, H-29$_a$), 4.47 (*brs*, H-29$_b$), 3.18 (*m*, H-3), 109.4 (C-29), and 79.1 ppm (C-3) assignable respectively to protons and carbons of a methylene and oxymethine groups at positions 29 and 3 of a lup-20(29)-en-3-ol class of triterpenes (Mahato and Kundu, 1994). Furthermore, one characteristic signal of carbon C-20 of lupeol structure was observed on ^{13}C NMR spectrum at δ 148.2 ppm while seven singlet signals relevant for angular methyl groups appeared on the ^{1}H NMR spectrum between 0.8-1.7 ppm. All the NMR data including ^{1}H (Figure 1 in Appendix), ^{13}C (Figure 2 in Appendix), ^{1}H ^{1}H COSY (Figure 3 in Appendix), HSQC (Figure 4) and HMBC (Figure 5 in Appendix) were in agreement with those published for lupeol (Figure 4-31) previously isolated from the same species (Poumale *et al.*, 2011).

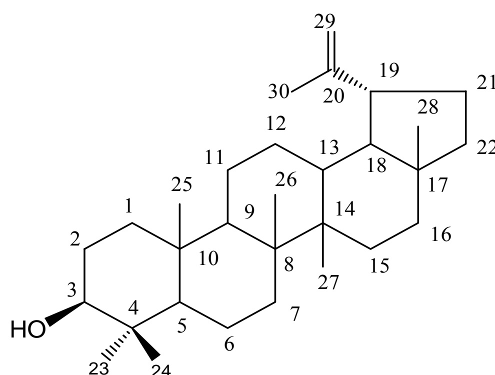

Figure 4-31The structure of AFL1 (lupeol) isolated from the leaves of *F. lutea*.

4.3.2 Structure Elucidation of AFL2 or Stigmasterol (2)

AFL2 was obtained as a white powder. The presence of total number of 30 carbons on its ^{13}C NMR spectrum (Figure 6 in Appendix) including signals due to six angular methyl groups was relevant for this compound to be a phytosterol. The ^{13}C NMR spectrum displayed signals at δ 140.7, 138.3, 129.2, 121.7 and 71.8 ppm corresponding respectively to carbons C-5, C-22, C-23, C-6 and C-3 of stigmasterol structure. The ^{1}H NMR spectrum (Figure 7 in Appendix) exhibited signals at δ 5.32 (*brd*, 2.2; 3.0 Hz, 1H), 5.17 (*dd*, 8.5 Hz, 1H), 5.00 (*dd*, 8.5 Hz, 1H) and 3.50 (*m*, 1H) corresponding to protons H-6, H-22, H-23 and H-3 respectively. Furthermore, signals due to methyl groups were observed on its ^{1}H NMR between 0.90 and 0.60 ppm. The ^{1}H and ^{13}C NMR (Figure 6 and 7 in Appendix) as well as the ^{1}H ^{1}H COSY (Figure 8 in Appendix), HSQC (Fig 9 in Appendix) and HMBC (Figure 10 in Appendix) data were in agreement with those of previously reported stigmasterol (Figure 4-32) ((Forgo and Kövér, 2004). This compound (Figure 4-32) which is very common in plant kingdom is isolated for the first time from this species.

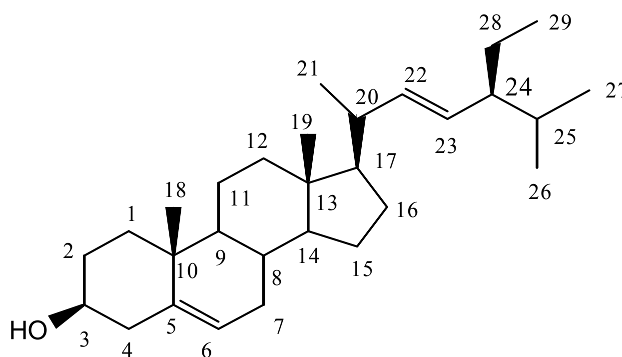

Figure 4-32The structure of compound AFL2 (stigmasterol) isolated from the leaves of *F. lutea.*

4.3.3 Structure Elucidation of AFL3 or α-Amyrin Acetate (3)

AFL3 was obtained as oil. Its ^{13}C NMR spectrum (Figure 11 in Appendix) exhibited a total number of 30 carbons with 4 characteristic downfields displayed at δ 170.9, 139.7, 124.4 and 81.0 assignable to a triterpene skeleton with one carbonyl acetate (CH_3CO), one ethylenic double bond (C=CH) and one oxymethine (HCOH) groups. The ^{1}H NMR spectrum (Figure 12 in Appendix) exhibited characteristic signals at δ 5.12 (*t*, 3.7 Hz, 1H), 4.49 (*m*, 1H) and 2.02 (*s*, 3H) due to protons at positions C-12, C-3 and acetyl group from α-amyrin acetate (Mahato and Kundu, 1994). All these data above as well as DEPT (Figure 13 in Appendix), ^{1}H ^{1}H COSY (Figure 14 in Appendix), HSQC (Figure 15 in Appendix) and HMBC (Figure 16 in Appendix) were similar to those published for α-amyrin acetate (Figure 4-33) previously isolated from the same species (Poumale *et al.*, 2011).

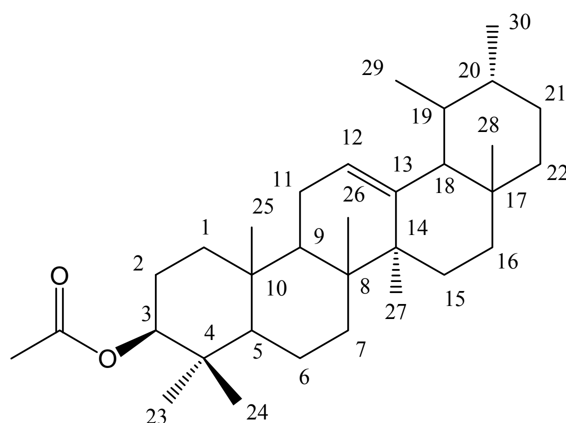

Figure 4-33The structure of compound AFL3 (α-amyrin acetate) isolated from the leaves of *F. lutea*.

4.3.4 Structure elucidation of AFL4 or Epicatechin (4)

AFL4 was obtained as a yellowish powder. Its ^{1}H NMR spectra (Figure 17 in Appendix) showed singlets at δ 8.10, 7.94, 7.79 and 7.74 ppm assignable to four phenolic protons. The ^{1}H NMR spectrum also exhibited a broad singlet, one multiplet and two doublet of doublet signals at δ 4.87 (*brs*, 1H), 4.20 (*m*, 1H), 2.85 (*dd*, 4.6, 16.5 Hz, 1H) and 2.72 (*dd*, 3.3, 16.7 Hz, 1H) attributable to protons H-2, H-3, H-4_b and H-4_a, respectively. Furthermore, the ^{1}H NMR spectrum showed two sets of aromatic protons: the first one at δ 6.01 (*d*, 2.3 Hz, H-6) and 5.91 (*d*, 2.3 Hz, H-8) corresponding to A ring, and the second one at δ 7.04 (*d*, 2.0 Hz, H-2'), 6.88 (*d*, 8.4 Hz, H-5') and 6.83 ppm (*dd*, 2.0, 8.4 Hz, H-6') imputable to B ring protons. The ^{13}C NMR spectrum (Figure 18) exhibited the characteristic flavan-3-ol signals at δ 79.4, 66.9 and 28.9 ppm corresponding to C-2 (OCH), C-3 (COH) and C-4 (CH_2), respectively (Morimoto *et al.*, 1985; Pan *et al.*, 2003). Moreover, the ^{13}C NMR spectrum exhibited 12 aromatic carbons at δ 145.2, 145.3, 157.1, 157.5 and 157.6 ppm attributable respectively to five oxygenated carbons C-3', C-4', C-5, C-7 and C-9, and at δ 132.2, 115.2, 115.4, 119.3, 96.1, 96.0 and 99.8 corresponding to carbons C-1', C-2', C-5', C-6', C-6, C-8 and C-10, respectively. Unambiguous assignments for the ^{1}H and ^{13}C NMR for this compound were made by the combination of DEPT (Figure 19 in Appendix), ^{1}H-^{1}H COSY (Figure 20 in Appendix), HSQC (Figure 21 in Appendix) and HMBC (Figure 22 in Appendix) spectra. All the spectroscopic data above were in agreement with those reported for epicatechin (Figure 4-34) previously isolated and characterized by Jiang and co-workers from pericarp tissues of lychee fruit (Zhao *et al.*, 2006). The isolation of this compound is reported here for the first time from this species.

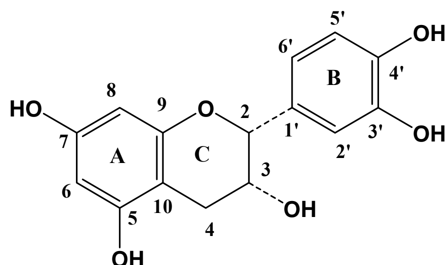

Figure 4-34The structure of AFL4 (epicatechin) isolated from the leaves of *F. lutea*.

4.3.5 Structure elucidation of AFL5 or Epiafzelechin (5)

AFL4 was obtained as yellowish powder. Its ^{13}C NMR and DEPT spectra (Figure 23 and 24 in Appendix) exhibited signals at δ 78.7, 66.1 and 28.1 ppm corresponding to carbons C-2, C-3 and C-4 characteristic for a flavan-3-ol skeleton. This was confirmed by the presence of important signals on its ^{1}H NMR spectrum (Figure 24 in Appendix) at δ 4.87 (*brs*, H-2), 4.15 (*m*, H-3), 2.80 (*dd*, 4.4, 16.8 Hz, H-4$_a$) and 2.62 (*dd*, 3.4, 16.6 Hz, H-4$_b$) due to protons at positions C-2, C-3 and C-4, respectively. Two sets of aromatic protons were observed on its ^{1}H NMR spectrum: the first set appeared as two doublets at δ 5.98 (*d*, 2.2 Hz, H-8) and 5.87 (d, 2.2 Hz, H-6) due to the ring-A while the second one appeared as AA'BB' system at δ 7.28 (*d*, 8.4 Hz, H-2'/H-6') and 6.76 (*d*, 8.4 Hz, H-3'/H-5') corresponding to B-ring. Furthermore, the ^{1}H NMR spectrum also displayed three downfield broad signals between 9.5 and 8.9 ppm assignable to three hydroxyl groups at C-4', C-7 and C-5. The broad singlet multiplicity of proton H-2 was indicative to this proton to be in *cis* configuration with proton H-3. Moreover, the ^{13}C NMR spectrum exhibited signals at δ 156.9, 156.2, 130.4 and 99.0 ppm corresponding to carbons C-7/C-4', C-5/9, C-1' and C-10, respectively. All these data were in agreement with those published for epiafzelechin (Figure 4-35) (Kpegba *et al.*, 2010). This compound is isolated here for the first time from this species.

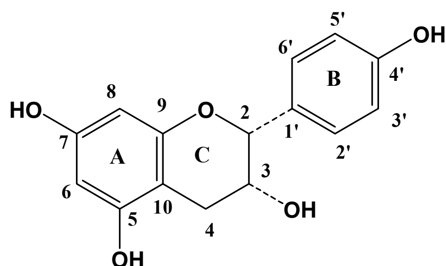

Figure 4-35The structure of AFL5 (epiafzelechin) isolated from the leaves of *F. lutea*.

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4.3.6 Inhibition of α-glucosidase activity by the compounds

The concentration of the compounds isolated from the ethyl acetate fraction of *F. lutea* leading to inhibition of 50% of α-glucosidase (sucrase) activity (EC_{50}) is presented in Table 4-8. Epicatechin was the most potent with an EC_{50} value of 5.72 ± 2.7 µg/ml and this was followed by epiafzelechin (EC_{50} value = 7.64 ± 37.5 µg/ml), with no significant difference between them. The lupeol was the least active (EC_{50}>1000) among the compounds isolated.

Table 4-8The EC_{50} sucrase activity of compounds from ethyl acetate fraction of *F. lutea*

Compound	EC_{50} (µg/ml)
Lupeol	>1000
Stigmasterol	115.71 ± 11.6[a]
α-Amyrin acetate	335.82 ± 22.6[a]
Epicatechin	5.72 ± 2.6[b]
Epiafzelechin	7.64 ± 4.9[b]

[a,b]No significant difference between extracts with same value, but significant difference *p* 0.05 between different values.

4.3.7 Glucose uptake in C2C12 muscle cells

The effect of the compounds isolated from the ethyl acetate fraction of *F. lutea* at different concentrations (15 µg/ml – 250 µg/ml) on glucose uptake in C2C12 muscle cells is presented in Figure 4-36. Of the five isolated compounds only epicatechin and epiafzelechin significantly (*p* 0.001) enhanced glucose utilisation activity in C2C12 muscle. Epiafzelechin increased glucose uptake in a dose responsive manner with the highest uptake (33.4 1.8%) resulting at a concentration of 250 µg/ml. Similarly epicatechin increased glucose uptake in a concentration dependent manner with the highest uptake of 40.7 ± 1.9% at 63 µg/ml.

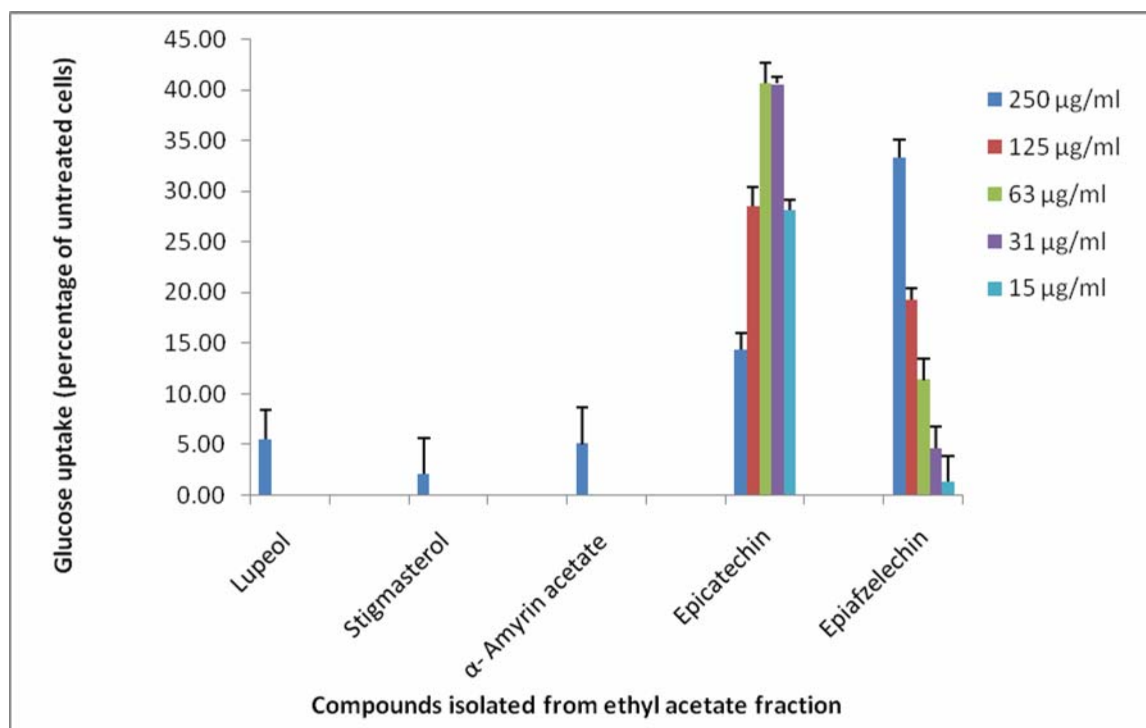

Figure 4-36Glucose uptake in C2C12 muscle cells (as percentage of untreated cells control cells ± standard error of mean, n=9) exposed to the fractions of acetone extract of *F. lutea.*

The effect of epiafzelechin on glucose uptake by the muscle cells, in the presence of insulin was subsequently evaluated. Epicatechin was not evaluated due to time constraints. The uptake of glucose by C2C12 cells treated with epiafzelechin at different concentrations (15 µg/ml – 250 µg/ml) in medium containing twodifferent concentrations of insulin (1 µM and 10 µM) is presented in Figure 4-37. The insulin-mediated glucose uptake of C2C12 exposed to epiafzelechin at different insulin concentrations of 1 µM and 10 µM was 33.2 ± 0.5% and 34.5 ± 1.1% respectively compared to epiafzelechin alone (33.4 ± 1.8%) at the highest epiafzelechin concentration (250 µg/ml).

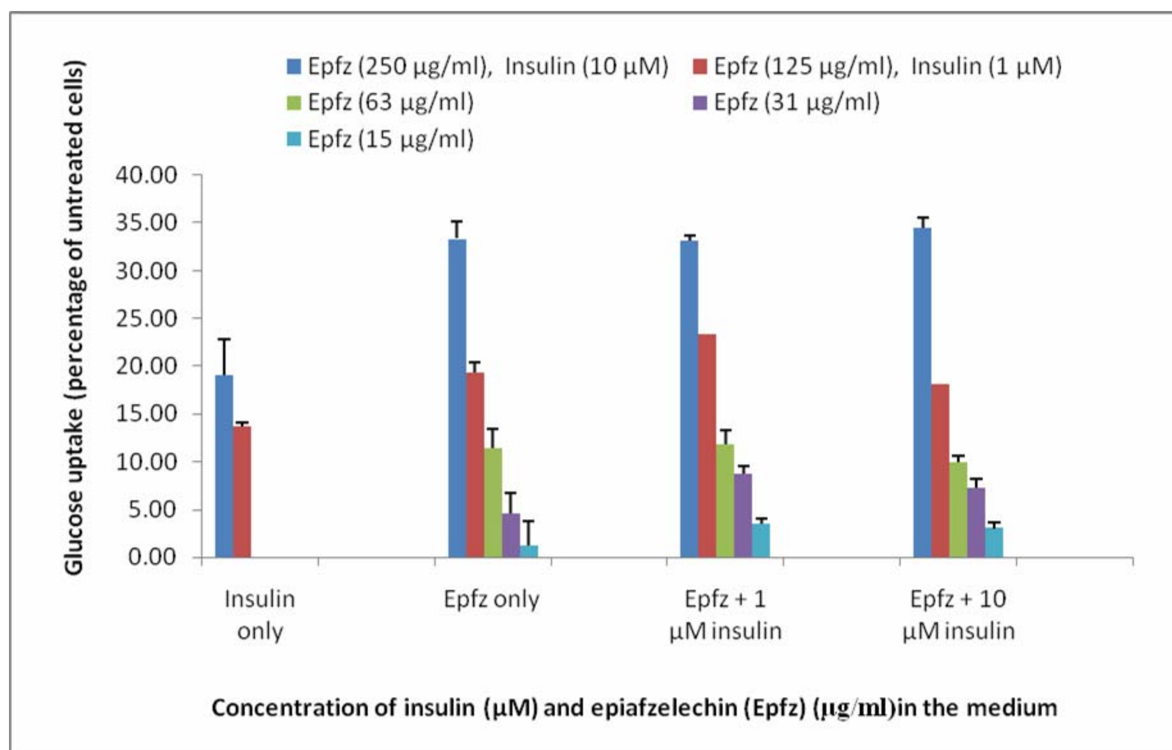

Figure 4-37Glucose uptake in C2C12 muscle cells (as percentage of untreated control cells ± standard error of mean, n=9) exposed to the epiafzelechin at different concentrations (represented by the different colours) in medium containing different concentrations of insulin (on the x-axis).

4.3.8 Glucose uptake in H-4-11-E liver cells

The effect of the compounds isolated from the ethyl acetate fraction of *F. lutea* at different concentrations (15 µg/ml – 250 µg/ml) on glucose uptake in H-4-II-E liver cells is presented in Figure 4-38. Of the five isolated compounds only epicatechin and epiafzelechin significantly (p 0.001) increased glucose uptake in a concentration dependent manner with epicatechin enhancing glucose uptake of 46.7 ± 1.2% at the concentration of 250 µg/ml and this was followed by epiafzelechin with an uptake of 32.4 ± 1.5% at the same concentration.

The effect of epiafzelechin on glucose uptake by the H-4-II-E liver cells, in the presence of insulin was subsequently evaluated. The uptake of glucose by C2C12 cells treated with epiafzelechin at different concentrations (15 µg/ml – 250 µg/ml) in medium containing two different concentrations of insulin (1 µM and 10 µM) is presented in Figure 4-39. Epicatechin was not evaluated due to time constraints. The insulin-mediated glucose uptake in H-4-II-E liver cells exposed to epiafzelechin at different insulin concentrations of 1 µM and 10 µM was 36.9 ± 1.0% and 37.9 ± 0.9% respectively when compared to epiafzelechin alone (32.4 ± 1.5%) at the highest concentration (250 µg/ml).

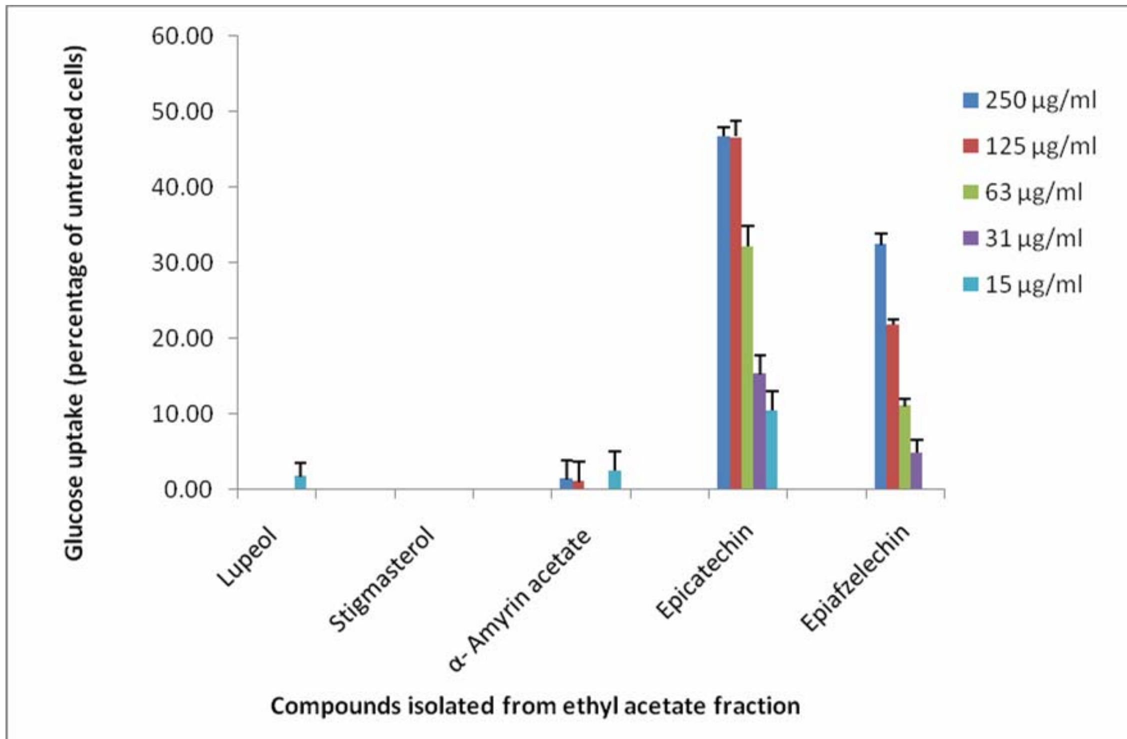

Figure 4-38Glucose uptake in H-4-11-E rat liver cells (as percentage of untreated control cells ± standard error of mean, n=9 for) exposed to the fractions of acetone extract of *F. lutea*.

