EVALUATION OF COMBINATION EFFECT ONTOXICITY AND ANTIDIABETIC POTENTIAL OF *GYMNEMA SYLVESTRE*R. Br. AND *COMBRETUM MICRANTHUM* Fam. LEAF EXTRACTS

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DECLARATION

I declare that the work in this thesis entitled "Evaluation of Combination Effect onToxicity andAntidiabeticPotential of *Gymnema sylvestre*R. Br.and *Combretum micranthum*Fam. Leaf Extracts" has been carried out by me in the Department of Biochemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Aminu Ibrahim	Signature	Date

CERTIFICATION

This thesis entitled "EVALUATION OF COMBINATION EFFECT ONTOXICITY AND ANTIDIABETIC POTENTIAL OF *GYMNEMA SYLVESTRE*R. Br. AND *COMBRETUM MICRANTHUM*Fam. LEAF EXTRACTS" by Aminu Ibrahim meets the regulations governing the award of the degree of Doctor of Philosophy (PhD) in Biochemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to the following for their care, support and love.

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ABSTRACT

Plants like *Gymnema sylvestre* R.Br. ("periploca of the woods" in English; "Kafisuga" in Hausa) (Asclepiadaceae) and *Combretum micranthum*, Fam. (Géézà in Hausa) (Combretaceae)) are used in combination for the management of diabetes mellitus (DM) in the North-Eastern part of Nigeria with little or no scientific basis. It is thus the aim of this research to validate the antidiabeticactivities of *G. sylvestre* (GS) and*C. micranthum* (CM) leaf extracts, individually and in combined form (GSCM). The study assayed for acute toxicity (LD_{50}) and sub-chronic toxicity (various markers) indices. *In vivo*antidiabetic potential wasanalyzed by following fasting blood glucose (FBG), feed and water intakes, lipid profile, serum insulin and liver hexokinase levels. Fasting blood glucose, inhibitory activities of α-amylase and α-glucosidase and phytochemical contents were used to initially direct the column chromatography fractionation. Further fractionation and isolation (Preparative-TLC) was guided by FBG and the characterization/identification of compounds done byUltra-High-Performance Liquid Chromatography Electrospray Ionization Quadrupole-Time-of-Flight Tandem Mass Spectrometry (UHPLC-ESI-Q-TOF-MS/MS), NMR and FTIR. Both plants were found to be relatively safe (LD_{50} > 5000mg/kg) in mice. Sub-chronic toxicity studies revealed that GSCM (1:1) reversed body weight loss of the diabetic rats at 600mg/kg b.w. dose when compared with control after 3 weeks of administration. The increases in relative organ weights (liver and kidney) were reversed significantly ($P < 0.05$);Hb, MCHand MCHC increased; serum total protein increased; levels of liver MDA, SOD, ROSandCAT and serum ALTand ALP showed a general reduction trend with a concomitant increase in liver GSH, GSSG andGSH-Px when "diabetic + treatment" groups were compared with the diabetic control. Moreover, GS and CM revealed good FRAP and radical scavenging activities against ABTS, DPPH and Nitric Oxide with *C. micranthum* being significantly better - both plants also showed good total flavonoids and total phenolic contents. Feed and water intake were reversed in the treated groups with percentage reductions of between 28 and 57 (for feed) and 14 and 39 (for water), with GSCM (1:1) displaying some dose-dependency. Fasting blood glucose for the 21 days experiment showed some significant reductions with the "diabetic + 400GS" group giving the highest percentage of 65. Serum Total Cholesterol, LDL, VLDL and TG in the "diabetes + treatment" groups showed general reduction whilst HDLincreases when compared with diabetic control – VLDL, TG andHDL displayed dose-dependency in the GSCM (1:1) groups. The antihyperglycaemic activities may be associated with glycosides and phenolic compoundslike coumarins which act via insulin-mimicking effect and inhibition of carbohydrate hydrolysing enzymes. in conclusion, *G. sylvestre* and *C. micranthum* aqueous leaf extracts could be relatively safe, have hypoglycaemic activities and the $(1:1)$ combination of a phenolic-rich fraction from GS and the compound 2,3,4,5-Tetrahydroxy-cyclohexane ester glucoside from CM at a dose of 10mg/kg b.w. displayed significantly synergistic hypoglycaemic effect comparing to the fractions used separately and has a better glucose-lowering effect than glibenclamide at 6mg/kg b.w. The constituents of the plants, separately and in a 1:1 combined form, could thus be beneficial in the management of diabetes mellitus.

TABLE OF CONTENTS

Title Page…… i

Declaration…… ii

Certification…… iii

Dedication…….. iv

Acknowledgements…… v

Abstract…….. vi

Table of Contents…….. vii

List of Figures…… xii

List of Tables……. xiv

List ofPlates…….. xviii

List of Appendices……. xix

List of Abbreviations……. xx

Chapter One…… 1

1.0 INTRODUCTION ……. 1

1.1 Statement of Research Problem……..... 5

1.2 Justification……. 6

1.3 Aim and Objectives……..... 6

Chapter Two…….**.....** 8

2.0 LITERATURE REVIEW……..... 8

2.1 Diabetes Mellitus…… 8

2.1.1 Types of diabetes mellitus……. 8

2.1.2 Insulin-dependent diabetes mellitus (IDDM) type 1…… 12

2.1.3 Non-insulin-dependent diabetes mellitus (NIDDM): type 2…… 17

2.1.4 Incidence/Prevalence of diabetes mellitus……. 19

2.1.5 Therapeutic intervention for hyperglycaemia…… 19

2.1.6 New frontiers in diabetes therapy…….. 28

2.2 Toxicity and Medicinal Properties of some Phytochemicals..... 30

2.2.1 Phenylpropanoids…….. 31

2.2.2 Coumarins…….. 32

2.2.3 Polyphenols—Tannins and Flavonoids…… 32

2.2.4 Glycosides…….. 37

2.2.5 Anthraquinones…… 37

2.2.6 Terpenes……. 38

2.2.7 Saponins……. 39

2.2.8 Alkaloids……... 40

2.3 Antidiabetic Plants with Antioxidants Properties…… 42

2.3.1 Oxidative stress…… 42

2.3.2 Antioxidants…….. 43

2.4 *Gymnema sylvestre* and *Combretum micranthum* - Ethnomedicinal Uses…… 48

2.4.1 *Gymnema sylvestre*…… 48

2.4.2 Combretum micranthum…… 50

2.5 Herb Combinations in Treatment/Management of Diseases…….. 52

2.6 Isolation and Characterization of Bioactive Compounds fromPlant Extracts…… 55

Chapter Three……..... 60

3.0 MATERIALS AND METHODS……... 60

3.1Materials…… 60

3.1.1Plant material collection, identificationand processing …..…… 60

3.1.2 Animals …… 60

3.1.3 Chemicals, reagents and equipment…….. 61

3.2Methods…… 61

3.2.1 Extracts preparations…… …… 61

3.2.2Phytochemical screening and antioxidant properties…..…….. 61

3.2.3 Acute toxicity …… 67

3.2.4 Sub-chronic studies…… 68

3.2.5 Preliminary screening for antidiabetic activity…….……... 69

3.2.6Antidiabetic studies…….. 70

3.2.6.3 Analyses……. 73

3.2.7Bioassay-guided studies…… 90

3.2.8 *In vitro* alpha-amylase and alpha-glucosidase inhibition assay…… 93

3.2.9 *In vivo* bioassay screening of fractions …… 94

3.2.10Further bioassay-guided fractionation - preparative TLC..…… 95

3.2.11 Identification of compounds…….. 96

3.2.12 Molecular docking…….. 98

3.2.13 Statistical analysis…… 100

Chapter Four…….…….. 101

4.0 RESULTS…… 101

4.1 Extractions Yields……... 101

4.2 Phytochemical Analyses …….. 101

4.3 Acute Toxicity…… 106

4.4 Sub-chronic Toxicity…….. 106

4.4.1 Body weight changes…… 106

4.4.2 Feed and water intakes…….. 106

4.4.3 Haematological changes……. 106

4.4.4 Relative organs weights……. 106

4.4.5 Liver function tests…… 114

4.4.6 Renal function tests…… 114

4.4.7*In vitro* Antioxidant (Radical Scavenging) Activity…… 114

4.4.8 *In vivo* Antioxidant Activity……. 114

4.5 Preliminary Antidiabetic Screening…….. 114

4.6 Antidiabetic Action of GS and CM…….. 120

4.6.1 Feed and water intakes……... 120

.2 Fasting blood glucose…… 120

.3 Lipid profile……. 120

.4 Serum insulin and liver hexokinase…….. 125

4.6.5 *In vivo*antioxidant activity in STZ diabetic rats…….. 125

4.6.6 Body weight changes in STZ diabetic rats…… 132

4.6.7 Haematological changes in STZ diabetic rats…… 132

4.6.8 Relative organs weights in STZ diabetic rats…… 132

4.6.9 Liver function tests in STZ diabetic rats…… 138

4.6.10 Renal function tests in STZ diabetic rats…… 138

4.7 Alpha-amylase and Alpha-glucosidase Inhibition……... 138

Bioassay – Guided Studies……. 142

4.8.1 Fractionation by column chromatography and pooling of fractions …… 142

.2 Further fractionation (preparative TLC)……..…….. 147

Alpha-amylase and Alpha-glucosidase Inhibition…..…….. 147

4.10 Isolation and Characterization of Compounds……..... 152

4.10.1 Ultra-high-performance liquid chromatography – mass spectroscopy
alyses……... 152

4.10.2 Fourier transform infra-red spectroscopy (FTIR) ……..… 152

4.10.3 Nuclear magnetic resonance spectroscopy (NMR) ……. 152

4.11 Molecular Docking Studies……... 152

Chapter Five…… 159

5.0 DISCUSSION…….. 159

Chapter Six…….. 171

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS……..... 171

6.1 Summary……. 171

6.2Conclusion…….. 172

6.3 Recommendations…….. 172

REFERENCES…….. 174

APPENDICES……. 193

LIST OF FIGURES

Figure 2.1: Structure of Caffeic Acid ……..…….... 31

Figure 2.2: Structure of Aesculetin…….. 32

Figure 2.3: Structure of Ellagic Acid ……... 33

Figure 2.4: Structure of Epicatechin…… 34

Figure 2.5: Structure of Apigenin…… 35

Figure 2.6: Structure of Kaempferol…… 35

Figure 2.7: Structure of Rheum-emodin…… 38

Figure 2.8: Structure of Glycyrrhizic Acid…….. 39

Figure 2.9: Production of Various Reactive Oxygen Species, The Role in Tissue Damage, and Their Detoxicationand Toxication……. 42

Figure 4.1: Total Flavonoids, Total Phenolics, Total Antioxidants and Ferric Reducing Antioxidant Power of Aqueous Leaf Extracts of *G. sylvestre*and *C. micranthum*…… 105

Figure 4.2: Relative Organ Weights of Normal Rats Administered *G. sylvestre* Aqueous Leaf Extract…… 111

Figure 4.3: Relative Organ Weights of Normal Rats Administered *C. micranthum* Aqueous Leaf Extract……. 112

Figure 4.4: Relative Organ Weights of Normal Rats Administered 1:1 Combination of *G. sylvestre* and *C. micranthum* Aqueous Leaf Extracts……. 113

Figure 4.5: Radical Scavenging Activities of Aqueous Leaf Extracts of *G.sylvestre* and *C. micranthum*…… 117

Figure 4.6: Serum Insulin Level (µIU/ml) In Streptozotocin-induced Diabetic Rats Administered*G. sylvestre, C. micranthum,* and their (1:1) Combination for 21 Days……. 126

Figure 4.7: Liver Hexokinase Level (U/mg protein) In Streptozotocin-Induced Diabetic Rats Administered *G. sylvestre, C. micranthum,* and their (1:1) Combination for 21 Days 127

Figure 4.8: Relative Organ Weights of Streptozotocin-Diabetic Rats Administered Different Doses of *G. sylvestre* Aqueous Leaf Extract…….. 135

Figure 4.9: Relative Organ Weights of Streptozotocin-Diabetic Rats Administered Different Doses of *C. micranthum* Aqueous Leaf Extract……. 136

Figure 4.10: Relative Organ Weights of Streptozotocin-Diabetic Rats Administered Different Doses of 1:1 Combination of *G. sylvestre* and *C. micranthum* Aqueous Leaf Extracts…….. 137

Figure 4.11: In vitro α- amylase and α-glucosidase Inhibition Activities of *G. sylvestre* and *C. micranthum* aqueous and methanolic Leaf Extracts……... 141

Figure 4.12: *In vitro*α- amylase and α-glucosidase Inhibition Activities of *G.sylvestre* (Subfraction D4) and *C. micranthum* (Subfraction G4) methanolic Leaf Extracts…… 149

Figure 4.13: *In vitro*α- amylase and α-glucosidase inhibitory activity of 1:1 combination (Subfractions D4:G4) of *G. sylvestre* and *C. micranthum*Methanolic Leaf Extracts…… 150

Figure 4.14: Structure/Name of Compound Isolated from Sub-fraction G4 of *Combretum micranthum* Leaf Extract…… 154

LIST OF TABLES

Table 3.1: Timetable of Solvent GradientProlfile of LC-MS Run…….. 97

Table 4.1: Extraction Yield for *G. sylvestre* and *C. micranthum* Leaf Extracts…… 102

Table 4.2:Qualitative Phytochemical Composition of Leaf Extracts of *G. sylvestre* and *C. micranthum*…… 103

Table 4.3:Quantitative Phytochemical Contents of Extracts of *G. sylvestre* and *C. micranthum* Leaves……... 104

Table 4.4: Percentage (%) Change in Body Weight of Normal Rats Administered *G.sylvestre*, *C.micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts…… 107

Table 4.5: Mean and Percentage (%) Change in Feed Intake of Normal Rats Administered *G. sylvestre* (GS), *C. micranthum* (CM) and 1:1 Combination (GSCM) of Their Aqueous Leaf Extracts for 21 Days…….. 108

Table 4.6: Mean and Percentage (%) Change in Water Intake of Normal Rats Administered *G. sylvestre* (GS), *C. micranthum* (CM) and 1:1 Combination (GSCM) of Their Aqueous Leaf Extracts for 21 Days…….. 109

Table 4.7: Haematological Indices of Normal Rats Administered *G. sylvestre*, *C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts for 21 days……. 110

Table 4.8: Liver Function Indices of Normal Rats Administered *G. sylvestre*, *C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts for 21 Days….. 115

Table 4.9: Renal Function Indices of Normal Rats Administered *G. sylvestre*, *C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts for 21 Days……. 116

Table 4.10: Some Liver Antioxidant Indices of Normal Rats Administered *G. Sylvestre*, *C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts…… 118

Table 4.11: Effect of Administration of Aqueous Leaf Extracts of *G. sylvestre*, *C. micranthum* and their Combinations (GSCM) on Mean Fasting Blood Glucose Level (mg/dl) and the Percentage (%) Reductions in FBG of Alloxan-induced Diabetic Mice for Four Hours…….. 119

Table 4.12: Mean and Percentage (%) Change in Feed Intake of Streptozotocin-Induced Diabetic Rats Administered Different Doses of *G. sylvestre* (GS), *C. micranthum* (CM) and 1:1 Combination (GSCM) of Their Aqueous Leaf Extracts For Twenty-One (21) Days…….. 121

Table 4.13: Mean and Percentage (%) Change in Water Intake of Streptozotocin-Induced Diabetic Rats Administered Different Doses of *G. sylvestre* (GS), *C. micranthum* (CM) and 1:1 Combination (GSCM) of Their Aqueous Leaf Extracts for 21 Days……... 122

Table 4.14: Percentage (%) Changes in Mean Fasting Blood Glucose of Streptozotocin-Diabetic Rats Administered Different Doses of *G.sylvestre*, *C.micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts for 21 Days… 123

Table 4.15: Lipid Profile of Streptozotocin-Diabetic Rats Administered Different Doses of *G.sylvestre*, *C.micranthum*and 1:1 Combination of Their Aqueous Leaf Extracts for 21 Days……. 124

Table 4.16: Some Liver Antioxidant Indices of Streptozotocin-Diabetic Rats Administered *G. sylvestre*, *C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts……. 128

Table 4.17: Relationship between % Decrease in FBG, Insulin, Alpha-Amylase/Alpha-Glucosidase and Hexokinase in Rats Administered *Gymnema sylvestre* and *Combretum micranthum* Aqueous Leaf Extracts…….. 129

Table 4.18: Relationship between % Decrease in FBG of Rats and *In vitro* Antioxidant Capacity of *Gymnema sylvestre* and *Combretum micranthum* Leaves… 130

Table 4.19: Relationship between % Decrease in FBG, *In vivo* Antioxidant/Peroxidation Status in Rats Administered *Gymnema sylvestre* and *Combretum micranthum* Aqueous Leaf Extracts……. 131

Table 4.20: Percentage (%) Change in Body Weight of Streptozotocin-Diabetic Rats Administered Different Doses of *G.sylvestre*, *C.micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts…….. 133

Table 4.21: Haematological Indices of Streptozotocin-Diabetic Rats Administered Different Doses of *G.sylvestre,C.micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts…….. 134

Table 4.22: Liver Function Indices of Streptozotocin-Diabetic Rats Administered *G. sylvestre*, *C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts…… 139

Table 4.23: Renal Function Indices of Streptozotocin-Diabetic Rats Administered *G. sylvestre*, *C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts... 140

Table 4.24: Mean Fasting Blood Glucose and their Percentage (%) Changes for Alloxan-induced Diabetic Mice Administered Different Solvent Partitioned Fractions of *G. sylvestre* and *C. micranthum* Aqueous Leaf Extracts for Four Hours 143

Table 4.25: Mean Fasting Blood Glucose and Their Percentage (%) Changes of Alloxan-Induced Diabetic Mice Administered Different Fractions (40mg/kg body weight) of Aqueous/Methanolic Leaf Extracts of *G. sylvestre* for Four Hours…… 144

Table 4.26: Mean Fasting Blood Glucose and Their Percentage (%) Changes of Alloxan-Induced Diabetic Mice Administered Different Fractions (40mg/kg body weight) of Aqueous/Methanolic Leaf Extracts of *C. micranthum* For Four Hours….. 145

Table 4.27: Mean Fasting Blood Glucose and Their Percentage (%) Changes of Alloxan-Induced Diabetic Mice Administered Fractions D and G (and their 1:1 Combinations) at 40mg/kg body weight of Aqueous/Methanolic Leaf Extracts of *G. sylvestre* and *C. micranthum* Respectively for Four Hours……. 146

Table 4.28: Mean Fasting Blood Glucose and their Percentage (%) Changes of Alloxan-induced Diabetic Mice Administered Subfractions (10mg/kg body weight) of *G. sylvestre* (Fractions D1-D5), *C. micranthum* (Fractions G0-G5) and 1:1 Combinations of Most Active Subfractions (D4:G4) Leaf Extracts for Four Hours... 148

Table 4.29: Relationship between % Decrease in FBG and Inhibitory Activities of Alpha-Amylase/Alpha-Glucosidase in Rats Administered *Gymnema sylvestre* (sub-fraction D4), *Combretum micranthum*(sub-fraction G4)and their 1:1 combination (D4:G4)Methanol Leaf Extracts…….. 151

Table 4.30: Compound Composition of Subfraction 4 of Fraction D [N-Hexane:Ethylacetate (20:80)] for *G. sylvestre* Aqueous/Methanolic Leaf Extract Using LC/MS in the Negative Ion Mode……. 153

Table 4.31: Compound Composition of Sub-fraction 4 of Fraction G [Ethylacetate:Methanol (30:70)] For *C. micranthum*Aqueous/Methanolic Leaf Extract Using LC/MS In The Negative Ion Mode…… 154

Table 4.32: ICM docking scores for compounds (ligands) from sub-fractions D4 and G4 of *G. sylvestre* and *C. micranthum* respectively into the active site of Human Pancreatic alpha-amylase (PDB ID= 4W93)……... 155

Table 4.33: ICM docking scores for compounds (ligands) from sub-fractions D4 and G4 of *G. sylvestre* and *C. micranthum* respectively into the active site of Alpha-glucosidase (PDB ID= 3WY2)…….. 157

Table 4.34: ICM docking scores for compounds (ligands) from sub-fractions D4 and G4 of *G. sylvestre* and *C. micranthum* respectively into the active site of Phosphorylated-insulin receptor tyrosine kinase (PDB ID= 1IR3)…… 158

LIST OF PLATES

Plate I: *Gymnema sylvestre* Plant – Leaves and Stem ……. 49

Plate II: *Combretum micranthum* Plant – Leaves and Stem ……..…… 51

LIST OF APPENDICES

ppendix I: Reagent Composition of Commercial Kits …….. 193

ppendix II: Computation tables for enzyme activities …… 198

Appendix III: Details of Fractionations of GS and CM Leaf Extracts/Fractions 199

Appendix IV: TLC Plates of Fractions/Sub-fractions of GS and CM Leaf Extracts 202

Appendix V: LCMSChromatograms and Mass Spectra of Sub-fractions of GS and CM Leaf Extracts…….. 206

Appendix VI: FTIRSpectra and Principal Functional Groups of Sub-fractions of GS and CM Leaf Extracts…….. 209

Appendix VII: ^{1}H NMR and ^{13}C NMR Spectra and the Spectral Data of Sub-fractions of CM Leaf Extracts…….. 213

Appendix VIII: Docking Poses of Isolated/Predicted Compounds from Sub-fractions of GS and CM Leaf Extracts…….. 216

Appendix IX: Selected Publications and Papers Presented At Conferences…….. 224

LIST OF ABBREVIATIONS

AAS Atomic absorption spectrophotometry

ABTS 2,2-azino-bis-(3-ethylenebenzothiozoline-6-sulfonic acid

ALT/GPT Alanine aminotransferase/Glutamic pyruvic transaminase

AOAC Association of official Analytical Chemists

AST/GOT Aspartate aminotransferase/Glutamic oxaloacetic transaminase

b.w. Body weight

CATCatalase

CHARMmChemistry at Harvard Macromolecular Mechanics force field

CM *Combretum micranthum*

DPPH 1,1-diphenyl-2-picrylhydrazyl

EDTAEthylenediaminetetraacetic acid

FBG Fasting Blood Glucose

FDA Food and Drug Administration of USA

FRAP Ferric Reducting Antioxidant Power

FTIR Fourier Transform Infrared Spectroscopy

GS *Gymnema sylvestre*

GSCM 1:1 Combination of *Gymnema sylvestre* and *Combretum micranthum*

GSH Glutathione-reduced

GSH-PxGlutathione Peroxidase

GSSG Glutathione-oxidized

Hb Haemoglobin

HDL High Density Lipoprotein

HXK Hexokinase

IDDM Insulin Dependent Diabetes Mellitus

LDL Low Density Lipoprotein

MCH Mean Cell Haemoglobin

MCHC Mean Cell Haemoglobin Concentration

MCV Mean Cell Volume

MDA Malondialdehyde

MMFF Merck Molecular Force Field

MODY Maturity – onset diabetes of the youth

NIDDM Non – insulin dependent diabetes mellitus

NMR Nuclear magnetic resonance spectroscopy

$NO^{\bullet}$ Nitric oxide radical

OECD Organization for Economic Cooperation and Development

OGTT Oral Glucose tolerance Test

PCV Packed cell volume

PDB Protein Data Bank

Prep-TLC Preparative Thin Layer Chromatography

RBC Red blood cell

RCSB Research Collaboratory for StructuralBioinformatics

ROS Reactive oxygen species

ROW Relative organ weight

SOD Superoxide Dismutase

TBARS Thiobarbituric Reactive Substances

TC Total Cholesterol

TG Triglycerides

TLC Thin Layer Chromatography

TPP Total plasma peroxides

UHPLC-ESI-Q-TOF-MS/MS Ultra-High-Performance Liquid Chromatography Electrospray Ionization Quadrupole-Time-of-Flight Tandem Mass Spectrometry

VLDL Very Low Density Lipoprotein

WBC White blood cell

CHAPTER ONE

1.0 INTRODUCTION

Diabetes mellitus (DM) is defined as a syndrome characterized by hyperglycaemia and disturbances of carbohydrate, fat and protein metabolisms as a result of absolute or relative deficiency in insulin action and/or secretion (Kuzuya *et. al.*, 2002; Marx, 2002; Liu *et. al.*, 2013). Diabetes mellitus is broadly classified into two, viz; Insulin Dependent Diabetes Mellitus (IDDM) and Non-Insulin Dependent Diabetes Mellitus (NIDDM).