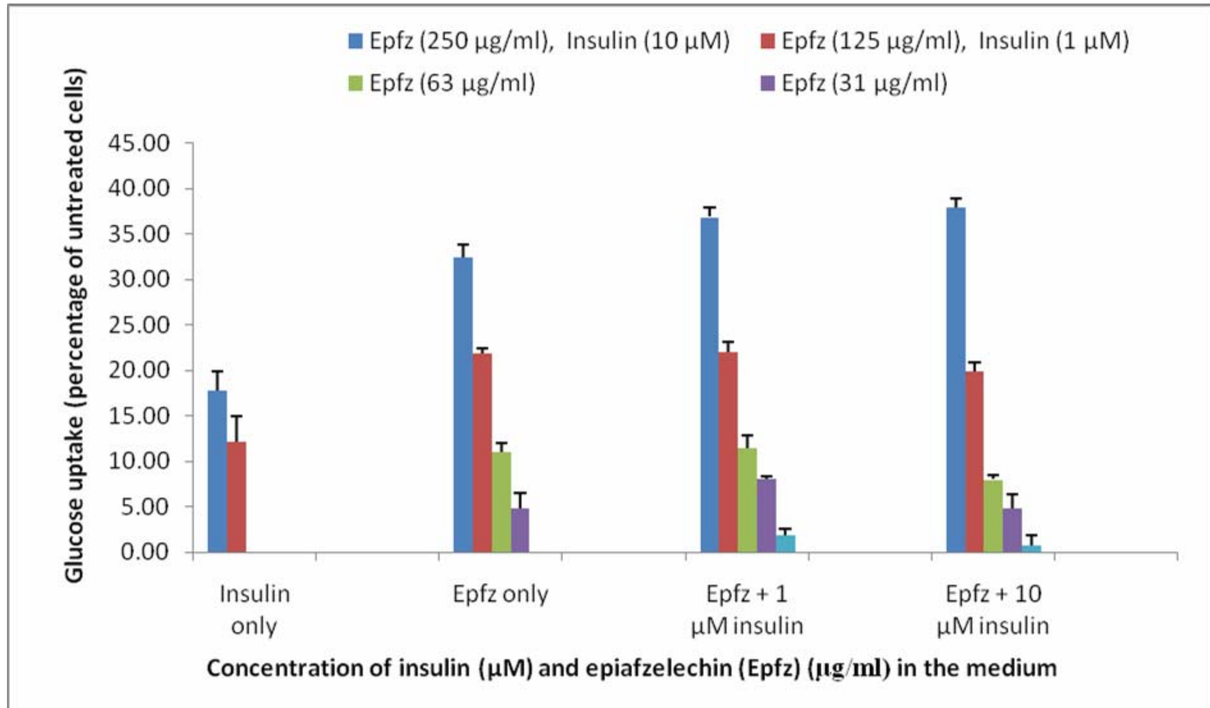

Figure 4-39Glucose uptake in H-4-II-E rat liver cells (as percentage of untreated control cells ± standard error of mean, n=9) exposed to the compound epiafzelechin in medium containing different concentrations of insulin.

4.3.9 Insulin secretion in RIN-m5F pancreatic cells

Epiafzelechin was evaluated at different concentrations (62.5 µg/ml – 500 µg/ml) for its ability to stimulate insulin secretion in RIN-m5F pancreatic β-cells and was compared with the untreated control cells. Epicatechin was not evaluated due to time constraints. The RIN-m5F pancreatic cells exposed to the epiafzelechin resulted in a dose related increase in insulin secretion (Figure 4-40). The insulin secreted significantly (*p* 0.001) increased from 47.1 ± 10.2% at the concentration of 62.5 µg/ml to 123.9 ± 19.2% at the concentration of 500 µg/ml.

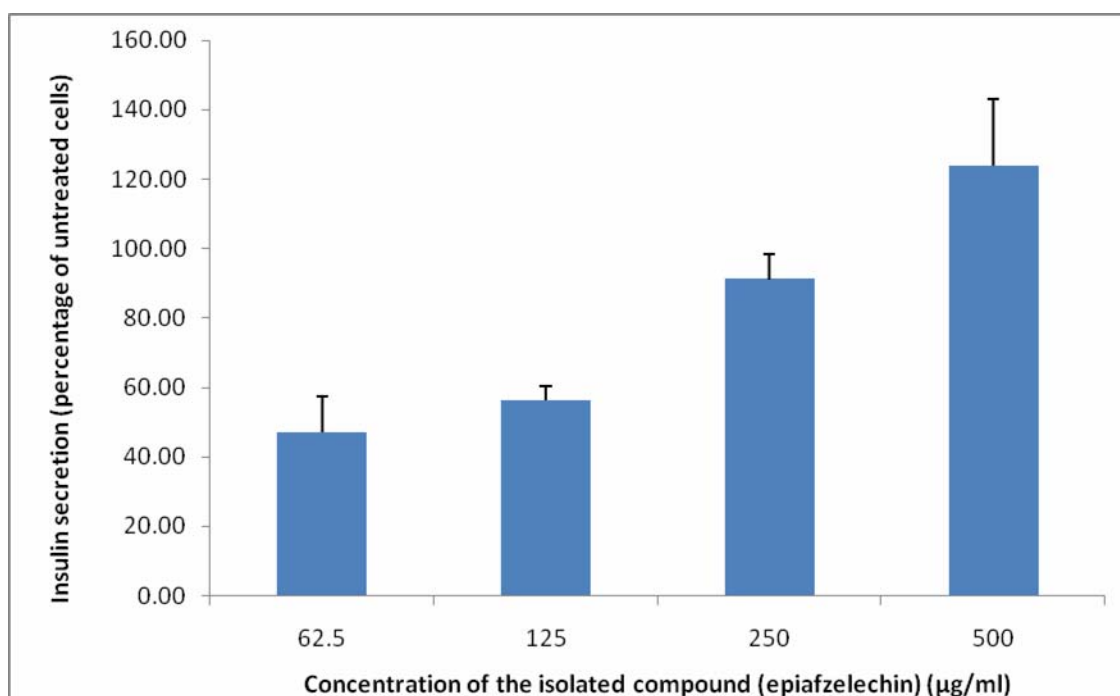

Figure 4-40Insulin secreted in RIN-m5F pancreatic cells (as percentage of untreated control cells ± standard error of mean, n=6) exposed to the isolated compound (epiafzelechin).

The effect of the isolated compound (epiafzelechin) on the viability of RIN-m5F pancreatic β-cells after insulin secretion assay was investigated (Figure 4-41). The result showed that the RIN-m5F cells exposed to epiafzelechin had dose related decrease in cell viability from 106.4 ± 1.6% to81.1 ± 0.3% as concentration was increased from 62.5 µg/ml to 500 µg/ml. The correlation coefficient between the viability of RIN-m5F pancreatic β-cells and insulin secretion by the ethyl acetate fraction of the extract of *F. lutea* R^2 was 0.66 (Figure 4-42). This indicates that the stimulation of RIN-m5F pancreatic β-cells by epiafzelechin to secrete insulin may be attributed in part to disruption of cell membrane.

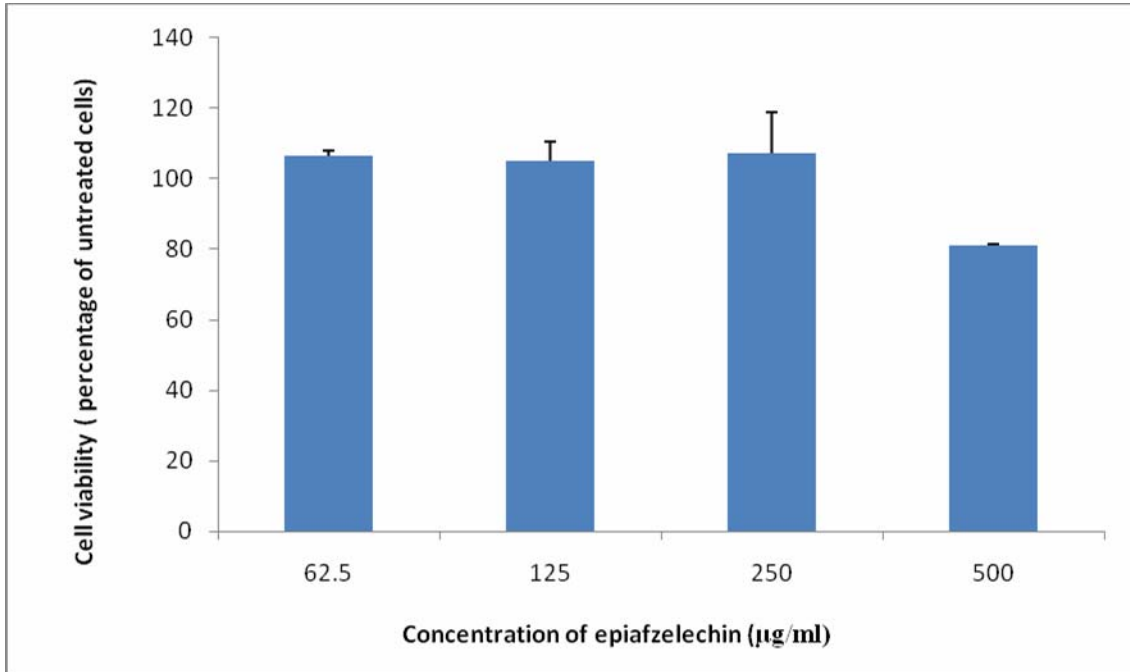

Figure 4-41Effect of epiafzelechin on RIN-m5F pancreatic cell viability (as percentage of untreated control cells± standard error of mean, n=6) after stimulating insulin secretion.

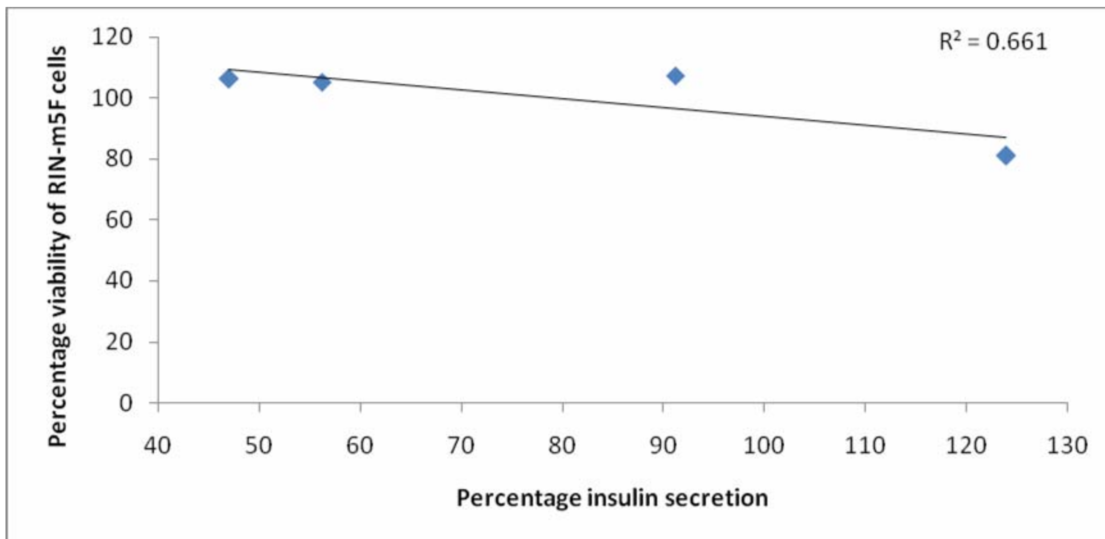

Figure 4-42 The correlation between percentage cell viabilty of RIN-m5F pancreatic β-cells and percentage insulin secretion by epiafzelechin.

4.4 *In vivo* study

4.4.1 The effect of different diets on body weight

Prior to the start of treatments, four mice died from the high caloric diet. Histological evaluation of the mice indicated that they died from ulcerative dermatitis. This condition is known to occur in mice fed a high fat diet most likely due to deficiency in the antioxidant vitamins. Due to the prior allocation of animals to treatment groups, the study was unbalanced with all treatment groups having ten animals, with the exception of the high caloric diet treatment group which has 6 miceThe initial weights of the CD1 mice (mean ± S.E.M.) at period 0 (Figure 4-43) before commencement of treatment was 40.45 ± 2.35 g (high calorie diet control), 41.02 ± 1.12 g (high calorie diet with treatment), 44.77 ± 0.48 g (normal diet control) and 42.39 ± 1.88 g (normal diet with treatment). The mice were hereafter fed their respective diet for about 7 weeks and the result of the different diet on body weight is presented in Figure 4-43. The body weight of the control mice fed the high calorie diet and the normal diet gradually increased throughout the study more than that of their comparative treatment groups, with the high calorie diet having the greater increase. Conversely, the body weight of mice on treatment in conjunction with a high calorie diet and normal diet with (ethyl acetate fraction) showed a gradual decrease in body weight throughout the study, with the mice on normal diet having the greatest reduction. Unfortunately none of these differences were significant (p   0.05) between the treatments and their controls. Similarly there was no significant difference (p   0.05) between the two treatment groups (high calorie diet with treatment group and the normal diet with treatment group) nor between the untreated groups (high calorie diet group and normal diet group). The final weight of mice at the end of the treatment plan was 44.14 ± 4.67 g (high calorie diet group), 40.88 ± 0.92 g (high calorie diet with treatment group), 45.90 ± 2.10 g (normal diet group) and 39.54 ± 1.34 (normal diet with treatment group).

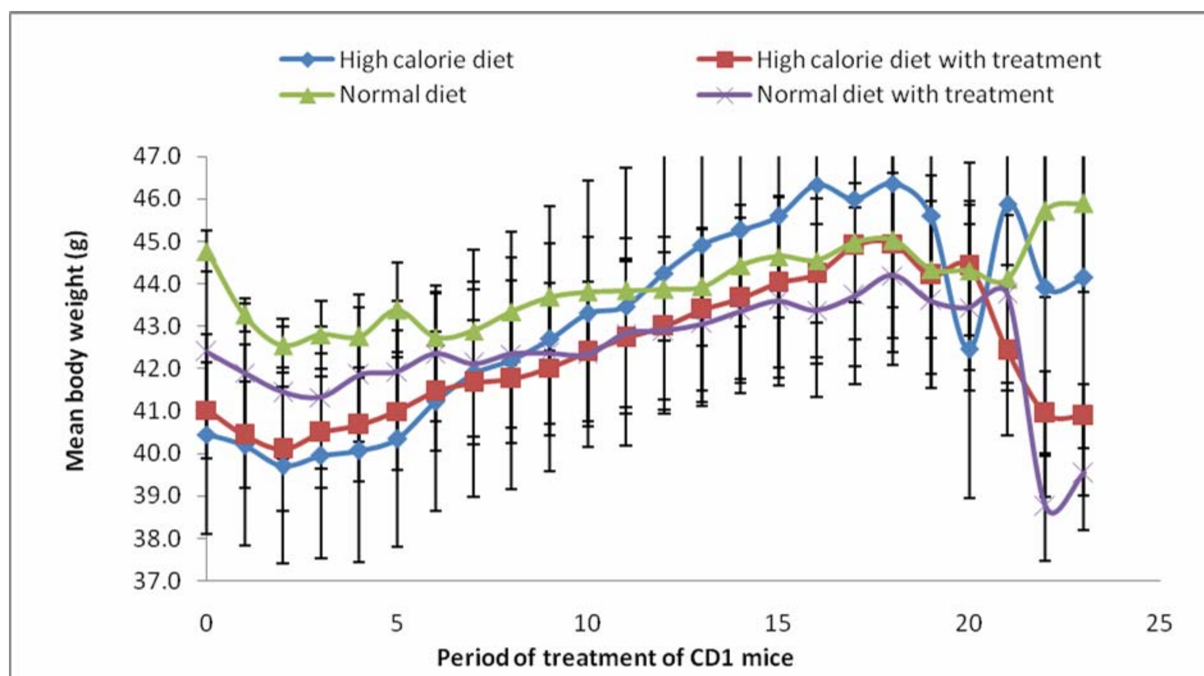

Figure 4-43The effect of high calorie and normal diet with and without treatment (the ethyl acetate fraction of *F. lutea*) on body weight of CD1 mice (mean ± S.E.M.). The initial body weight at period 0 was when obesity state was attained by mice prior to commencement of treatment for about 7 weeks.

4.4.2 The effect of different diets on food intake

After induction of obesity the mean food intake before commencement of treatment was 9.50 ± 1.42 g (high calorie diet group), 9.40 ± 1.01 g (high calorie diet with treatment group), 10.50 ± 1.12 g (normal diet group) and 10.60 ± 1.20 g (normal diet with treatment group). The mice were fed their respective diet for about 7 weeks and the result of food intake is presented in Figure 4-44. The mice placed on normal diet with and without treatment (ethyl acetate fraction of *F. lutea*) ate more food than those on high calorie diet with and without treatment. The food intake in mice on normal diet group was not significantly different (p 0.05) from that of mice placed on normal food with treatment. Similarly, food intake in mice placed on high calorie diet was not significantly different from those on high calorie with treatment throughout the study. Furthermore, there was no significant difference between the food intake in mice fed normal diet with treatment and mice fed high calorie diet with treatment, nor was there any significant difference between there controls. The Final food intake at the end of the study was 10.60 ± 1.07g (high calorie diet group), 9.00 ± 0.98 g (high calorie diet with treatment group), 14.00 ± 0.53 g (for the normal diet group) and 12.20 ± 0.98 g (normal diet with treatment group).

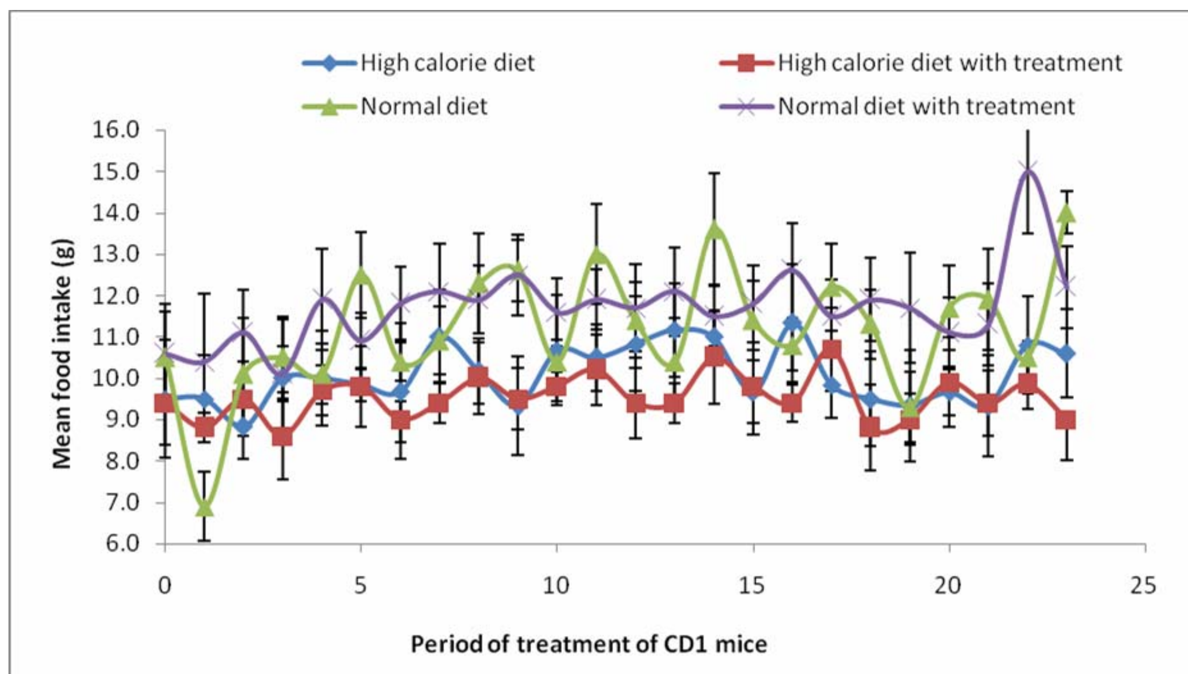

Figure 4-44The effect of high calorie and normal diet with and without treatment (the ethyl acetate fraction of *F. lutea*) on food intake of CD1 mice ((mean ± S.E.M.). Food intake at period 0 was when obesity state was attained by mice prior to commencement of treatment for about 7 weeks.

4.4.3 The effect of different diets on faecal weight

The faecal output based on weight is presented in Figure 4-45. The initial faecal weight prior to commencement of treatment was 2.00 ± 0.36 g (high calorie diet group), 2.10 ± 0.28 g (high calorie with treatment group), 1.80 ± 0.20 g (normal diet group) and 2.30 ± 0.26 g (normal diet with treatment group). Mice were fed their respective diet for about 7 weeks and the result of faecal weight (Figure 4-45) showed that the faecal weight for the mice on normal diet was higher than for those on high calorie diet.

A closer look also showed that mice on diet with treatment had higher faecal output than those on diet without treatment. At the end of the study the final faecal output for the mice were 2.80 ± 1.14 g (high calorie group), 1.67 ± 0.33 g (high calorie with treatment group), 6.25 ± 0.30 g (normal diet group) and 4.60 ± 0.28 (normal diet with treatment group). The high calorie diet group was significantly different to both groups on the normal diet (p   0.01) and tended towards significance against the non-treated high caloric diet group (p = 0.075).

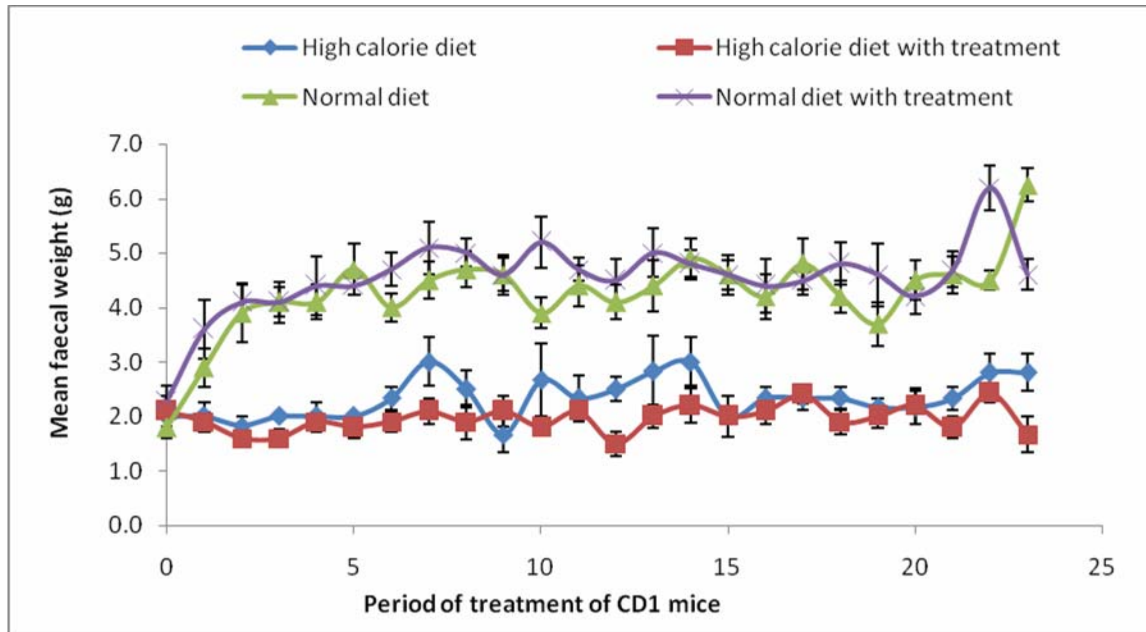

Figure 4-45The effect of high calorie and normal diet with and without treatment (the ethyl acetate fraction of *F. lutea*) on faecal output ((mean ± S.E.M.). Faecal output at period 0 was when obesity state was attained by mice prior to commencement of treatment for about 7 weeks.

4.4.4 The effect of high calorie diet on blood glucose concentration

The fasting blood glucose and glucose tolerance tests were performed on all the obese CD1 mice prior to commencement of treatment (Figure 4-46). The average fasting blood glucose concentration was 8.43 ± 1.16, 8.83 ± 0.57, 8.90 ± 0.88 and 8.82 ± 0.58 mM respectively for obese mice placed in high calorie diet, high calorie diet with treatment, normal diet and normal diet with treatment groups. There was no significant difference (p 0.05) among the treatment groups in the fasting blood glucose concentrations prior to treatment. GTT were performed on all the mice after ip injection of glucose (2 g/kg) and the average blood glucose concentrations at 5 min was 16.15 ± 4.26, 11.85 ± 1.86, 13.00 ± 2.12 and 15.68 ± 2.22 mM respectively for mice placed in high calorie diet, high calorie diet with treatment, normal diet and normal diet with treatment groups. The CD1 mice placed in the high calorie with treatment group had the lowest GTT value followed by those in the normal diet group but there was no significant difference (p 0.05) among them, those mice placed in normal diet and treatment group had the highest GTT values, followed by those in normal diet group (Figure 4-46). The blood glucose concentrations of all the mice except mice place in high calorie with treatment group continued to rise until after 90 min (Figure 4-46) when it declined to 20.92 ± 3.57, 24.26 ± 2.33 and 24.99 ± 2.09 mM respectively for mice place in high calorie diet, normal diet and normal diet with treatment groups while mice place in high calorie diet with treatment group had a decline in blood glucose concentration (18.69 ± 3.89 mM) after 30 min of glucose injection which went up again at 90

min (22.29 ± 3.82 mM). There was no significantly difference (p 0.05) in the blood glucose concentrations were present between the treatment groups.

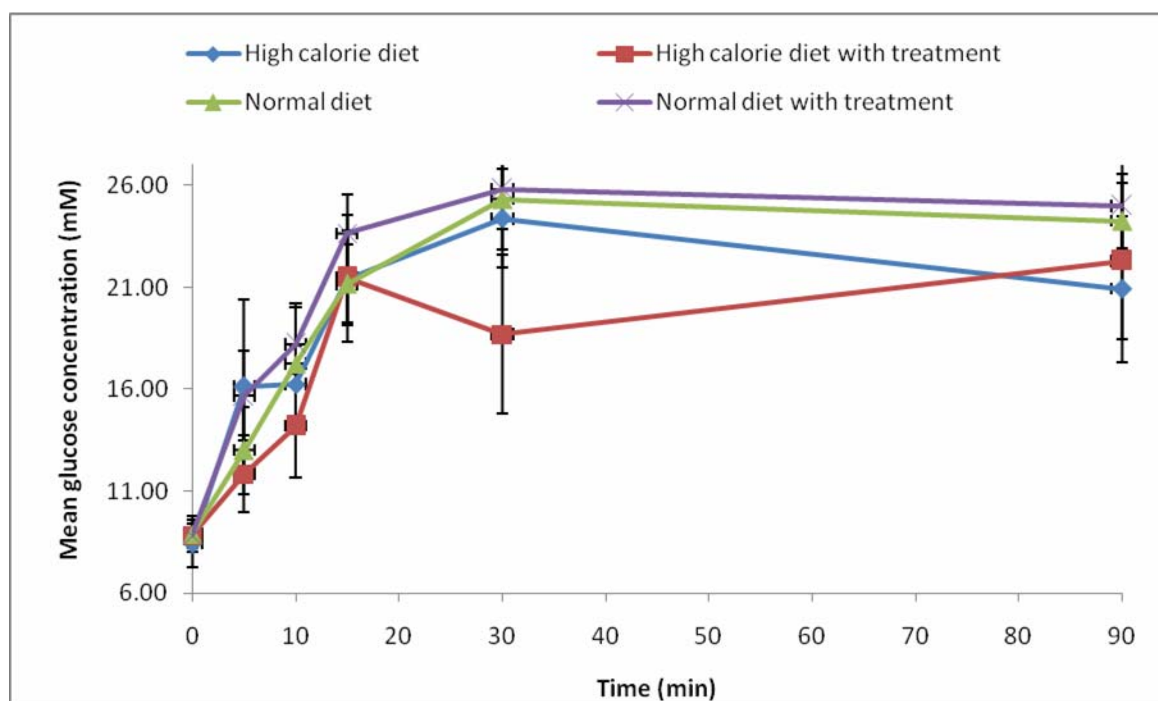

Figure 4-46The effect of high calorie diet on blood glucose concentrations of CD1 mice ((mean ± S.E.M.). Fasting blood glucose concentrations and glucose tolerance tests (GTT) at period 0 when obesity state was attained by CD1 mice prior to commencement of treatment.

The blood glucose concentrations of all the CD1 mice were evaluated again after 6 weeks of treatment (Figure 4-47) by performing the fasting blood glucose test and GTT. The average fasting blood glucose concentrations for the CD1 mice was 8.72 ± 1.16, 7.71 ± 0.57, 7.05 ± 0.88 and 7.71 ± 0.58 mM respectively for the animals in high calorie diet, high calorie diet with treatment, normal diet and normal diet with treatment groups. All the mice had fasting blood glucose concentration lower than 8 mM except the mice in the high calorie treatment group, but no significant difference (p 0.05) in the fasting blood glucose concentrations was observed among them. GTT were performed on all the mice after ip injection of glucose (2 g/kg) and the average blood glucose concentrations at 5 min was 15.72 ± 4.42, 12.23 ± 1.86, 10.96 ± 2.12 and 13.95 ± 2.22 mM respectively for mice in high calorie diet, high calorie diet with treatment, normal diet and normal diet with treatment groups. The average blood glucose concentration of the mice after 90 min of glucose administration declined for all the mice 22.97 ± 3.57 mM (mice on high calorie diet), 18.04 ± 3.82 mM (mice on high calorie with treatment), 13.22 ± 2.33 mM (mice on normal diet) and 13.38 ± 2.09 mM (mice on normal diet with treatment), with mice on normal diet and normal diet and treatment having the lowest GTT values13.22 ± 2.33 mM and 13.38 ± 2.09 mM respectively.

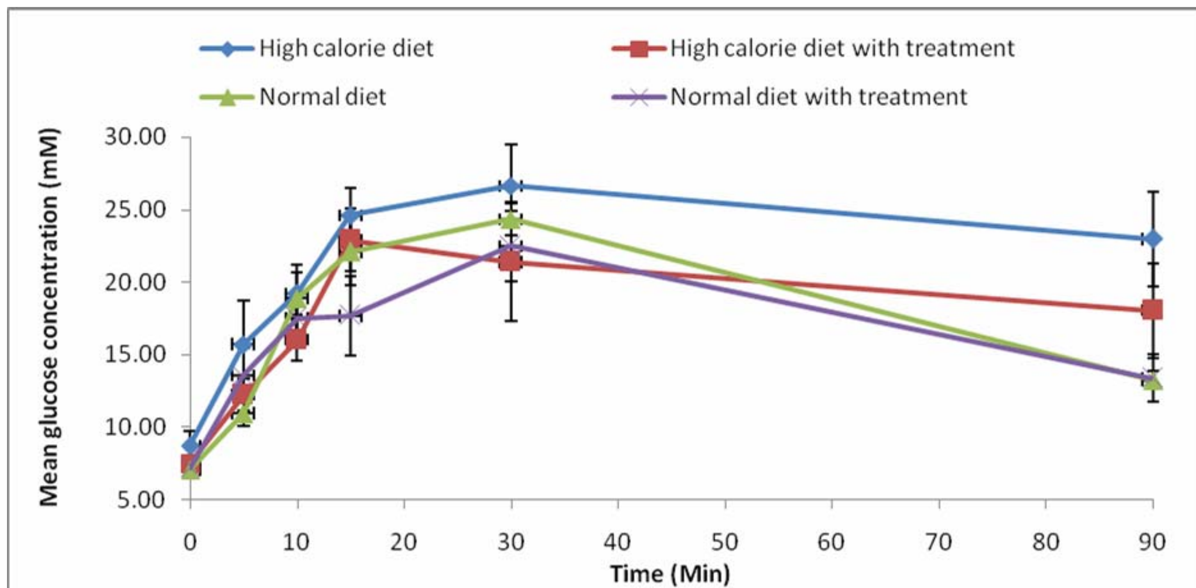

Figure 4-47The effect of different diets on blood glucose concentrations of CD 1 mice ((mean ± S.E.M.). Fasting blood glucose concentrations and glucose tolerance tests (GTT) of CD1 mice after 6 weeks of treatment.

4.4.5 The effect of different diets on full blood count parameters

The result of the haematological parameters (full blood count) is presented in Table 4-9 with no significant difference (p 0.05) between treatment groups. It was observed that the mice fed diets with treatment had lower haemoglobin, platelet and lymphocytes values than those of their respective controls. Also observed was that the RDW, WCC and neutrophils of the mice fed diet with treatment had higher values than those fed diet without treatment (Table 4-9).

4.4.6 The effect of different diets on serum chemistry parameters

The result of the serum chemistry parameters is presented in Table 4-10 with no significant difference (p 0.05) between them. All values are with the normal range for the CD1 mice. Even though values are within the normal range the alkaline phosphatase activity (ALP) and alanine aminotransferase (ALT) respectively for CD1 mice on high calorie diet with treatment and on high calorie diet. In addition, the blood glucose concentrations for the CD1 mice on diet with treatment are higher than their respective controls (Table 4-10).

4.4.7 The effect of diets on gross pathological changes in CD1 mice.

The liver, kidneys, pancreas, heart and blood vessel to the legs were collected for histological pathological examination, and there were no morphological changes observed in the kidneys, heart and blood vessel to the legs. The most consistent morphological changes in the animals were the

hypertrophic changes in the endocrine islet cells of Langerhans within the pancreas and metabolic-induce vacuolation and swelling of the hepatocytes in the liver (hepatosis). Excessive fat depots were also observed in several of the animals and excessive fat is one of the predisposing factors for diabetes. These morphological changes were induced by the high calorie diet. There was no line of demarcation between the morphological changes in mice and the diet consumed.

Table 4-9The effect of diets (with or without ethyl acetate fraction of *F. lutea*) on haematological parameters of mice

Full blood count	Group			
	High Calorie diet	High Calorie diet with Treatment	Normal diet	Normal diet with Treatment
Hb (g/l)	125.2 ± 2.94	118.6 ± 6.60	127.3 ± 3.90	124.5 ± 3.42
RCC x 10e12/l	8.6 ± 0.25	8.3 ± 0.26	8.7 ± 0.17	8.6 ± 0.14
Ht (l/l)	0.4 ± 0.01	0.38 ± 0.02	0.4 ± 0.01	0.4 ± 0.11
MCV (fl)	46.4 ± 0.52	46.4 ± 1.08	46.9 ± 0.51	46.7 ± 1.14
MCH (g/dl RC)	14.6 ± 0.17	14.2 ± 0.50	14.7 ± 0.19	14.8 ± 0.29
MCHC (g/dl RC)	31.5 ± 0.26	30.6 ± 0.46	31.3 ± 0.15	31.7 ± 0.50
RDW (%)	13.3 ± 0.44	14.7 ± 0.57	13.9 ± 0.30	15.2 ± 0.36
WCC (10e9/l)	2.5 ± 0.56	3.0 ± 0.33	1.9 ± 0.42	3.8 ± 0.65
Neuts (mat) %	21.7 ± 3.12	30.8 ± 3.91	19.4 ± 1.87	23.8 ± 3.18
N (mat) abs x 10e9/l	0.5 ± 0.12	0.9 ± 0.11	0.4 ± 0.13	0.8 ± 0.15
N (immat) %	0	0	0	0
N (immat) abs x 10e9/l	0.00	0.00	0.00	0.01 0.01
Lymph %	72.8 ± 2.20	58.7 ± 5.07	70.3 ± 3.31	68.0 ± 2.81
Lymph abs x 10e9/l	1.8 ± 0.43	1.8 ± 0.26	1.3 ± 0.22	2.3 ± 0.37
Mono %	4.2 ± 0.83	7.9 ± 1.95	6.0 ± 1.68	4.5 ± 0.81
Mono abs x 10e9/l	0.09 ± 0.02	0.2 ± 0.05	0.2 ± 0.08	0.14 ± 0.03
Eos %	1.3 ± 0.67	2.8 ± 0.91	2.9 ± 0.88	2.3 ± 0.69
Eos abs x 10e9/l	0.03 ± 0.01	0.08 ± 0.03	0.05 ± 0.16	0.07 ± 0.02
Baso %	0	0.1 ± 0.11	1.4 ±1.4	0.09 ± 0.09
Baso abs x 10e9/l	0.00	0.00	0.02 ± 0.02	0.003 ± 0.003
Plt C x 10e9/l	1133.8 ± 118.30	1037.8 ± 121.23	1231.0 ± 54.81	1174.3 ± 80.77
MPV (fl)	6.5 ± 0.24	6.5 ± 0.17	6.7 ± 0.15	6.1 ± 0.28

Table 4-10The effect of diets (with or without ethyl acetate fraction of *F. lutea*) on serum chemistry parameters of mice

Serum chemistry parameters	Group			
	High Calorie	High Calorie with Treatment	Normal diet	Normal diet with Treatment
Total protein (g/l)	51.3 ± 1.52	52.1 ± 0.90	52.2 ± 0.92	50.1 ± 0.94
Albumin (g/l)	25.2 ± 1.85	23.6 ± 1.56	24.6 ± 1.97	23.4 ± 1.06
Globulin (g/l)	26.2 ± 1.25	28.7 ± 1.00	27.9 ± 1.55	26.7 ± 1.02
Albumin/globulin ratio	1.0 ± 0.0	0.9 ± 0.11	0.9 ± 0.10	1.0 ± 0.0
ALT (U/l)	21.2 ± 1.33	150.2 ± 122.62	79.0 ± 30.50	76.6 ± 28.10
ALP (U/l)	128.7 ± 30.51	57.7± 14.44	54.5 ± 4.83	52.3 ± 3.57
Glucose (mmol/l)	9.0 ± 1.07	11.3 ± 0.91	9.3 ± 0.56	10.0 ± 0.62
Cholesterol (mmol/l)	4.7 ± 0.56	4.8 ± 0.36	3.9 ± 0.23	3.7 ± 0.24
Urea (mmol/l)	4.5 ± 0.34	4.1 ± 0.42	9.3 ± 0.50	8.4 ± 0.36
Creatinine (mmol/l)	9.0 ± 0.0	10.1 ± 1.11	9.0 ± 0.0	8.7 ± 0.27
Triglycerides (mmol/l)	1.3 ± 0.33	1.0 ± 0.17	1.3 ± 0.21	1.7 ± 0.20

Chapter 5

5 Discussion

5.1 Selection of solvent for extraction of plants

Ten *Ficus* species were selected based on availability and accessibility and were each extracted with acetone, chloroform and hexane.This did differ from traditional practice as health practitioners would prepare herbal remedies with either water or alcohol (Kelmanson*et al.*, 2000). Unfortunately these solvents extract mainly polar compounds thus limiting the amount of extractable active compounds derivable form plants. Additionally polar plants extracts are known to contain glucose and other monosaccharide sugar, which if present would complicate carbohydrate hydrolysing enzyme inhibition assays (Ali *et al.*, 2006). The presence of sugars can interfere with bioassays that measure glucose concentrations. For instance, the assay for α-amylase enzyme activity involves incubating the enzyme with standard concentration of starch as substrate, resulting in the release of the reducing disaccharide maltose (Bernfeld, 1955). In the presence of the α-amylase inhibitor, the amount of maltose released will be reduced and quantified as a percentage of the starting concentration. However, if exogenous carbohydrates are present, this can result in an inaccurate result as the starting concentration of starch could be increased to an unknown level.

Acetone was also specifically included as it is an intermediately polar solvent extractant. Martini and Eloff (1998) showed in their study that the polarity of an extracting solvent plays an important role in the quantity of mass extracted. The authors said that the polar and intermediate polar solvents have higher extracting abilities than the non-polar solvents. They said further that acetone always extracted a greater variety of compounds and the larger the variety of compounds that can be extracted by solvents, the better the chance that biologically active components will also be extracted (Eloff, 1998a).

5.2 Efficacy of crude extracts of the ten *Ficus* species

The most challenging goal in the management of diabetes mellitus is to achieve blood glucose levels as close to normal as possible (Tiwari and Rao, 2002). Postprandial hyperglycaemia is the earliest metabolic abnormality to occur in Type II diabetes mellitus (Lebovitz, 1998). Postprandial blood glucose levels may be elevated while fasting plasma glucose is normal, constituting an early stage of Type II diabetes referred to as postprandial diabetes (Baron, 1998). Alpha-amylases are endoglucanases, which hydrolyse the internal α-1,4 glucosidic linkages in starch, and α-glucosidase (sucrase), one of the glucosidases located in the brush border surface membrane of intestinal cells, is a key enzyme for carbohydrate digestion and absorption. These enzymes have been recognized as therapeutic targets for modulation of postprandial hyperglycaemia. It has also been speculated that these enzymes could be target site for the selected plants as they are all used by the oral route in

traditionally. For this study the inhibitory effectiveness of the acetone extract of the leaves of ten *Ficus* species against α-amylase and α-glucosidase were investigated and their possible relationship with total polyphenolic content and antioxidant activity was also studied. This is because the polyphenolic extracts from a number of plants have been known to be effective inhibitors of intestinal α-glucosidase and α-amylase enzymes, demonstrating their potential therapeutic effect on post-meal blood glucose levels (McDougall *et al.*, 2005).

The ten *Ficus* species were generally potent inhibitors of activity of the porcine pancreatic α-amylase but weaker inhibitors of activity of the rat small intestinal α-glucosidase. The extract of *F. sycomorus* inhibited the activity of α-glucosidase with the lowest EC_{50} of 217 ± 69 µg/ml albeit non-significantly to the EC_{50} of *F. lutea*. Some authors have shown that the extracts of other *Ficus* species such as *F. racemose* (Ahmed and Urooj, 2010), *F. benghalensis* (Ahmed *et al.*, 2011) and *F. deltoidea* (Farsi *et al.*, 2011) inhibited the activity of sucrase with an EC_{50} of 367 ± 15.2 µg/ml and 239 ± 14.3 µg/ml respectively for cold and hot water extract of *F. racemosa*, and 193 ± 21.6 µg/ml and 141 ± 22.1 µg/ml respectively for cold and hot water extract of *F. benghalensis*. This was in agreement with our results. Furthermore, studies also showed the activity of α-amylase is inhibited with an EC_{50} of 0.94 ± 0.15% and 0.58 ± 0.15% respectively for the cold and hot water extracts of *F. racemosa,* (Ahmed and Urooj, 2010) and, 4.4 and 125 µg/ml respectively for the cold and hot water extracts of *F. benghalensis* (Ponnusamy *et al.*, 2011), which was once again in agreement with our results.

While the active agent(s) responsible for the effect seen being unknown, it was speculated that the effect could be due to the presence of the polyphenolic compounds. From previous studies it was observed that the potency with which flavonoidsinhibited the activity of porcine pancreatic α-amylase and rat small intestinal α-glucosidase are different (McDougall *et al.*, 2005;Tadera *et al.*, 2006). Strawberry and raspberry extracts were the most effective inhibitors of α-amylase followed by blueberry and blackcurrant. Although these extracts also inhibited rat intestinal α-glucosidase activity, the order of effectiveness was different than forα-amylase as blueberry and blackcurrant were the most effective followed by strawberry and raspberry (McDougall *et al.*, 2005). It would therefore appear that rat intestinal α-glucosidase is generally weakly inhibited by many flavonoids, while flavonoids are often potent inhibitors of porcine pancreatic α-amylase (Tadera *et al.*, 2006). It is therefore possible that the extracts of the *Ficus* species used in this study demonstrated potent α-amylase inhibitory activity because they contain more of the flavonoid groups. To test this hypothesis, the correlation between total polyphenolic content and inhibitory of α-amylase and α-glucosidase activity was ascertained. For all ten *Ficus* species the correlation coefficient varied between 0.81 and

0.85, indicating the likelihood of the polyphenols in the acetone extracts being partly responsible for the inhibition of the activity of the enzymes.

To better evaluate study the relationship between polyphenolic content and enzyme interaction, the kinetics of inhibition for the most active extract (*F lutea*) was evaluated. With the polyphenols being present, it was speculate that the interaction with the enzymes would be non-competitive (Dixon and Webb 1999). As expected the extract of *F. lutea* showed partial non-competitive inhibition against porcine pancreatic α-amylase and α-glucosidase. Thisshowed that polyphenols (tannins) non-selectively bind to and precipitate proteins (digestive enzymes) thereby decreasing the efficiency of the enzymes.

To further establish the polyphenolic compounds as the main compounds responsible for the enzyme inhibitory effects seen, the antioxidant activity of the plant extracts was also evaluated. The extracts of *F. glumosa, F. sycomorus* and *F.lutea* both possessed good antioxidant activities with correlation with polyphenolics content being 0.62. Based on the presence of polyphenolic compounds and anti-oxidant activity, it was speculated the high molecular weight polyphenols, such as catechins, pelargonidins or leucopelargonidin may be possible active ingredients derivatives in addition to the flavonoids (Manian *et al.*, 2008). This was based on findings of Ivanova *et al.* (2005), who found that not all polyphenolic compounds possess $ABTS^+$ radical scavenging activities with activity being restricted to the higher molecular weight moleculesin this class (Hagerman *et al.* 1998). Another important finding of this study was the antioxidant activity seen was in agreement with other studies where the *F. glumosa*(Madubunyi *et al.*, 2012), *F. sycomorus* (Abdel-Hameed, 2009) and *F.lutea* (Marwah*et al.*, 2006) have very good DPPHscavenging activities with reported IC_{50} of 79.5 ± 1.77 µg/ml and 11.9 ± 0.3 µg/ml respectively for *F. sycomorus* and *F. lutea*. This was important as it suggests that the secondary metabolites responsible for the observed effect may be part of the natural constituent of the plant and not a stress produced mediator.

In the *in vitro* glucose uptake assays in primary cell cultures, only the extracts of *F. lutea* significantly increased in a dose related manner glucose uptake into primary muscle cell cultures, while the extracts of *F. lutea* and *F. glumosa* significantly increased in a dose related manner glucose uptake into primary fat cell culture. The extract of *F. lutea* enhanced glucose uptake into primary muscle and fat cell cultures at basal glucose concentration i.e. in the absence of insulin possible through the involvement of residual insulin receptor binding within the primary cultures preparation (Gray *et al.*, 2000), or possibly that the extract of *F. lutea* enhanced glucose uptake into the primary cultured muscle and fat cells via the same pathway as the insulin (Gray *et al.*, 2000), therefore enhancing

glucose utilisation activities in the primary cell cultures in an insulin-mimetic manner. Similarly, in the *in vitro* glucose uptake assays in the established cell lines, only the extracts of *F. lutea* significantly increased a dose related uptake of glucose into C2C12 muscle and H-4-II-E liver cells but not the 3T3-L1 pre-adipocytes in the absence of insulin. The effect was also in part enhanced by the addition of insulin to the cell wells. Based on these results the antidabetic activity of the extracts of *F. lutea* could be related to the enhancement of glucose uptake by the muscle and the liver cells.

Based on the specific effect seen in the different cell culture it is believed that the effect seen was due to an increase in the translocation of GLUT4 transported to the cell membrane. This was based on the increase in glucose uptake in the primary muscle, primary fat cell culture, C2C12 muscle and H-4-II-E liver with an absence of uptake in non-differentiated adipocytes. At the molecular level the only known differences between these cells is the absence of the GLUT4 receptor in the pre-adipocytes. Based on the speculation of activity being related to the presence of polyphenolic compounds (as discussed above), a similar effect was evident *in vitro* with plant polyphenols in muscle and liver cultures (Cazarolli *et al.*, 2008). The polyphenolic compounds are believed to enhance glucose uptake through the stimulation of the GLUT4 receptor translocation to the plasma membrane in a an insulin mimic manner by directly acting on specific components of insulin signalling transduction pathway (Pinent *et al.*, 2004) or by elevation of GLUT4 gene expression and protein levels (Liu *et al.*, 2006). For this study we believe that the former may explain the evident effect as the concurrent administration of insulin failed to significantly enhance glucose uptake. From normal physiology, the mechanism controlling the GLUT4 receptor translocation from the intra-cellular environment is mainly through the insulin receptor activity. With the number of GLUT4 receptors being fixed, it is most likely that the phenomenon of tacchyphylaxis resulted i.e. a maximum effect was based on the limited number of GLUT4 receptors present as opposed to the stimulatory potential of the extract in combination with insulin. This result was similar to those observed withresveratrol, a wine polyphenol, which when used in the absence of insulin enhanced muscular uptake of glucose but when added simultaneous to insulin led to a time dependent diminishing of glucose uptake in C1C12 muscle cells (Deug *et al.*, 2008). The authors concluded that resveratrol stimulated muscular glucose uptake via insulin independent and insulin dependent pathways in a time dependent manner (Deug *et al.*, 2008).