IDDM, also referred to as type 1, is characterized by absence of circulating insulin; plasma glucagon is inappropriately elevated, and the pancreatic β-cells fail to respond to all insulinogenic stimuli (Paoli *et. al.*, 2013; Mahendran *et. al.*, 2014). The prominent diagnostic feature of DM is a fasting blood glucose above 7 mmol/l or a random blood glucose greater than 10 mmol/l repeated on two occasions (King, 2015). IDDM generally occurs before age 30, hence sometimes referred to as juvenile – onset diabetes, but can also be found in adult life, hence another form known as maturity –onset diabetes of the youth (MODY).

NIDDM, also called adult-onset diabetes or type 2 diabetes is mainly a problem of a "target-organ" disorder resulting in ineffective insulin action that can secondarily influence pancreatic β-cell function (Paoli *et. al.*, 2013; Mahendran *et. al.*, 2014). Thus insulin resistance is the fundamental pathogenic factor for NIDDM and is one of the targets for antidiabetic medication (Mohler *et. al.*, 2009; Jiang and Morahan, 2016). Approaches are made to reduce the hyperglycaemia associated with DM using drugs such as sulfonylureas, which stimulates pancreatic islet cells to secrete insulin; glucosidase inhibitors, which interfere with glucose absorption and insulin itself, which suppresses glucose production and augments glucose utilization (Mollar, 2001). Though it is important that glycaemic

control should be achieved as rapidly as possible to minimize the impact of glucose toxicity, it is also necessary to provide therapy to control other related risk factors, including oxidative stress, dyslipidaemia, mitochondrial dysfunction, vascular complications, etc. (Duckworth, 2001; Jain and Saraf, 2010). Diabetics present with varying complications ranging from the acute ones like ketoacidosis and infections and the chronic ones like cardiovascular, haematological, renal, ocular, neuropathic, skin complications and foot ulceration (Reaven, 1988; WHO, 1994; Karam, 1998).

DM can be found in almost all populations throughout the world but the incidence and prevalence varies from place to place. Globally, the number of people that have been diagnosed with diabetes has exploded in the past two decades. In 2000, 151 million people in the world were reported to be diabetic (Zimet *et. al.*, 2001; Wild *et. al.*, 2004; Roglic *et. al.*, 2005). It has been projected that 221 million people will be diabetic in 2010 and 324 million by 2025 withthe global prevalence of diabetes estimated at 9% in 2014 (WHO, 2014).

In addition to the conventional regimens, many hypoglycaemic plants are being studied (and even used) for the management of DM (Upendra *et. al.*, 2010; Liu *et. al.*, 2013; Paoli *et. al.*, 2013; Mahendran*et. al.*, 2014). In view of the high cost,limited supply and storage conditions of insulin preparations, coupled with adverse effects associated with the use of oral hypoglycaemic drugs like sulphonylureaswhichexacerbates hyperinsulinaemia, causing weight gain andthe biguanides being weak hypoglycaemics (Bengston *et. al.*, 1972; Shen and Bressler, 1976a and b; Karam, 1998; Mohler *et. al.*, 2009), interest has now shifted to research on the untapped wealth of medicinal plants with potential hypoglycaemic effects. Some of the plants that have been in use in traditional medicine as oral hypoglycaemics include the leaves of *Gymnema sylvestre*, flowers of

Musa sapientum, Momordica charantia fruits, *Carica papaya, Strophanthus hispidus, Dioscorea cayenensis, Triplochiton scleroxylon*and *Terminalia macroplera* (Evans, 1999; Pari and Maheshwara, 1999; Grover *et. al.*, 2002; Prohp and Onoagbe, 2011) as well as *Combretum micranthum* (Mazza andMiniati, 1993). These plants contain, as active components which confers the hypoglycaemic activity, substances like polysaccharides, steroids, alkaloids and some steroidal-saponins (Evans, 1999); ferulic acid, chlorogenic acid, caffeic acid, coumaric acid, cinnamic acid, eugenol and berberine have also shown antidiabetic effects when they were tested in two different cell lines at basal level (Prabhakar and Doble 2009, 2011a,b,c). Moreover, It has been found that herbal preparations often used by diabetic patients (Dunning, 2003; Peng *et. al.*,2004; Chavez *et. al.*, 2006), contain biflavonoids, which not only confer per se antidiabetic effect but also eliminate hyperglycaemia-induced oxidative stress, and thereby help to attenuate secondary complications of diabetes (Dias *et. al.*, 2005). Most of these phytochemicals act at various locations in the insulin signaling cascade and improve their effectivity. They also have been shown to reduce the lipid content probably through regulation of their synthetic pathways (Umar *et. al.*, 2014). Another study reported the inhibitory effects of polyphenols on carbohydrates hydrolyzing enzymes. These include the green tea polyphenols that inhibit the activities of α-glucosidase and sucrase, berry polyphenols that inhibit the activity of α-glucosidase and α-amylase as well as sweet potato polyphenols which inhibit the activity of α-glucosidase (Bello *et. al.*, 2011).

It has been adopted that an effective antidiabetic compound should have both hypoglycemic and antioxidant properties, with minimal or no side effects asseveral African medicinal plants were reported to have both hypoglycemic and antioxidant activities (Atawodi, 2005). Studies indicated that *Gymnema sylvestre* and *G. montanum*, possess

potent antioxidant and antidiabetic potentials due to their possession of polyphenolic compounds and their ability to inhibit the activities of α- glucosidase, α- amylase and lipases (Bello *et. al.,* 2011). Bello *et. al.,* (2011), also reported that the extract of *Leptadenia hastata* (Pers.) Decne may possess significant inhibitors of α-glucosidase and may thus be effective in reducing postprandial hyperglyceamia. Moreover, *C. micranthum* have been reported to be a potential herbal antidiabetic (Welch, 2010).

Adverse effects from herbal remedies are reported; e. g. sub-chronic study showed that *Gouania longipetala* methanol leaf extract significantly increased relative liver weight, liver enzyme markers and serum urea at the dose of 10 mg/kg on day 90 - the extract also revealed degenerative histopathological changes in the kidney and liver at same dose in rats (Ezeja *et. al.,* 2014), but their frequency and severity are unknown (Prabhakar *et. al.,* 2014). However, some plants also showed beneficial effects as consumption of the aqueous extract of *H. sabdariffa* calyces by *T. congolense*–infected rats prevented fall in systemic antioxidant reserves and alleviated disease–induced damage to red blood cells, as well as hepatic and renal structures (Umar *et. al.,* 2009). To overcome these drawbacks scientific study of herbal remedies and their potential to cause interactions when used in combination need to be thoroughly understood and systematically studied (Inamdar *et. al.,* 2007; Prabhakar *et. al.,* 2014). Many of the phytomedicines in the drug market are whole extracts of plants because it is known that various components of individual or mixtures of herbs act in synergy and hence are a vital part of their therapeutic efficacy (Lin and Sun 2010). Two drugs that produce almost similar effect when used together may sometimes produce enhanced, same or diminished effect. In this interaction there might be three different types of behaviors namely synergy, antagonistic and additive/indifferent.

When using a combination therapy, the dosing of both the compounds is very important. These combinations can show one type of behavior at one concentration mixture and other type at another concentration.

1.1Statement of Research Problem

Drugs with plants as raw materials are increasingly favoured by people all over the world (Fisher and Ward, 1994; Barnes *et. al.*, 1999; De Smet, 2002) for their unique advantages in preventing and curing diseases, rehabilitation and health care, especially in Europe, the United States, Africa and many Asian countries. According to statistics, in the United States alone, there are more than 15 million people using herbal preparations in varying degrees, including Chinese herbal medicines, as either a therapy or an adjuvant therapy for various diseases, with the annual cost of approximately 30 billion U.S. dollars (Eisenberg *et. al.*, 1998). In the West African subcontinent, Nigeria inclusive, domestic medicinal plant market is of substantial economic importance (Agbaje *et. al.*, 2009; Idu *et. al.*, 2010). A volume of approximately 655 metric tons worth 2.7 million USD is offered for sale annually (Quiroz *et. al.*, 2014).

Herbal medicine may be particularly valuable in helping the body cope with the side effects of chemotherapeutic agents (Taixiang *et. al.*, 2005; Mok *et. al.*, 2007)However, which herbs in what dosages and how the concurrent administration should be handled are questions largely unanswered. In sub-saharan Africa (Northern Nigeria inclusive), the use of multiple herbs as decoctions or in different combinations to treat chronic non-communicable diseases like diabetes mellitus and hypertension is common (Agbaje *et. al.*, 2009; Idu *et. al.*, 2010). However, some of these plants, when used together, can have either positive or negative effects on the individual. Due to this, the current study looks at two of such plants (GS and CM) used concomitantly for the management of diabetes

mellitus in the Northern part of Nigeria, so as to find the right combination which shall be beneficial to the patients. Very few or virtually no work has been done to address this problem, which shall go a long way in tackling economic and medical challenges associated with the disease, diabetes. So, the hypotheses is that, the right combination of isolated compounds from GS and CM leaf extracts have better hypoglycaemic and hypolipidaemic properties compared to when they have been used alone/separately in diabetic rats.

1.2Justification

Total recovery from diabetes has yet to be reported and this explains the intensive studies done on it (El-Shafey, *et. al.*, 2013). The International Diabetes Federation estimates that there are over 300 million people around the world with diabetes. This total is expected to reach close to 500 million within 20 years (WHO, 2014).

Moreover, scientific study of herbal remedies and their potential to cause interactions when used in combination need to be thoroughly understood and systematically studied so as tounderstand whether there is synergy, antagonism and/or additivity. In addition, there are very little or no scientific work done to look into the right combination of these two herbs (GS and CM) and how effective or otherwise is such a combination when compared to the plants used individually as well as to an orthodox oral hypoglycaemic commonly prescribed, hence the need for the current study.

1.3Aim and Objectives

The aim of this study is to evaluate the combined antidiabetic properties of the aqueous leaf extracts of *G. sylvestre* and *C. micranthum* and their toxicity risks.

The specific objectives are:

I. To determine the median lethal dose and sub-chronic toxicities of *G. sylvestre*and*C. micranthum* leaf extracts in mice.

II. To determine *in vitro* and *in vivo* antioxidant activity of *G. sylvestre*and*C. micranthum*Leaf Extracts.

III. To screen individually, *G. sylvestre*and*C. micranthum* leaf extracts and their combinations for anti-diabetic action in mice.

IV. To assess anti-diabetic action of *G. sylvestre* and *C. micranthum* leaf extracts and their combination in streptozotocin-induced diabetic rats.

V. To determine *in vitro*anti-diabetic effect and deduce a probable mode of action of *G. sylvestre*and*C. micranthum* leaf extracts.

VI. To isolate and characterize the antidiabetic principle(s) in GS and CM using bioassay-guided fractionation of the aqueous extracts.

VII. To use a molecular docking approach to identify potential antidiabetic phytochemicals/compounds found in the fractions of GS and CM.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Diabetes Mellitus

Diabetes mellitus (DM), a chronic metabolic diseasecharacterized by a deficiency in the pancreas insulin production and/or by peripheral insulin resistance, can be referred as a global epidemic disease (WHO, 1985; Turnbridge and Home, 1991; WHO, 1994; Kuzuya *et. al.*, 2002; Marx, 2002; Liu *et. al.*, 2013).Additional symptoms of diabetes mellitus include excessive thirst (polydipsia), glucosuria, polyuria, lipaemia and hunger (polyphagia) (Porchezhian and Dobriyal, 2003; Azeez, *et. al.*,2010; Bishop, *et. al.*, 2010). If left untreated the disease can lead to fatal ketoacidosis.

Criteria, which clinically establish an individual as suffering from diabetes mellitus, include:

1.having a fasting plasma glucose level in excess of 126mg/dL (7mmol/L). Normal levels should be less than 100mg/dL (5.6mmol/L) or:

2. having postprandial glucose levels in excess of 200mg/dL (11mmol/L) at two times points during an oral glucose tolerance test, OGTT, one of which must be within 2 hrs of ingestion of glucose (King, 2015).

2.1.1 Types of Diabetes Mellitus

Diabetes mellitus is a heterogeneous clinical disorder with numerous causes. Two main classifications of diabetes mellitus exist, idiopathic and secondary.

Idiopathic diabetes is divided into two main types; insulin dependent and non-insulin-dependent. Insulin-dependent diabetes mellitus, IDDM (more commonly referred to as type 1 diabetes) is defined by the development of ketoacidosis in the absence of insulin therapy. Type 1 diabetes most often manifests in childhood (hence, also called juvenile onset diabetes) and is the result of an autoimmune destruction of the β-cells of the pancreas. Non-insulin-dependent diabetes mellitus, NIDDM (more commonly referred to as type 2 diabetes) is characterized by persistent hyperglycaemia but rarely leads to ketoacidosis. Type 2 diabetes generally manifests after age 40 and therefore has the obsolete name of adult onset-type diabetes. Type 2 diabetes can result from genetic defects that cause both insulin resistance and insulin deficiency (WHO, 2014; King, 2015). There are two main forms of type 2 diabetes:

1. Late onset associated with obesity.

2. Late onset not associated with obesity.

There is a strong correlation between obesity and the onset of type 2 diabetes with its associated insulin resistance. It should be pointed out that in the United States the proportion of the population under 40 that can be clinically defined as obese now exceeds 25%. In Nigeria, the prevalence of overweight individuals ranged from 20.3%–35.1%, while the prevalence of obesity ranged from 8.1%–22.2% (Chukwuonye *et. al.*, 2013; Akarolo-Anthony *et. al.*, 2014; Okafor *et. al.*, 2014) and that hypertension, obesity and dyslipidaemia are common features in persons with newly diagnosed DM (Edo and Edo, 2016).Many children are obese and are developing type 2 diabetes at an alarming epidemic rate. The dramatic rise in obesity in the US has led to an equally alarming increase in the

percentage of the population who suffer from the metabolic syndrome. The metabolic syndrome is a clustering of atherosclerotic cardiovascular disease risk factors, one of which involves insulin resistance characteristic in type 2 diabetes. It should be pointed out that obesity alone does not always lead to insulin resistance as some individuals who are obese do not experience insulin resistance and conversely, some individuals who manifest insulin resistance are not obese. These latter observations point to the added role of genetics in the acquisition of insulin resistance(WHO, 2014; King, 2015).

Secondary Diabetes Mellitusinclude:

1. **Maturity onset type diabetes of the young (MODY)** was previously considered to be a third form of type 2 diabetes. However, with the discovery of specific mutations leading to MODY, it is now classified under secondary or other specific types of diabetes. MODY is characterized by onset prior to age 25. All cases to date have shown impaired β-cell function. Patients may also exhibit insulin resistance and late β-cell failure (King, 2015).

2. **Pancreatic disease:** Pancreatectomy leads to the clearest example of secondary diabetes. Cystic fibrosis and pancreatitis can also lead to destruction of the pancreas.

3. **Endocrine disease:** Some tumors can produce counter-regulatory hormones that oppose the action of insulin or inhibit insulin secretion. These counter-regulatory hormones are glucagon, epinephrine, growth hormone and cortisol.

a. Glucagonomas are pancreatic cancers that secrete glucagon.

b. Pheochromocytomas secrete epinephrine.

c. Cushing syndrome results from excess cortisol secretion.

d. Acromegaly results in excess growth hormone production.

4. **Drug-induced diabetes**; treatment with glucocorticoids and diuretics can interfere with insulin function.

5. **Anti-insulin receptor** autoantibodies (Type B insulin resistance).

6. **Mutations in the insulin gene.**

7. **Mutations in insulin receptor gene** which lead to the syndromes listed below. Two clinical features are common in all syndromes that result from mutations in the insulin receptor gene: acanthosis nigricans and hyperandrogenism (the latter being observed only in females).

a. Donohue syndrome (also referred to as Leprachaunism)

b. Rabson-Mendenhall syndrome

c. Type A insulin resistance

8**. Gestational diabetes**; this syndrome sets in during pregnancy and usually resolves itself following childbirth (Metzger *et. al*., 2008; Miettinen *et. al.*, 2014).

9. **Many other genetic syndromes** have either diabetes or impaired glucose tolerance associated with them; lipoatrophic diabetes, Wolfram syndrome, Down syndrome, Klinefelter syndrome (XXY males), Turner syndrome, myotonic dystrophy, muscular

dystrophy, Huntington disease, Friedreich ataxia, Prader-Willi syndrome, Werner syndromeand Cockayne syndrome(Kuzuya *et. al.*, 2002; Strojek, 2003 WHO, 2014; King, 2015).

2.1.2 Insulin-dependent diabetes mellitus (IDDM) -Type 1

2.1.2.1 Etiology of type 1 diabetes

Type 1 diabetes has been shown to be the result of an autoimmune reaction to antigens of the islet cells of the pancreas. There is a strong association between IDDM and other endocrine autoimmunities (e.g. Addison disease). Additionally, there is an increased prevalence of autoimmune disease in family members of IDDM patients.

Types of Autoantibodies

1. Islet cell cytoplasmic antibodies: The primary antibodies found in 90% of type 1 diabetics are against islet cell cytoplasmic proteins (termed ICCA, islet cell cytoplasmic antibodies). In non-diabetics ICCA frequency is only 0.5%–4%. The presence of ICCA is a highly accurate predictor of future development of IDDM. ICCA are not specific for the β-cells and recognize antigens in other cell types in the islet. However, the autoimmune attack appears to selectively destroy β-cells. Therefore, the antibodies may play a primary role in the destruction of islet cells. It is an equally likely possibility that the production of anti-islet antibodies occurs as a result of the destruction of β-cells. Whether a direct cause or an effect of islet cell destruction, the titer of the ICCA tends to decline over time (Kuzuya *et. al.*, 2002; Strojek, 2003; WHO, 2014; King, 2015).

2. Islet cell surface antibodies: Autoantibodies directed against cell-surface antigens (ICSA) have also been described in as many as 80% of type 1 diabetics. Similar to ICCA, the titer of ICSA declines over time. Some patients with type 2 diabetes have been identified that are ICSA positive.

3. Specific antigenic targets of islet cells: Antibodies to glutamic acid decarboxylase (GAD) have been identified in over 80% of patients newly diagnosed with IDDM. Like ICCA, anti-GAD antibodies decline over time in type 1 diabetics. There are two GAD genes in humans identified as GAD1 and GAD2. The GAD isoforms produced by these two genes are identified as GAD67 (GAD1 gene: GAD67) and GAD65 (GAD2 gene: GAD65) which is reflective of their molecular weights. Both the GAD1 and GAD2 genes are expressed in the brain and GAD2 expression also occurs in the pancreas. The presence of anti-GAD antibodies (both anti-GAD65 and anti-GAD67) is a strong predictor of the future development of IDDM in high-risk populations. Anti-insulin antibodies (IAA) have been identified in IDDM patients and in relatives at risk to develop IDDM. These IAA are detectable even before the onset of insulin therapy in type 1 diabetics. IAAs are detectable in around 40% of young children with IDDM.

2.1.2.2 Pathophysiology of type 1 diabetes

The autoimmune destruction of pancreatic β-cells leads to a deficiency of insulin secretion. It is this loss of insulin secretion that leads to the metabolic derangements associated with IDDM. In addition to the loss of insulin secretion, the function of pancreatic α-cells is also abnormal. There is excessive secretion of glucagon in IDDM patients. Normally, hyperglycaemia leads to reduced glucagon secretion. However, in patients with IDDM,

glucagon secretion is not suppressed by hyperglycaemia. The resultant inappropriately elevated glucagon level exacerbates the metabolic defects due to insulin deficiency (Altan, 2003; King, 2015). The most pronounced example of this metabolic disruption is that patients with IDDM rapidly develop diabetic ketoacidosis in the absence of insulin administration. If somatostatin is administered to suppress glucagon secretion, there is a concomitant suppression in the rise of glucose and ketone bodies. Particularly problematic for long term IDDM patients is an impaired ability to secrete glucagon in response to hypoglycaemia. This leads to potentially fatal hypoglycaemia in response to insulin treatment in these patients.

Although insulin deficiency is the primary defect in IDDM, in patients with poorly controlled IDDM there is also a defect in the ability of target tissues to respond to the administration of insulin. There are multiple biochemical mechanisms that account for this impairment of tissues to respond to insulin. Deficiency in insulin leads to elevated levels of free fatty acids in the plasma as a result of uncontrolled lipolysis in adipose tissue. Free fatty acids suppress glucose metabolism in peripheral tissues such as skeletal muscle. This impairs the action of insulin in these tissues, i.e. the promotion of glucose utilization. Additionally, insulin deficiency decreases the expression of a number of genes necessary for target tissues to respond normally to insulin such as glucokinase in liver and the GLUT 4 class of glucose transporters in adipose tissue. The major metabolic derangements which result from insulin deficiency in IDDM are impaired glucose, lipid and protein metabolism.