The extract of *F. lutea*mayalso possible also contain also glucokinin, a plant insulin-like protein. The presence of this insulin-like protein (glucokinin) has been discovered and extracted from plants with antidiabetic activity (Banting *et al.*, 1922; Xavier-Filho *et al.*, 2003). Glucokinin exhibit similar metabolic functions as insulin from animals and initiate metabolic activities in carbohydrate

metabolism (Sangeetha and Vasanthi, 2009). Plant insulin is found to be effective in regulating blood glucose by mimicking insulin signal in animals. For this insulin-like protein to be effective it has to be ingested together with protease inhibitors to protect it from hydrolysis in the digestive track (Sangeetha and Vasanthi, 2009).

The potential of the acetone extract of *F. lutea* to stimulate insulin secretion in RIN-m5F pancreatic cell line under basal condition was investigated. Results revealed that the acetone extract of *F. lutea* was capable of stimulating the pancreatic β-cells to increase the release of insulin in dose responsive manner. The extracts of *F. lutea* stimulated insulin secretion (4.58 fold) in the RIN-m5F pancreatic cells at the highest concentration in comparison to the lowest concentration while cell viability was reduced by 17.8%. The positive control, glibenclamide stimulated 1.19 fold increase insulin secretion. While our result thus suggests that the extract of *F. lutea*couldpossess insulin secretagogue properties, the effect may be due to cell membrane lysis and release of stored insulin granules. The presences of cytotoxic compounds such as glycosides in plant extracts have been demonstrated experimentally to compromise cell membranes by cell lysis which in turn could lead to the release of insulin (Persaud *et al.*, 1999). Studies have shown that the extract of *Gymnema sylvestre,* a plant that plays key role in Ayurvedic medicine stimulates insulin release *in vitro* but the mechanism is by cell membrane disruption (Persaud *et al.*, 1999).

However, despite the promising *in vitro* activity, the ability of the plant extracts to inhibit enzymes activity permanently does indicate a potential for the extract to be toxic. For this study we selected a hepatocyte and renal cell culture as these two cell types represent the most susceptible cells in the body due to their high metabolic activity, high perfusion and special transport systems that allow for the bioaccumulation of toxins. In general the ten *Ficus* species showed that the extracts were generally more toxic to the Vero kidney cells than to the C3A liver cells. However the extracts of *F. lutea* and *F. polita* were generally more toxic to the C3A liver cells than to the Vero kidney cells. Although the extracts of the *Ficus* species seemed to contain toxins that are selectively nephrotoxic, in order to avoid overestimation or underestimation of the cytotoxicity, it was suggested that more than one assay should be used to determine cell viability in *in vitro* studies, as this would increase the reliability of the results obtained (Fotakis and Timbrell, 2006). Safety of plant medicine needs to be carefully considered, investigated and validates (Halberstein, 2005), more especially when traditional use of plant extracts have been reported to cause deaths due to toxic effects of some extracts (Winslow and Kroll, 1998).

5.3 Isolation of the active components from the acetone extract of *F. lutea*

Since the extract of *F. lutea*was rich in polyphenols, possessedantioxidant activity, was a potent inhibitor of α-amylase and α-glucosidase activity, enhancedglucose uptake in cells and was a possible stimulator of insulin release, it was chosen for further studies. The active extract of *F. lutea* was fractionated using solvents of various polarities from the least non-polar (hexane) to the most to polar (water) to yield six fractions (hexane, chloroform, dichloromethane, ethyl acetate, n-butanol and water fractions). The fractions were subjected to evaluation in the α-amylase and α-glucosidase inhibition assays. These assays were selected for bio-guided fractionation as the crude extract demonstrated high inhibitory activity in previous tests (sections 4.1.5 and 4.1.6). In addition, the inhibition of these enzymes has been suggested as a means of modulating post-prandial hyperglycaemia. The n-butanol fraction was the most active in the inhibition of the α-amylase enzyme, followed by the ethyl acetate fraction and the opposite for the inhibition of α-glucosidase enzyme activity. The inhibition of α-glucosidase (sucrase) activity of the ethyl acetate and n-butanol fractions are more potent than that of the crude acetone extract of *F. lutea* with the ethyl acetate fraction being the most potent, while forα-amylase activity the n-butanol fraction was not as active as the crude extract of *F. lutea*. For the α-glucosidase (sucrase) activity, it appeared that the fractionation process resulted in potentization while for α-amylase activity was loss. The loss of activity was difficult to explain, except perhaps that the effect was non-specific and due to the additive effect of a number of different compounds of different polarity.

All the fractions of the acetone extract of *F. lutea* were generally less toxic than the crude extract except the ethyl acetate and n-butanol fractions. Although the ethyl acetate fraction was relatively less toxic, it more cytotoxic against the Vero kidney cells than the crude extract of *F. lutea* while the n-butanol fraction was more toxic against the C3A liver cells than the crude extract indicating that the toxic compounds responsible for the cytotoxicity of the extract of F. lutea against the C3A liver cells reside in the n-butanol fraction. The ethyl acetate (100.1 ± 1.5 mg/g dry weight of extract) and the n-butanol (79.58 ± 0.5 mg/g dry weight of extract)fractions of *F. lutea* contained the highest polyphenolic compounds. Sincethepolyphenols are known to interact with proteins whether dietary protein or enzymes to form complexes with and precipitate them (Gyémánt *et al.*, 2009), this characteristic of polyphenols may also account for toxic activity of the ethyl acetate and n-butanol fraction.

The ability of the fractions of acetone extract of *F. lutea* to enhance glucose uptake in C2C12 muscle cells and H-4-II-E liver cells was evaluated. The ethyl acetate and n-butanol fractions were the only fractions that significantly increased in a dose related manner glucose uptake, with the ethyl acetate

fraction being superior. When insulin and the ethyl acetate fraction were added simultaneously to the C2C12 muscle and H-4-II-E liver cells, there was neither synergistic nor additive effect rather there was an inhibition of insulin mediate glucose uptake. This result once again supported the presence of synergismof the extract.

The potential of the ethyl acetate fraction of the acetone extract of *F. lutea* to stimulate insulin secretion in RIN-m5F pancreatic cell line under basal condition was investigated. Resultsindicatethat the ethyl acetate fraction was capable of stimulating the pancreatic β-cells to increase the release of insulin in dose responsive manner. The maximum stimulation of insulin secretion got lower with potentiation. The ethyl acetate fraction stimulated insulin secretion of 3.49 fold in RIN-m5F pancreatic cells with a reduction in cell viability by about 41.7%. This result once again tends to suggests that the ethyl acetate fraction probably increasesinsulin releasethrough cell lysis.

5.4 The isolated compounds from the ethyl acetate fraction of *F. lutea*

Five polyphenolic compounds were isolated from the ethyl acetate fraction of *F. lutea*. Of the five compounds were isolated, epicatechin and epiafzelechin were isolated from this plant for the first time. All the compounds isolated except epiafzelechin have been reported in literature to have antidiabetic activity. As far as could be established, this is the first study to demonstrate that epiafzelechin has hypoglycaemic activity. In this study, epicatechin, epiafzelechin and stigmasterol in decreasing order were potent inhibitors of sucrase activity and their potency was superior to that of the crude extract of *F. lutea* as well as to the ethyl acetate fraction. Furthermore, epicatechin and epiafzelechin also enhanced superior glucose uptake in C2C12 muscle cells and H-4-II-E liver cells above those of the crude extract and the fraction and their mechanism of action was probably via the insulin-mimetic mode of action.

In the*in vitro* assay, lupeol and α-Amyrin acetate isolated from the ethyl acetate fraction of *F. lutea* did not inhibit sucrase activity nor enhance glucose uptake. Deutschlander *et al.* (2011) found that the lupeol isolated from *Euclea undulate* in *in vitro* assay inhibited α-glucosidase activity whereas Rahman *et al.* (2008) and Mbaze *et al.* (2007) found that the lupeol isolated from *C. intybus* and *Fagara tessmannii* respectively did not *in vitro* inhibit α-glucosidase activity. Oral administration of α-Amyrin acetate from *F. bengalensis* (Singh *et al.*, 2009) and *F. racemosa* (Narender *et al.*, 2009) was seen to lower blood glucose profile in streptozotocin-induced diabetic rat model but the mechanism of action was not provided. Oral administration of stigmasterol isolated from *B. monospera* decrease serum glucose concentration with a concomitant increase in insulin level (Panda *et al.*, 2009). Also in their study Deutschlander *et al.* (2011) reported epicatechin to be a potent *in*

*vitro*inhibitor of α-glucosidase activity as well as increasing glucose uptake in C2C12 muscle cells which was in agreement with our result.

When insulin and the epiafzelechin were added simultaneously to the C2C12 muscle and H-4-II-E liver cells, there was no change in glucose uptake of the cells compared to the compound in the absence of insulin indicating that the pathway by which epiafzelechin enhanced insulin mediated glucose uptake may be similar to insulin. These results was similar to those observed by Ueda *et al.* (2008), where epigallocatechin gallate stimulated a dose dependent increase in glucose uptake of L6 muscle cells with no synergism being present with insulin.

5.5 *In vivo* assay

Animals have long been used in *in vivo* diabetic experiment as alternative to the use of humans. Numerous methods exist for inducing hyperglycaemia and the diabetogenic agents often used are grouped into different classes including chemical agents, biological agents, peptides, potentiators, steroids and high calorie diet (Matteucci and Giampietro, 2008). In this study inducing obesity and its associated conditions such as insulin resistance and type II diabetes was used. It was conducted in normal male CD1 mice placed on high calorie diet *ad libitum* continuously until they became either obese or were for a total period of 13 weeks. Hereafter they were placed in one of the four treatment plans. Our result showed that the feeding of animals with high calorie can lead to increase in body mass and induce obesity. This was manifested by increased body weight and elevated blood glucose levels of most of the mice suggesting they were already in pre-diabetic state when the first glucose tolerance test was conducted. The histopathology report also suggested that the animals were prediabetic as the animals had moderate to excessive fat deposit in the abdominal cavity, mild to moderate cell swelling with vacuolated changes within the cytoplasm of the hepatocytes with or with accumulation of fatty acids suggesting metabolic-induced fatty acid changes of the liver and the enlargement of the pancreatic islets of β-cell (hypertrophic changes in the islets of the β-cell). Metabolic induced cytoplasmic vacuolation of the liver has been reported by some authors (Samuthasaneeto *et al.*, 2007; Dhibi *et al.*, 2011) who found that high fat diets and obesity enhanced the mobilisation of free fatty acid from adipose tissue and transportation into the hepatocytes. This swelling of the hepatocytes may lead to expulsion of intracellular content, indicating cell necrosis (Dhibi *et al.*, 2011), or disruption of the plasma membrane resulting into the leakage of some hepatic enzyme such as ALP and ALT into the extracellular fluid where they can be detected at abnormal levels in the serum (Dashti *et al.*, 2002). This was observed in our study asthe mice on high calorie diet and high calorie diet with treatment groups had high ALP and ALT values respectively compared to those in other groups indicative of liver damage. Hypertrophy changes in islet of β-cell have been

associated with obesity (Nugent *et al.*, 2012). The islet of β-cell being an important controlling unit of metabolism is dynamic and reactive to changes in secretory demand. Metabolic changes due to insulin insensitivity and loss of glucose control are visible by changes in islet structure.

Studies have found that feeding of CD1 mice with high calorie diet for a period of about 12 week promotes about 80% body weight gain relative to the baseline weigh (Breslin *et al.*, 2010; Hou *et al.*, 2010). The addition of the ethyl acetate fraction of *F. lutea*to the high calorie diet and normal diet of mice did not significantly reduce body weight. Hou *et al.* (2010) found a similar effect in their study in which there was lack of statistical significance between the group receiving a normal standard diet and high carbohydrate – high fat diet (HC-HF) as well as between the groups of the HC-HF diet and metformin administration. This was blamed on small number of animals in each group (n=5) as well as internal variations. FurthermoreJayaprakasam*et al.* (2006) also observed that there was no significant body weight difference between C57BL/6 mice fed high fat diet and those fed high fat diet with ursolic acid and this was similarly observed in this study. However, there was significant body weight difference between C57BL/6 mice fed high fat diet and high fat diet with anthocyanin for (Jayaprakasam *et al.*, 2006). The findings of our study are therefore difficult to explain, as our ethyl acetate fraction of *F. lutea*was rich in polyphenolic compounds. This lead to a conclusion that the sample sized used in the study may not have been sufficiently high to demonstrate such an effect.

In terms of food intake Jayaprakasam *et al.* (2006) and Tsuda *et al.* (2003) noted in their studies with C57BL/6 mice that the animals fed normal calorie diet consumed more food by weight than mice on the high fat diet with no significant difference in the caloric intake of animals. However, Hou *et al.* (2010) observed in their study with CD1 mice that the animals on high fat diet significantly consumed more food than that on normal diet and on high fat diet with metformin treatment. Jayaprakasam *et al.* (2006) observed further that the addition of anthocyanin to the high fat diet did not affect food intake nor did the addition of ursolic acid when compared to group fed high fat diet alone. This implies that anthocyanin is not an appetite suppressant as observed with the ethyl acetate fraction of *F. lutea*used in this study.

In the study by Okada *et al.* (2012) the faecal weight of rat fed normal diet with adzuki bean extracts was significantly higher than rat fed normal diet alone. Similarly the faecal weight of rat fed high calorie diet with adzuki bean extracts was significantly higher than rat fed high calorie diet alone. However, no significant difference was observed in the faecal weight of rat fed normal diet only and high fat diet alone or between those fed normal diet with treatment and high calorie with treatment. In

this study there was less faeces forthe fat diet group. This may be due to the higher nutritional value of the food, thereby results in lower waste and thus lower faecal output.

No changes in GTT were evident for this study despite positive results *in vitro*. This was an unexpected finding as, Cherian *et al.* (1992) in their study demonstrated that pelargonidin 3-O-rhamnoside isolated from the bark of *Ficus bengalensis* improved glucose tolerance in diabetic rats. This improvement in glucose tolerance was related to the compound's ability to stimulate insulin release *in vitro*. Similarly, in the study by Jayaprakasam *et al.* (2006) a mixture of pure delphinidin, cyaniding and pelargonidin 3-O-galactoside from the fruit of *Cornus mas* improved glucose tolerance in fed high fat diet and was similar to the control group fed normal standard diet. Jayaprakasam *et al.* (2006) also noted that even though ursolic acid did not significantly decrease body weight in high fat diet mice, glucose level was corrected in all the tested animals. In this study the glucose levels of all the CD1 mice after the treatment period were not significantly different and the glucose levels (Table 4-10) were within the expected values for mice of their age group (River, 1986).

The failure of an observed effect may be due to several factors. The dose is certainly a factor as the dose administer to the animals (1 mg ethyl acetate fraction/kg food) may contain very low amount of active compounds to produce physiological response. A physiological plasma concentration of not exceeding 10 µmol/l should be attained and sustained after the consumption of extract to exert biological effects (Williamson and Manach 2005). An oral dose of epigallocatechin gallate (EGCG) of 50 mg/kg body weight given to volunteers yielded a peak plasma concentration of about 0.15 µmol/l 1 h after consumption (Williamson and Manach 2005). Another contributing factor may be the mode of administration. Although the number and the concentration of the active compounds present in the ethyl acetate is unknown,direct oral administration of the fraction could have elicited better result than the addition of the fraction to diet. Oven drying the experimental food to which treatment has been added at 70°C could have effect of the compounds. Nonetheless the most likely reason is the bioavailability of the active compounds. Manach *et al.* (2004) in their review noted that many excellent results in*in vitro* assays failed in *in vivo* assays mainly because many polyphenols probably have lower intrinsic activity or because they are poorly absorbed from the intestine, highly metabolised or rapidly eliminated. They said further that the metabolites that are found in the blood and target organs after digestion or hepatic modification most often differ from the native substances in terms of biological activity. It was speculated that most polyphenols are not absorbed and metabolised probably because of the hydrophilicity which restrict their ability to penetrate the gut wall by passive diffusion (Manach *et al.*, 2004). In addition because most polyphenols are in the glycosylated forms and glycosylation influences absorption, it was also speculated that most of the

glycosides resist acid hydrolysis in the stomach and thus remain in their intact form on reaching the duodenum Manach *et al.*, 2004).

Chapter 6

6 Conclusion

This study investigated the potential antidiabetic activity of ten *Ficus* species, focussing on digestive enzymes in relation to polyphenol content and antioxidant activity, glucose uptake in muscle, fat and liver; insulin secretion and safety through cytotoxicity assay. All acetone extracts of the ten *Ficus* species had α-amylase and α-glucosidase enzyme inhibitory activity. The crude acetone extract of *F. lutea* was the most active in all the assays. The acetone extract of *F. lutea* was the most effective in stimulating glucose uptake in the primary muscle and fat cell culture as well as in established cell lines of C2C12 muscle and H-4-II-E liver but not in undifferentiated 3T3-L1 pre-adipocyte in the absence and presence of insulin indicating an insulin-mimetic and insulin sensitising as a possible mode of action. The extract of *F. lutea* in addition enhanced insulin secretion in RIN-m5F pancreatic cell line. Although the extract of *F. lutea* was relatively less cytotoxic to Vero kidney cells it was more cytotoxic to C3A liver cells when compared to the other *Ficus* species.

The extract of *Ficus lutea,* being the most active, was fractioned by solvent-solvent fraction into six fractions. These fractions were subjected to the above assays and ethyl acetate fraction was the most active fraction ininhibiting α-glucosidase (sucrase) activity in a manner that was more potent than the crude, with good correlated withits polyphenol content. The ethyl acetate fraction was superior to the crude extract in enhancing glucose uptake in C2C12 muscle and H-4-II-E liver in the absence of insulin. The mechanism through which the ethyl acetate fraction enhanced glucose uptake is uncertain but it is probably similar to that for insulin. The ethyl acetate fraction was relatively more cytotoxic to Vero kidney cells than the other fractions and the crude extract.

Five compounds, namely lupeol, stigmasterol, α-amyrin acetate, epicatechin and epiafzelechin were isolated from the ethyl acetate fraction of *F lutea*, with all of the compounds except epiafzelechin previously known to possess antidiabetic activity. The ethylacetate fraction was also evaluated for its weight reducing potential in obese mouse model. Unfortunately no *in vivo* activity was discernible.

Type II diabetes affects many metabolic pathways in different tissues which are potential targets for drug treatment (Van de Venter *et al.*, 2008). Conversely, the extract of *F. lutea* consists of some phytochemicals that has been shown to possess antidiabetic activities. It can then be speculated that the mechanisms underlying the antidiabetic activity of the extract of *F. lutea* includes the inhibition of α-amylase and α-glucosidase activities (enzymes responsible for the breakdown of carbohydrate), enhancing of glucose uptake in muscle, fat and liverand by stimulating the secretion of insulin. In conclusion, this study is the first to report on the *in vitro* antidiabetic activity of the extract of *F. lutea*.

6.1 Future Work

More extensive *in vitro* cytotoxicity evaluation of the extract of *F. lutea* and *in vivo* acute and chronic toxicity assay is needed to ascertain its safety especially the target organ for toxicity.

As the n-butanol fraction of the extract of *F. lutea*inhibited α-amylase and potential to stimulate glucose uptake in muscle and liver cell lines, it may be worthwhile to investigate its stimulatory effect in insulin stimulated glucose uptake in muscle, liver and fat cells.

A repeat of the failed obesity induced diabetic animal study may reveal new finding perhaps with animals being treated with oral gavage dose of the ethyl acetate fraction of the extract of *F. lutea*. The oral dosing of animals may allow for higher concentration of the ethyl acetate fraction to be in contact with target tissues/organs.

Since Type II diabetes is a multi-faceted disease a general evaluation of the hypoglycaemic activity of the extract of *F. luteain vivo* in a natural diabetes rodent could give an indication of its potential as an antidiabetic agent.In further work the following aspects may be addressed:

- To what extent will the complications of type II diabetes be reduced by *F. lutea* extracts and do the extracts of *F. lutea* reduce the extent of oxidative stress leading to these complications?
- Does ingestion of polyphenolic compounds present in *F. lutea* influence endogenous antioxidant enzymes and non-enzymatic reactions?
- Could the measurement of glycated haemoglobin (HbA1c) in the rodent blood be a better way of evaluating the management of type II diabetes in a mouse obesity model subjected to the *F. lutea* extract?

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Appendix

NMR Spectra of compound isolated from *Ficus lutea*

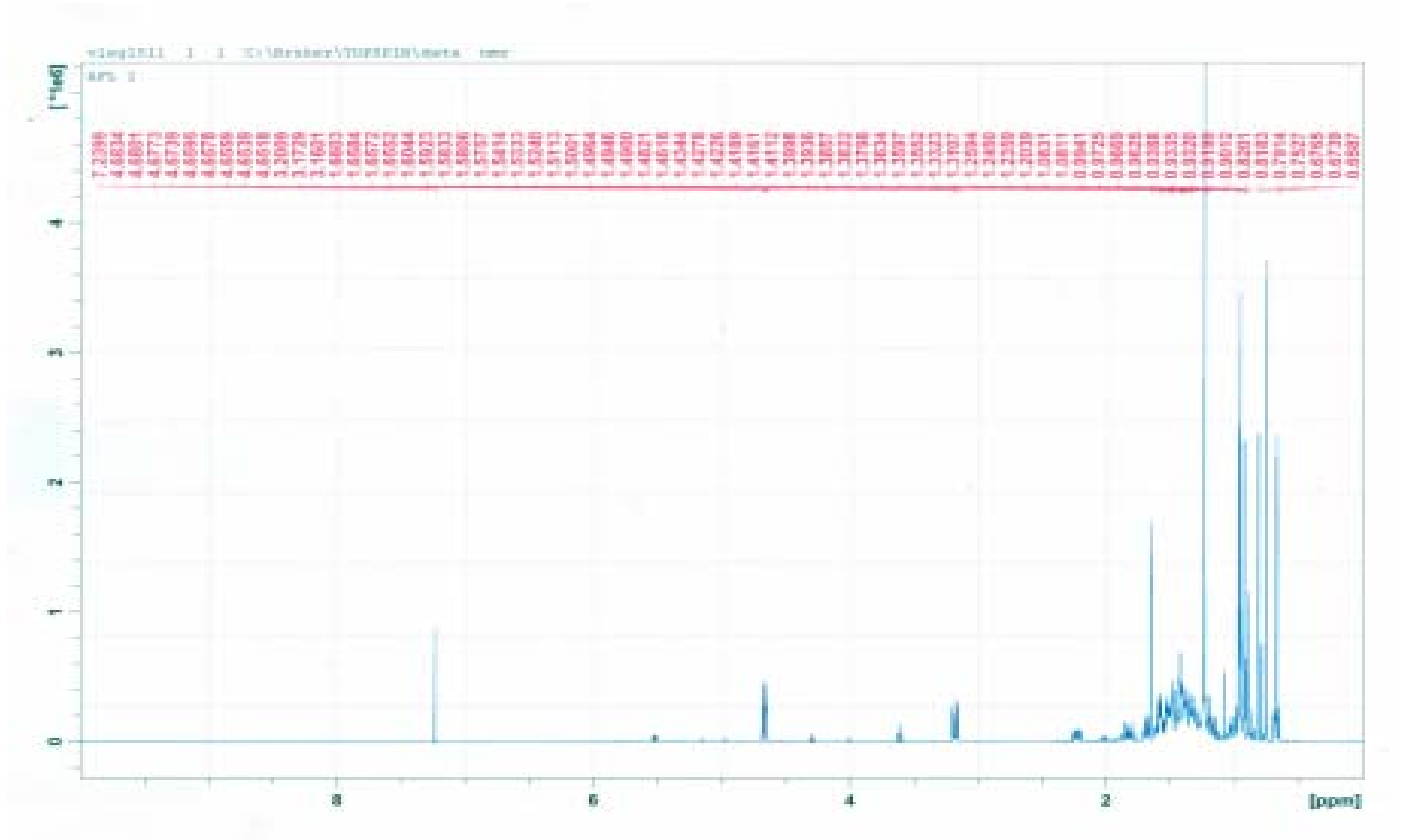

Figure 1: ^{1}H-NMR (500 MHz, $CDCl_3$) of AFL1 or Lupeol (**1**)

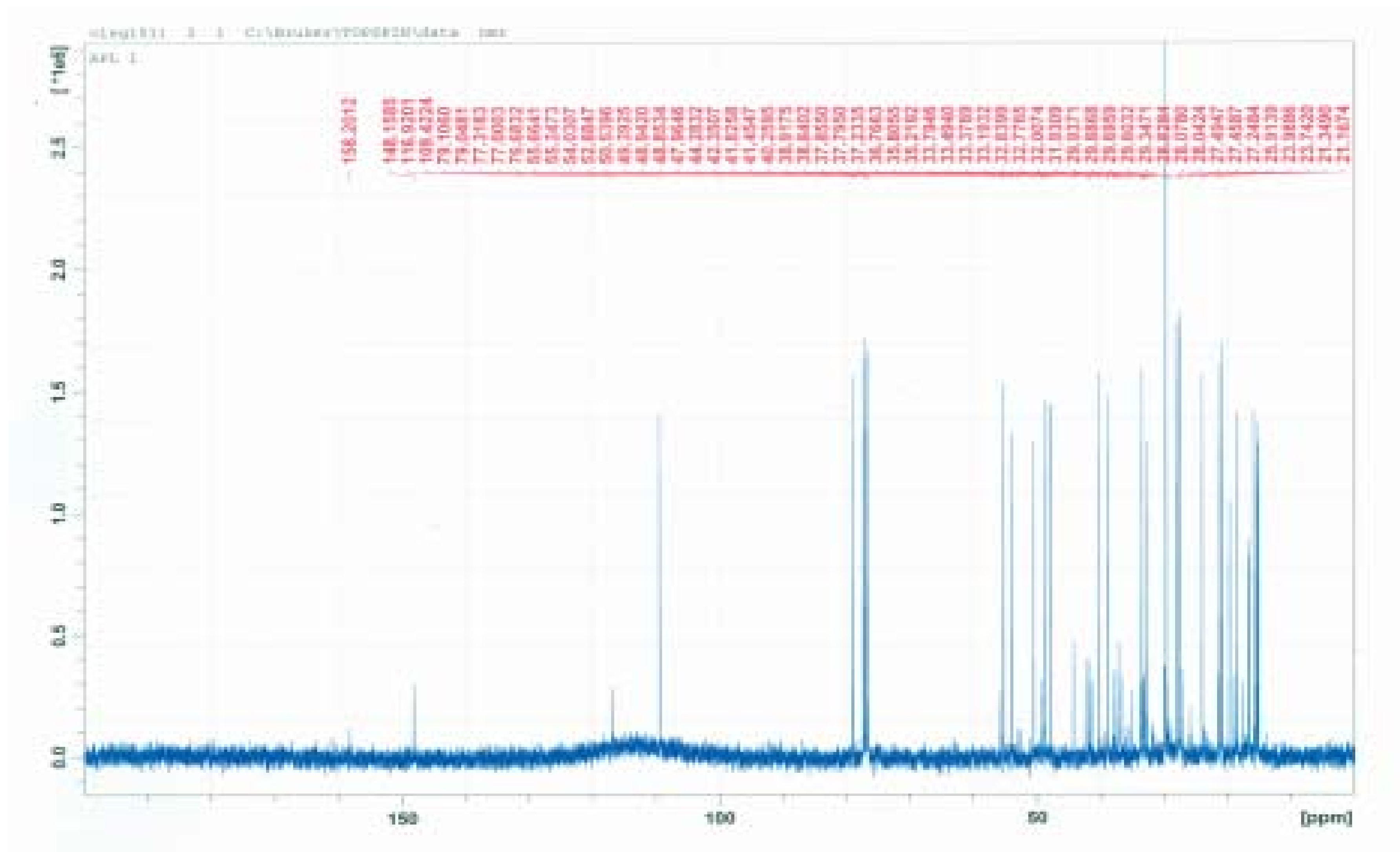

Figure 2: ^{13}C-NMR (125 MHz, $CDCl_3$) Spectrum of AFL1 or Lupeol (**1**)

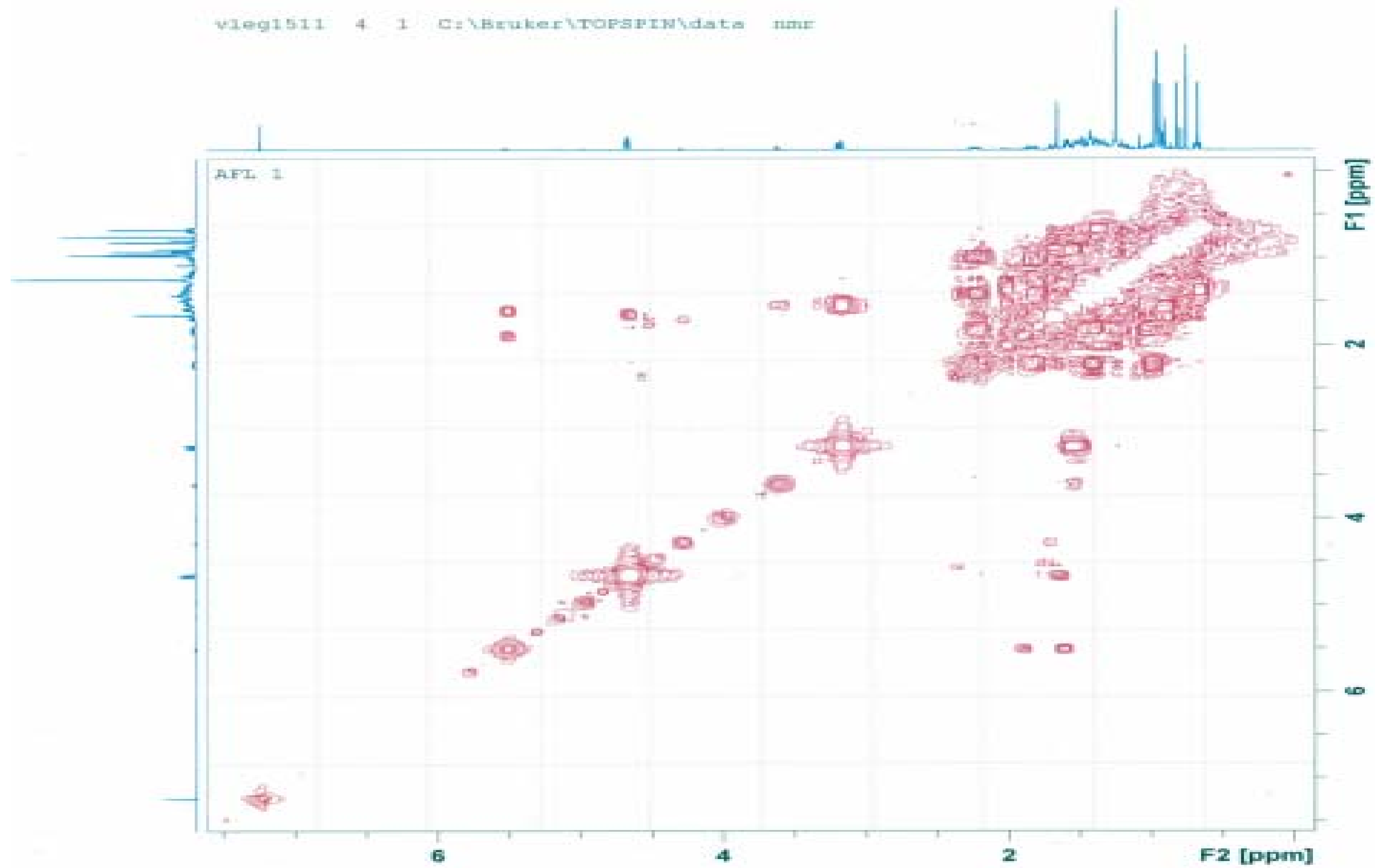

Figure 3:^{1}H ^{1}H COSY (500 MHz, $CDCl_3$) Spectrum of AFL1 or Lupeol (**1**)

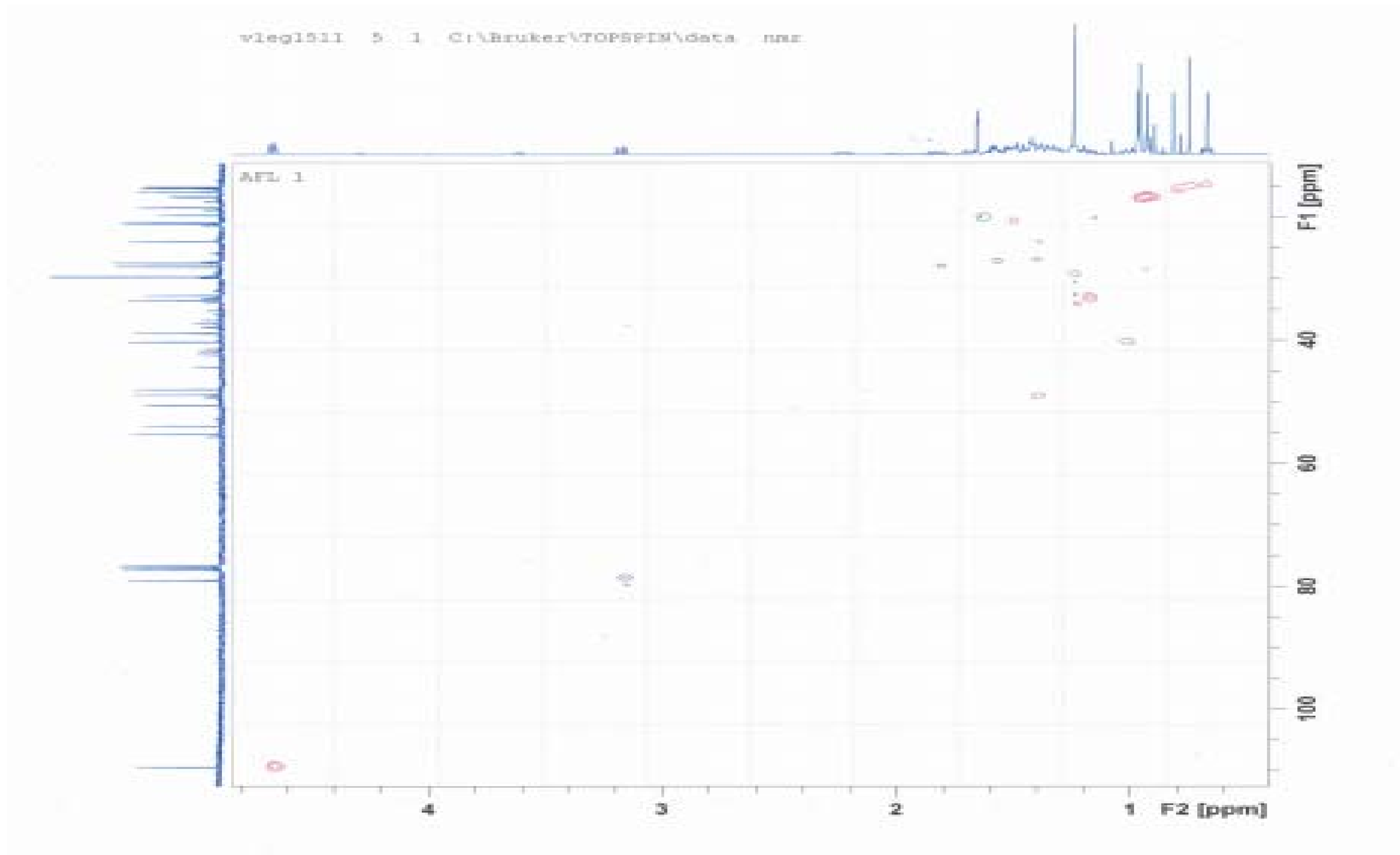

Figure 4: HSQC (500 MHz :[1]H and 125 MHz : [13]C, $CDCl_3$) Spectrum of AFL1 or Lupeol (**1**)

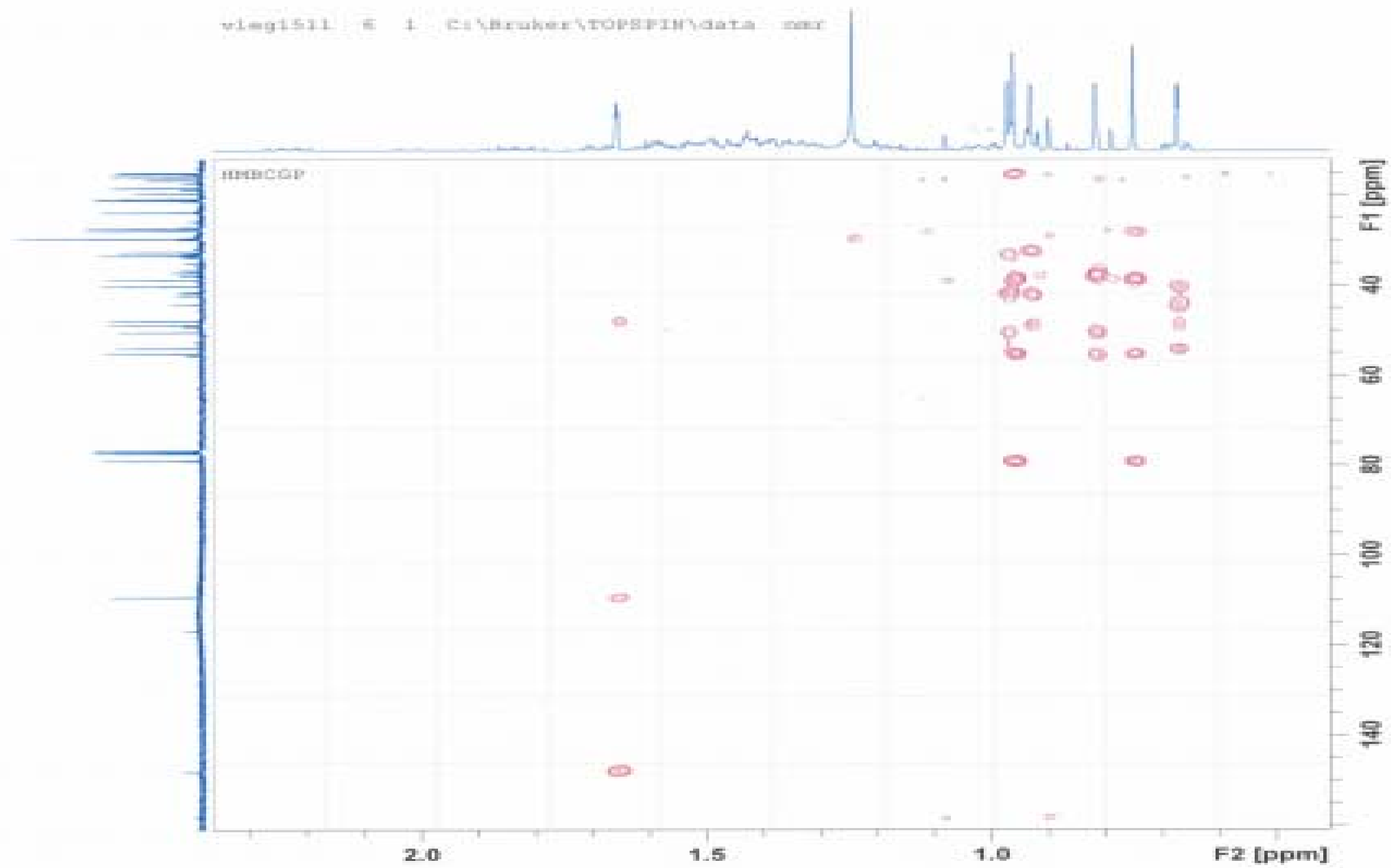

Figure 5: HMBC (500 MHz: [1]H and 125 MHz :[13]C, $CDCl_3$) Spectrum of AFL1 or Lupeol (**1**)

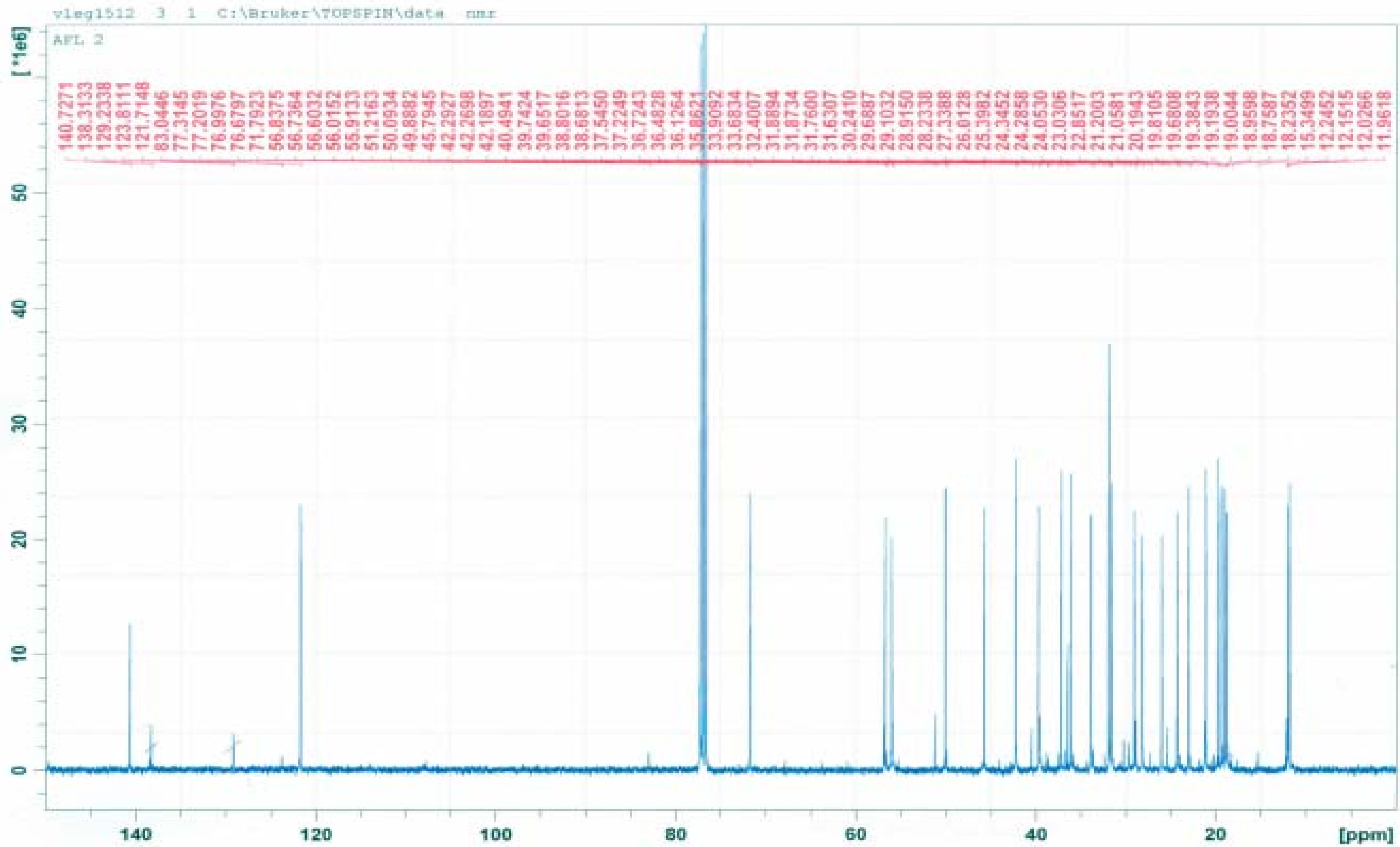

Figure 6: ^{13}C-NMR (125 MHz, $CDCl_3$) Spectrum of AFL2 or Stigmasterol (**2**)

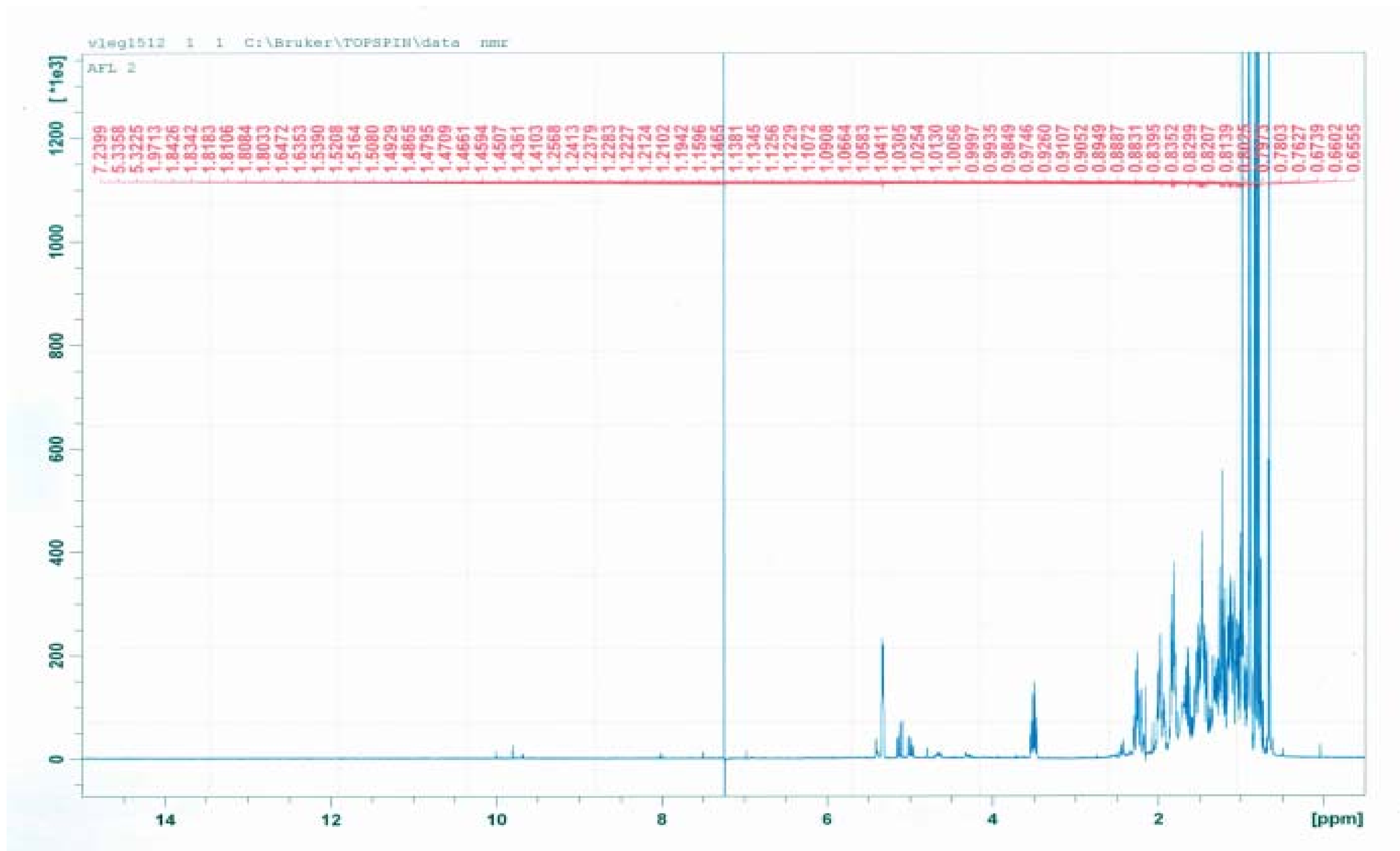

Figure 7:^{1}H-NMR (500 MHz, $CDCl_3$) Spectrum of AFL2 or Stigmasterol (**2**)