Glucose Metabolism: Uncontrolled IDDM leads to increased hepatic glucose output. First, liver glycogen stores are mobilized then hepatic gluconeogenesis is used to produce glucose. Insulin deficiency also impairs non-hepatic tissue utilization of glucose. In

particular in adipose tissue and skeletal muscle, insulin stimulates glucose uptake. This is accomplished by insulin-mediated movement of glucose transporter proteins to the plasma membrane of these tissues. Reduced glucose uptake by peripheral tissues in turn leads to a reduced rate of glucose metabolism. In addition, the level of hepatic glucokinase is regulated by insulin. Therefore, a reduced rate of glucose phosphorylation in hepatocytes leads to increased delivery to the blood. Other enzymes involved in anabolic metabolism of glucose are affected by insulin (primarily through covalent modifications). The combination of increased hepatic glucose production and reduced peripheral tissues metabolism leads to elevated plasma glucose levels. When the capacity of the kidneys to absorb glucose is surpassed, glucosuria ensues. Glucose is an osmotic diuretic and an increase in renal loss of glucose is accompanied by loss of water and electrolytes, termed polyuria. The result of the loss of water (and overall volume) leads to the activation of the thirst mechanism (polydipsia). The negative caloric balance which results from the glucosuria and tissue catabolism leads to an increase in appetite and food intake (polyphagia) (Altan, 2003; King, 2015).

Lipid Metabolism: One major role of insulin is to stimulate the storage of food energy following the consumption of a meal. This energy storage is in the form of glycogen in hepatocytes and skeletal muscle. Additionally, insulin stimulates hepatocytes to synthesize triglycerides and storage of triglycerides in adipose tissue. In opposition to increased adipocyte storage of triglycerides is insulin-mediated inhibition of lipolysis. In uncontrolled IDDM there is a rapid mobilization of triglycerides leading to increased levels of plasma free fatty acids. The free fatty acids are taken up by numerous tissues (however, not the brain) and metabolized to provide energy. Free fatty acids are also taken up by the liver.

Normally, the levels of malonyl-CoA are high in the presence of insulin. These high levels of malonyl-CoA inhibit carnitine palmitoyltransferase I, the enzyme required for the transport of fatty acyl-CoA's into the mitochondria where they are subject to oxidation for energy production. Thus, in the absence of insulin, malonyl-CoA levels fall and transport of fatty acyl-CoA's into the mitochondria increases. Mitochondrial oxidation of fatty acids generates acetyl-CoA which can be further oxidized in the TCA cycle. However, in hepatocytes the majority of the acetyl-CoA is not oxidized by the TCA cycle but is metabolized into the ketone bodies, acetoacetate and β-hydroxybutyrate. These ketone bodies leave the liver and are used for energy production by the brain, heart and skeletal muscle. In IDDM, the increased availability of free fatty acids and ketone bodies exacerbates the reduced utilization of glucose furthering the ensuing hyperglycaemia. Production of ketone bodies, in excess of the organisms ability to utilize them leads to ketoacidosis. In diabetics, this can be easily diagnosed by smelling the breath. A spontaneous breakdown product of acetoacetate is acetone which is volatilized by the lungs producing a distinctive odour.

Normally, plasma triglycerides are acted upon by lipoprotein lipase (LPL), an enzyme on the surface of the endothelial cells lining the vessels. In particular, LPL activity allows fatty acids to be taken from circulating triglycerides for storage in adipocytes. The activity of LPL requires insulin and in its absence a hypertriglyceridemia results.

Protein Metabolism: Insulin regulates the synthesis of many genes, either positively or negatively that then affect overall metabolism. Insulin has a global effect on protein metabolism, increasing the rate of protein synthesis and decreasing the rate of protein degradation. Thus, insulin deficiency will lead to increased catabolism of protein. The

increased rate of proteolysis leads to elevated concentrations in plasma amino acids. These amino acids serve as precursors for hepatic and renal gluconeogensis. In liver, the increased gluconeogenesis further contributes to the hyperglycaemia seen in IDDM (Altan, 2003; King, 2015).

2.1.3 Non-insulin-dependent diabetes mellitus (NIDDM) - Type 2

2.1.3.1 Etiology of type 2 diabetes

Type 2 diabetes refers to the common form of idiopathic NIDDM. Type 2 diabetes is not an autoimmune disorder; however, there is a strong genetic correlation to the susceptibility to this form of diabetes. The susceptibility genes that predispose one to NIDDM have not been identified in most patients. This is due in part to the heterogeneity of the genes responsible for the susceptibility to type 2 diabetes. Obesity is a major risk factor that predisposes one to type 2 diabetes. Genetic studies in mice and rats have demonstrated a link between genes responsible for obesity and those that cause diabetes mellitus (King, 2015).

2.1.3.2 Pathophysiology of type 2 diabetes

Unlike patients with type 1 diabetes, those with type 2 diabetes have detectable levels of circulating insulin. On the basis of oral glucose tolerance testing the essential elements of type 2 diabetes can be divided into 4 distinct groups; those with normal glucose tolerance, clinical diabetes (called impaired glucose tolerance), diabetes with minimal fasting hyperglycaemia (fasting plasma glucose <140 mg/dL), and diabetes mellitus in association with overt fasting hyperglycaemia (fasting plasma glucose >140 mg/dL). Diabetics with

impaired glucose tolerance and elevated plasma insulin indicate that these individuals are resistant to the action of insulin. In the progression from impaired glucose tolerance to diabetes mellitus the level of insulin declines indicating that patients with type 2 diabetes have decreased insulin secretion.

Additional studies have subsequently demonstrated that both insulin resistance and insulin deficiency is common in the average type 2 diabetic patient (Edo *et. al.*, 2014). Many experts conclude that insulin resistance is the primary cause of type 2 diabetes; however, others contend that insulin deficiency is the primary cause because a moderate degree of insulin resistance is not sufficient to cause type 2 diabetes. As indicated above, most patients with the common form of type 2 diabetes have both defects.

The major clinical complications of type 2 diabetes are the result of persistent hyperglycaemia which leads to numerous pathophysiological consequences. As the glucose level rises in the blood the blood becomes more viscous which makes circulation of the blood in the small capillaries difficult. The reduced circulation results in progressive vascular complications leading to diabetic retinopathy (referred to as diabetic blindness), peripheral neuropathy (resulting in numbness in the extremities and tingling in fingers and toes), poor wound healing, and erectile dysfunction. In addition to these major clinical complications, the body reacts by increasing the level of glucose excretion by the kidneys leading to frequent urination which is called polyuria. As the glucose is excreted there is a concomitant loss of water to maintain normal osmolarity of the urine. The water loss leads to excessive thirst called polydypsia.

Assessment of therapeutic efficacy in the treatment of the hyperglycaemia in type 2 diabetes is accomplished by routine measurement of the circulating levels of glycosylated hemoglobin, designated as the level of HbA_{1c}, often designated as just A1C. HbA_1 is the major form of adult hemoglobin in the blood and the "c" refers to the glycosylated form of the protein. Since hemoglobin is present in red blood cells and these cells have a limited life span of 120 days in the circulation, measurement of HbA_{1c} levels is a relatively accurate measure of the amount of glucose in the blood and the length of time the level has been elevated.

2.1.4Incidence/prevalence of diabetes mellitus

More than 220 million people worldwide are affected by diabetes and its incidence is expected to increase to 400 million by 2030 (Wild *et. al.,* 2004; Roglic *et. al.,* 2005). The prevalence of this disease in low and middle-income countries accounts for 80% of diabetes deaths (WHO, 2014). The prevalences of both type 1 and type 2 diabetes are increasing.Type 2 diabetes is increasing far more rapidly, driven by decreasing life expectancy and the epidemic of obesity.

2.1.5Therapeutic intervention for hyperglycaemia

Many, if not all, of the vascular consequences of insulin resistance are due to the persistent hyperglycaemia seen in type 2 diabetes. For this reason a major goal of therapeutic intervention in type 2 diabetes is to reduce circulating glucose levels. There are many pharmacologic strategies to accomplish these goals.

2.1.5.1 The Thiazolidinediones (TZDs): The TZDs, such as rosiglitazone (Avandia®) and pioglitazone (Actos®) have proven useful in treating the hyperglycaemia associated with

insulin-resistance in both type 2 diabetes and non-diabetic conditions. The TZDs function as agonists for the transcription factor,peroxisome proliferator-activated receptor-γ(PPARγ). PPARγ is a member of the superfamily of nuclear receptor transcription factors. In addition to PPARγ there are the closely related members, PPARα and PPARβ/δ. PPARγ exists as a heterodimer with the nuclear retinoid X receptors, RXRs. The heterodimer binds to PPAR response elements (PPREs) in a number of target genes. Without ligand bound the heterodimer is associated with a co-repressor complex that includes a histone deacetylase. Deacetylated histone keeps DNA in a transcriptionally repressed state. When ligand binds to PPARγ the co-repressor complex dissociates and a co-activator complex containing histone acetylase associates resulting in chromatin structural changes and transcriptional activation. The net effect of the TZDs is a potentiation of the actions of insulin in liver, adipose tissue and skeletal muscle, increased peripheral glucose disposal and a decrease in glucose output by the liver. Genes shown to be affected by PPARγ action include those encoding glucokinase, GLUT4, malic enzyme, lipoprotein lipase, fatty acyl-CoA synthase and adipocyte fatty acid binding protein. PPARγ is primarily expressed in adipose tissue and thus it was at first difficult to reconcile how a drug that was apparently acting only in adipose tissue could lead to improved insulin sensitivity of other tissues. The answer to this question came when it was discovered that the TZDs stimulated the expression and release of the adipocyte hormone (adipokine), adiponectin. Adiponectin stimulates glucose uptake and fatty acid oxidation in skeletal muscle. In addition, adiponectin stimulates fatty acid oxidation in liver while inhibiting expression of gluconeogenic enzymes in this tissue. These responses to adiponectin are exerted via activation of AMPK. The significance of PPARγ as a diabetes target is apparent not only from the observed effects of drugs that activate the receptor but also from genome wide screens showing that mutations in the

PPARγ gene are correlated to familial insulin resistance (Altan, 2003; Mohler *et. al.*, 2009; King, 2015).

Recent studies have identified a critical role for an enzyme (phosphatidic acid phosphatase, PAP1) involved in overall triglyceride and phospholipid homeostasis as a critical target of the PPARγ signaling pathway. Lipin-1 has been shown to interact with PPARγ co-activator 1α (PGC-1α) and PPARα. The interactions of lipin-1 with these other transcription factors leads to increased expression of fatty acid oxidizing genes such as carnitine palmitoyl transferase-1, acyl CoA oxidase, and medium-chain acylCoA dehydrogenase (MCAD). In addition to the obvious role of lipin-1 in TAG synthesis, evidence indicates that the protein is also required for the development of mature adipocytes, coordination of peripheral tissue glucose and fatty acid storage and utilization, and serves as a transcriptional co-activator. The latter function has significance to diabetes as it has been shown that some of the effects of the TZDs are exerted via the effects of lipin-1. (Altan, 2003; Mohler *et. al.*, 2009; King, 2015).

2.1.5.2 Targeting glucagon-like peptide-1 (GLP-1): The primary metabolic responses to GLP-1 release from the enteroendocrine L-cells of the gut are inhibition of glucagon secretion and enhancement of glucose-dependent insulin release from the pancreas, both effects lead to decreased glycaemic excursion. The hormonal action of GLP-1 is rapidly terminated as a consequence of enzymatic cleavage by dipeptidylpeptidase IV (DPP IV or DPP4). Recent clinical evidence has shown that either infusion of GLP-1 or inhibition of DPP4 can result in dramatic reductions in plasma glucose concentrations, reductions in HbA_{1c} and improvement in pancreatic β-cell function. Thus, both represent potential targets

for the prevention of the hyperglycaemia associated with diabetes and impaired insulin function.

There are advantages and disadvantages with the current therapeutic approaches to targeting GLP-1 action in diabetic patients. Current use of GLP-1 mimetics and/or GLP-1 receptor (GLP-1R) agonists focus on peptides or modified peptides and these must be injected. The need for chronic injection as a means of therapy always runs into the problem of patient compliance. One of the most promising GLP-1R agonists that has recently been approved for use is **βYETTA®** (also written as BYETTA) developed by Amylin Pharmaceuticals and Eli Lilly and Co. BYETTA is composed of **exenatide** which is the lizard salivary peptide called exendin-4. Exenatide is 53% identical to GLP-1 at the level of amino acids and binds to and activates the GLP-1R. The advantage of exenatide as a therapeutic is that it is resistant to cleavage and inactivation by DPP4. In a recent trial in patients with type 2 diabetes, BYETTA was shown not only to lower blood glucose levels and HbA_{1c}, but patients also had an associated weight loss (Altan, 2003; Mohler *et. al.*, 2009; King, 2015).

Another GLP-1R agonist is **Victoza®** (liraglutide) which was developed by Novo Nordisk. Victoza is a once-a-day injectable recombinant DNA produced modified GLP-1 protein complex. The protein is a fatty acid-linked derivative of human GLP-1 that is resistant to DPP4 cleavage. The 16-carbon fatty acyl-chain (palmitic acid) addition to the protein allows liraglutide to bind to albumin in the blood which prevents its excretion via the kidneys. Liraglutide has been shown to have a half-life of 11-13 hours making it ideal for once-a-day injection. Results of clinical studies demonstrated significant reductions in HbA_{1c} levels in liraglutide treated patients. Victoza was approved for use in the United

States in January 2010. One problematic side effect of Victoza treatment is pancreatitis which occurs in patients with a higher frequency than with other diabetes treatments.

Although targeting compounds that can inhibit the enzymatic action of DPP4 would seem like ideal candidates for treating the hyperglycaemia of uncontrolled diabetes, there are several unknowns associated with DPP4 inhibition. One of these issues is the fact that GLP-1 and GIP are only two of the many known substrates for DPP4 cleavage. Thus, prolonged inhibition of DPP4 enzymatic activity may have unexpected consequences unrelated to control of hyperglycaemia. Despite the potential for as yet unknown effects, the DDP4 inhibitor developed by Merck, **Januvia®** (sitagliptin), has recently been approved for use alone or in combination with either metformin or the thiazolidinediones. Treatment of patients with Januvia as the only therapeutic agent for 18 weeks produced significant reductions of HbA_{1c}, along with an improvement of β-cell function and no change in body weight (Altan, 2003; Mohler *et. al.*, 2009; King, 2015).

A second generation DPP4 inhibitor developed by Novartis called **Glavus®** (vildagliptin) has recently received approvable status from the US FDA. Glavus administration is associated with significantly increased pancreatic β-cell function and reduced HbA_{1c} levels without hypoglycaemia or other adverse events. Another drug in the DPP4 inhibitor class to receive US FDA approval is **Onglyza®** (saxagliptin) made by AstraZeneca and Bristol-Myers Squibb. Onglyza is designed as a once-daily orally administered tablet.

The major clinical advantage to the use of DPP4 inhibitors is that the ones in use or in current trials are orally delivered. Compliance in patients is much higher with orally

delivered drugs than with those that require injection (Altan, 2003; Mohler *et. al.*, 2009; King, 2015).

2.1.5.3 The Biguanides: The biguanides are a class of drugs that function to lower serum glucose levels by enhancing insulin-mediated suppression of hepatic glucose production and enhancing insulin-stimulated glucose uptake by skeletal muscle. Metformin (Glucophage®) is a member of this class and is currently the most widely prescribed insulin-sensitizing drug in current clinical use. Metformin administration does not lead to increased insulin release from the pancreas and as such the risk of hypoglycaemia is minimal. Because the major site of action for metformin is the liver its use can be contraindicated in patients with liver dysfunction. The drug is ideal for obese patients and for younger type 2 diabetics.

Evidence on the mode of action of metformin shows that it improves insulin sensitivity by increasing insulin receptor tyrosine kinase activity, enhancing glycogen synthesis and increasing recruitment and transport of GLUT4 transporters to the plasma membrane. Additionally, it has been shown that metformin affects mitochondrial activities dependent upon the model system studied. Metformin has a mild inhibitory effect on complex I of oxidative phosphorylation, has antioxidant properties, and activates both glucose 6-phosphate dehydrogenase, G6PDH and AMP-activated protein kinase, AMPK. The importance of AMPK in the actions of metformin stems from the role of AMPK in the regulation of both lipid and carbohydrate metabolism. In adipose tissue, metformin inhibits lipolysis while enhancing re-esterification of fatty acids. The activation of AMPK by metformin is likely related to the inhibitory effects of the drug on complex I of oxidative phosphorylation. This would lead to a reduction in ATP production and, therefore, an

increase in the level of AMP and as a result activation of AMPK. In fact, since the cells of the gut will see the highest doses of metformin they will experience the greatest level of inhibited complex I which may explain the gastrointestinal side effects (nausea, diarrhea, anorexia) of the drug that limit its utility in many patients (Altan, 2003; Mohler *et. al.*, 2009; King, 2015).

2.1.5.4 The Sulfonylureas: The sulfonylurea and meglitinide classes of oral hypoglycaemic drugs are referred to as endogenous insulin secretagogues because they induce the pancreatic release of endogenous insulin. Sulfonylureas function by binding to and inhibiting the pancreatic ATP-dependent potassium channel that is normally involved in glucose-mediated insulin secretion.Binding of a sulfonylurea inhibits the efflux of potassium ions through the channel and results in depolarization. Depolarization opens a voltage-gated calcium channel and results in calcium influx and the release of preformed insulin. Sulfonylureas have no significant effects on circulating triglycerides, lipoproteins or cholesterol.

Sulfonylureas are metabolized by the liver and, with the exception of acetohexamide, the metabolites are either weakly active or inactive. The metabolites are excreted by the kidney and in the case of the second-generation sulfonylureas, partly excreted in the bile.

Idiosyncratic reactions are rare, with skin rashes or hematologic toxicity (leukopenia, thrombocytopenia) occurring in less than 0.1% of cases. The second-generation sulfonylureas have greater affinity for their receptor compared with the first-generation agents. The correspondingly lower effective doses and plasma levels of the second-generation drugs therefore lower the risk of drug-drug interactions based on competition for

plasma binding sites or hepatic enzyme action (Altan, 2003; Mohler *et. al.*, 2009; Katzung and Trevor, 2015; King, 2015).

The sulfonylureas have been used in the US for nearly 50 years. The first generation sulfonylureas (tolbutamide, acetohexamide, chlorpropramide and tolazamide) are not routinely prescribed any longer in the US. The second generation sulfonylureas include glipizide (Glucotrol®), glimepiride (Amaryl®) and glyburide (DiaBeta®, Micronase®, Glynase®). Because all of these drugs can induce pronounced hypoglycaemia, treatment is initiated with the lowest possible dose and carefully monitored until the dose is found that results in a fasting plasma glucose (FPG) of 110-140mg/dL.

2.1.5.5 The Meglitinides: As indicated, the meglitinides repaglinide (Prandin®) and nateglinide (Starlix®) are non-sulfonylurea insulin secretagogues that are both fast acting and of short duration. Like the sulfonylureas, meglitinides therapy results in significant reduction in FPG as well as HbA_{1c}. The mechanism of action of the meglitinides is initiated by binding to a receptor on the pancreatic β-cell that is distinct from the receptors for the sulfonylureas. However, meglitinides do exert effects on potassium conductance. Like the sulfonylureas, the meglitinides have no direct effects on the circulating levels of plasma lipids.

2.1.5.6 The α-Glucosidase inhibitors: α-glucosidase inhibitors such as acarbose (Precose®) and miglitol (Glyset®) function by interfering with the action of the α-glucosidases present in the small intestinal brush border. The consequence of this inhibition is a reduction in digestion and the consequent absorption of glucose into the systemic circulation. The reduction in glucose uptake allows the pancreatic β-cells to more

effectively regulate insulin secretion. The advantage to the use of the α-glucosidase inhibitors is that they function locally in the intestine and have no major systemic action. Hypoglycaemia does not usually occur with the use of α-glucosidase inhibitors but they are effective at reducing fasting plasma glucose (FPG) levels and levels of glycosylated hemoglobin (HbA_{1c}). The common adverse side effects of these inhibitors are abdominal bloating and discomfort, diarrhea and flatulence.

2.1.5.7 SGLT2 antagonists: A new class of orally administered compounds that targets renal glucose transport and inducers of glucosuria are currently being tested for efficacy in type 2 diabetes treatment. In the kidney, glucose is filtered at the glomerulus and then reabsorbed via active transport in the proximal convoluted tubule. Two sodium-glucose co-transporters (SGLT1 and SGLT2) have been identified as responsible for this renal glucose reabsorption. The SGLT proteins are members of the solute carrier 5 family of membrane transporters, and thus, SGLT1 is SLC5A1 and SGLT2 is SLC5A2. SGLT1 is found in other tissues and accounts for approximately 10% of the renal glucose reabsorption. SGLT2 is expressed exclusively in the S1 segment of the proximal tubule and is responsible for 90% of the renal glucose reabsorption. Therefore, it is postulated that selective inhibition of the renal SGLT2 activity should result in greatly enhanced glucose release in the urine. Several drugs (all of which carry the suffix gliflozin: empagliflozin, canagliflozin, dapagliflozin, ipragliflozin) that inhibit SGLT2 are currently under investigation. Canagliflozin (**Invokana™**), dapagliflozin (**Farxiga™**), and empagliflozin (**Jardiance™**) are the only drugs in this class currently approved by the FDA for the treatment of type 2 diabetes. The advantage of these drugs is that they are taken orally so compliance will be higher than

injected type 2 diabetes drugs. In addition, these drugs are formulated as once-a-day tablets (Altan, 2003; Mohler *et. al.*, 2009; Katzung and Trevor, 2015; King, 2015).

2.1.6New frontiers in diabetes therapy

Several new approaches are being taken in the search for treatments for diabetes. These include the development of newer drugs that target the same pathways as described in the sections above including, but not limited to, new classes of DPP4 antagonists and GLP-1 receptor agonists. Additional, potentially exciting targets, include the hepatic-derived fibroblast growth factor 21 (FGF21), the renal sodium-glucose transporter-2 (SGLT2), the NAD^+-dependent deacetylase SIRT1 or sirtuin 1, and the lipid binding G-protein coupled receptor GPR119.

2.1.6.1 FGF21 agonists:Recent evidence demonstrated that expression of the fibroblast growth factor family member, FGF21, was significantly elevated in mice fed a high-fat, low-carbohydrate ketogenic diet. Additionally, in mice with experimentally induced reduction in FGF21 expression there was an associated lipemia, reduced ketogenesis and a resultant fatty liver. Conversely, administration of FGF21 to diabetic animals resulted in reductions in the levels of fasting glucose and serum lipids. These results indicate that FGF21 plays a key role in regulating the expression of genes involved in hepatic lipid homeostasis and that activation of FGF21 activity could prove to be a significant tool in the treatment of the disrupted metabolic status in diabetic individuals (Altan, 2003; Mohler *et. al.*, 2009; Katzung and Trevor, 2015; King, 2015).