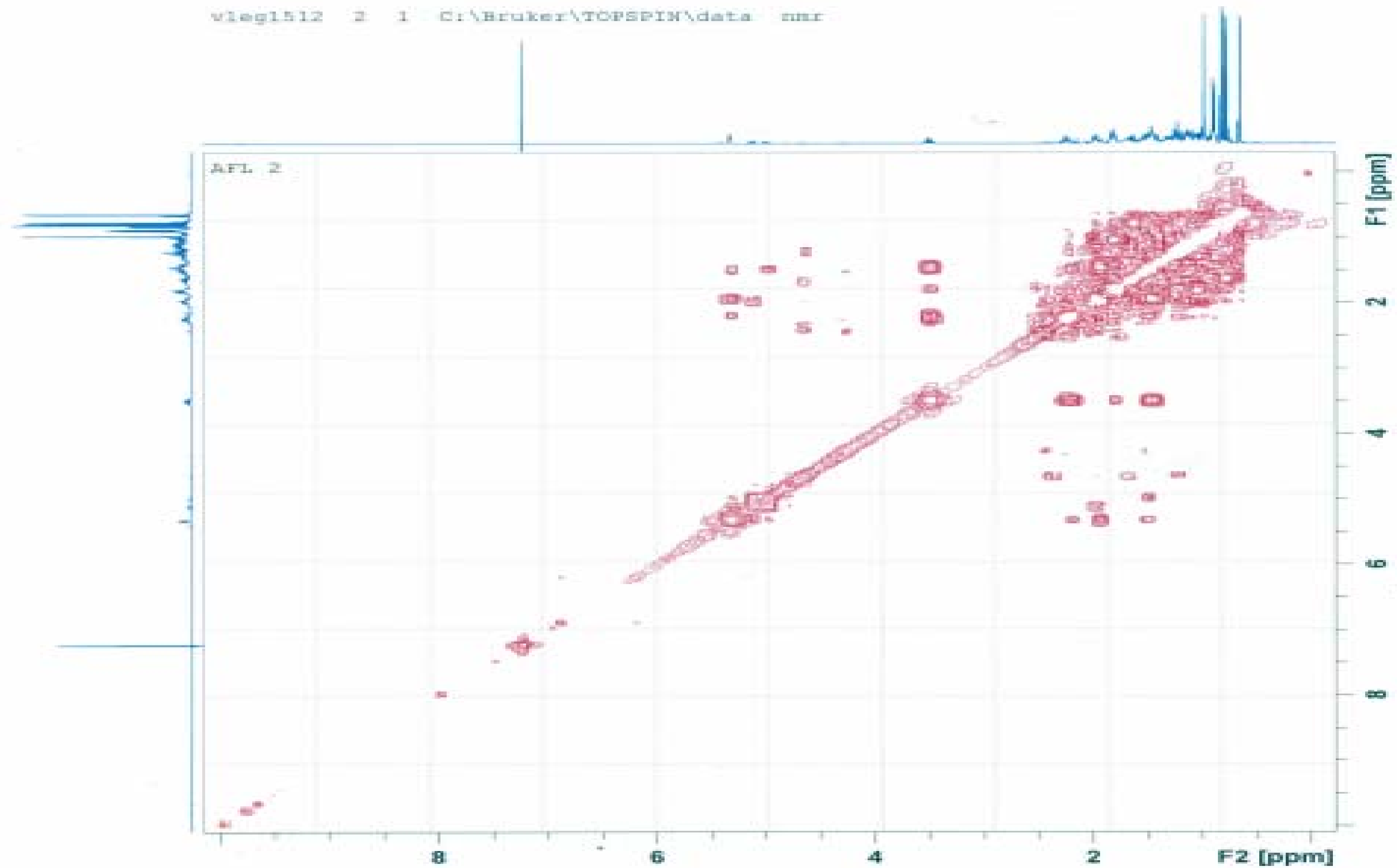

Figure 8: ^{1}H ^{1}H COSY (500 MHz, $CDCl_3$) Spectrum of AFL2 or Stigmasterol (**2**)

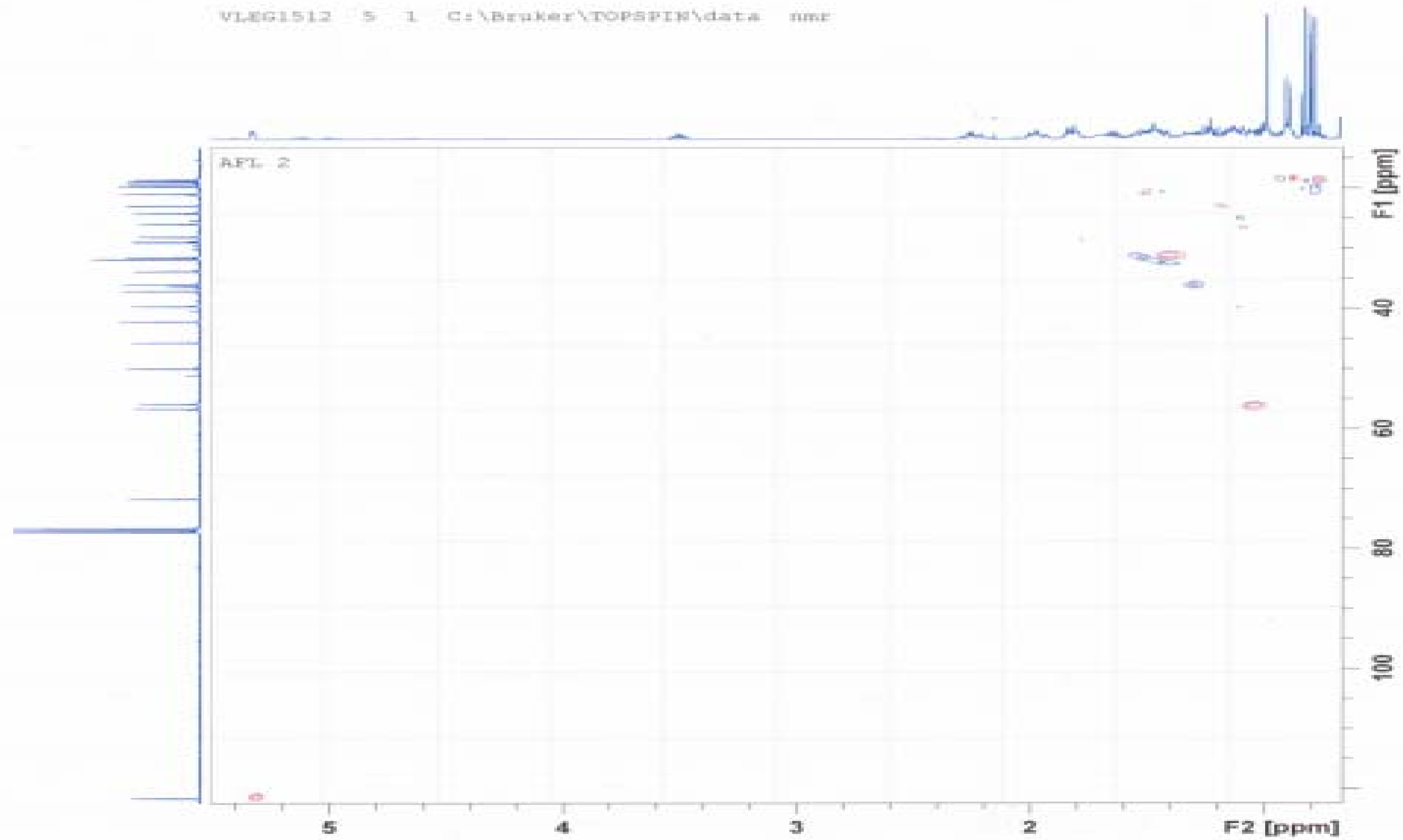

Figure 9: HSQC (500 MHz :[1]H and 125 MHz : [13]C, $CDCl_3$) Spectrum of AFL2 or Stigmasterol (**2**)

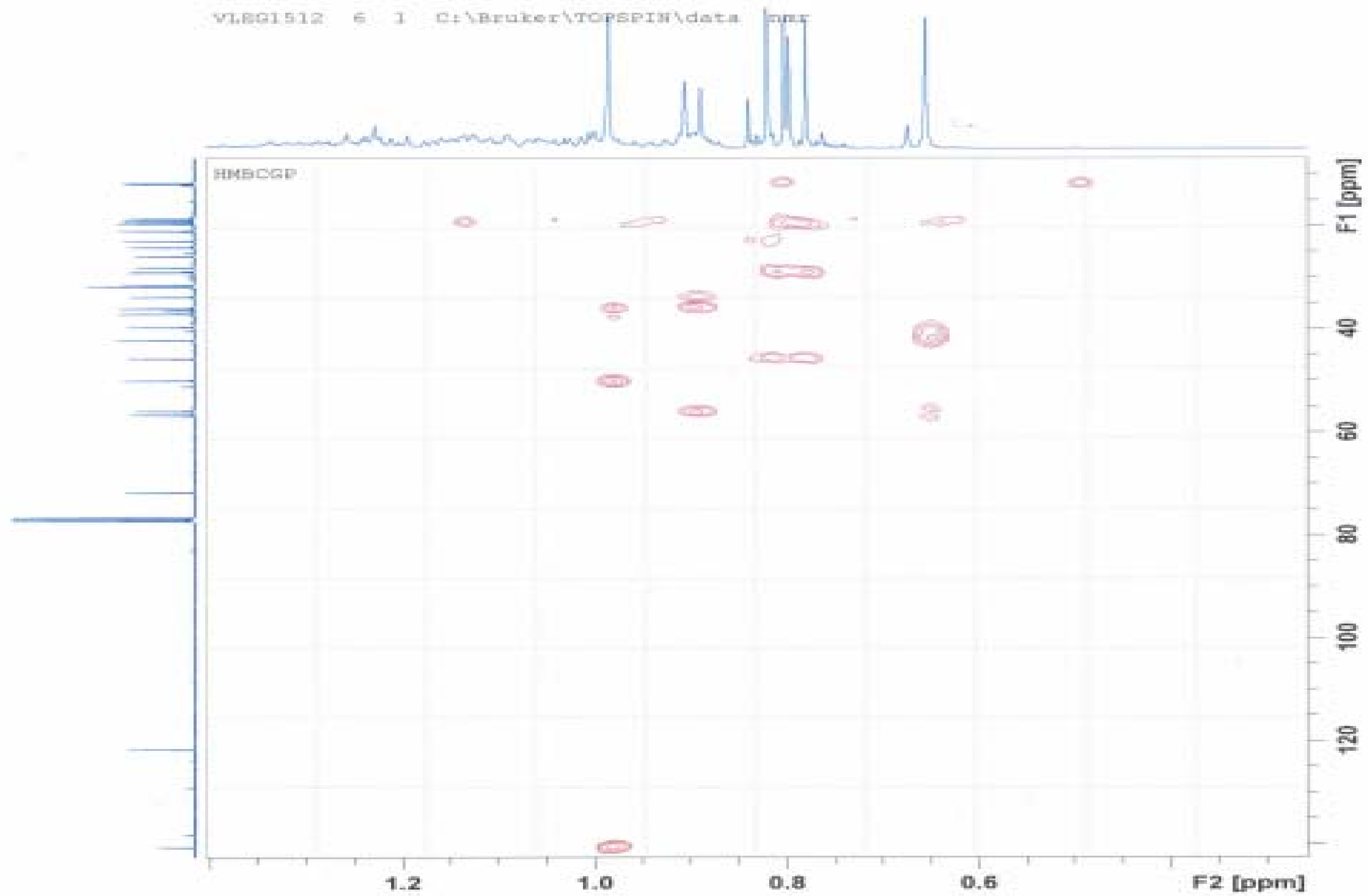

Figure 10: HMBC (500 MHz : ^{1}H and 125 MHz : ^{13}C, $CDCl_3$) Spectrum of AFL2 or Stigmasterol (**2**)

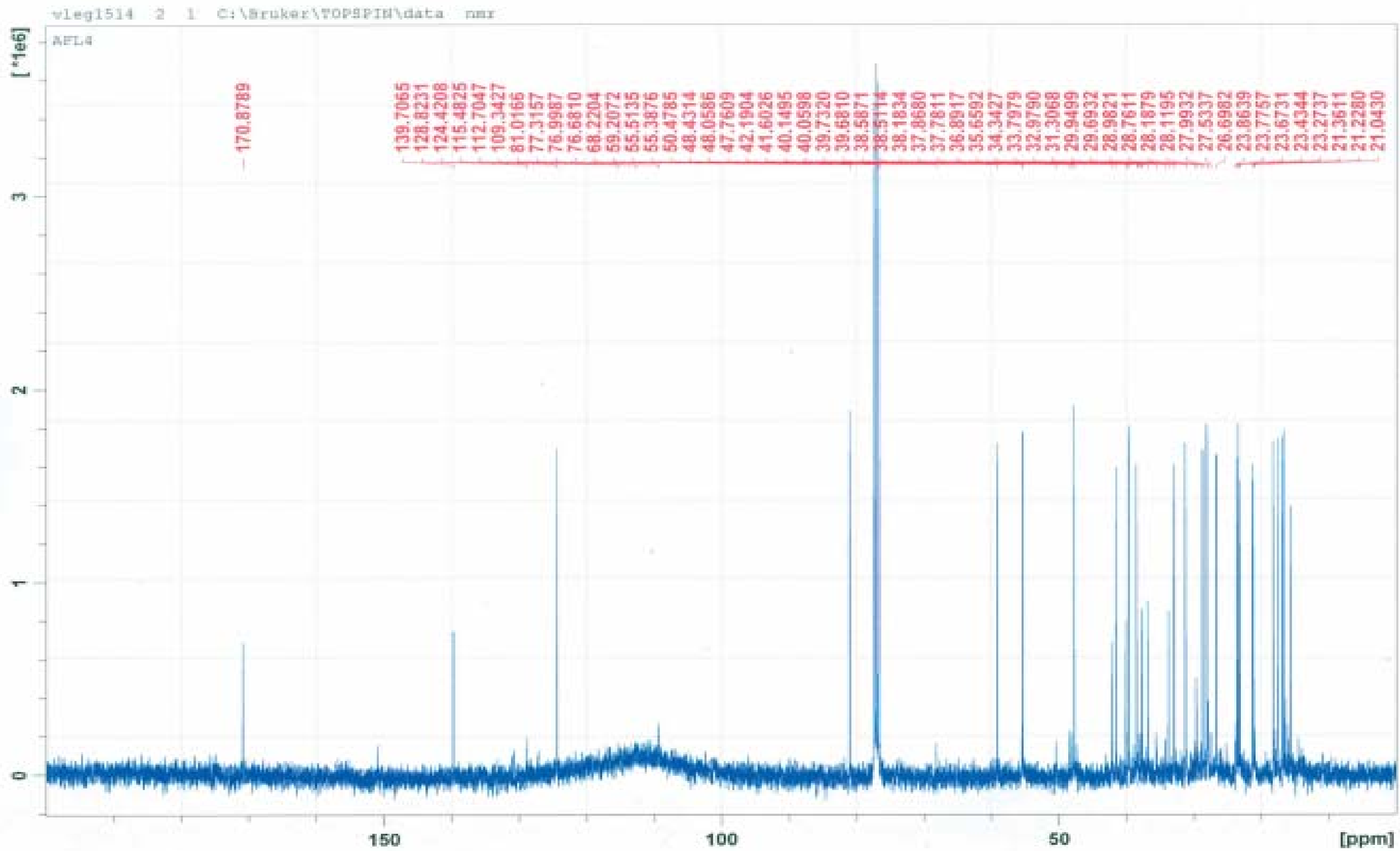

Figure 11:^{13}C-NMR (125 MHz, $CDCl_3$) Spectrum of AFL3 or α-amyrin acetate (**3**)

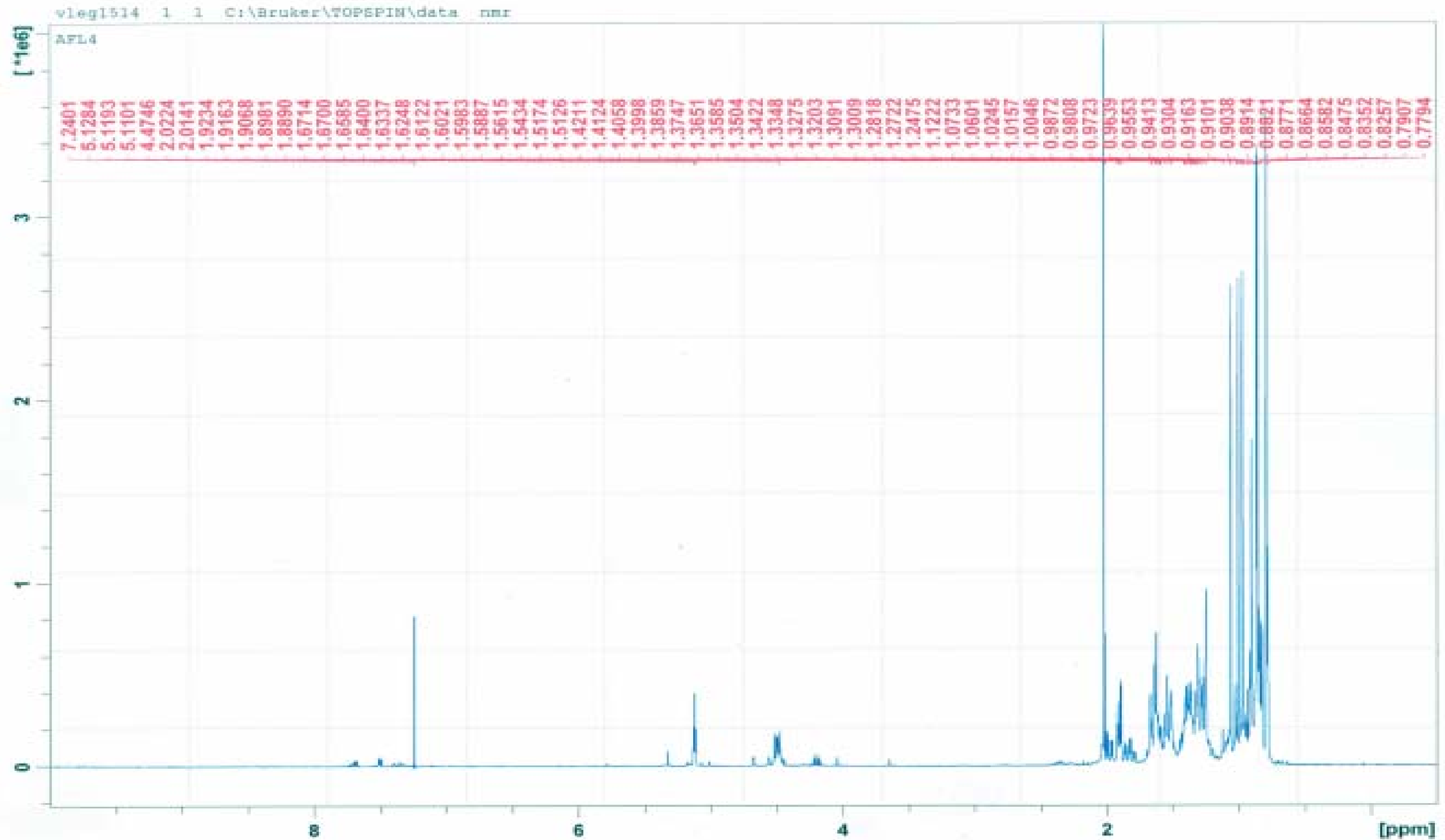

Figure 12: ^{1}H NMR (500 MHz, $CDCl_3$) Spectrum of AFL3 or α-amyrin acetate (**3**)

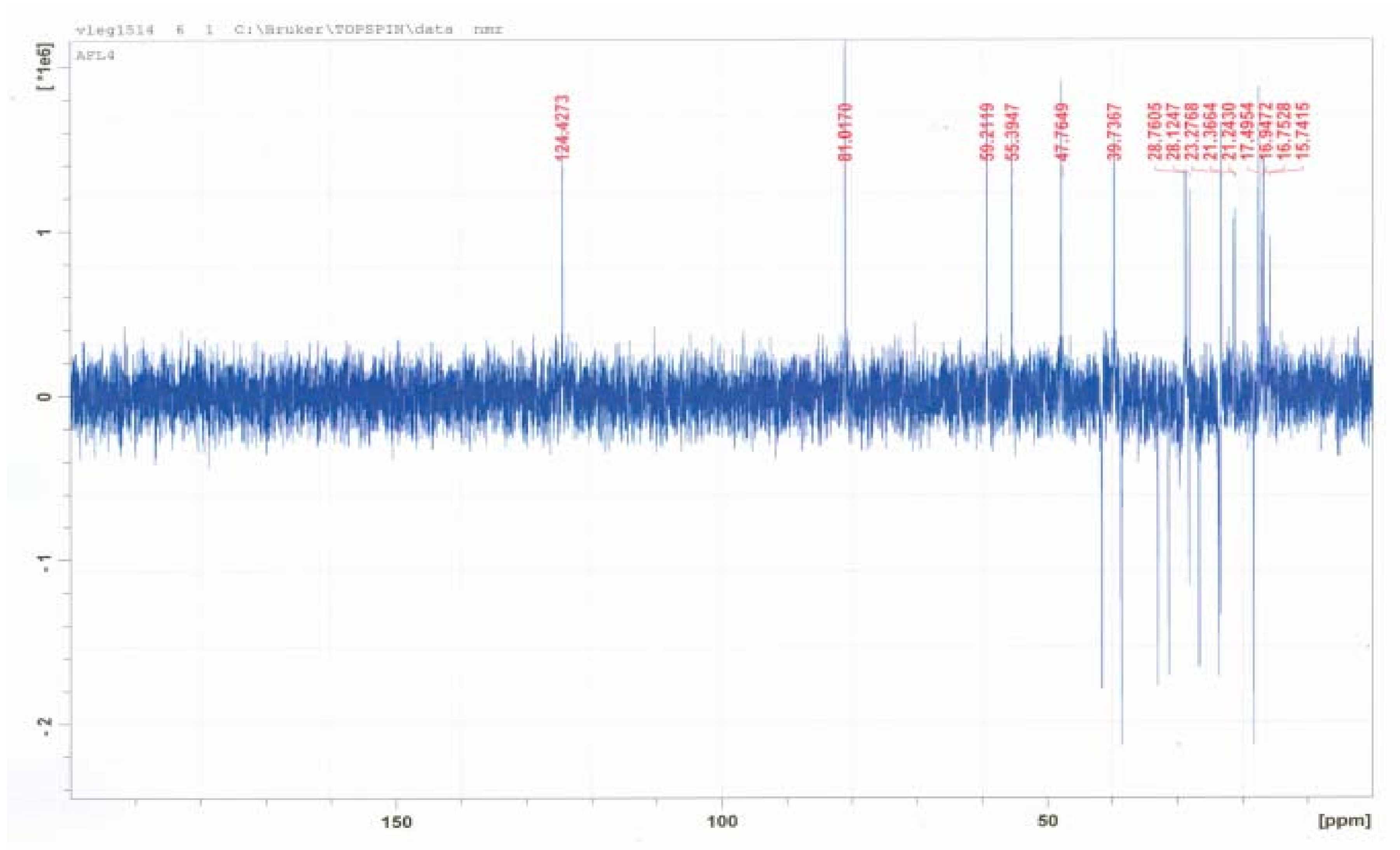

Figure 13: DEPT (125 MHz, $CDCl_3$) Spectrum of AFL3 or α-amyrin acetate (**3**)