2.1.6.2 SIRT1 activators: SIRT1 or sirtuin 1 is the homolog of the yeast (*S. cerevisiae*) Sir2 gene (Sir refers to **S**ilent mating type **I**nformation **R**egulator). SIRT1 is a member of

the sirtuin family of proteins (seven members; SIRT1 through SIRT7) that are characterized by a sirtuin core domain and grouped into four classes. The yeast sirtuin proteins are known to regulate life-span extension, epigenetic gene silencing and suppress recombination of ribosomal DNA (rDNA). SIRT1 is an NAD^+-dependent deacetylase that modulates the activities of proteins that are in pathways downstream of the beneficial effects of calorie restriction. SIRT1 catalyzes a reaction where hydrolysis of NAD^+ is coupled to the deacetylation of acetylated lysines in target proteins. These target proteins include histones, transcription factors and transcription factor co-regulators. The NAD^+ is hydrolyzed to nicotinamide (which is a strong inhibitor of SIRT1 activity) and *O*-acetyl-ADP ribose. Principal pathways involved in glucose homeostasis and insulin sensitivity are affected by SIRT1 activity. In skeletal muscle, a major site of insulin-induced glucose uptake, SIRT1 and AMPK work in concert to increase the rate of fatty acid oxidation in periods of decreased nutrient availability (Altan, 2003; Mohler *et. al.*, 2009; Katzung and Trevor, 2015; King, 2015).

The plant-derived compound, resveratrol (a polyphenolic compound), is a known activator of SIRT1 function. The effects of resveratrol have been shown to increase mitochondrial content, ameliorate insulin resistance and prolong survival in laboratory mice fed a high-fat diet. Recent studies on the action of SIRT1 agonists have demonstrated that compounds that activate SIRT1, but that are structurally unrelated to resveratrol, also improve insulin sensitivity in adipose tissue, liver and skeletal muscle resulting in lower plasma glucose. The actions of these compounds in laboratory studies indicate the potential efficacy of a therapeutic approach to type 2 diabetes that includes activators of SIRT1 activity (Altan, 2003; Mohler *et. al.*, 2009; Katzung and Trevor, 2015; King, 2015).

2.1.6.3 GPR119 agonists: The fatty acid-sensing receptor, GPR119, is a G_s-type G-protein coupled receptor. GPR119 is expressed at the highest levels in the pancreas and fetal liver with expression also seen in the gastrointestinal tract, specifically the ileum and colon. GPR119 is a member of the class A family (rhodopsin-type) of GPCRs. GPR119 binds long-chain fatty acids including oleoylethanolamide (OEA), lysophosphatidylcholine (LPC), various lipid amides, and retinoic acid. OEA is the most potent ligand and likely represents the endogenous ligand for GPR119. The demonstration that OEA is the most active endogenous ligand for GPR119 is of particular interest since previous work has demonstrated that OEA, when administered to laboratory animals, causes a significant reduction in food intake and body weight gain. These effects of OEA are the result of the activation of the nuclear receptor PPARα, increased expression of fatty acid translocase, and modification of feeding behavior and motor activity. In addition, activation of GPR119 in the pancreas is correlated with enhanced glucose-stimulated insulin secretion (GSIS) and activation of the receptor in the gut results in increased secretion of the incretin hormones GLP-1 and GIP. These observations indicate that GPR119 activation is associated with a dual mechanism of reducing blood glucose: acting directly through pancreatic β-cells to promote GSIS and in the gut via the stimulation of the incretins GLP1 and GIP both of which increase insulin release from the pancreas in response to food intake. Currently there are several small molecule agonists of GPR119 in clinical trials being tested for their efficacy in treating the hyperglycaemia of type 2 diabetes as well as for their efficacy in treating obesity (Altan, 2003; Mohler *et. al.*, 2009; Katzung and Trevor, 2015; King, 2015).

2.2 Toxicity and Medicinal Properties of some Phytochemicals

Given that many of the pharmaceutical drugs in use are derived from plants directly or indirectly, it is obvious that at least some plants contain compounds with pharmacological activity that can be harnessed as medicinal agents. Increasingly, the public—and particularly the medical establishment—are demanding herbalists and other complementary therapists provide scientific evidence for the efficacy and safety of their practices.

2.2.1Phenylpropanoids

These are C6C3 compounds, made up of a benzene ring with a three carbon side chain. The most important are the hydroxycinnamic acids: caffeic acid, p-coumaric acid, ferulic acid and sinapic acid. They can be derived from different stages of the shikimic acid pathway. These acids are of much benefit therapeutically and are non-toxic. They may also occur as glycosides. By modification of their C3 side chains or changes in substitution patterns of their aromatic nucleus, hydroxycinnamic acids are able to form a host of secondary compounds including phenolic ethers, lignans, coumarins, glycosides, dimers such as rosmarinic acid and curcumin from the roots of turmeric, and the depsides cynarin and chlorogenic acid.

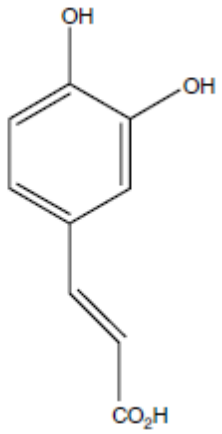


Figure 2.1: Structure of Caffeic Acid (Pengelly, 2004)

Smallanthus sonchifolius was found to be rich in 4,5-di-O-caffeoylquinic acid (CQA) and 3,5-di-O-CQA and displayed a good α-amylase and α-glucosidase inhibition (Russo et. al, 2015); several other phenolic acids,chiefly *p*-coumaric, caffeic, ferulic and sinapic acids were reported to posess antidiabetic activity (Pandey and Rizvi, 2009).

2.2.2Coumarins

Coumarins are lactones of hydroxycinnamic acids, with cyclic C_6C_3 skeletons. Most simple coumarins are substituted with OH or OCH_3 at positions C-6 and C-7. They often occur in glycosidic form, for example aesculin is the glycoside of aesculetin.

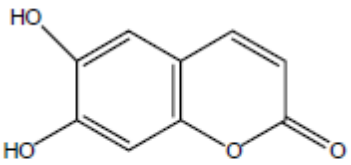


Figure 2.2: Structure of Aesculetin (Pengelly, 2004)

The distribution of coumarins is widespread. Originally isolated from tonka beans, they are abundant in particular plant families, for example Rubiaceae—Asperula; Poaceae—Avena; Fabaceae—Medicago, Melilotus; Rutaceae—Ruta, Citrus spp., Murraya; Apiaceae—Angelica, Ammi. Dicoumaral (bishydroxycoumarin), used as a medical drug, was originally derived from Melilotus (sweet clover). Coumarins in general have antimicrobial and fungicidal activity but several others have been found to have antidiabetic potentials by reducing blood glucose levels (Patience *et. al.*, 2014).

2.2.3Polyphenols—Tannins and Flavonoids

Tannins

Tannins represent the largest group of polyphenols. They are widely distributed in the bark of trees, insect galls, leaves, stems and fruit. Tannins were originally isolated from the bark

and insect galls of oak trees. They are the chief plant constituents responsible for astringency. Tannins are high-molecular-weight compounds (500–5000) containing sufficient phenolic hydroxyl groups to permit the formation of stable cross-links with proteins, and as a result of this cross-linking enzymes may be inhibited. Almost all tannins are classified as either hydrolysable tannins or condensed tannins—some plants contain both kinds.

Hydrolysable tannins

Hydrolysable tannins are derived from simple phenolic acids, particularly gallic acid, which is linked to a sugar by esterfication. The gallic acid groups are usually bonded to form dimers such as ellagic acid. Hydrolysable tannins break down on hydrolysis to give gallic acid and glucose or ellagic acid and glucose, known as **gallotannins** and **ellagitannins** respectively.

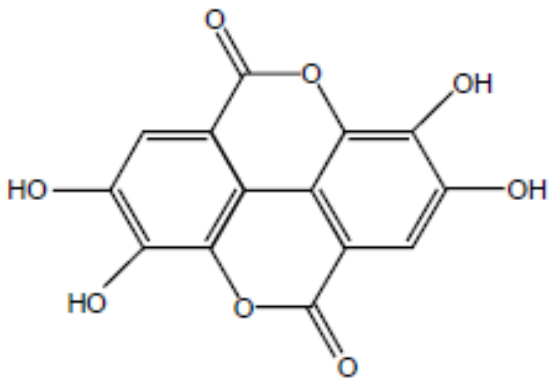


Figure 2.3: Structure of Ellagic Acid (Pengelly, 2004)

Condensed tannins

Condensed tannins or phlobotannins are polymers of flavan-3-ols (catechins) and flavan-3,4-diols (leucoanthocyanins). The basic catechin-type molecules are of low molecular weight—hence they are not true polyphenols and have low astringency.

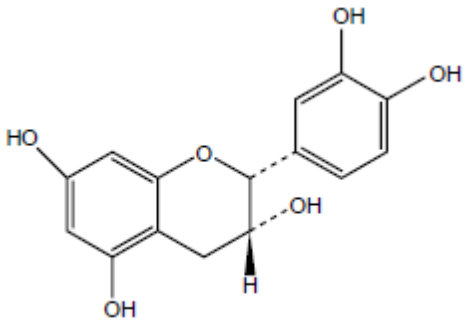


Figure 2.4: Structure of Epicatechin (Pengelly, 2004)

Oligomeric procyanidins (OPCs) are a major subcategory of condensed tannins in which two or more molecules of catechin and/or epicatechin are linked by simple carbon bonds. OPCs are present in popular beverages such as green and black teas and red wine. Green tea is especially rich in the beneficial epigallocatechin, which is a potent antioxidant and vascular tonic.

Flavonoids

These compounds occur as yellow and white plant pigments (Latin flavus = yellow). Flavonoids occur both in the free state and as glycosides. Their chemical structure is based on a C15 skeleton consisting of two benzene rings connected by a three-carbon chain, that is, C6–C3–C6.

The three-carbon chain is generally closed to form a heterocyclic ring (the C-ring). Flavonoids are products of both the shikimic acid and acetate pathways, being formed by the condensation of a phenylpropanoid precursor with three malonyl coenzyme A units. Almost all plants studied have been shown to contain a series of closely interrelated flavonoids with different degrees of oxidation and/or hydroxylation patterns. While there are numerous structural classes within the flavonoids, the most commonly occurring are flavones (e.g. apigenin) and flavonols (e.g. quercetin).

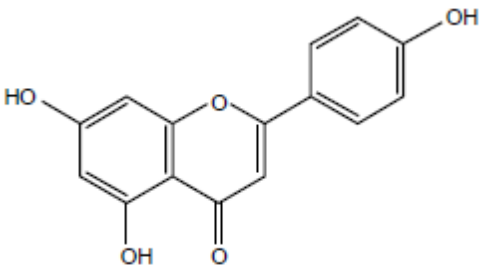


Figure 2.5: Structure of Apigenin (Pengelly, 2004)

Experiments have proven flavonoids affect the heart and circulatory system and strengthen the capillaries. They are often referred to as 'biological stress modifiers' since they serve as protection against environmental stress. They are also known to have synergistic effects with ascorbic acid. Their protective actions are mainly due to membrane stabilizing and antioxidant effects. Therapeutic effects of flavonoids such as antioxidant, antiviral, hepatoprotective, anti-atheromatous, anti-inflammatory and antihypertensive have been widely reported, though it must be remembered these effects are dependent on their degree of absorption. Rutin and hespiridin among others are effective in reducing permeability of blood capillaries and are widely used for peripheral vascular disorders.

A study indicates that the intake of flavonoids is inversely associated with mortality from coronary heart disease and, to a lesser extent, myocardial infarct. These flavonoids include **quercetin**, **kaempferol** and **myricitin** as well as the catechin-type condensed tannins found in black tea.

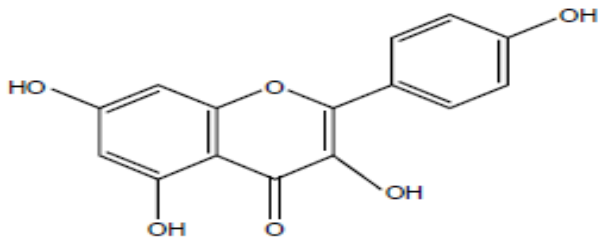


Figure 2.6: Structure of Kaempferol (Pengelly, 2004)

Epidemiological studies with dietary flavonoids have also demonstrated an inverse association with incidence of stroke. Antitumour activity has been demonstrated in flavonoids. **Nomilin** in citrus induces glutathione-S-transferase (GST), which aids in detoxification of carcinogens. Quercetin inhibits cytotoxic T lymphocytes *in vitro*. *In vitro*studies also suggest flavonoids such as **tangeretin** and **nobiletin** from citrus fruits may inhibit mutagenesis produced by mutagens from cooked food, thereby playing a role in prevention of carcinogenesis.

Animal studies show many flavonoids protect liver cells from damage induced by toxins such as carbon tetrachloride. **Silymarin**, the flavonolignan complex from *Silybum marianum,* is an inducer of phase 1 detoxication, and protects liver mitochondria and microsomes from lipid peroxidation. This protection also occurs with the flavonoids quercetin and taxifolin. Silymarin causes hepatic regeneration and increases hepatic glutathione *in vivo*.

The presence of numerous aromatic hydroxyl groups on flavonoids enables them to easily attach to enzyme surfaces averting potent inhibition of some enzyme systems in humans, for example aldose reductase (which causes diabetic cataracts) hasquercetrin as a very potent inhibitor of this enzyme;others include nepetrin and its glycoside nepitin from *Rosinarinus officinalis,* as well as glycosides of luteolin and kaempferol (Pengelly, 2004). Several polyphenol-containing plants have also been found to have antidiabetic activities (Büyükbalci and El 2008; Pandey *et. al.*, 2009; Tang *et. al.*, 2011; Ahmed *et. al.*, 2014; Russo *et. al.*, 2015; Stanley *et. al.*, 2016; Tafesse *et. al.*, 2017).

Flavonoids offer the advantage of a high margin of safety and lack of side effects such as ulcerogenicity over the classical anti-inflammatory drugs. Diseases associated with

increased permeability of blood capillaries include diabetes, chronic venous insufficiency, haemorrhoids, scurvy, varicose ulcers and bruising.

2.2.4Glycosides

Glycosides are a group of compounds characterized by the fact that chemically they consist of a sugar portion (or moiety) attached by a special bond to one or more non-sugar portions. Chemically they are hydroxyls of a sugar that are capable of forming ethers with other alcohols, or esters with acids. Glycosides are broken down upon hydrolysis with enzymes or acids to:

1. a sugar moiety = glycone; and

2. a non-sugar moiety = aglycone/active portion.

These may be phenol, alcohol or sulfur compounds. The bond between the two moieties may involve a phenolic hydroxyl group in which case an O-glycoside is formed. In other cases carbon (C-glycosides), nitrogen (N-glycosides) or sulfur (S-glycosides) may be involved. Glycosidic bonds are of extra significance since they link monosaccharides together to form oligosaccharides and polysaccharides.

Most glycosides can be classed as ‘prodrugs’ since they remain inactive until they are hydrolysed in the large bowel with the help of specialized bacteria, leading to the release of the aglycone—the truly active constituent. Classification of glycosides is based on the nature of the aglycone, which can be any of a wide range of molecular types, including phenols, quinones, terpenes and steroids.

2.2.5Anthraquinones

Anthraquinonesare yellow-brown pigments, most commonly occurring as O-glycosides or C-glycosides. Their aglycones consist of two or more phenols linked by a quinone ring. Hydroxyl groups always occur at positions 1 and 8, that is, 1,8-dihydroxyanthraquinones.

The main anthraquinone-containing plants are cascara sagrada (*Rhamnus purshiana*), senna, rhubarb, aloes, dock and St John's wort. **Rheum-emodin** is a typical simple anthraquinone from rhubarb root (*Rheum palmatum*).

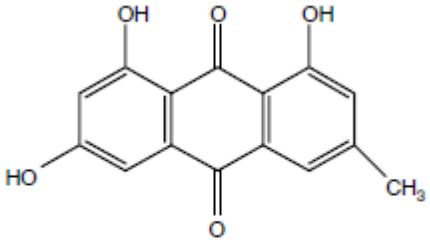


Figure 2.7: Structure of Rheum-emodin (Pengelly, 2004)

The composition of glycosides and their derivatives in antraquinone containing plants determines their effectiveness as laxatives. The gentlest acting laxatives in this group belong to the buckthorns (*Rhamnus catharticus* and *R. frangula*) and rhubarb (*Rheum palmatum*). Aloes (*Aloe barbadensis*) and *Senna* spp. are the other commonly used laxative agents in this class.

2.2.6Terpenes

Terpenoids, or terpenes, comprise one of the most important groups of active compounds in plants with over 20 000 known structures. All terpenoid structures may be divided into isoprene (five-carbon) units containing two unsaturated bonds. They are synthesised from acetate via the mevalonic acid pathway. During the formation of the terpenes, the isoprene units are linked in a head to tail fashion. The number of units incorporated into a particular terpene serves as a basis for the classification of these compounds, as shown below:

Isoprene C_5H_8; Terpene $(C_5H_8)n$; Monoterpene $C_{10}H_{16}$; Essential oils, e.g. menthol, iridoids; Sesquiterpene $C_{15}H_{24}$ e.g. bitter principles, especially sesquiterpene lactones;

Diterpene $C_{20}H_{32}$e.g.resin acids, bitter principles; Triterpene $C_{30}H_{48}$e.g.saponins, steroids; Tetraterpene $C_{40}H_{64}$e.g.carotenoids; and Polyterpene $(C_5H_8)n$ e.g.rubber.

2.2.7Saponins

Saponins are compounds whose active portions form colloidal solutions in water, which produce lather on shaking and precipitate cholesterol. They occur as glycosides whose aglycones are triterpenoid or steroidal structures. The combination of lipophilic (fat-soluble) aglycones at one end of the molecule and hydrophilic (water-soluble) sugars at the other end gives them the ability to lower surface tension, producing the characteristic detergent or soap-like effect on membranes and skin.

Triterpenoid saponins

The most widely distributed triterpenoid aglycone is oleanolic acid, which forms a pentacyclic (containing five carbon rings) structure referred to as the oleanane-type ring system. **Glycyrrhetic acid** from liquorice root is an example. Other triterpenoid ring systems include ursane and lupane types as well as the dammarane type—represented by **ginsenosides** from *Panax ginseng* and **jujubosides** from *Ziziphus jujube.*

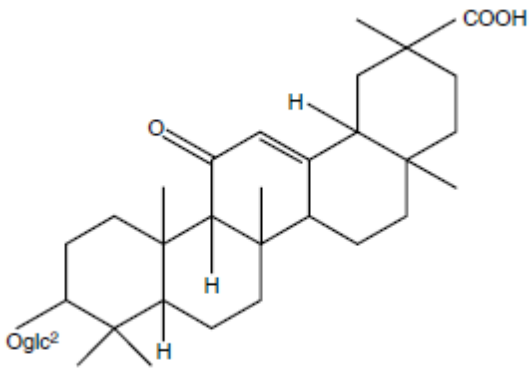


Figure 2.8: Structure of Glycyrrhizic Acid (Pengelly, 2004)

2.2.8Alkaloids

Typical alkaloids are alkaline organic compounds containing one or more nitrogen atoms, each connected to at least two carbon atoms within a heterocyclic ring system. They have limited distribution in plants, so that vitamins and hormones are excluded even though they may structurally comply with the definition. Most alkaloids are derived at least partly from various amino acids as their direct precursors, while a few are derived from isoprene units (terpenoids).

Alkaloids are found in 15–30% of all flowering plants and are particularly common in certain families, such as Fabaceae, Liliaceae, Ranunculaceae, Apocynaceae, Solanaceae and Papaveraceae. The most widely occurring alkaloids are caffeine and berberine.

While the higher plants are the major source of alkaloids, they are also known to occur in lower plants such as horsetails, in algae, fungi, microorganisms, insects and even the organs of mammals. Over 10 000 different alkaloids have been isolated from over 300 plant families. They may be found in roots, rhizomes, leaves, bark, fruit or seeds.

Most alkaloids are soluble in organic solvents such as chloroform, ether and alcohol, but are practically water insoluble. Water-soluble alkaloids include **ephedrine** and colchicine. Alkaloidal salts are generally soluble in water and alcohol. All alkaloid names end in ‘ine’.

Examples of some of the more dramatic actions of alkaloids are:Analgesics/narcotics—morphine; Mydriatics—atropine; Miotics—pilocarpine; Hypertensives—ephedrine; Hypotensives—reserpine; Bronchodilator—lobeline; Stimulants—strychnine; Antimicrobials—berberine; Antileukemic—vinblastine.

Because they are so reactive, even at small doses, most alkaloid rich plants are used sparingly, if at all, in Western herbalism. Indeed, use of many of the alkaloidal species is restricted by law, or listed on poison schedules. However, alkaloids—either as isolated

compounds or their semi-synthetic derivatives—are widely used in pharmaceutical medicines. Much of our understanding of the mechanisms of neurotransmitters and receptor sites comes from research into the pharmacodynamics of alkaloids. The widespread use of terms such as nicotinic and muscarinic receptors supports this idea.

Alkaloids are a large and diverse group of chemical compounds that defy easy classification. They are commonly grouped together according to their ring structures. Two major divisions can be made:

1. **Heterocyclic alkaloids**—regarded as most typical

2. **Non-heterocyclic alkaloids**—also known as protoalkaloids or biologicalamines, e.g. ephedrine, colchicine.

Studies have shown that compounds belonging to the phytochemical classes like tannins, saponins, terpenoids, anthraquinones, glycosides and alkaloids have antidiabetic properties by significantly lowering the blood glucose levels (Büyükbalci and El 2008; Pandey *et. al.*, 2009; Tang *et. al.*, 2011; Ahmed *et. al.*, 2014; Russo *et. al.*, 2015; Stanley *et. al.*, 2016; Cunha *et. al.*, 2017; Tafesse *et. al.*, 2017).

Plants used in traditional medicine are expected to have low toxicity by the consumers. The latest surveys have indicated that many medicinal plants used as traditional medicine showed undesirable effects. Therefore emphasies should be given to elucidate the safe use of any plant for medicinal purposes. The possible toxic effects resulting from the short term and long term use of such medicinal plants have raised an alarm. To increase the confidence in their safety to the consumers especially for use in the development of pharmaceuticals, the data of the acute and sub-acute toxicity studies on medicinal plants should be obtained. The most critical part of any medicinal plant extract used in animal or

human is the evaluation of its toxic effects. Hence a systemic approach in evaluating the efficacy and safety profile in such plants is needed (Shah *et. al.*, 2016).

2.3 Antidiabetic Plants with Antioxidants Properties

2.3.1 Oxidative Stress

The production of reactive oxygen species (ROS) may lead to a cycle of oxidation and reduction (redox cycle) with electrons being donated to oxygen to yield superoxide. The superoxide produced may then be metabolized to hydrogenperoxide by superoxide dismutase.The hydrogen peroxide is further metabolized to water by catalase (Figure 2.9).