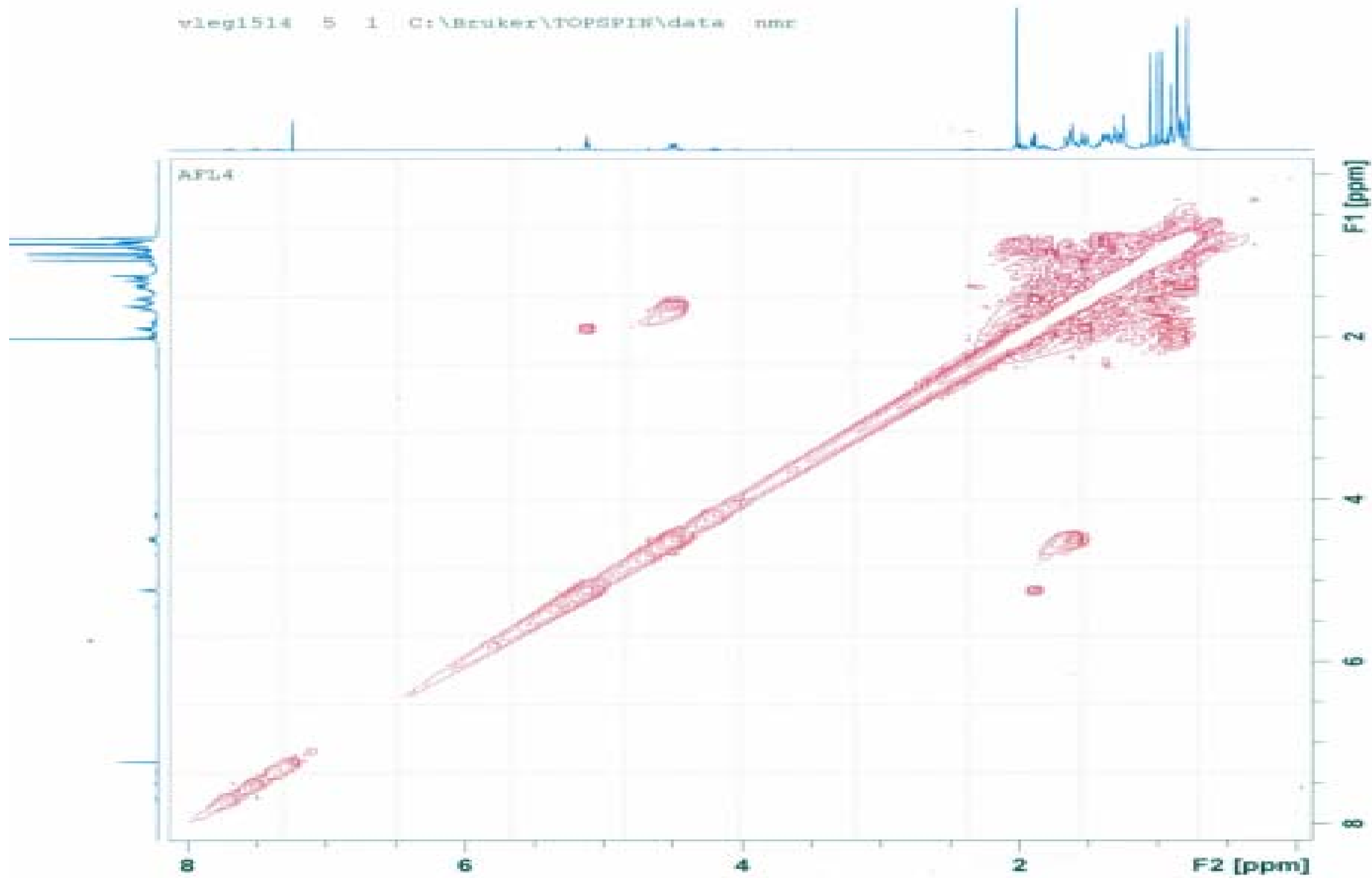

Figure 14: ^1H ^1H COSY (500 MHz, $CDCl_3$) Spectrum of AFL3 or α-amyrin acetate (**3**)

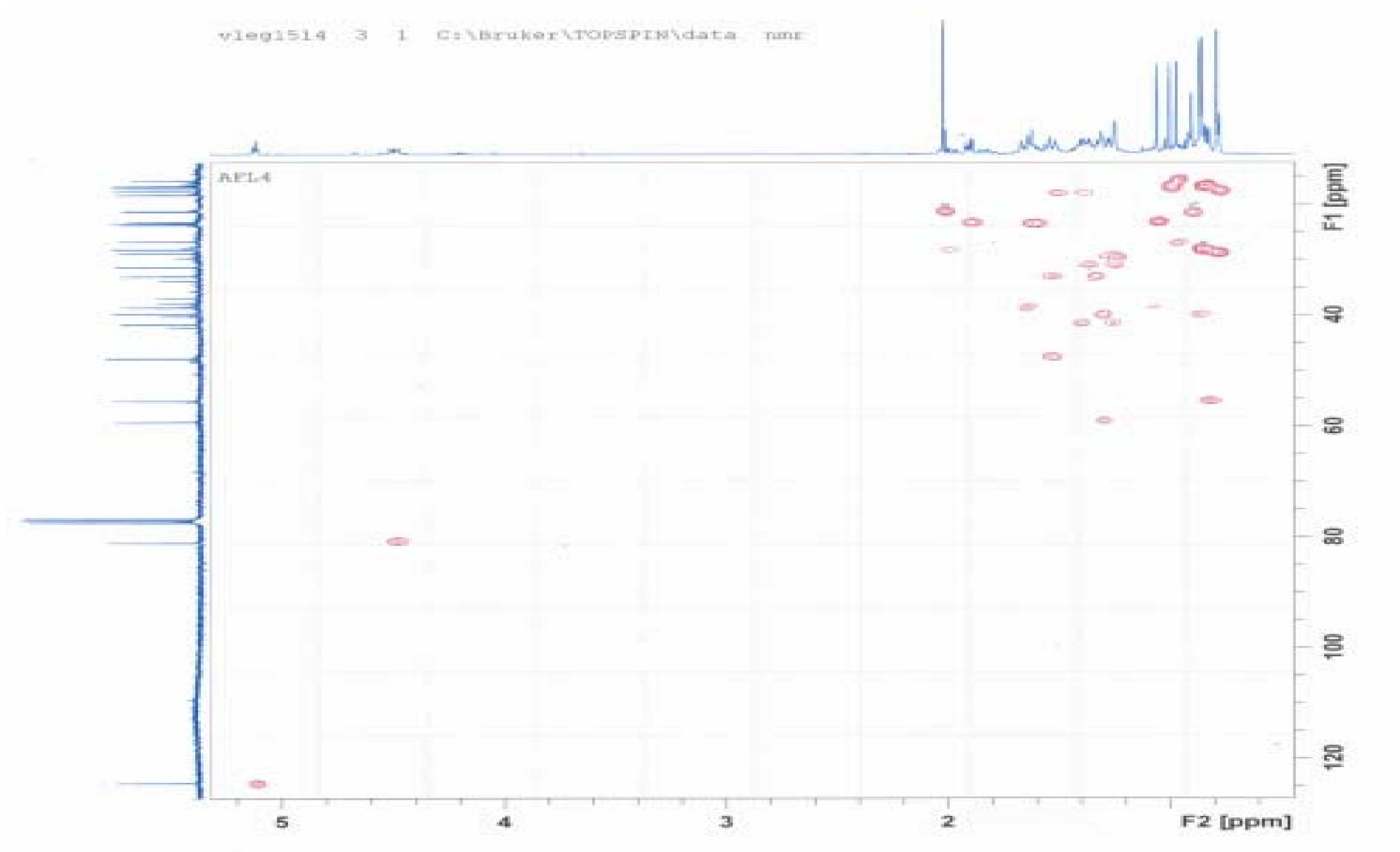

Figure 15: HSQC (500 MHz :[1]H and 125 MHz : [13]C, $CDCl_3$) Spectrum of AFL3 or α-amyrin acetate (**3**)

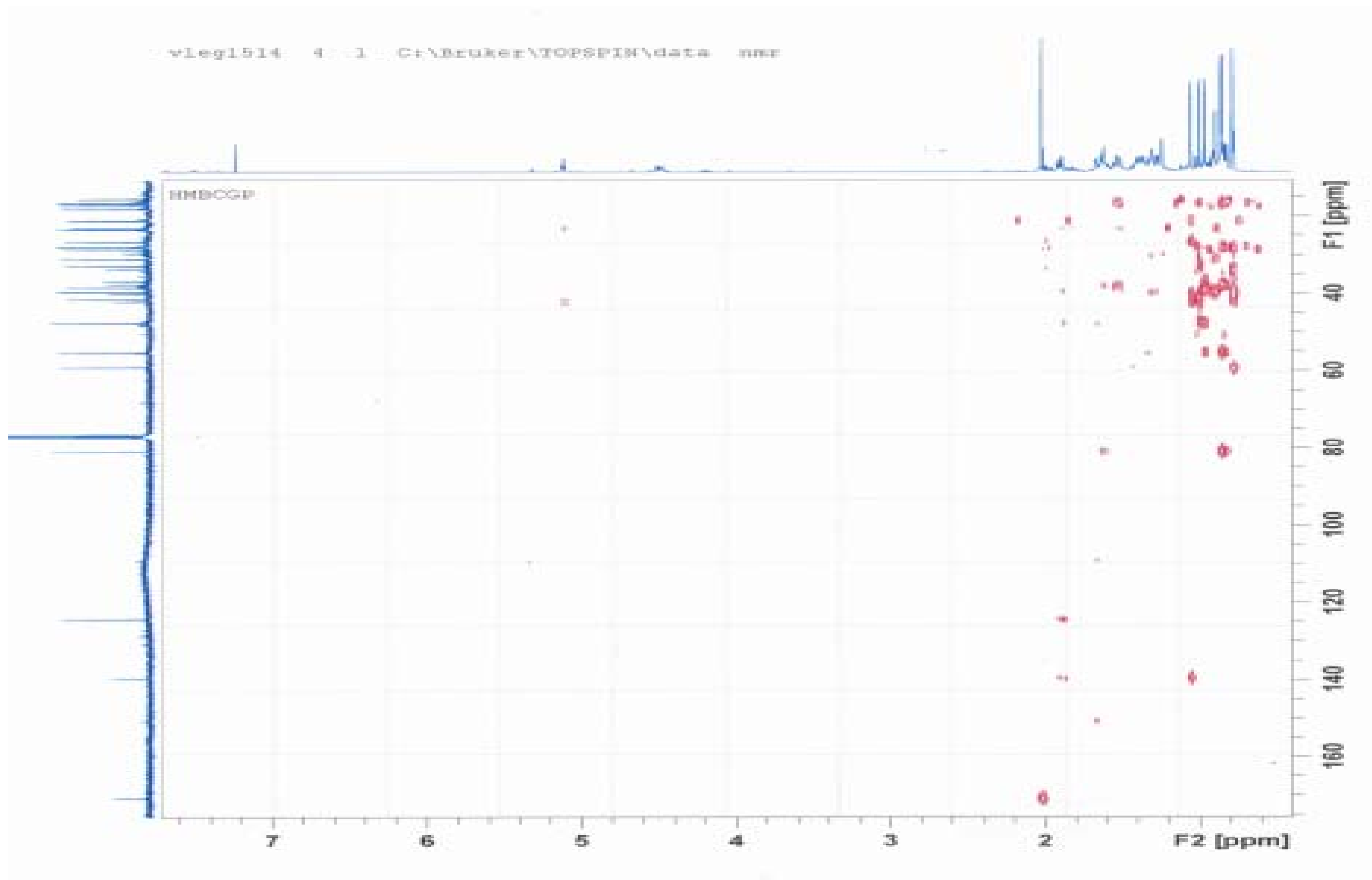

Figure 16: HMBC (500 MHz :[1]H and 125 MHz : [13]C, $CDCl_3$) Spectrum of AFL3 or α-amyrin acetate (**3**)

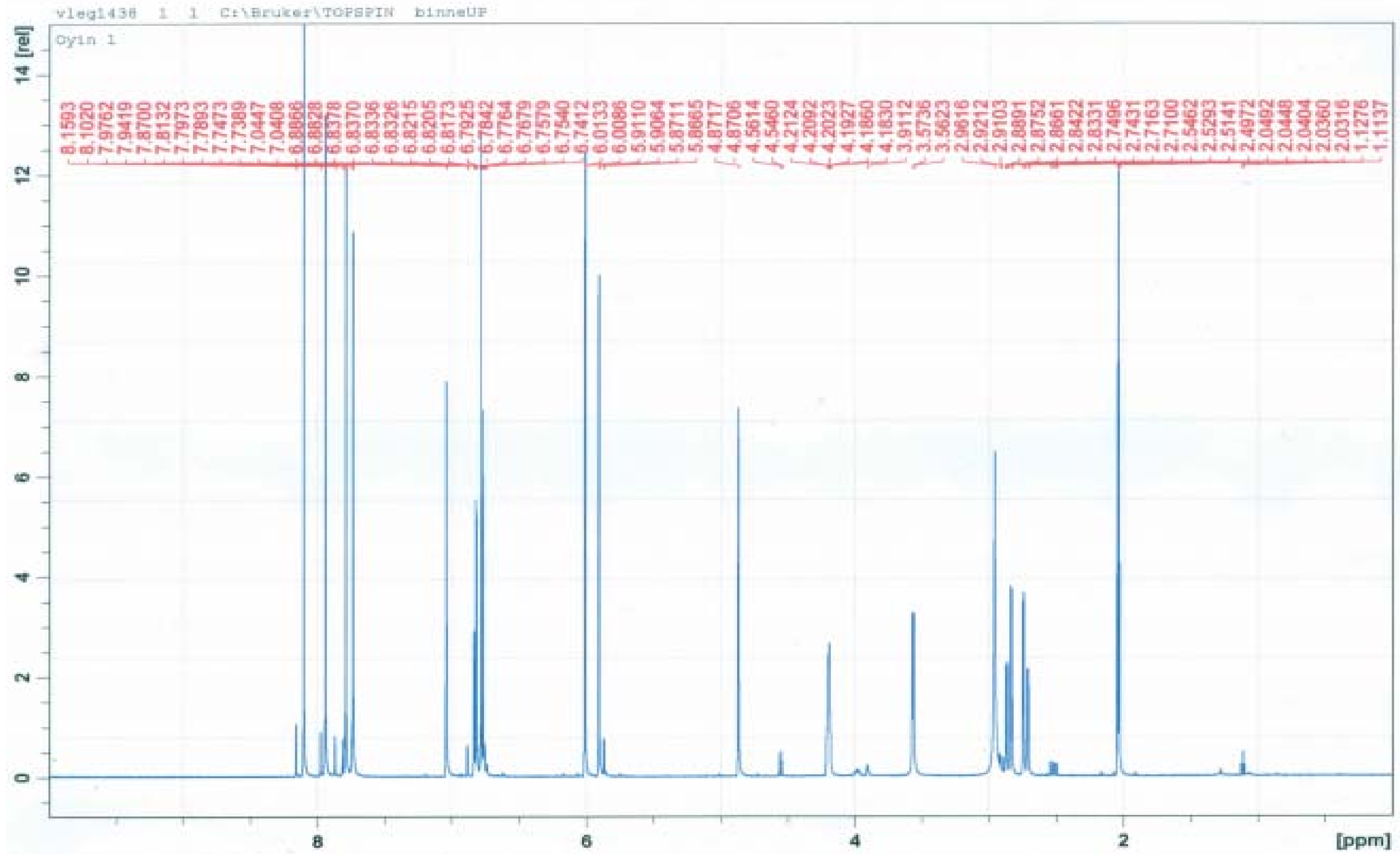

Figure 17: ^1H NMR (500 MHz, $CDCl_3$) Spectrum of AFL4 or Epicatechin (**4**)

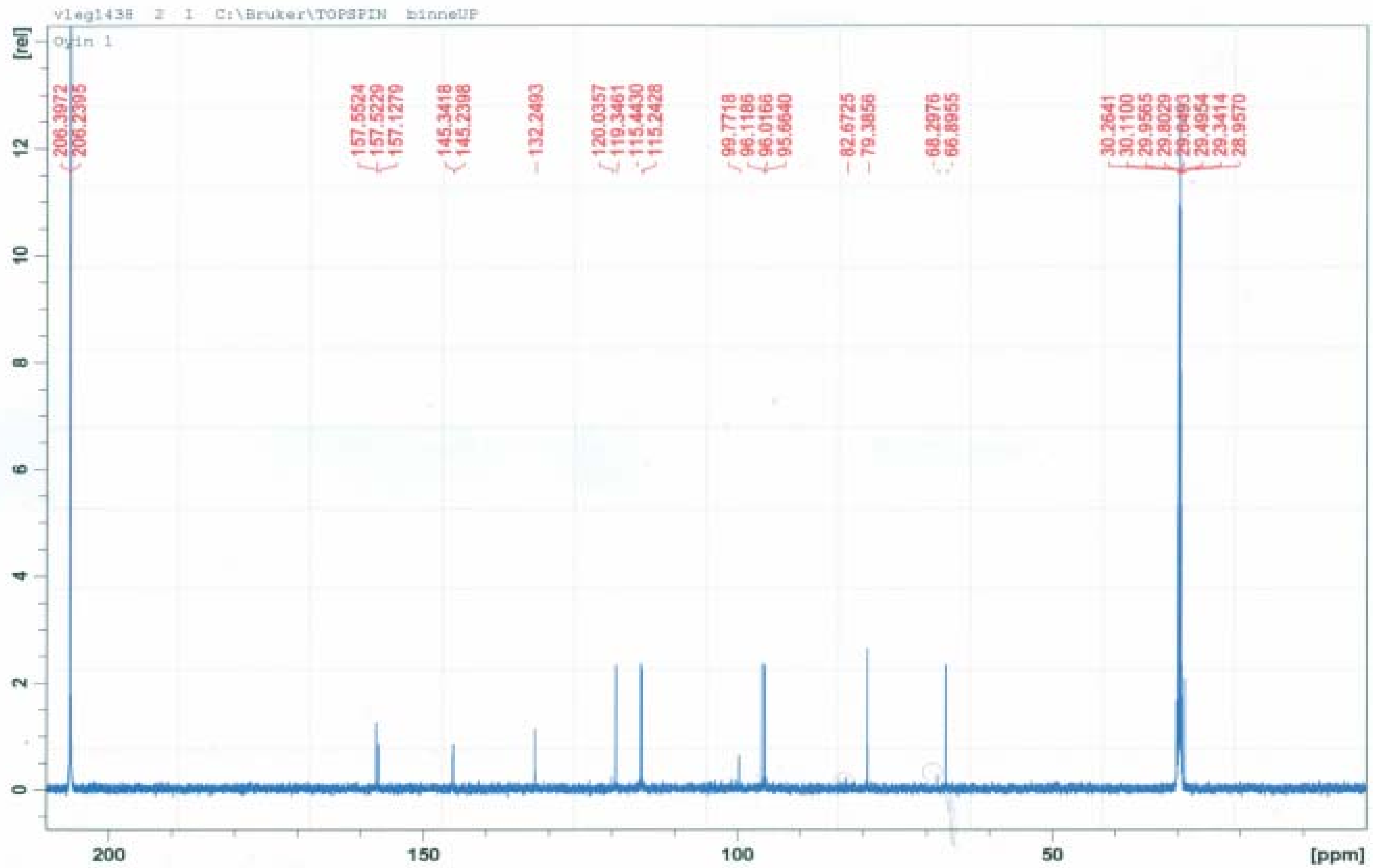

Figure 18: ^{13}C-NMR (125 MHz, $CDCl_3$) Spectrum of AFL4 or Epicatechin (**4**)

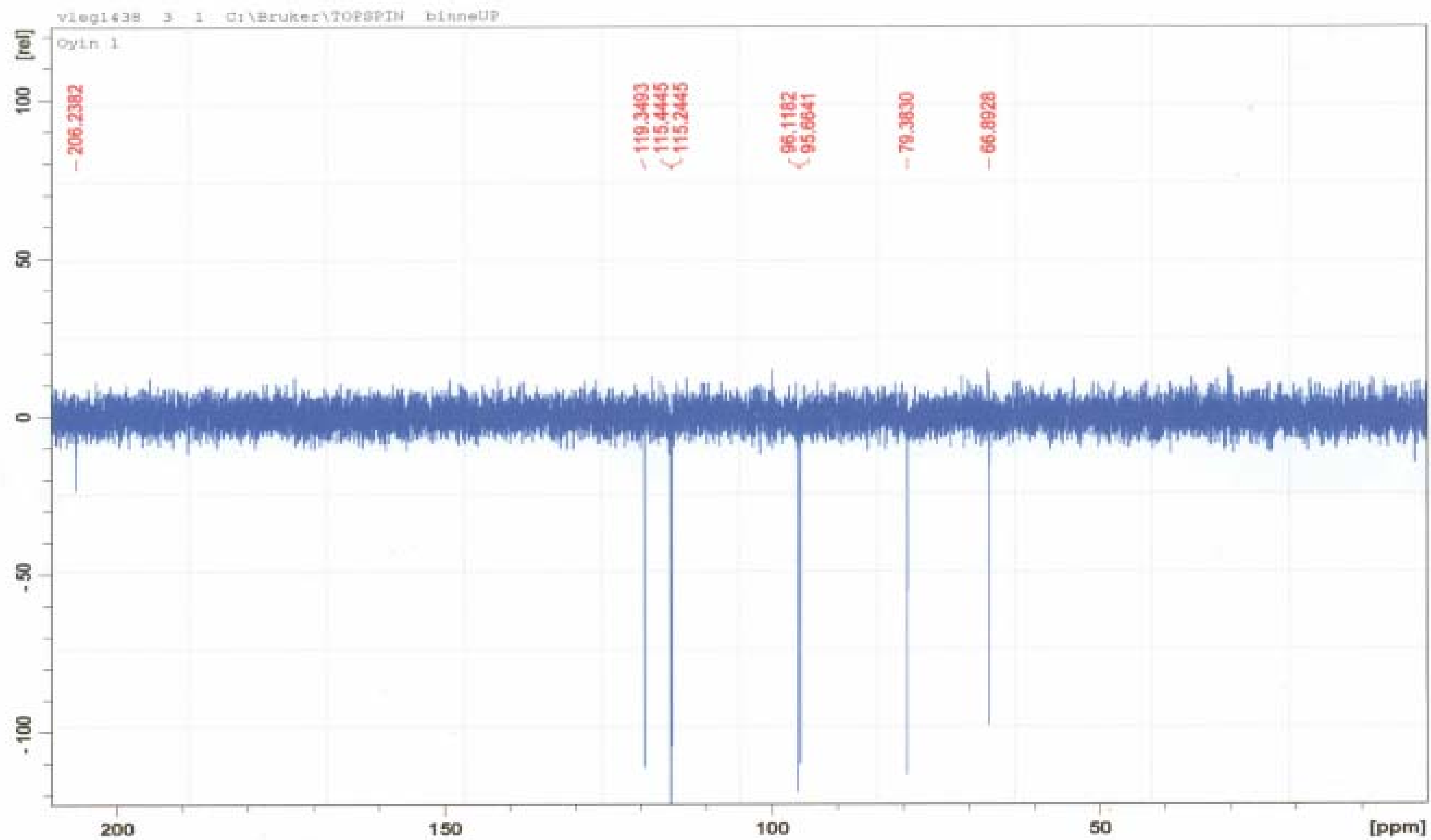

Figure 19: DEPT (125 MHz, $CDCl_3$) Spectrum of AFL4 or Epicatechin (**4**)