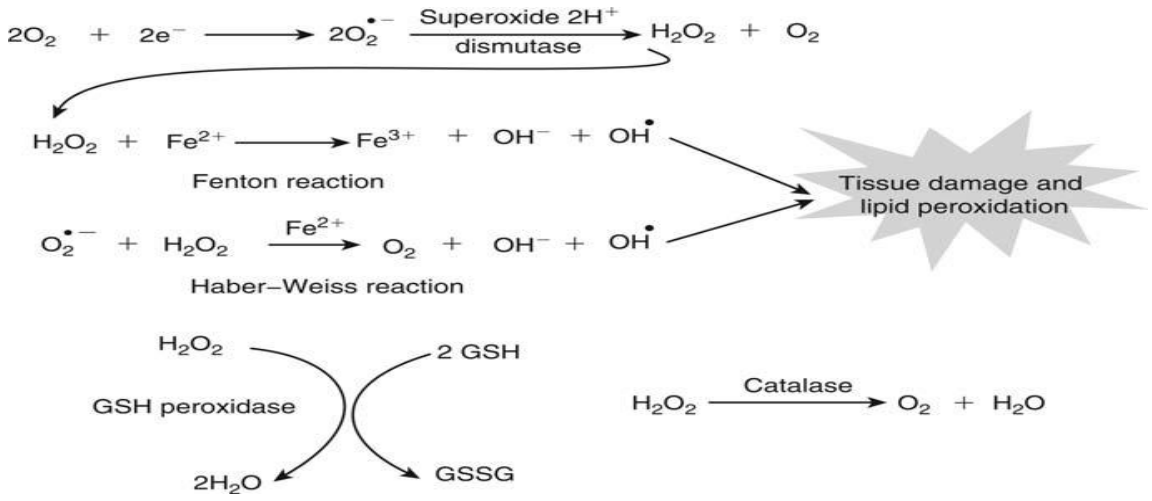


Figure 2.9: Production of various reactive oxygen species, the role in tissue damage, and their detoxication and toxication. Reactive species shown are hydrogen peroxide, superoxide, and hydroxyl radical.Adopted from Timbrell (2009).

However, under certain circumstances, such as in the presence of transition metal ions,hydroxyl radicals may result from either the Haber–Weiss reaction or the Fenton reaction,which cause lipid peroxidation and cell injury (Figure2.9).Hydroxyl radicals are

cytotoxic and can be involved in the production of further activeoxygen species such as singlet oxygen.

If a large amount of the toxic substance is present, then the detoxication processespresent are overwhelmed. Excess superoxide is produced, reduced GSH and NADPH are depleted, and hydroxyl radicals and singlet oxygen are formed. This is the condition known as oxidative stress. As well as causing lipid peroxidation, ROS will also cause DNA damage and damage to proteins (Timbrell,2009).

2.3.2Antioxidants

Antioxidants protect cells against the damaging effects of reactive oxygen species otherwisecalled, free radicals such as singlet oxygen, super oxide, peroxyl radicals, hydroxyl radicalsand peroxynite which results in oxidative stress leading to cellular damage. Natural antioxidants play a key role in health maintenance and prevention ofthe chronic and degenerative diseases, such as atherosclerosis, cardiac and cerebral ischema,carcinogenesis, neurodegenerative disorders, diabetic pregnancy, rheumatic disorder, DNAdamage and ageing. Antioxidants exert their activityby scavenging the 'free-oxygen radicals' thereby giving rise to a fairly 'stable radical'. Thefree radicals are metastable chemical species, which tend to trap electrons from themolecules in the immediate surroundings. These radicals if not scavenged effectively intime, they may damage crucial biomolecules like lipids, proteins including those present inall membranes, mitochondria and, the DNA resulting in abnormalities leading to diseaseconditions. Thus, free radicals are involved in a number of diseasesincluding: tumour inflammation, hemorrhagic shock, atherosclerosis, diabetes, infertility,gastrointestinal ulcerogenesis, asthma, rheumatoid arthritis, cardiovascular disorders, cysticfibrosis, neurodegenerative

diseases (e.g. Parkinsonism, Alzheimer's diseases), AIDS andeven early senescence. The human body producesinsufficient amount of antioxidants which are essential for preventing oxidative stress. Freeradicals generated in the body can be removed by the body's own natural antioxidantdefences such as glutathione or catalases. Therefore this deficiency had to be compensated by making use of natural exogenous antioxidants, such as vitamin C, vitamin E, flavones, β-carotene and natural products in plants.

Plants contain a wide variety of free radicals scavenging molecules including phenols,flavonoids, vitamins, terpenoids that are rich in antioxidant activity. Many plants, citrus fruits and leafy vegetables are the source ofascorbic acid, vitamin E, carotenoids, flavanols and phenolics which possess the ability to scavenge the free radicals in human body. Significant antioxidant properties have been recorded in phytochemicals that are necessary for the reduction in the occurrence of many diseases. Many dietary polyphenolic constituents derived from plants are more effective antioxidants *in vitro* than vitamins E or C, and thus might contribute significantly to protective effects *in vivo.* Methanol extract of Cinnamon contains a number of antioxidant compounds which can effectively scavenge reactive oxygen species including superoxide anions and hydroxyl radicals as well as other free radicals *in vitro*. The fruit of Cinnamon, an under-utilized and unconventional part of the plant, contains a good amount of phenolic antioxidants to counteract the damaging effects of free radicals and may protect against mutagenesis (Doughari,2012).

Several plants showed both antioxidants andantidiabeticeffects. Examples include,

Acacia Arabica belongs to the family Leguminosae.Seeds and bark of the plant was demonstrated for itsantihyperglycemic and antioxidant activity. Seeds showedhypoglycemic effect by initiating the release of insulin frompancreatic β- cells. Hydroalchoholic and chloroformextracts of bark showed antidiabetic and antioxidant

activityin alloxan induced diabetic rats.*Aegle marmelos* (L.) correa ex Roxb.belongs to thefamily Rutaceae. Aqueous extract of leaf showed antihyperglycemic activity in Streptozotocin induced diabeticrats. Leaf extract increases glucose uptake in glucoseinduced hyperglycemic rats.*Allium cepa* L. belongs to the family Amaryllidaceae.Fruits of Allium cepa reported for its strong antidiabetic andantioxidant activity. Aqueous extract of fruits demonstratedantidiabetic, hepatoprotective and antioxidant activity onalloxan induced diabetic rabbits.*Aloe vera* (L.) Burm.F. belongs to the family Aloaceae.Hypoglycemic activity of the whole plant studied on normalfasted rats, oral glucose loaded rats and Streptozotocin induced diabetic ratsand leaf pulp extracts are studied intype I and type II diabetic rats and its activity may be due tocarbohydrate hydrolyzing enzymes and antioxidant activity.*Alpinia galangal* Willd belongs to the familyzingiberaceae. Dried rhizomes of this plant showedantidiabetic and antioxidant potential. Ethanol extractof dried rhizomes showed potent free radical scavengingactivity and antidiabetic activity in both *invitro* and *invivo*models. Further it demonstrated inhibition of β-glucosidaseenzyme.*Ampelodesma mauritanica* Durand.belongs to the familyPoaceae. Roots of this plant were reported for its strongantioxidant and antidiabetic activity (Mazumder *et. al.*, 2012). Methanolic extract ofroots showed antioxidant effect and antihyperglycemic effectin normal glycemic mice.

Annona squamosa L. belongs to the family Annonaceae.Leaves of *Annona squamosa* is known for its strongantioxidant activity. Ethanolic extract of leaf lowers bloodglucose level in normal, streptozotocin diabetic rats andalloxan treated rabbit.*Artanema seamoides* Benth.belongs to the familyScrophulariaceae. Aerial parts of the plant studied for itsantidiabetic and antioxidant activity. Methanol extractof aerial parts demonstrated antidiabetic activity bysensitizing the insulin receptor or stimulating the secretionof insulin

from beta cells of islets of langerhans in pancreasof steptozotocin (STZ) induced diabetic rats and it wassupported by improved *in vivo* antioxidant status.

Ethanol extract of the whole plant,*Asparagus racemosus* Willd.(Liliaceae)demonstrated antidiabetic,antioxidant and antihyperglycemic activity in Streptozotocin (STZ) induced diabetic rats.*Asystasia gangetica* belongs to the family Acanthaceae;leaves of *Asystasia gangetica* were reported for itsantioxidant and antidiabetic activity. 70% ethanolic extractof leaves showed potent antioxidant and antidiabetic activityin alloxan induced diabetic albino rats (Mazumder *et.al*, 2012).

Basella subra belongs to the family Basellaceae.Antidiabetic and antioxidant studies were performed on theleaves of *Basella subra*. Aqueous extract of leaves effectivelyreduced the oxidative stress induced by streptozotocin (STZ)and potential reduction in blood sugar level.*Benincasa hispida* belongs to the family Cucurbitaceae.Hydroalchocolic and chloroform fruit extracts of *Benincasahispida* demonstrated antidiabetic activity in alloxaninduced diabetic ratsand it was found to be effective inoxidative stress by decreasing malondialdehyde levels andincreasing superoxide dismutase and vitamin C levels.

Cassia auriculata (L.) belongs to the family Leguminosae.Aqueous flower extract of this plant suppresses enhancedgluconeogenesis during diabetes and enhance utilization of glucose through increased glycolysis and it also inhibitsthe alpha glucosidase enzyme.

Cinnamomum tamala Fr. Nees belongs to the familyLauraceae. Leaves of *Cinnamomum tamala* were studiedfor its antioxidant and antidiabetic activity. Aqueous extractof leaves showed antihyperglycemic as well as antioxidantactivities in streptozotocin (STZ) induced rats.

Dodonaea viscose Linn.belongs to the family Sapindaceae.Leaves and aerial parts were reported for antioxidant and antidiabetic activity. In STZ induced diabetic rats, *D. viscosa* reduced glucose levels in dose dependent manner. Altered levels of lipids, TBARS, non enzymatic and enzymatic antioxidant levels were restored (Mazumder *et. al.*, 2012).

Eucalyptus camaldulensis Dehnh.belongs to the familyMurtaceae. Leaves of *Eucalyptus camaldulensis* werestudied for its carbohydrate hydrolyzing enzymes inhibitionpotential and for its antioxidant activity. The essential oilisolated from the plant demonstrated antioxidant activityand the same inhibited alpha amylase and alpha glucosidaseinhibition.

Eucalyptus globules belongs to the family Myrtaceae.Aqueous extract of leaves of *Eucalyptus globules* restores theantioxidant power, due to the improved hyperglycaemia in streptozotocin (STZ) induced diabetic rats and it reduce theplasma glucose level too.

Eugenia jambolanum belongs to the family Myrtaceae.Ethanolic extract of seeds of *Eugenia jambolanum* showedhypoglycemic, better glucose tolerance activity andincreases the tissue antioxidant levels in streptozotocin (STZ)induced diabetic rats.*Eurphorbia hirta* belongs to the family Euphorbiaceae.Stems of *Eurphorbia hirta* reported for its strong antioxidantand antidiabetic activity. Ethanol and petroleum etherextracts of stems showed antioxidant, antihyperglycemic andhyperlipidemic activity.

Ficus racemosa Linn belongs to the family Moraceae.Fruits of *Ficus racemosa* is known for its strong antioxidantpotential and the same was studied for its antidiabeticactivity. Ethanolic extract of fruits demonstratedhypoglycemic activity and *in vitro* antioxidant activity.

Glinus oppositifolius belongs to the family Molluginaceae.Methanol extract of leaves of *Glinus oppositifolius*showed moderate antioxidant activity and significantantihyperglycemic activity in alloxan induced diabetic rats.

Gymnema sylvestre belongs to the family Asclepiadaceae.Ethanol extract of the leaves exhibited strong antioxidantactivity and hypoglycemic activity in streptozotocin (STZ) induced diabetic rats. The leaves extract of *Gymnema sylvestre* stimulates *in vitro* insulin secretion using MIN6-β-cell line and isolated human islets of langerhans. Aqueousleaf extract of the same plant showed hypolipidemic andhypoglycemic activity in alloxan induced diabetic rats at 400and 800 mg/ kg b.w. (Mazumder *et. al.*, 2012). Several other studies reported plant and plant compounds with both antioxidant and antidiabetic properties (Büyükbalci and El 2008; Pandey *et. al.*, 2009; Tang *et. al.*, 2011; Mishra *et. al.*, 2013; Ahmed *et. al.*, 2014; Ali *et. al.*, 2015; Russo *et. al.*, 2015; Stanley *et. al.*, 2016; Tafesse *et. al.*, 2017).

2.4 *Gymnema sylvestre a*nd *Combretum micranthum*- Ethnomedicinal Uses

Plants (*Gymnema sylvestre* R.Br. ("periploca of the woods" in English; "Kafi suga" in Hausa) (Asclepiadaceae) and *Combretum micranthum*, Fam. (Géézà in Hausa) (Combretaceae)) are used for the management of Diabetes Mellitus in the North-Western part of Nigeria (Atiku *et. al.*, 2005; Chika and Bello, 2010).

2.4.1*Gymnema sylvestre*

G. sylvestre R.Br. is a perennial, woody climber belonging tofamily Asclepiadaceae or the "milk weed" family. Thegenus consist of 40 species, some of which like *G. sylvestre*,*G. montanum*, *G. yunnanense*, and *G. inodorum* havemedicinal properties. The plant is found in tropicaland subtropical regions; it is well distributed in parts of centraland southern India and in the southern part of China, tropicalAfrica, Malaysia, and Sri Lanka. *G. sylvestre*is slowgrowing herb, found ideally in tropical and subtropical humidclimate and common in hills of evergreen forests (Tiwari *et. al.*, 2014).



PlateI:*Gymnema sylvestre* Plant – Leaves and Stem (https//www.motherherbs.com, 02/08/2017).

The leaves of *G. sylvestre*contain triterpene saponins belonging to oleanane and dammarane classes.The major constituents like gymnemic acids and gymnemasaponins are members of oleanane type of saponins while gymnemasides are dammarane saponins. Other phytoconstituents include anthraquinones, flavones, hentriacontane, pentatriacontane,phytin, resins, tartaric acid, formic acid, butyric acid, lupeol, β-amyrin related glycosides, stigmasterol, and calcium oxalate (Sinsheimer *et. al.*, 1970).The presence of alkaloids has been detected in plant extracts. Leaves of *G. sylvestre*have acidic glycosides and anthraquinones and their derivatives.The major secondary metabolites in *Gymnema* includes a group of nine closely related acidic glycosides, the main are gymnemic acid A–D and found in all parts of the plant (Tiwari *et. al.,* 2014). The mode of action of the gymnemic acid is through stimulation of insulin secretion from pancreas. It also exerts a similar effect by delaying the glucose absorption in the blood. Similarly, in the intestine it attaches to the receptor present in external layer of intestine, thereby preventing the absorption of sugar molecules by intestine, leading to reduction in blood sugar levels

(Tiwari *et. al.,* 2014). Experimental trials confirmed the hypoglycemic effect of *G. sylvestre* on beryllium nitrate and streptozotocintreated rats (Grover *et. al.*, 2002; Tiwari *et. al.,* 2014). There was a slight increase in body weight and protein and a significant decrease in fasting blood glucose in diabetic rats treated with *G. sylvestre*and the effectswerequite similar to insulin and glibenclamide treated mice (Grover *et. al.*, 2002). LC/MS analysis revealed the presence of antihyperglycemic compounds like gymnemagenin and gymnemicacids in *G. sylvestre* extract and the level of lipid peroxidationreduced by 31.7% in serum, 9.9% in liver, and 9.1%in kidney in diabetic rats fed with the ethanolic extract (Kang *et. al.*, 2012).

2.4.2 Combretum micranthum

*Combretum micranthum*Fam. (Géézà in Hausa) belong to the family of Combretaceae. It is a widely knownethnomedicinal plant used in West Africa for treating several conditionsand in Northwestern Nigeria; it is used for managing diabetes mellitus. Ethanol extract of CM leaves is reported to be richin polyphenols (tannins, flavonoids and other components) constituents known to possess various beneficialpharmacological properties such as antioxidant, antimutagenic,anticarcinogenic, hypolipidemic and cardioproprotectiveactivities (Chika and Bello, 2010)The antioxidant, antimicrobial, antidiarhoeal as well asanti-inflammatory properties of the plant have also been documented (Touré *et. al.,* 2011; Abdullahi *et. al.,* 2014).

The Combretaceae family consists of as many as 600 species of trees, shrubs and lianas in 18 – 20 genera, depending on reports, and is found in tropical and sub-tropical regions, mostly of Africa and India. Five genera are commonly found in southern and western Africa, four of which are tree species and the two largest of which are *Combretum* and *Terminalia*. The other genera are *Anogeissus, Buchenavia, Bucida, Calopyxis,*

Calycopteris, Conocarpus, Dansiea, Guiera, Laguncularia, Lumnitzera, Macropteranthes, Melostemon, Pteleopsis, Quisqualis, Strephonema, Terminaliopsis and Thiloa. The most commonly occurring genus, *Combretum*, includes almost 400 species found all across Africa, many of which are widely used in African traditional medicine (Welch, 2010).



Plate II: *Combretum micranthum* Plant – Leaves and Stem (https//www.jircas.affrc.go.jp, 02/08/17).

Combretum micranthum is an undomesticated shrub species found in the Tiger bush region of western Africa. Tiger bush is a patterned area of vegetation consisting of alternating bands of trees or shrubs and bare ground that forms stripes when viewed from above. The patterns follow lines of equal elevation and occur on low slopes in arid and semi-arid regions, such as sub-Saharan, or Sahelian, West Africa. *C. micranthum* is common on cultivated and fallow ground, throughout the continent, but it appears to be dominant in sub-Saharan Africa, from Sudan to Nigeria, from Gambia to Congo, with higher concentrations in Senegal, Mali, and Burkina Faso. The leaf of *C. micranthum* is used as a popular herbal infusion or tea and is an ethnomedicinal plant widely used in West Africa to

treat many diseases. In traditional medicine, it is used for the treatment of bruises and sprains by rubbing in root powder with shea butter or palm oil. The roots are also used, in a decoction that is either drank as a treatment for guinea worm infestations, or as a wash in the treatment for open wounds. The most common use of *C. micranthum* is for diuretic and digestion purposes, including gastrointestinal problems, colic, and vomiting, in which a beverage is brewed from the dried leaves. The treatment of diabetes is also an interesting effect of *C. micranthum* tea (Welch, 2010).

2.5 Herb Combinations in Treatment/Management of Diseases

Though it is important that glycaemic control should be achieved as rapidly as possible to minimize the impact of glucose toxicity, it is also necessary to provide therapy to control other related risk factors, including oxidative stress, dyslipidaemia, mitochondrial dysfunction and vascular complications (Prabhakar *et. al.*, 2014). Hence, a combination therapy becomes necessary to combat the multiple risk factors in diabetics. A judicious selection of suitable agents for combination therapy which can provide most metabolic benefits to the patient with diabetes should be considered. Thus, a combination therapy (having morethan two pharmacodynamic agents), to treat the conditions is necessary. Such a strategy can help to:

(1) Attain rapid and long term glycemic control;

(2) Combat other risk factors which arise as a consequence of the disease;

(3) Treat the multiple risk factors that are responsible for the onsetand development of diabetes;

(4) Provide a mild anti-inflammatory effect – since inflammationis both the cause and consequence of the condition.

Many of the phytomedicines in the drug market are whole extracts of plants because it is known that various components of individual or mixtures of herbs act in synergy and hence are a vital part of their therapeutic efficacy. Two drugs that produce almost similar effect, when used together may sometimes produce enhanced, same or diminished effect. In this interaction there might be three different types of behaviours namely synergy, antagonistic and additive/indifferent. Different types of synergism in combination therapy are possible and they include:

(1) Increasing the efficacy of the therapeutic agent,

(2) Decreasing the dosage but increasing or maintaining the sameefficacy. Decreased dosage could lead to reduction in toxicity,

(3) Minimizing or slowing down the development of drug resistance,

(4) Providing selective synergism against target (efficacy synergism) versus host (toxicity antagonism).

(5) Enhancing the pharmacokinetic–pharmacodynamic properties.

Researches have shown that Phytochemicals when combined together, interact synergistically with each other in *in vivo* and *in vitro* conditions (Prabhakar and Doble 2009, 2011a,b,c; Atangwho *et. al.,* 2012). When herbs are used in combination, the effects can be complicated as various interactions can occur among the individual components. The most desirable interactions are those which can result in additional therapeutic benefit. This is often the intended or expected outcome when using combination therapy (Auwal *et. al.*, 2017). However, due to the presence of multiple components in the herbal products, the effects arising from herb-herb or herb-drug interactions are often unpredictable and complicated (Che *et. al.,* 2013). Several cinnamic acid derivatives when used in combination with metformin, increase glucose uptake by L6 myotubes and 3T3-adipocytes.

The combinations also increase the expression of genes which are involved in the insulin cascade and decrease the expression of genes involved in the secondary complications such as fatty acid synthase and HMGCoA reducase (Prabhakar and Doble, 2011a,b). Ferulic acid used in combination with these two antidiabetic drugs in the streptozotocin induced diabetic Wistar rats, increases the blood glucose and lipid profile when compared to the diabetic rats. The combination also improves their liver and kidney functions and increases the regeneration of pancreatic β-cells (Prabhakar *et. al.* 2013). According to an ethnobotanical literature on traditional phytotherapy, species like *Asparagus racemosus, Butea monosperma, Cathanranthus roseus, Coccinia indica, Gymnema sylvestre, Syzygium cumini and Momordica charantia* are used, singly or in combinations, by communities for the treatment of diabetes (Ayyanar *et. al.*, 2008). Moreover, methanolic extract (75%) of *Terminalia chebula, Terminalia belerica, Emblica officinalis* and their combination named 'Triphala'(equal proportion of above three plant extracts) were found to inhibit lipid peroxide formation, scavenge hydroxyl and superoxide radicals in vitro and an oral administration of the extracts (100 mg/kg body weight) reduced blood sugar level in normal and in alloxan (120 mg/kg) diabetic rats significantly within 4 hours (Sabu and Kuttan, 2002). A combination of *Abroma augusta* and *Curcuma Ionga*have shown antidiabetic activity and also reduced oxidative stress in diabetes - it also restored other general parameters, like TBARS, GSH, SOD and CAT activities in diabetic animals. The results were statistically analyzed and indicated that combination of herbal extracts showed better efficacy as compared to individual herbal plant extracts used (Halim, 2002).

Two methods namely, isobologram (Tallarida, 2001) and combination index (Zhao *et. al.*, 2004) have been used to determine synergy while using combination therapy in treating diabetics. The first method uses an isobologram, which is a graphical representation of the

interaction of two chemicals (drugs, phytochemicals or combination of both). The other method, explain the interaction of two compounds based on a calculated constant known as combination index (CI), estimated as

$$CI = \left[\frac{C_a}{IC_a}\right] + \left[\frac{C_b}{IC_b}\right]$$

where Ca and C_b are the concentrations of compounds A and B used together to achieve a fixed effect (ED_{50}). ICa and IC_b are the concentrations of A and B, respectively required individually to achieve the same effect. A CI value of less than, equal to, and more than one, indicates synergy, additivity, and antagonism respectively between these two compounds (Zhao *et. al.*, 2004; Ting-Chao, 2010).

2.6 Isolation and Characterization of Bioactive Compounds fromPlant Extracts

Due to the fact that plant extracts usually occur as a combination of various types of bioactive compounds orphytochemicals with different polarities, their separation still remains a big challenge for the process of identification and characterization of bioactive compounds. It is a common practice in isolation of these bioactive compounds that a number of different chromatographic separation techniques such as thin layer chromatography (TLC), column chromatography, flash chromatography, Sephadex chromatography and high-performance liquid chromatography (HPLC), should be used to obtain pure compounds. The structures of compounds are elucidated and then subjected to biological activity. Beside that, non-chromatographic techniques such as immunoassay, which use monoclonal antibodies (MAbs), phytochemical screening assay, Fourier-transform infrared spectroscopy (FTIR), can also be used to obtain and facilitate the identification of the bioactive compounds. TLC is a simple, quick, and inexpensive

procedure that gives the researcher a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the Rf of a compound is compared with the Rf of a known compound. Additional tests involve the spraying of phytochemical screening reagents, which cause color changes according to the phytochemicals existing in a plants extract; or by viewing the plate under the UV light. This has also been used for confirmation of purity and identity of isolated compounds (Lopes *et. al.,* 1999; Sasidharan *et. al.*, 2011; Kim *et. al.,* 2015).

Due to the high separation performance and sensitivity and structural identification ability, ultra-high performance liquid chromatography-quadrupole-time of flight mass spectrometry (UHPLC-Q-TOF-MS) has been applied more and more in the study of herbal components. Whether the predicted metabolites exist in the plants can be determined by searching their molecular formulae or exact masses using liquid chromatography-mass spectrometry (LC-MS). Using this method, the trace components could be identified too. However, it will still take a long time to manually calculate the molecular formulae or exact masses. Nowadays, there are several commercial software products for the identification of drug metabolites, e.g., Metabolite ID (Agilent Technologies, Santa Clara, CA, USA), LightSight (Applied Biosystems LLC and MDS Inc., Carlsbad, CA, USA), Profiler-M (Phenomenome Discoveries Inc. Saskatoon,Canada) and CFM-ID web server. Metabolite identification software cannot only offer the essential function for automatically identifying and confirming metabolites from data acquired on MS and MS/MS spectra, but also typically contains a built-in editable biotransformation database. Using the metabolites software the only step need to set in the whole identification process is to import a structure or formula of a parent compound into the biotransformation database, and then the predicted molecular formula and masses of metabolites are calculated automatically followed by applying the

fundamental algorithms of all related mass signals in the acquired MS data. Finally, the predicted metabolites are further confirmed using weighted relevance criteria including isotopic pattern matching, mass defect, extracted compound chromatography (ECC) responses and MS/MS fragment pattern matching. Compared with the traditional manual identification, metabolites software is a simpler, rapid and automatic method for predicting and identifying metabolites. The databases were established using the MassHunter Metabolite ID software, which mainly includes three parts, the name of the metabolite (Name), the formula and mass of the modifier (Formula and Mass), molecular formula and mass of the assumptive metabolite (Result formula and result mass) (Satheeshkumar *et. al.*, 2014; Gu *et. al.,* 2015; Qiao *et. al.,* 2015).

NMR is another powerful tool for determining the 3D structures of compounds and requires that the compound be in solution, rather than crystalline. It provides information about the number and types of atoms in the molecule and the electronic environment around these atoms. The principle behind NMR is that, when a molecule is placed in a strong external magnetic field, certain nuclei of atoms within the molecule, such as ^{1}H, ^{13}C, ^{15}N, ^{19}F, and ^{31}P, will resonate as they absorb energy at specific frequencies that are characteristics of their electronic environment. Because most drug and protein molecules are composed of hydrogen, carbon, nitrogen, fluorine, and phosphorus, NMR is ideally suited to unravel structural information of drugs and proteins (Rick, 2009). An NMR spectrum shows the types of environment around the nuclei (atoms) and the ratios of these nuclei. For 1 H NMR, the hydrogen nuclei of a compound can resonate downfield (higher frequency) or upfield (lower frequency) relative to a standard called tetramethylsilane (TMS). The NMR spectrum also provides information about the number of nuclei within each distinct environment. This is given by the area under each resonant peak representing the relative

number of nuclei of each type. Furthermore, the surrounding nuclei also cause a splitting pattern. For example, one H surrounded by n other H neighbors will have its resonance peak split into n + 1 peaks (Rick, 2009).

NMR spectroscopy provided most of the experimentaldata that enabled the complex equilibria ofinterconverting forms of reducing sugars to be unravelled. As the sensitivity of NMR methodshas improved, in conjunction with the availabilityof ^{13}C-labeled compounds, even minor componentsof those equilibria have become observable, andthe conformational space occupied by carbohydrateshas become better defined. Conversely,when NMR experiments are used to determine thestructures of carbohydrates it is necessary to beaware of all possible molecular permutations tointerpret the spectra. TheNMR spectra of carbohydrates must therefore beinterpreted as a representation of a kaleidoscope ofchemical and biologic diversity. Despite the fact that most resonances are clusteredbetween ~δ 3.4 and ~δ 4.0, ^{1}H spectra ofcarbohydrates do contain some well-resolved signals,including those of anomeric protons (~δ 4.4 – ~δ 5.5),acetyl (~δ 2.0 – ~δ 2.1) and methyl (~δ 1.2) groups,and other protons that are influenced by specific functionality,including amino groups, phosphorylation,sulphation, glycosylation, and acetylation, or the lackof functionality as in deoxysugars (Bubb, 2003). Apart from this diagnostic chemical shift data,comparison of the integrated intensities of anomericprotons can reveal the number of monosaccharideresidues. At a given magnetic field strength, ^{13}C resonances of saccharides are much more dispersed than their ^{1}H counterparts. Guidelines for assignment of the spectra of oligosaccharides, and in general applicable to ^{13}C data for polysaccharides, may be summarized as follows:

1. In the absence of structural modification, a sugar residue in an oligosaccharide has ^{13}C

chemical shifts that are usually within 0.3 ppm of the corresponding resonances in the free monosaccharide.

2. Glycosylation leads to high frequency shifts of 4–10 ppm for the carbons at the anomeric and linked positions. [Note, in this respect, that the anomeric carbon resonances of lactose embrace a chemical shift range that is comparable to that of all other carbons excluding C-6].

3. Resonances of carbon atoms that are adjacent to each carbon in the glycosyl linkage are in general shifted to slightly lower frequency.

4. The chemical shift of an anomeric carbon in an α-linkage (~δ 97– δ 101) is in general less than that of an anomeric carbon in a β-linkage (~δ 103–δ 105), but the exceptions to this rule recommend determination of $^{1}J_{C\text{-}1,H\text{-}1}$ as the most reliable indication of anomeric configuration.

5. ^{13}C nuclei in furanose sugars are less shielded than in their configurationally related pyranose counterparts (Bubb, 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 *Materials*

3.1.1Plant material collection, identificationand processing

Fresh samples of the plants *Gymnema sylvestre* (GS) and *Combretum micranthum* (CM) werecollected from Shira Local Government Area (N 11° 27’ 29” and E 10° 2’ 48” or Latitude: 11.46 and Longitude: 10.05) of Bauchi State in Nigeria on 26th February 2015. The plants were identified at the herbarium unit of Department of Biological Sciences, Bayero University, Kano (B.U.K.) and voucher specimens, with number BUKHAN 0349 (for *Gymnema sylvestre*) and BUKHAN 0272 (for *Combretum micranthum*)were then deposited for future reference.

The leaves of the plants *Gymnema sylvestre* (GS) and *Combretum micranthum* (CM) used for the study were plucked from the stem, washed and then dried under shade. The dried samples were then ground into powder in a laboratory mortar using a pestle. The powdered samples were then kept in a closed plastic container for use in the experiment.

3.1.2 Animals

Adult male wistar rats (150 – 200 g body weight) and mice (15 – 25 g body weight) were purchased from the Department of Pharmacology and Therapeutics of A.B.U., Zaria. They were kept in standard metal animal cages at room temperature in the animal house of Biological Sciences Department, B.U.K., allowed free access to food and water and allowed to acclimatize for a week prior to use.The protocol of the study was according to international guidelines i.e. the Organization for Economic Cooperation and Development (OECD) Test Guidelines (TG407) (OECD, 2006).

3.1.3 Chemicals, reagents and equipment

All the chemicals and reagents used for this work were of analytical grade and purchased from reputable chemical companies, e.g. SIGMA-ALDRICH-FLUKA. The laboratory equipment were also of standard quality.

3.2 Methods

3.2.1 Extracts preparations

Six hundred grams of the air-dried samples of each plant were cold extracted by maceration with 6L of distilled water.The extracts were then filtered through Whatman No. 1 paper.The filtrate of the aqueous extract was then evaporated initially, in a hot-air oven at 40°Cand then lyophilisedunder vacuum at -80°C and a pressure of 0.06 mbar for 72 hours.The dried/powdered extracts were weighed, percent yield calculated and stored at -20°Cfor further analyses (Kong *et. al.*, 2014).The extracts were subjected to phytochemical analyses, *in vitro* antioxidants, and preliminary antidiabetic screening studies.

3.2.2Phytochemical screening and antioxidant properties

The GS and CM extracts were screened qualitatively for phytochemical constituents utilizing standard methods of analysis (Vishnoi, 1979; Sofowora, 1993; Trease and Evans, 2002) to test for tannins, terpenoids, saponins, flavonoids and alkaloids.Quantitative Phytochemical assay (Harborne, 1994) was carried out to determine the contents of saponins, flavonoids, alkaloids, tannins, total phenolics and total flavonoids.

3.2.2.1Qualitative phytochemical tests

i. Test for tannins:Exactly 0.5 g of each plant extract was stirred with 10 ml of distilled water and then filtered. Three drops of 1% alcoholic ferric chloride solution were added to 2 ml of the filtrate; occurrence of a blue-black, green or blue-green precipitate indicated the presence of tannins (Trease and Evans, 2002).

ii. Borntrager's test:Exactly 0.2 g of each plant extract to be tested was shaken with 10 ml of benzene and then filtered. Five millilitres of the 10% ammonia solution was then added to the filtrate and shaken. Appearance of a pink, red or violet colour in the ammoniacal (lower) phase was taken as the presence of free anthraquinones (Sofowora, 1993).

iii. Test for terpenoids:Exactly 0.2 g of each plant extract was dissolved in ethanol. To it 1 ml of acetic anhydride was added followed by the addition of 5 drops of conc. H_2SO_4. A change in colour from pink to violet showed the presence of terpenoids (Sofowora, 1993).

iv. Test for saponins: One (1) gram of each plant extract was boiled with 5 ml of distilled water and filtered. To the filtrate, 3 ml of distilled water was further added and shaken vigorously for 5 minutes. Frothing which persisted for 5 minutes on warming was taken as an evidence for the presence of saponins (Sofowora, 1993).

v. Ferric chloride test for flavonoids: Exactly0.5g of each plant extract was boiled with distilled water and then filtered. To 2 ml of the filtrate, three drops of 10% ferric chloride solution were then added. A green-blue or violet colouration indicated the presence of a phenolic hydroxyl group (Trease and Evans, 2002).

vi. Test for alkaloids:Exactly0.2 g of each plant extract was stirred with 5 ml of 1% aqueous HCl on water bath and then filtered. The filtrate was divided into two portions; 1 ml of each portion was taken into 2 separate test tubes. To the first portion, three drops of Dragendorff's reagent was added; occurrence of orange-red precipitate was taken as positive. To the second 1 ml, Mayer's reagent was added and appearance of buff-coloured precipitate was an indication for the presence of alkaloids (Sofowora, 1993).

3.2.2.2Quantitative phytochemical assays (Harborne, 1994)

i. Saponins: Twenty (20) g of plant sample was put into a conical flask after which 200 ml of 20 % aqueous ethanol added. The mixture was heated on a hot water bath, at $55^{O}C$, for 4

hours with continuous stirring, after which the mixture was filtered and the residue re-extracted with a further 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over a water bath at 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether added and then shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated three times and 60 ml of n-butanol added. The combined n-butanol fractionwas washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated on a water bath at 90°C. After evaporation, the sample was dried in an oven to a constant weight; the saponin content was calculated as percentage of the starting material.

ii. Flavonoids:Ten (10) g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol, at room temperature. The whole solution was filtered through Whatman filter paper No 1. The filtrate was later transferred to a crucible and evaporated to dryness over a water bath; the dry content was weighed to a constant weight. The flavonoid content was calculated as a percentage of the starting material, i.e. $\frac{A}{B} * 100$

Where A = Initial weight of sample; B = Final weight of sample.

iii. Alkaloids:Five(5) g of the plant sample was weighed into a 250 ml beaker and 200ml of 10% acetic acid in ethanol was added. The reaction mixture was covered and allowed to stand for 4 hours and filtered. The extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop-wise to the extract until precipitation is complete. The whole solution was allowed to settle and the precipitate collected, washed with dilute ammonium hydroxide and then filtered; the residue being the alkaloid was dried and weighed to a constant mass (Evans, 1999). The alkaloid content was calculated as a percentage of the starting material, i.e. $\frac{A}{B} * 100$,

where A = Initial weight of sample; B = Final weight of sample.

iv. Tannins/Pseudotannins:Five (5g) of the plant sample was placed into a stoppered conical flask; 25ml of 0.1N iodine and 4% sodium hydroxide (10ml) were then added. The resulting mixture was kept in the dark and acidified with 4% sulphuric acid (10ml). The mixture was then titrated with 0.1N sodium thiosulfate solution using starch solution as an indicator. The titre value corresponds to the sum of tannins and pseudotannins concentration **A**. Another 5g of the plant extract was placed in a stoppered conical flask followed by 15ml of 1% gelatin. The volume was made up with distilled water. Aliquot of 20ml was measured into a volumetric flask; 25ml of 0.1N iodine and 10ml of 4% sodium hydroxide then added, mixed and kept in the dark for 15 minutes. The mixture was diluted with distilled water (10ml) and acidified with 10ml of 4% sulphuric acid. This was finally titrated with 0.1N sodium thiosulphate solution using starch solution as indicator. The titration value obtained, correspond only to pseudotannins concentration **B**. A blank experiment was carried out simultaneously using distilled water (El-Olemy *et. al.*, 1994).The tannins and pseudotannins content of the sample were then calculated using the following formula:

% of Pseudotannins = ([Blank - Exp A] × 0.029× 100)/5(volume taken) = [A]

% of Pseudotannins = ([Blank - Exp B] × 0.029× 100)/5(volume taken) = [B]

% of true tannins = [A] - [B]

v.Estimation of total phenolic (pyrocatechol gallic acid) compounds

Total soluble phenolics in the GS and CM and GSCM were determined with Folin-Ciocalteu reagent according to the method of Naskar *et. al.*(2010). Briefly, 1mg of each extract wasput into 100ml Erlenmeyer flask then 46ml of distilled water added. Afterwards, 1ml of Folin – Ciocalteu reagent (FCR) was added to this mixture and after 3minutes, 3ml

of Na_2CO_3 (2%) added. Subsequently, the mixture was shaken on a shaker for 2 hours at room temperature then absorbance measured at 760nm. All the tests were performed in triplicate and the results averaged. The concentration of total phenolic compounds in extracts was determined as microgram of pyrocatechol equivalent by using Eqs. (2) and (3) that were obtained from standard pyrocatechol/gallic acid graph.

Absorbance = 0.001 × pyrocatechol (μg) + 0.0033 ……..(2)

Absorbance = 0.0053× gallic acid (μg) -0.0059 …….(3)

vi.Estimation of total flavonoid content

The total flavonoids content was determined using the method as in Naskar *et. al.* (2010). Two (2) ml of 2 % aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extract solution (0.1 mg/ml). Absorbance readings at 415 nm using spectrophotometer were taken after 10 minutes against a blank sample consisting of a 2 ml extract solution with 2 ml methanol without $AlCl_3$. The total flavonoid content was determined using a standard curve with quercetin (25 - 200 μg/2ml methanol) as the standard. Total flavonoid content is expressed as μg of quercetin equivalents (QE)/mg of extract.

3.2.2.3*In vitro*antioxidant activity

i.DPPH radical scavenging activity: DPPH radical scavenging activity was measured using the method as described in Naskar *et. al.* (2010). Three (3) ml of reaction mixture containing 0.2 ml of DPPH (100 μM in methanol) and 2.8 ml of test solution, at various concentrations (5, 10, 20, 40, 80, 160 320 μg/ml) of the extract/fractions was incubated at 37°C for 30 min, absorbance of the resulting solution was measured at 517 nm using spectrophotometer. The percentage inhibition of DPPH radical was calculated by

comparing the results of the test with those of the control (not treated with extract) using the following equation:

Percentage inhibition = (1– absorbance of test/absorbance of control) × 100 …….. (1)

ii.Nitric oxide: In aqueous solution at physiological pH, sodium nitroprusside generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be measured by the Griess reaction(Naskar *et. al.,* 2010). One (1) ml of 10 mM sodium nitroprusside was mixed with 1 ml of test solution of different concentrations (80, 160, 320, 500, 800, 1000 µg/ml) in phosphate buffer (pH 7.4) and the mixture incubated at 25°C for 150 min. From the incubated mixture, 1 ml was taken out, 1 ml of Griess' reagent (1% sulphanilamide, 2% o-phosphoric acid 0.1% naphthyl ethylene diamine dihydrochloride) added to it. Absorbance of the chromophore formed by the diazotization of nitrite with sulfanilamide subsequent coupling with naphthyl ethylene diamine dihydrochloride was read at 546 nm and percentage inhibition calculated by comparing the results of the test with those of the control using Eq. (1) above.

iii. Ferric reducingantioxidant power (FRAP): Reducing power of the test samples was determined on the basis of the ability of their antioxidant principles to form colored complex with potassium ferricyanide, TCA, $FeCl_3$. It was measured by the method reported by Naskar *et. al.* (2010); 1 ml of different concentrations (25, 50, 100, 200, 400 µg/ml) of the extract fractions was mixed with potassium ferricyanide (2.5 ml, 1%), 2.5 ml of phosphate buffer (pH 6.6). The mixture was incubated at 50°C for 20 min. Then 2.5 ml TCA (10%) was added to it and centrifuged at 3000 rpm for 10 min; 2.5 ml of supernatant was taken out and to this 2.5 ml water, 0.5 ml $FeCl_3$ (0.1%) was added and absorbance measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power. The reducing capacities were calculated as the slope from the line of best fit of the

absorbance against concentration (of ascorbic acid) using the linear regression curve (Ahmed *et. al.*, 2014).

iv.ABTS scavenging effects: The antioxidant effect of the leaf extracts was also studied using ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation decolourisation assay according to the method of Shirwaikar *et. al.* (2006). Briefly, ABTS radical cations ($ABTS^{+}$) were produced by reacting ABTS solution (7mM) with 2.45mM ammonium persulphate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. Aliquots (0.5ml) of the extracts were added to 0.3ml of ABTS solution and the final volume was made up to 1ml with ethanol. The absorbance was read at 745nm in a spectrophotometer and the per cent inhibition was calculated using the formula.

Inhibition (%) = (Control – Test) × 100/ Control

v. Total antioxidant capacity

Total antioxidant capacity (TAC) of the samples was determined using a novel automated measurement method, developed by Erel (2005) with modification – ascorbic acid was used in place of trolox. In this method, the characteristic color of $ABTS^{*+}$ is bleached by antioxidants present in the sample. This reaction can be monitored spectrophotometrically and the bleaching rate is inversely related to the TAC of the sample. The reaction rate is calibrated with ascorbic acid, as a traditional standard, and the assay results are expressed in ascorbic acid equivalent, µg/g dry weight of plant sample.

3.2.3 Acute toxicity

The median lethal dose (LD_{50}) of aqueous leaf extracts of the plants was determined in mice using a modified method of Lorke (Lorke, 1983) as described in the work of Adewole and Ojewole (2009). Mice were fasted for 16hours and randomly divided into groups of eight mice each. Stepwise, graded doses of GS and CM (separately) extracts (25, 50, 100,

200, 400, 800, 1600 and 3200 mg kg^{-1} b.w.) were administered orally to the mice in each of the 'test' groups. Each of the mice in the 'control' group was given distilled water (3 ml kg^{-1}b.w.) only. The mice in both the 'test' and 'control' groups were then allowed free access to food and drinking water, and observed over a period of 48hours for signs of acute toxicity which include changes in feed and fluid intake, body weight, respiratory, GIT, CNS symptoms, behavioural patterns and mortality. The number of deaths (caused by the extract in each group) within this period of time was noted and recorded. Log dose-response plots were subsequently constructed for the plants' extract, from which the $LD_{50}$was determined. A tenth ($1/10^{th}$) of the LD_{50} of each extract/fractions was considered as the highest dose for the sub-chronic (long term) study (Kumar *et. al.*, 2009). Other dose levels ($1/5^{th}$, $1/15^{th}$ and $1/20^{th}$ of the LD_{50}) were considered to enable determination of dose-response relationships.

3.2.4 Sub-chronic studies

Experimental design

According to the OECD guideline, if the acute toxicity test at one dose level of at least 5000mg/kg b.w. produced no observable toxic effect then the full study using three dose levels is considered not necessary. As a result, total of 24 rats were used for this study and they were divided into four groups with six rats per group, using a single dose ($1/10^{th}$ of the LD_{50}) as outlined below:

Group 1, normal untreated rats (0.5 mL water/100 g body weight/rat/day);

Group 2, normal rats treated with GS alone (600 mg/kg b.w.);

Group 3, normal rats treated with CM alone (600 mg/kg b.w.);

Group 4, normal rats treated with 1:1 GSCM (600 mg/kg b.w.);

All experiments were carried out in overnight fasted rats. The crude aqueous plant extracts were given orally in aqueous solution daily using an intragastric tube for 21 days. Animal sacrifice, preparation of serum and toxicity assays were conducted as in sections 3.2.6.2 and 3.2.6.3.

3.2.5 Preliminary screening for antidiabeticactivity

3.2.5.1 Alloxan induction of diabetes

Alloxan, a well- known diabetogenic agent is widely used to induce diabetes in animals. The drug and its reduction product dialuric acid establish a redox cycle with the formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide. Thereafter, highly reactive hydroxyl radicals are formed by Fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of β cells (Kumar *et. al.*, 2012).