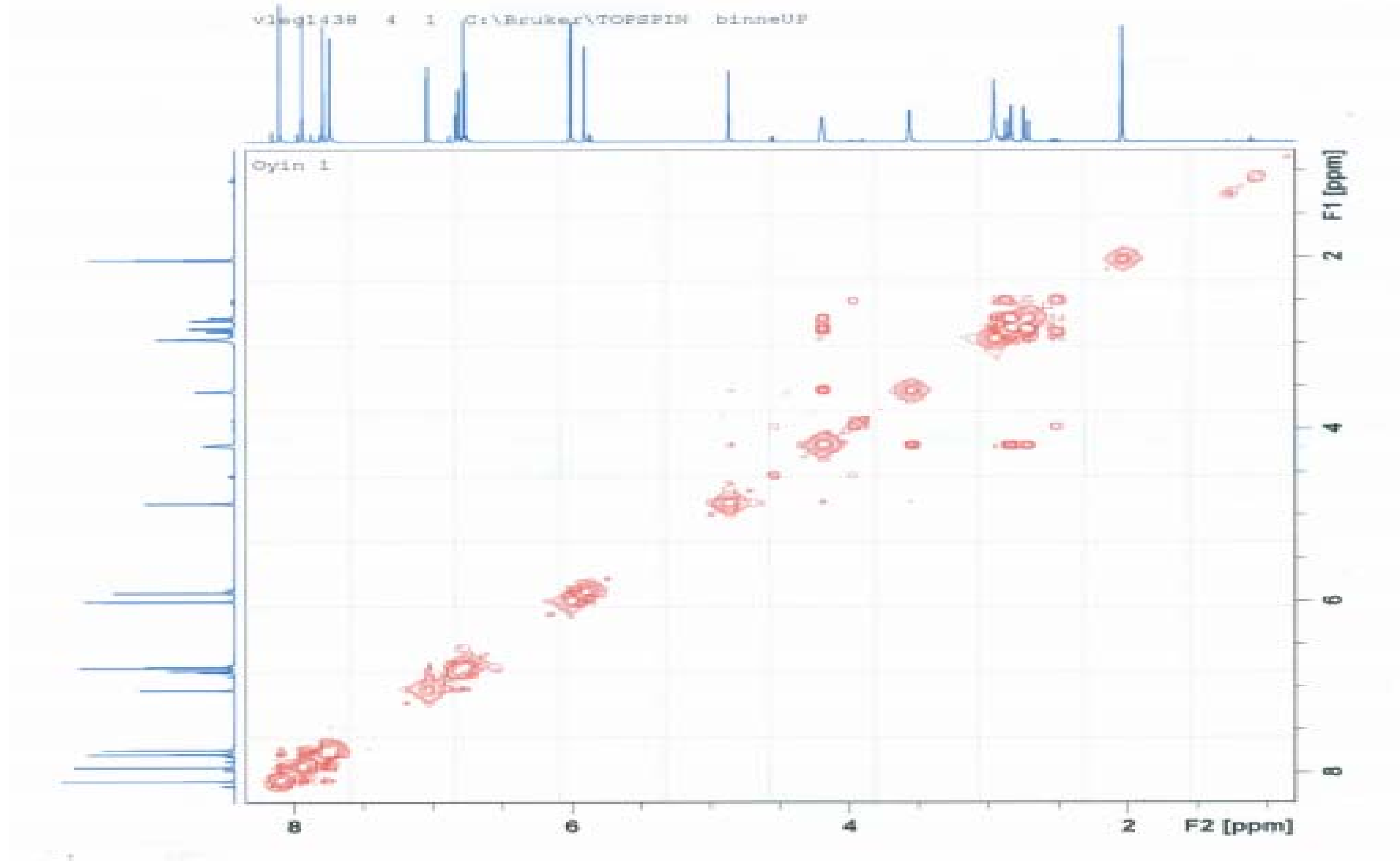

Figure 20:^{1}H ^{1}H COSY (500 MHz, $CDCl_3$) Spectrum of AFL4 or Epicatechin (**4**)

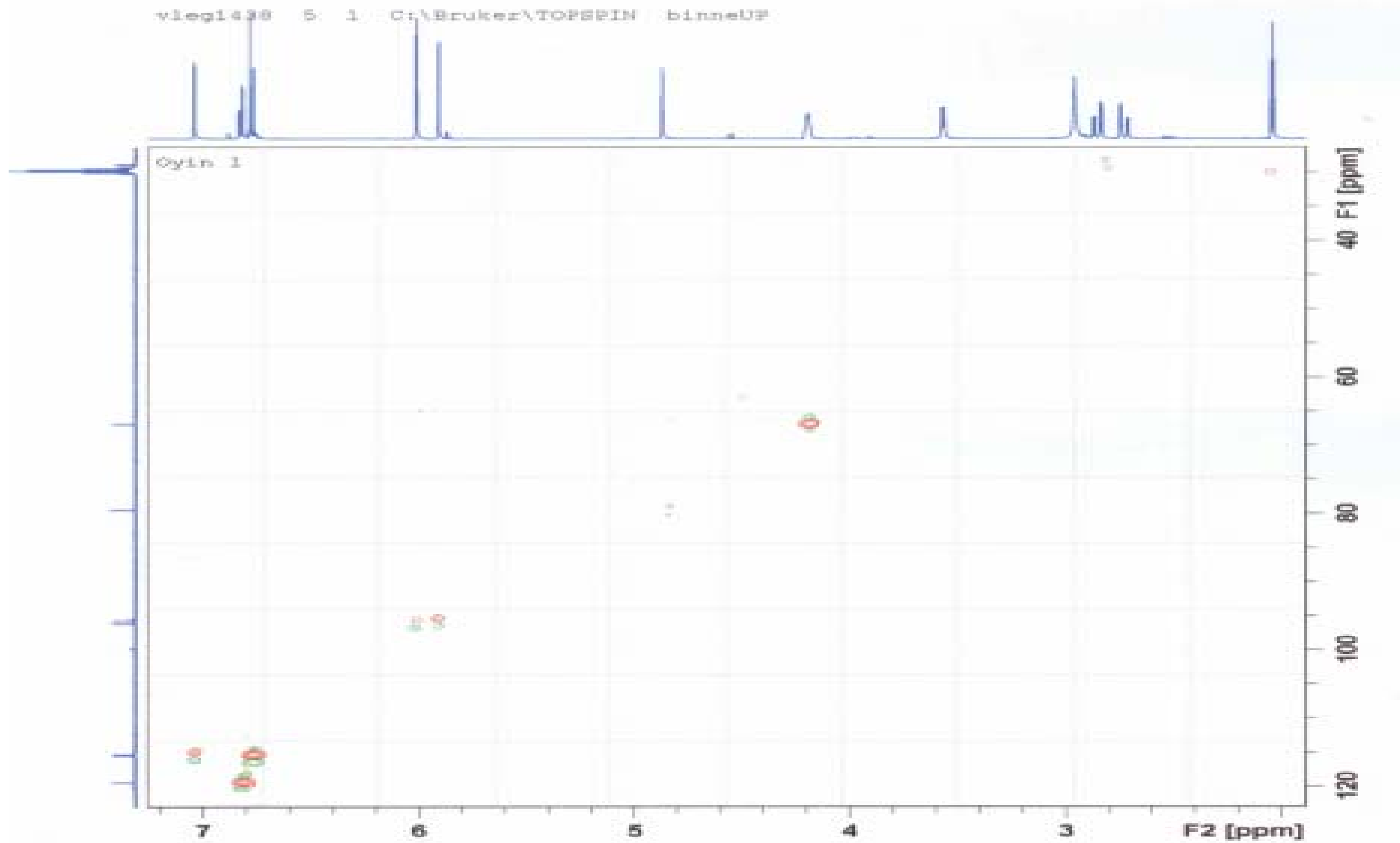

Figure 21: HSQC (500 MHz :[1]H and 125 MHz : [13]C, $CDCl_3$) Spectrum of AFL4 or Epicatechin (**4**)

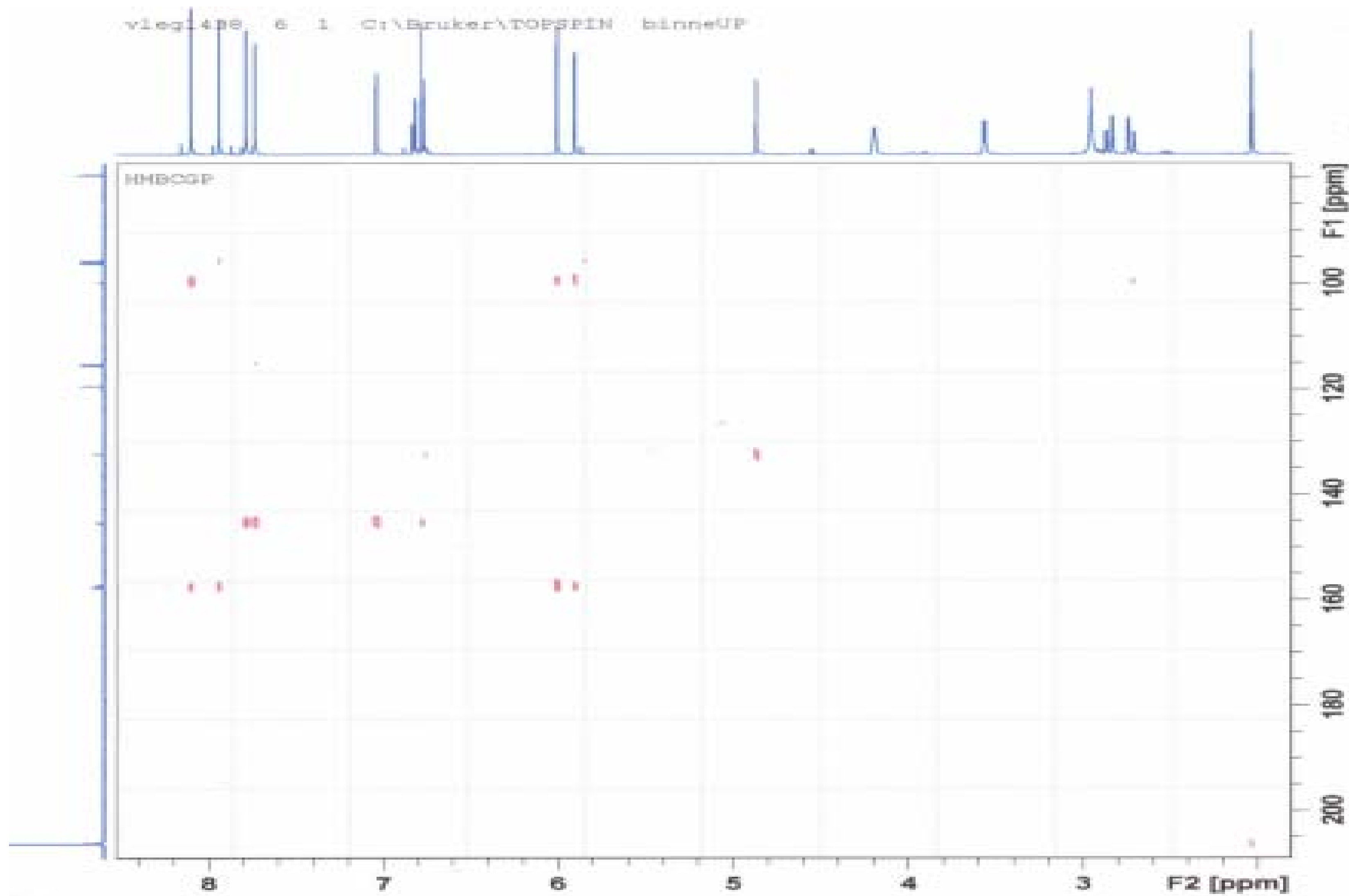

Figure 22: HMBC (500 MHz :^{1}H and 125 MHz : ^{13}C, $CDCl_3$) Spectrum of AFL4 or Epicatechin (**4**)

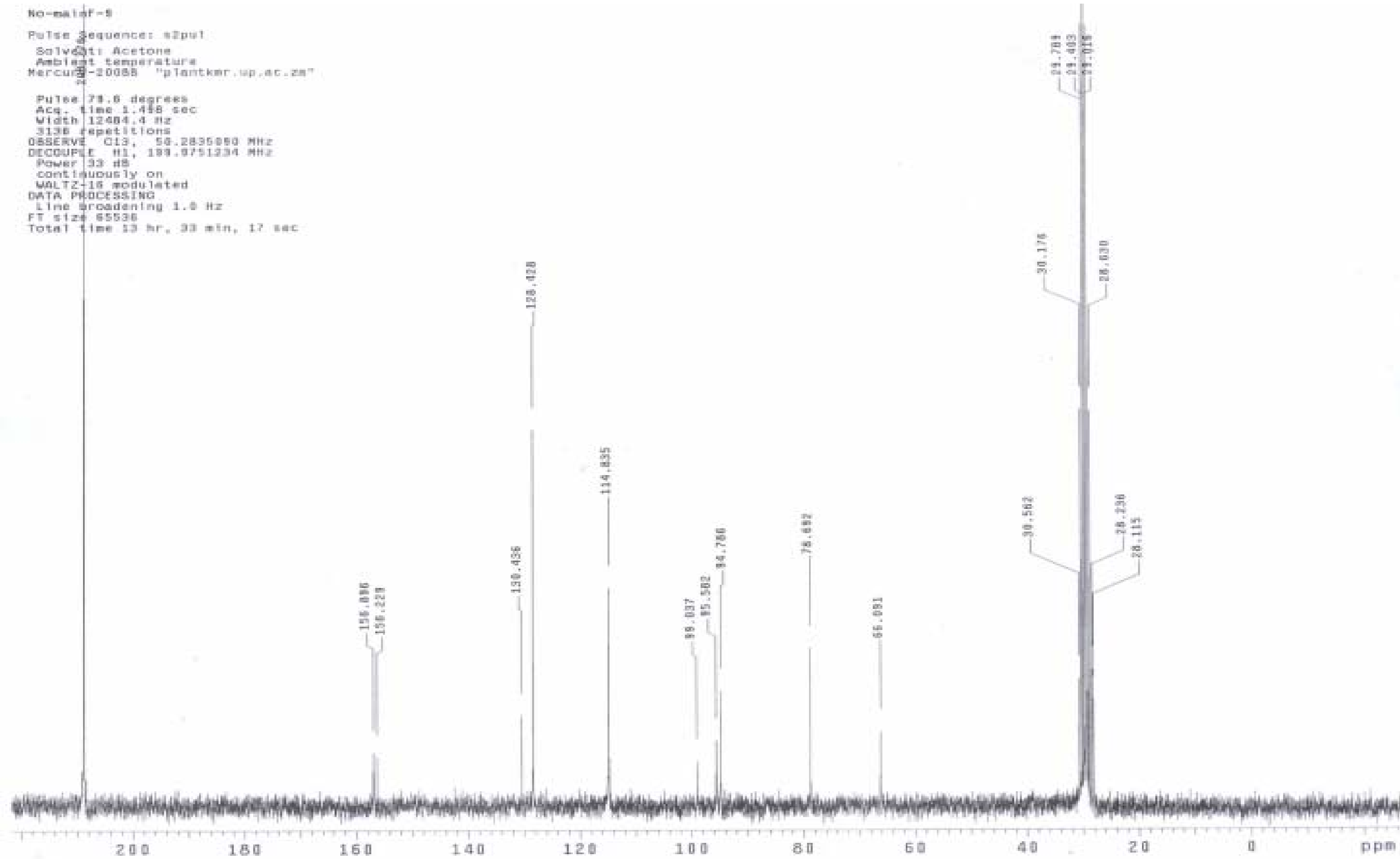

Figure 23: ^{13}C-NMR (125 MHz, $CDCl_3$) Spectrum of AFL5 or Epiafzelechin (**5**)

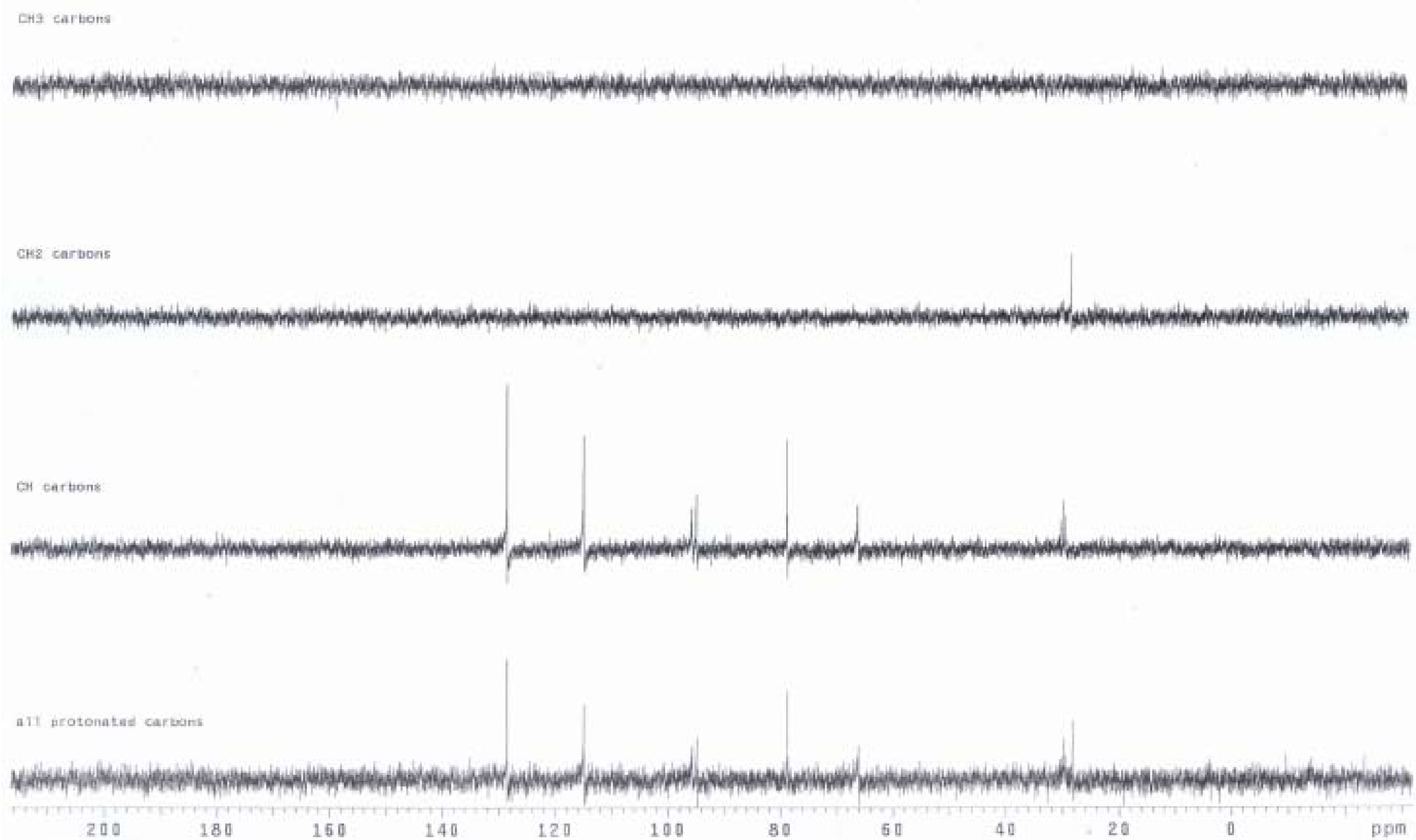

Figure 24: DEPT (125 MHz, $CDCl_3$) Spectrum of AFL5 or Epiafzelechin (**5**)

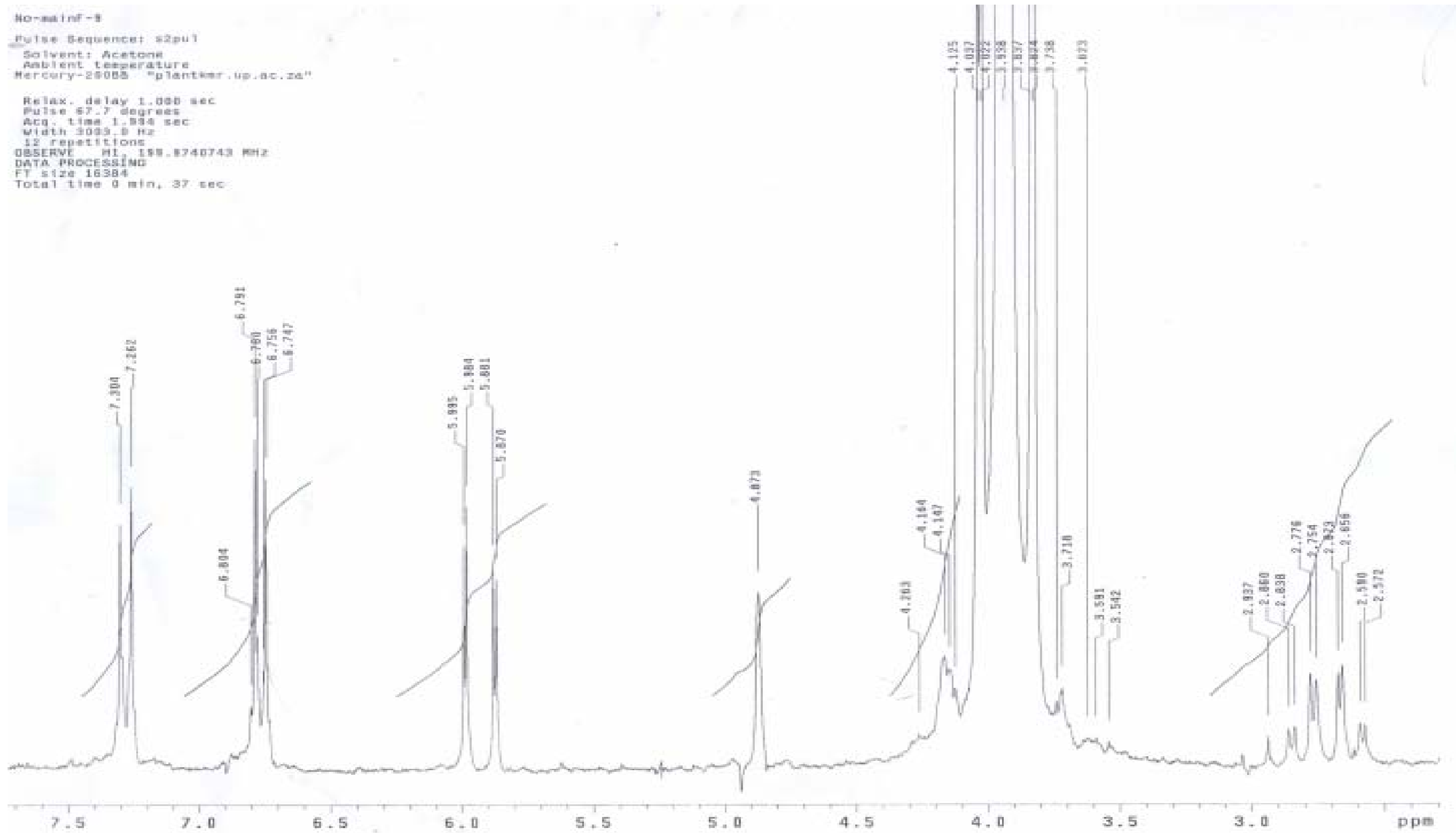

Figure 25: ^{1}H NMR (500 MHz, $CDCl_3$) Spectrum of AFL5 or Epiafzelechin

RE: Permission to use pictures of Ficus species

Wednesday, November 14, 2012 5:17 AM

From:

"Simon Van Noort" <svannoort@iziko.org.za>

To:

"Oyinlola Olaokun" <oyinolaokun@yahoo.com>

Dear Oyinlola

Yes you are welcome to use the images in your PhD thesis with appropriate acknowledgment: Images copyright Simon van Noort (Iziko Museums of South Africa).

Note that Ficus thonningii does not occur in South Africa. The species previously known as F. thonningii in South Africa is now split into F. burkei and F. petersii. Ficus thonningii in the strict sense probably only occurs further north in tropical Africa (See Burrows & Burrows 2003)

Kind regards

Simon

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