Alloxan induction was conducted according to the method of Adoum *et. al.*(2011); Atiku *et. al.*(2010); Swami*et. al.*, (2010); Chatzigeorgiou*et. al.* (2009) with slight modifications.A stock solution was prepared by dissolving alloxan monohydrate (1.2g) in normal saline (12ml). Diabetes was induced, after 12 hour fasting of the animals, by single intraperitoneal injection of alloxan monohydrate (100mg/kg). The animals were allowed 48 hours of rest for blood glucose stabilization before the administration of the extracts and their fractions. The initial blood glucose of each of the micewas measured.

Because Alloxan is capable of producing fatal hypoglycaemia as a result of massive pancreatic insulin release, micewere treated with 20% glucose solution (5 to 10 ml) orally after 6 hours. The micewere then kept for the next 24 hours on 5% glucose solution to prevent severe hypoglycaemia (Stanley and Venugopal, 2001). After 48 hours, mice with moderate diabetes having hyperglycaemia (i.e., with blood glucose levels of above 200

mg/dl) were chosen for the experiment (Karthic *et. al.*, 2012; Williamson *et. al.*, 1996; Huralikuppi, 1991).

3.2.5.2 Experimental design for preliminary antidiabetic studies

A total of 24 mice were used for this phase of the experiment and they were divided into eight groups with three animals per group as outlined below:

Group 1, diabetic mice treated with GS aqueous (600 mg/kg b.w.);

Group 2, diabetic mice treated with CM aqueous (600 mg/kg b.w.);

Group 3, diabetic mice treated with GSCM 1:1 (600 mg/kg b.w.);

Group 4, diabetic mice treated with GSCM 1:2 (600 mg/kg b.w.);

Group 5, diabetic mice treated with GSCM 2:1 (600 mg/kg b.w.);

Group 6, normal untreated mice;given only distilled water (10 ml/kg)

Group 7, diabetic untreated mice; given only distilled water (10 ml/kg) and

Group 8, diabetic mice treated with glibenclamide (6 mg/kg b.w.) (Ramkumar *et. al.*, 2011).

All experiments were carried out in overnight fasted mice. The plant extracts and the drug glibenclamide were given orally as aqueous solutions using an intragastric tube and the FBG monitored for a sampling time of 4 hours. The 1:1 combination of theaqueous extract gave the highest FBG reduction and was selected for the antidiabetic (long-term) studies.

3.2.6 Antidiabetic studies

3.2.6.1 Streptozotocin induction of diabetes

Streptozotocin,also known as streptozocin, Izostazin, or zanosar (STZ) is a synthetic nitrosoureido glucopyranose derivative (2-Deoxy-2[(methylnitrosoamino)-carbonyl] amino]-Dglucopyranose) isolated from fermentations of *Streptomyces achromogenes* that is

classically an anti tumor antibiotic and chemically is related to other nitrosoureas used in cancer chemotherapy.

Insulin dependent diabetes mellitus(IDDM) was induced by a single intraperitonealinjection of streptozotocin (60mg/kg) in sterile citrate buffer (pH 4.5; 0.1M) to rats (Pellegrino *et. al.*, 1998; Chatzigeorgiou *et. al.*, 2009) afer an overnight fast.

3.2.6.2Experimental design (antidiabetic studies)

The aqueous plant extracts were used for this phase of the study. A total of 72 rats were used for this phase of the experiment and they were divided into twelve groups with six rats per group as outlined below:

Group 1, normal untreated rats (given only 0.5ml/100g b.w. water);

Group 2, diabetic control rats (given only 0.5ml/100g b.w. water);

Group 3, diabetic rats treated with GS alone (600 mg/kg b.w.);

Group 4, diabetic rats treated with CM alone (600 mg/kg b.w.);

Group 5, diabetic rats treated with GSCM (1:1) (600 mg/kg b.w.);

Group 6, diabetic rats treated with GS alone (400 mg/kg b.w.);

Group 7, diabetic rats treated with CM alone (400 mg/kg b.w.);

Group 8, diabetic rats treated with GSCM (1:1) (400 mg/kg b.w.);

Group 9, diabetic rats treated with GS alone (200 mg/kg b.w.);

Group 10, diabetic rats treated with CM alone (200 mg/kg b.w.);

Group 11, diabetic rats treated with GSCM (200 mg/kg b.w.); and

Group 12, diabetic rats treated with glibenclamide (6 mg/kg b.w.) (Ramkumar *et. al.*, 2011).

All experiments were carried out in overnight fasted rats. The aqueous plant extracts and the drug glibenclamide were given orally in aqueous solution daily using an intragastric tube for 21 days.

Screening for antidiabetic activity of the different extracts of GS and CM and the combined GSCM were done. Animals of Groups 1 and 2 were given 0.5 ml water/100g body weight/day for the 21 days to keep all the animals under the same experimental condition and stress imposition, if any, due to the administration of extracts and animal handling. Starting from Day 1 of treatment, fasting blood glucose levelswere measured weekly in all groups as detailed in the "biochemical assay" section. Also, feed and fluid intakes were monitored during the study period. On 22^{nd}day of the experiment,all the animals were sacrificed in the fasting state by light ether anesthesia followed by decapitation after recording the final body weight. Blood wascollected into clean tubes and the serum separated by centrifugation at 2800g for 5minutes for the estimation of serum insulin, biochemical parameters and toxicity markers as specified under the "biochemical assay" and "assay of markers of tissue damage" section. The liver of each sacrificed animal was dissected out immediately, weighed and stored at-20°C for the assessment of the activities of the enzymes hexokinase, catalase, superoxide dismutase and glutathione peroxidase; and the levels of reduced and oxidized glutathione, reactive oxygen species and thiobarbituric acid reactive substances in the liver homogenate prepared. Blood collected in EDTA bottleswas used for the quantification of haematologic parameters as described below.

3.2.6.3 Analyses

i.Body Weight

The body weight of each rat was assessed during the acclimatization period, once before commencement of dosing, once every 7 days during the dosing period and once on the day of sacrifice. The relative body weight (RBW) of each animal was then calculated using the formula RBW=[absolute body weight of one time interval (g) / body weight of rat on commencement of dosing day(g)] × 100 (Kuete *et. al.*, 2010).

ii.Haematology

XE-2100 performs haematology analyses according to the direct-current resistance and radio-frequency resistance (RF/DC) detection method, HydroDynamic Focusing (DC Detection), flow cytometry method (using the semiconductor laser) and SULFOLYSER (SLS-haemoglobin) method.

At the end of dosing period for the sub-chronic study, blood samples were collected from the ventral tail vein of each rat into EDTA tubes for haematological analysis using Haematological Auto-analyzer (SYSMEX XE-2100 Haematology Automated Analyser) (Stiene-Martin *et. al.*, 1998). The haematological parameters analyzed include: Packed cell volume (PCV), Red Blood Cell (RBC) count, White Blood Cell (WBC) count and Haemoglobin (Hb) concentration, Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC).

iii.Relative Organ Weight and Preparation of Homogenate Samples

After taking the blood, the abdominal cavity of each animal was opened and the liver and kidney quickly removed, cleaned with ice-cold saline, blotted, weighed and stored at −80°C. The liver tissues was thawed and homogenized and the supernatant used for Hexokinase (HXK), Catalase (CAT), Superoxide Dismutase (SOD), Glutathione

Peroxidase (GSH-Px) enzymes as well as Glutathione-reduced (GSH), Glutathione-oxidized (GSSG), Reactive Oxygen Species (ROS) and Thiobarbituric Reactive Substances (TBARS) assays as described below. The relative organ weight (ROW) of each animal was then calculated using the formula ROW = [absolute organ weight (g)/body weight of rat on day of sacrifice (g)] ×100 (Kuete *et. al.*, 2010).

iv. Biochemical Assays

a)Fasting Blood Glucose (FBG)Determination

Fasting blood glucose concentrations were determined by means of ACCU-CHEK Active® Glucometer and strips. When blood is applied to the test area on the strip, the glucose 6-P dehydrogenaseenzyme (Mut. 0-GDH 2) reacts with the blood glucose. The subsequent chemical reaction changes the colour of the test area. The meter registers this colour change and converts the signal obtained into a blood glucose result (glucose 6-P dehydrogenase enzyme method). Blood samples were obtained by repeated needle puncture of the tail tip veins;FBG was obtained 1 day before alloxan/STZ treatment, and subsequently weekly after induction of diabetes mellitus.

b)Serum Insulin Estimations

Serum insulin concentrations were determined by an enzyme-linked immunosorbent assay (ELISA), using a commercial kit (Accu-Bind ELISA Microwells; Monobind Inc. CA 92630; USA).

Immunoenzymometric assay (TYPE 3):

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (Ab),enzyme (conjugated and immobilized), with different and distinct epitope recognition, in excess, and native antigen (Ag). In this procedure, the immobilization takes place during the assay at thesurface of a microplate well through the

interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal insulin antibody. Upon mixing monoclonal biotinylated antibody, the enzyme-labeledantibody and a serum containing the native antigen,reaction results between the native antigen and the antibodies,without competition or steric hindrance, to form a solublesandwich complex. The interaction is illustrated by the followingequation:

$$^{Enz}Ab_{(M)} + Ag_{Ins} + {}^{Btn}Ab_{(M)} \; {}^{Enz}A \underset{K_{-a}}{\overset{K_a}{\leftrightarrow}} g_{Ins}\text{-}{}^{Btn}Ab_{(M)}$$

$^{Btn}Ab_{(M)}$ = Biotinylated Monoclonal Ab (Excess Quantity)

Ag_{Ins} = Native Antigen (Variable Quantity)

$^{Enz}Ab_{(M)}$= Enzyme labeled Monoclonal Ab (Excess Quantity)

$^{Enz}Ab_{(M)}$-Ag_{Ins}-$^{Btn}Ab_{(M)}$ = Antigen-Antibodies complex

K_a = Rate Constant of Association

k_{-a} = Rate Constant of Dissociation

Simultaneously, the complex is deposited to the well through thehigh affinity reaction of streptavidin and biotinylated antibody.This interaction is illustrated below:

$^{Enz}Ab_{(M)}$ - Ag_{Ins}–$^{Btn}Ab_{(M)}$ + <u>Streptavidin</u> cw→Immobilized complex

<u>Streptavidin</u> cw = Streptavidin immobilized on well

Immobilized complex = sandwich complex bound to the solid surface

After equilibrium is attained, the antibody-bound fraction isseparated from unbound antigen by decantation or aspiration.The enzyme activity in the antibody-bound fraction is directlyproportional to the native antigen concentration. By utilizingseveral different serum references of known antigen values, adose response curve can be generated from which the antigenconcentration of an unknown can beascertained.

Test Procedure:

Before proceeding with the assay, all reagents, serum references and controls were brought to room temperature (27^oC).The microplates' wells were formatted for calibrator, control and test; all samples were assayed in duplicate. Fifty (50µl) of the appropriate calibrators, controls, and test were pipetted into the assigned wells and 100µl of the insulin enzyme reagent added to each well. The microplate wasswirled gently for 30 seconds to mix andcovered with a plastic wrap. The contents were incubated for 120 minutes at room temperature (27^oC). Then the contents of the microplate discarded by decantation, i.e. by tapping and blotting the plate dry with absorbent paper. To the same microplate was then added 350µl of wash buffer, decanted (tapping and blotting) andrepeated two (2) additional times for a total three (3) washes. Then 100µl of working substrate solutionwas added to all the wells. This was incubated at room temperature for fifteen (15) minutes and 50µl of stop solution added to each well and mixed gently for 20 seconds. The absorbance in each well was read at 450nm (using a reference wavelength of 620 – 630nm to minimize well imperfections) in a microplate reader. The results were read within thirty (30) minutes of adding the stop solution.

Calculation of Results:

A dose response curve was used to ascertain the concentration of insulin in the unknown specimens (in µIU/ml).

c)Hexokinase Assay

The assay is based upon the reduction of NAD^+ through a coupled reaction with glucose-6-phosphate dehydrogenase and is determined spectrophotometrically by measuring the increase in absorbance at 340nm.

D-glucose + ATP $\xrightarrow{\text{Hexokinase/Mg}}$ glucose-6-P+ADP

Glucose-6-P + NAD $\xrightarrow{\text{Glucose 6-P Dehydrogenase}}$ gluconate-6-P + NADH + H^+

One unit of activity reduces one micromole of NAD^+ per minute at 30°C and pH 8.0 under specified conditions.Although there is a lack of specificity of the hexokinase, the overall assay is very specific for glucose because the linking enzyme is specific for glucose-6-phosphate and will react with neither fructose-6-phosphate nor mannose-6-phosphate without the incorporation of phosphoglucose isomerase to convert it to glucose-6-phosphate. Thus it is a requirement for the specific glucose assay that the hexokinase preparation is not contaminated with phosphoglucose isomerase.

Into each cuvette(the triplicates) was pipetted as follows:

Tris. $MgCl_2$ buffer	2.28 ml
0.67M Glucose	0.50 ml
16.5mM ATP	0.10 ml
6.8mM NAD	0.10 ml
G-6-PDH	0.01 ml

Incubation was carried out in the spectrophotometer at 30°C for 6-8 minutes to achieve temperature equilibration and establish blank rate, if any. At zero time, 0.1 ml of diluted hexokinase solution was added and mixed thoroughly. Increase in A340 for 3-4 minutes at 30°C was recorded.

Calculation: Hexokinase activity (Units/mg protein) $= \frac{\Delta A340/\text{min}}{6.22 \times \text{enzyme/ml reaction mixture}}$

d)Catalase activity (CAT)

This is based on coupled oxidation. Catalase or peroxidase combines with hydrogen peroxide as it is liberated, and the complex thus formed then brings about the oxidation of substances such as nitrite, ethanol, cytochrome C or manganese ions in the presence of p-cresol. The extent of these oxidations is measured spectrophotometrically (Clairborne, 1995).

Procedure:

Reagents	Volumes
Ammonium ferrous sulphate	2.5ml
Sorbitol	250µl
Xylenol Orange	100µl
H_2O_2	25µl
Sample	50µl

50µl of sample was mixed with the reagents listed above and incubated at room temperature for 30minutes. The absorbance was read at 560nm spectrophotometrically. The concentration of hydrogen peroxide generated was extrapolated from the standard curve.

e)Total Plasma Peroxide (TPP)/Reactive Oxygen Species (ROS)

Total Plasma Peroxide (TPP) levels were determined using the ferrous oxidation (FOX2) method (Miyazawa, 1989) with minor modifications (Yeni *et. al.*, 2005).The FOX2 system

is based on oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) by various types of peroxides contained within the plasma samples to produce a coloured ferric-xylenol orange complex whose absorbance can be measured.

Procedure:

Two hundred (200) µL of plasma + 1800µL FOX-2 reagent was incubated at room temperature (25^oC) for 30 mins. The mixture was then centrifuged at 3000rpm for 10 mins and absorbance of the supernatant measured at 560nm. The TPP/ROS content of the samples were determined as a function of the absorbance difference between test and blank tubes using a solution of H_2O_2 as standard and the results expressed as µmol/min/mg protein.

f)Reduced (GSH)and Oxidized (GSSG)Glutathione Levels

The method of Beutler *et. al.* (1963) was followed in estimating the level of reduced glutathione (GSH). The reduced form of glutathione comprises in most instances the bulk of cellular non-protein sulfhydryl groups. This method is therefore based upon the development of a relatively stable (yellow) colour when 5', 5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) is added to sulfhydryl compounds. The chromophoric product resulting from the reaction of Ellman reagent with the reduced glutathione, 2- nitro-5-thiobenzoic acid possesses a molar absorption at 412nm which was read at 412nm in a spectrophotometer.

Procedure:

An aliquot of the sample was deproteinised by the addition of an equal volume of 4% sulphursalicyclic acid. This was centrifuge at 10,000g for 5 minutes at 4^oC. Thereafter, 0.5ml of the supernatant was added to 4.5ml of Ellman's reagent. A blank was prepared with 0.5ml of the diluted precipitating agent and 4.5ml of Ellman's reagent. Reduced

glutathione, GSH, is proportional to the absorbance at 412nm. For GSSG determination, 2-vinylpyridine was used to derivatize GSH (to prevent oxidation of GSH to GSSG) (Puertas*et. al.*, 2012). Data are presented as Units (GSH / GSSG) per mg of total protein.

g)Superoxide Dismutase (SOD) Activity

The level of SOD activity was determined by the method of Misra and Fridovich (1972).The ability of superoxide dismutase to inhibit the auto-oxidation of epinephrine at pH 10.2 makes this reaction a basis for simple assay for dismutase. Superoxide (O^{2-}) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adenochrome and the yield of adenochrome produced per O^{2-} introduced increased with increasing pH and also increased with increasing concentration of epinephrine.

Procedure:

A 1ml sample was diluted in 9ml of distilled water to make a 1 in 10 dilution. An aliquot of 0.2ml of the diluted sample was added to 2.5ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3ml of freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5ml buffer, 0.3ml of substrate (adrenaline) and 0.2ml of water. The increase in absorbance at 480nm was monitored every 30 seconds for 150 seconds.

Calculation:$\text{Increase in absorbance per minute} = \text{A3} - \text{A0}/2.5$

Where A0 = absorbance after 30 seconds

A3 = absorbance after 150 seconds

$$\%\ \text{inhibition} = 100 - \left(\%\ \text{increase in absorbance for substrate} - \text{increase in absorbance of blank}\right)$$

One unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline. Results are expressed as units mg^{-1} protein.

h)Glutathione Peroxidase Assay

This is based on coupled oxidation. Catalase or peroxidase combines with hydrogen peroxide as it is liberated, and the complex thus formed then brings about the oxidation of substances such as nitrite, ethanol, cytochrome C or manganese ions in the presence of p-cresol. The extent of these oxidations is measured spectrophotometrically at 412nm (Rotruck *et. al.*, 1973).

Assay Protocol:

Phosphate buffer	500µl
NaN3	100µl
GSH	200µl
H_2O_2	100µl
Sample	500µl
Distilled water	600µl

The whole reaction mixture was incubated at 37°C for 3 minutes after which 0.5ml of TCA was added and thereafter centrifuged at 3000rpm for 5 minutes. To 1ml of each of the supernatants, 2ml of K_2HPO_4 and 1ml of DTNB was added and the absorbance was read at 412nm against a blank.The enzyme activity was calculated from the formula:

$$GPx\ activity = (OD\ of\ unknown\ /\ OD\ of\ Standard)x\ (Standard\ concentration\ /\ volume\ of\ serum\ in\ ml)x\ 1$$

i)Estimation of Malondialdehyde (MDA) (Niehaus and Samuelson,1968)

Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation.Malondialdehyde react with 2-thiobarbituric acid (TBA) in acidic pH to form a pink complex that can be measured spectrophotometrically at 532nm.

Procedure:

A 200µl of the sample was deproteinised with 0.5ml of Trichloroacetic acid (TCA) and centrifuged at 3000rpm for 10mins. One (1) ml of 0.75% TBA was added to 0.1ml of supernatant and boiled in water bath for 20 minutes at 100ºC and then cooledon iced water.

The absorbance was read spectrophotometrically at 535nm.

The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex at 1.56 x 10^5 cm^{-1} M^{-1}, and expressed in µmol TBARS mg^{-1} tissue protein.

j) Serum Total Cholesterol

Aliquots of serum were taken for determination of total cholesterol by enzymatic colorimetric assay method of Allain *et. al.* (1974).The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

$$\text{Chol Ester} + \text{H}_2\text{O} \xrightarrow{\text{Chol esterase}} \text{Chol} + \text{FFA}$$

$$\text{Chol} + \text{O}_2 \xrightarrow{\text{Chol oxidase}} \text{Cholestene} - 3 - \text{one} + \text{H}_2\text{O}_2$$

$$\text{H}_2\text{O}_2 + \text{Phenol} + 4 - \text{aminontipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + \text{H}_2\text{O}$$

$(\lambda = 546\text{nm})$

Into separate cuvettes were pipetted the following:

	Reagent blank (µl)	Standard (µl)	Sample (µl)
Distilled H_2O	10	-	-
Standard	-	10	-
Sample	-	-	10
Reagent	1000	1000	1000

The contents were mixed and incubated for 10 min at 25°C. The absorbance was measured (at 546nm) of the sample (A_{sample}) against the reagent blank within 60 minutes.

Calculation:

$$\text{Conc. of cholesterol in sample} = \left(\frac{\Delta\text{Asample}}{\Delta\text{Astandard}}\right) \text{ x Conc. of standard}$$

k) Triacylglycererol

Triglycerides concentration in the serum was determined according to the Burtis*et. al.* (2008) method as specified by the manufacturer of the kit. The triglycerides are determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen-peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

$$\text{Triglycerides} + H_2O \xrightarrow{\text{Lipases}} \text{glycerol} + \text{FFA}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol } 3-\text{P} + \text{ADP}$$

$$\text{Gly } 3-\text{P} + O_2 \xrightarrow{\text{GPO}} \text{DHAP} + H_2O_2$$

$$H_2O_2 + 4-\text{aminophenazone} + 4-\text{chlorophenol} \xrightarrow{\text{POD}} \text{quinoneimine} + \text{HCl} + 4\text{H2O}$$

($\lambda = 546\text{nm}$).The protocol was as follows.

The following were pipetted into test tubes:

	Reagent blank (µl)	**Standard (µl)**	**Sample (µl)**
Sample	-	-	10
Standard (CAL)	-	10	-
Reagent (RI)	1000	1000	1000

They were then mixed and incubated for 10 min.at 25°C. The absorbance was measured(at 546nm) of the sample (A_{sample}) and standard ($A_{standard}$) against the reagent blank within 60 minutes.

Calculation:

$$\text{Triglyceride concentration (mg/dl)} = \left(\frac{\text{Asample}}{\text{Astandard}}\right) \text{x standard conc.}$$

l) HDL-cholesterol

Serum high density lipoprotein (HDL)-cholesterol was assayed by precipitation of chylomicrons; Low density lipoproteins (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. Aftercentrifugation, the cholesterol concentration in the HDL (high density lipoprotein) fraction, which remains in thesupernatant, is determined ($\lambda = 546\text{nm}$) (Burtis*et. al.,* 2008).

Protocol:

1. **Precipitation**

   Into centrifuge tubes were pipetted the following:

Sample/standard	200 µl
Diluted/precipitant (RI)	500 µl

   Contents were mixed and allowed to sit for 10 minutes at room temperature (37°C). Then centrifuged for 10 minutes at 4000 rpm.

The clear supernatant was separated off within two hours and the cholesterol content determined as below.

2. Cholesterol CHOD – PAP Assay

The following were pipetted into cuvettes:

	Reagent blank SO	**Standard SI**	**Sample**
ddH_2O	50 µ1	-	-
Standard supernatant	-	50 µ1	-
Sample supernatant	-	-	50 µ1
CHOL reagent	500 µ1	500 µ1	500 µ1

Then mixed and Incubated for 10 min at 25°C. Absorbance was read at 546nm within 60 minutes.

Calculation:

$$\text{Concentration of HDL } \left(\frac{\text{mg}}{\text{dl}}\right) = \left(\frac{\Delta\text{Asample}}{\Delta\text{Astandard}}\right) \text{x Conc. of standard}$$

m)Low Density Lipoprotein (LDL)andVery-Low Density Lipoprotein (VLDL)

Estimation of low density lipoprotein (LDL)-cholesterol and very-low density lipoprotein (VLDL) was done by using empirical formula of Friedewald *et. al.* (1972) according to the respective formulae below:

$$LDL\ Cholesterol = Total\ Cholesterol - \frac{Triglyceride}{5} - HDL\ Cholesterol$$

$$VLDL = \frac{Triglycerides}{5}$$

n)Liver Marker Enzymes and Metabolites

Serum Aspartate Aminotransferase (AST) andserum Alanine Aminotransferase (ALT) were evaluated using the method of Reitman and Frankel (1957); serum alkaline phosphatase (ALP)according to the recommendations of the Deutsche Gesellschaft Fiir Klinische Chemie (DGKC), (1972); total protein (TP) using direct biuret method (Burtis*et. al.,* 2008)

while total bilirubin (TB) according to the method of Jendrassik and Grof (1938); all as described by Randox laboratories, using Randox kits (United Kingdom).

AST is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenylhydrazine ($\lambda = 546\text{nm}$).

α-oxoglutarate +L-aspartate $\xrightarrow{\text{AST}}$ L-glutamate + Oxaloacetate.

Protocol:Pipetted into test tubes are the following:

	Sample blank	**Sample**
Sample	-	0.1ml
Reagent I	0.5ml	0.5ml
These were then mixed and incubated for exactly 30 min. at 37°C. Then the following were added accordingly, mixed and allowed to stand for exactly 20min. at 25°C		
Reagent 2	0.5ml	0.5ml
Sample	0.1ml	-
Finally, sodium hydroxide was added as follows.		
Sodium Hydroxide	5.0ml	5.0ml

The contents were mixed and the absorbance of the sample (A_{sample}) read against the sample blank after 5 minutes.

Calculation:

The activity of AST in the serum was obtained from the table below (see appendix XII a).

ALT is measured by monitoring the concentration of pyruvate formed with 2,4-dinitrophenylhydrazine ($\lambda = 546\text{nm}$).

α-oxoglutarate +L-alanine $\xrightarrow{\text{ALT}}$L-glutamate + pyruvate.

Protocol:

Pipetted into test tubes were the following:

	Sample Blank	**Sample**
Sample	-	0.1ml
Solution RI	0.5ml	0.5ml
These were mixed and Incubated for exactly 30 min. at 37°C, then the following added.		
Solution R2	0.5ml	0.5ml
Sample	0.1ml	-
The contents were mixed again and allowed to stand for exactly 20 min. 25°C. Sodium hydroxide was later added as follows, mixed and the absorbance of the sample (A_{sample}) read against the sample blank after 5 minutes.		
Sodium Hydroxide	5.0ml	5.0ml

Calculation:

The activity of ALT in the serum was obtained from the table below (see appendix XII b).

Colorimetric Method for ALP: This is an optimized standard method according to the recommendations of the Deutsche Gesellschaft Fiir Klinische Chemie (DGKC, 1972).

p-nitrophenylphosphate + H2O$\xrightarrow{ALP}$phosphate + p-nitrophenol ($\lambda = 405nm$).

Protocol:Pipetted into cuvettes were the following:

	Semi – Micro method
Sample	0.02ml
Reagent (25°C)	1.00ml

Contents were mixed and initial absorbance and start timer simultaneously read. The absorbances and times were read again after 1, 2 and 3 minutes.

Calculation:

The ALP activity was calculated using the following formula:

U/I = 2760 X ΔA_{405} nm/Min.

Total Protein: Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a coloured complex ($\lambda = 546nm$) (Burtis*et. al.*, 2008).

Protocol:

The following were pipetted into cuvette:

	Reagent Blank	Standard	Sample
Distilled H_2O	0.02ml	-	-
Standard (CAL)	-	0.02ml	-
Serum or Plasma	-	-	0.02ml
RI	1.0ml	1.0ml	1.0ml

The contents were mixed and incubated for 30 min at 25°c. The absorbance of the sample (A_{sample}) and of the standard ($A_{standard}$) were measured against the reagent blank.

Calculation:

$$\text{Total Protein Conc.} = \left(\frac{\text{Asample}}{\text{Astandard}}\right) \text{x standard conc.}$$

Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction with diazotised sulphanilic acid ($\lambda = 578\text{nm}$) - Colorimetric method as described by Jendrassik and Grof (1938).

Protocol:

Reagents R1, R2, R3 and sample were mixed and allowed to stand for 10min at 25°C. Then R4 was added, mixed and allowed to stand for 30 min at 25°C, and then the absorbance of the sample against the sample blank (A_{TB}) read.

Calculation:

Total Bilirubin (mg/dl) = 10.8 x A_{TB} (578 nm)

o)Kidney function tests

Estimation of serum urea: This was done following the method of Bauer *et. al.*. (1982). Urea in serum is hydrolyzed to ammonia in the presence of urease. The ammonia is then measured photometrically by Berthelot's reaction. Urea + $H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$

NH_3 + hypochlorite + phenol → indophenol (blue compound) ($\lambda = 546\text{nm}$). The Protocol was as follows.Pipetted into test tubes were the following:

	Blank	Standard	sample
Sample	-	-	10µl

Standard (CAL)	-	10 µl	-
Distilled Water	10 µl	-	-
Reagent 1	100 µl	100 µl	100 µl
These were mixed and incubated at 37°C for 10 min. then R2 and R3 added as follows:			
Reagent 2	2.50ml	2.50ml	2.50ml
Reagent 3	2.50ml	2.50ml	2.50ml
These were also mixed Immediately and incubated at 37°C for 15 min.			

Absorbance of the sample (A_{sample}) and standard ($A_{standard}$) against the blank was then read.

Calculation:

[Serum Urea] (mg/dl)= $\frac{A_{sample}}{A_{standard}}$ x standard conc.

Estimation of serum creatinine: Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration($\lambda = 492nm$) (Butler, 1975).

Protocol:The following were pipetted into cuvettes:

	Standard Semi Micro	Sample Semi Micro
Working reagent	1.0ml	1.0ml
Standard solution	0.1ml	-
Sample	-	0.1ml

Then mixed and after 30 seconds the absorbance A_1 of the standard and sample read. Exactly 2 minutes later absorbance A_2 of standard and sample were also read.

Calculation:

$A_2 - A_1 = \Delta A$ sample or ΔA standard

Concentration of creatinine in serum was obtained by the following formula.

[Creatinine] (mg/dl)= $\frac{\Delta A \text{ sample}}{\Delta A \text{ standard}}$ x standard conc.

3.2.7 Bioassay-guided studies

3.2.7.1 Liquid–liquid partition of GS and CM aqueous extract

The dry,lyophilized extracts from leaves of GS and CM(100g each), were separately redissolved in 500 ml of 60% methanol. A successive partition of the extracts yielding hexane (4.59 g), ethyl acetate (4.2 g) and residual 60% methanol (33.10 g) GS fractions and of the CM extract affording hexane (13.52 g), ethyl acetate (7.44 g) and residual 60% methanol (26.60 g) fractions were subsequently subjected to tests of antidiabetic activity. The bioassay-guided fractionation was only carried out with the 60% methanol fractions of each plant extract (GS and CM) because they were the most active in reducing the FBG in the animals, gave the highest yield with varied phytochemical contents and good α-amylase and α-glucosidase inhibition (i.e. lowest IC_{50} values).

3.2.7.2 Column chromatographyof the 60% methanol fractions of GS and CM

i)Fractionation

This was done according to the work of Ode *et. al.*(2011).Twenty-five (25) g of the 60% methanol fraction of GS and CM found to have highest hypoglycaemic effect were subjected to column chromatography. Silica gel 60 - 120G was used as the stationary phase while varying solvent combinations of increasing polarity were used as the mobile phase. In the setting up of column chromatography, the lower part of the glass column (5.5 x 80cm) was stocked with glass wool with the aid of glass rod. The slurry prepared by mixing 450 g of silica gel and 500 ml of hexane was poured down carefully into the column. The tap of the glass column was left open to allow free flow of solvent. At the end of the packing process, the tap was locked. The column was allowed 24 h to stabilize, after which, the clear solvent on top of the silica gel was allowed to drain down to the silica gel meniscus. The sample was prepared by adsorbing 25 g of the extract to 50 g of silica gel (60 - 120G) in methanol and allowed to dry. Small amount of selide was added to the dry powder and stirred to mix very well, and then gently layered on top of the column. The

column tap was opened to allow the eluent to flow at the rate of 0.58ml per second. Elution was done with solvent systems of gradually increasing polarity using hexane, ethyl acetate and methanol. The following ratios of solvent combinations were sequentially used in the elution process; hexane:ethyl acetate 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40: 60, 30:70, 20: 80, and 10:90; ethylacetate: methanol 100:0, 70:30, 50:50, 30:70 and 0:100. The eluted fractions were collected in aliquots of 50 ml in conical flasks.

One hundred and thirty (130) fractions of GS were collected and monitored by TLC (mobile phase n-hexane/ethyl acetate 30:70), generating sixteen sub-fractions which were subjected to antidiabetic activity. Sub-fraction D (0.169 g, Rf = 0.66)showed the highest reduction trend in antidiabetic activity and, therefore, chosen for further fractionation and bioassay.

In the case of CM, 171 fractions were collected and monitored by TLC (mobile phase n-hexane/ethyl acetate 30:70), generating 7 sub-fractions which were subjected to antidiabetic activity. Sub-fraction G (2.8 g, Rf = 0.83) showed the highest reduction trend in antidiabetic activity and, therefore, chosen for further fractionation and bioassay. The 1:1 combinations (GSCM) of the plants' fractions were also used for the *in vivo* bioassay screening for antidiabetic activity.

iii)Further bioassay-guided fractionation with Preparative TLC and screening of the fractions

The sub-fractions D and G above were subjected to preparative thin-layer chromatography on glass plates with silica gel 60-120G using n-hexane/ethyl acetate (3:7) as mobile phase. Commercially – prepared TLC plates (Merck, Germany) were used for this study.

Each plate was viewed under daylight, ultra-violet lamp at 254 and 365 nm in a dark room as well as by iodine vapour.

For detection of phenols, sugars, steroids, and terpenes the plates were sprayed with a solution of freshly prepared 0.5ml p-anisaldehyde in 50ml glacial acetic acid and 1ml 97% sulphuric acid and heated to 105°C until maximum visualization of spots. Lichenconstituents, phenols, terpenes, sugars, and steroids turn violet, blue, red, grey or green.

For detection of sugars, plates were sprayed with a solution of freshly prepared 1ml p-anisaldehyde, 1ml 97% sulfuric acid in 18ml ethanol and heated at 110°C.Sugar phenylhydrazones produce green-yellow spots in 3 min. Sugars will produce blue, green, violet spots in 10min. Also detects digitalis glycosides.

iv) Elution of separated bands: The bands were removed from the plates with silica and extracted with absolute methanol. The eluent was separated from the adsorbent by filteringthrough a small No. 3 SINTAGLASS funnel, GALLENKAMP, England. This process was repeated until it was satisfactory that all the eluent has been collected as much as possible. The collected eluent in methanol wasevaporated to dryness at room temperature (37°C). The eluents were subjected to further bioassay test to confirm hypoglycaemic activity. Eluents that exhibited maximal *in vivo* hypoglycaemic ability (sub-sub-fractions D4 with Rf = 0.26 and G4 with Rf = 0.36)were then labelled as the active sub-fraction(s) and subjected to LC-MS, *in vitro* α-amylase/α-glucosidase inhibitory activities and NMR/FTIR analyses.

3.2.8*In vitro*alpha-amylase and alpha-glucosidase inhibition assay

The α-amylase and α-glucosidase inhibitory activities of samples were determined according to the method described by Kim*et. al.*(2000). For alpha glucosidase inhibition, yeast α-glucosidase was dissolved in 100 mM phosphate buffer, pH 7.0, containing bovine serum albumin 2 mg/ml and sodium azide 0.2 mg/ml which was used as enzyme source.

Paranitrophenyl- α-d-glucopyranosidewas used as substrate. Samples were weighed and serial dilutions of 50, 100, 150, 200, 250µg/ml were made up with equal volumes of dimethylsulfoxide and distilled water. Then 10µl of extract dilutions was incubated for 5 min with 50µl enzyme source. After incubation, 50µl of substrate was added and further incubated for 5 min at room temp. The pre substrate and post substrate addition absorbance was measured at 405 nm. The increase in absorbance on substrate addition was obtained. Each test was performed three times and the mean absorbance was used to calculate percentage α-glucosidase inhibition.

Acarbose was used as positive control with various concentrations 50, 100, 150, 200, 250 µg/ml. Percentage α glucosidase inhibition was calculated according to the following formula (Jung*et. al.*, 2006).

$$\text{Percentage of inhibition} = \frac{[(\text{Control } A_{405} - \text{Extract } A_{405})]}{\text{Control } A_{405}} \times 100$$

The alpha amylase inhibition activity of the plant was assayed by the same method used for alpha glucosidase inhibition activity. For alpha amylase inhibition assay the enzyme porcine pancreatic amylase and substrate Paranitro phenyl alpha D- maltopentoglycoside were used and the absorbance measured at 540nm (Jung *et. al.*, 2006). Percentage inhibition was calculated as follows:

$$\text{Percentage of inhibition} = \frac{[(\text{Control } A_{540} - \text{Extract } A_{540})]}{\text{Control } A_{540}} \times 100$$

3.2.9*In vivo* bioassay screening of fractions

3.2.9.1 Experimental design (liquid-liquid partition)

The fractions from the liquid-liquid parttition stage were used for this phase of the study. Mice of both sexes were weighed and assigned to groups of 3 mice each. All the mice were

fasted for 12h prior to commencement of the experiment. A total of 27 mice were used for this phase of the experiment and grouped as outlined below:

Group I (GShex): given GS hexane fraction (600 mg/kg b. w.) orally;

Group II (GSetac): given GS ethyl acetate fraction (600 mg/kg b. w.) orally;

Group III (GSmeoh): given GS 60% methanol fraction (600 mg/kg b. w.) orally;

Group IV (CMhex): given CM hexane fraction (600 mg/kg b. w.) orally;

Group V (CMetac): given CM ethyl acetate fraction (600 mg/kg b. w.) orally;

Group VI (CMmeoh): given CM 60% methanol fraction (600 mg/kg b. w.) orally;

Group VII (normal control – non diabetic): given only distilled water (10 ml/kg) orally;

Group VIII (negative control - diabetic): received only distilled water (10 ml/kg) orally;

Group IX (positive control - diabetic): received glibenclamide (6 mg/kg) orally;

All the blood glucose concentrations (for determining hypoglycaemic activity) in the mice were determined as described earlier.

3.2.9.2Experimental design (bioassay screening)

The sub-fractionsfrom the column chromatography stage were used for this phase of the study. Mice of both sexes were weighed and assigned to groups of 4 mice each. All the mice were fasted for 12h prior to commencement of the experiment. A total of 100micewere used for this phase of the experiment and grouped as outlined below:

Normal control group (non-diabetic), given only distilled water (10 ml/kg) orally;

Negative control (diabetic) group, receivedonly distilled water (10 ml/kg) orally;

Positive control (diabetic) group, received glibenclamide (6 mg/kg) orally;

Groups(A – O)were given oral treatments with 40 mg/kg each of the pooled *G. sylvestre*fractions (A – O) of the extracts respectively eluted from the chromatographic/TLC experiments.

Groups (B' – G') were given oral treatments with 40 mg/kg each of the pooled *C. micranthum* fractions (B – G) of the extracts respectively eluted from the chromatographic/TLC experiments.

Group Z were given oral treatments with 40 mg/kg of 1:1 combination of fractions D and G of *G. sylvestre* and *C. micranthum*.

All the blood glucose concentrations (for determining hypoglycaemic activity) in the mice weredetermined as described earlier.

3.2.10Further bioassay-guided fractionation -preparative TLC

3.2.10.1Experimental Design (Further Bioassay-Guided Screening)

Sub- fractions D and G from the fractionation abovewere selected for re-fractionation and further studies. Mice of both sexes were weighed and assigned to groups of 4 mice each. All the mice were fasted for 12h prior to commencement of the experiment. A total of 60 mice were used for this phase of the experiment and grouped as outlined below:

Group A^1 (normal control – non diabetic), given only distilled water (10 ml/kg) orally;

Group B^1 (negative control - diabetic), received only distilled water (10 ml/kg) orally;

Group C^1 (positive control - diabetic), received glibenclamide (6 mg/kg) orally;

Groups D^1, E^1, F^1, G^1, and H^1, were given oral treatments with 10 mg/kg body weight each of the sub-fractions of*G. sylvestre* (D1, D2, D3, D4, and D5) respectively eluted from the preparativeTLC experiments.

Groups I^1, J^1, K^1, L^1, M^1 and N^1 were given oral treatments with 10 mg/kg each of the sub-fractions of*C. micranthum* (G0, G1, G2, G3, G4 and G5) respectively eluted from the preparativeTLC experiments.

Group O^1 animals were given oral treatments with 10 mg/kg body weight of 1:1 combination of sub-sub-fractions D4 and G4 of *G. sylvestre* and *C. micranthum*.

All the blood glucose concentrations (for determining hypoglycaemic activity) in the mice weredetermined as described earlier.

3.2.11 Identification of compounds

3.2.11.1 UHPLC-ESI- Q-TOF-MS/MS

The UHPLC system (Agilent infinity 1290, USA) was incorporated with a binary pump (G4220A), an auto sampler (HiP - ALS, G4226A), degasser (G1322A), Thermostatted Column Compartment (G1316C) and a DAD detector (G4212A) with a light-pipe flow cell (recording at 280 and 320 nm). This was coupled to Ultra-High-resolution-Quadrupole-Time-of-Flight Mass Spectrometer (G6550A) (USA) equipped with an ESI source (DUAL AJS ESI) operating on Auto-MS/MS mode. The analysis was achieved in the negative ion mode in a mass range from m/z 100-2000. The ESI source parameters were: capillary voltage 3.5 KV; nebulizing gas pressure 2.4 bar; drying gas temperature 200.0°C, drying gas flow 14.0 l/min; iFunnel 1RF750.0 V; transfer time 50.0 µs; and pre-pulse storage 2.0 µs. The MS data were analyzed through Agilent Mass Hunter Qualitative Analysis software (B.06.00).

The UHPLC separation was achieved on a ZORBAX Eclipse Plus C18, Rapid Solution, 4.6x100mm, particle size 3.5micron column (Agilent Technologies, USA). Solvent A was water:methanol (60:40 v/v) and solvent B was acetonitrile (100%). Solvents were delivered at a total flow rate of 0.5 ml/min. The gradient profile was from 0% B (100%A) to 100% B (0%A) linearly in 50 min (Table 3.1). The injection volume was 2.00 µl. (Satheeshkumar *et. al.*, 2014; Karar and Kuhnert, 2015 – with modifications).

Table 3.1: Timetable of Solvent Gradient Prolfile of LC-MS Run

	Time	A	B	Flow	Pressure
1	5.00 min	100.00 %	0.00 %	--- mL/min	--- bar
2	10.00 min	80.00 %	20.00 %	--- mL/min	--- bar
3	15.00 min	60.00 %	40.00 %	--- mL/min	--- bar
4	20.00 min	40.00 %	60.00 %	--- mL/min	--- bar
5	25.00 min	20.00 %	80.00 %	--- mL/min	--- bar
6	30.00 min	0.00 %	100.00 %	--- mL/min	--- bar

3.2.11.2 Fourier Transform Infrared (FTIR) Spectroscopy

FTIR follows the Beer-Lambert law, thus the absorbance of a sample is directly proportional to the concentration, the pathlength and the molar absorptivity. Since the molar absorptivity of a molecule is constant and the measurement pathlength can be controlled, the absorbance of a sample is directly proportional to the concentration. The FTIR sampling methodologies used are attenuated total reflectance (ATR) and diffuse reflectance infrared Fourier transform (DRIFT) since it allows quick measurement of neat solids without any sample preparation. All measurements were carried out on an Agilent Cary 630 FTIR incorporating a diffuse reflectance sample interface. Data was recorded with a 30 second collection time (74 scans) at 4 cm^{-1}resolution with a scanning range of 4000 to 650cm^{-1}. The Cary 630 MicroLab PC software was used for data collection, and calibrations were developed using Agilent Resolutions Pro software (Seelenbinder and Higgins, 2011; Fadare *et. al.*, 2015).

3.2.11.3 Structural Elucidation (NMR)

Nuclear magnetic resonance (NMR) spectra were recorded on anAgilent Vnmrs4000 (400 MHz) spectrometer (Germany). Chemical shifts (δ) are expressed in ppm with reference to

the trimethylsilyl (TMS) signal. All solvents used for the analysis were deuterated HPLC-grade (Fisher Scientific, Springfield, NJ); HPLC-grade water was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA).

3.2.12Molecular docking

3.2.12.1 Ligand structures

All ligand structures were optimized by using Merck Molecular Force Field (MMFF) and the semi-empirical Austin Model (AM1) method, both of which are implemented in Spartan 14 software (version 14.1.4, Wavefunction Inc, http://www.wavefun.com/) in order to remove all strain from the molecular structure. In addition this will ensure a well defined conformer relationship among compounds of the study (Viswanadhan *et. al.*, 1989). From the setup calculation option on Spartan 14, the calculation was set to equilibrium geometry at the ground state using density functional theory at B3LYP (Becke88 three-parameter hybrid exchange potentials with Lee-Yang-Parr correlation potential) level of theory and 6-311G (d) basis set for the geometrical optimization of the cleansed structures i.e.B3LYP/6-311G (d) level of theory. After optimization, Spartan molecular descriptors were obtained from the display-output and display-properties option on Spartan 14 GUI. The fully optimized 3D structure without symmetry restrictions, were saved as SD file through the file option on the Spartan 14 GUI.

3.2.12.2 Enzyme structure

The X-ray crystal structures of transferase (phosphorylated insulin receptor tyrosine kinase) (PDB ID: 1IR3), Alpha-glucosidase (PDB ID: 3WY2) and Human pancreatic alpha-amylase (PDB ID: 4W93) with resolutions of 1.9Å, 1.47Å and 1.35 Å respectively were downloaded from RCSB Database(http://www.rcsb.org/pdb). A high degree of structural similarity (70%) between porcine pancreatic alpha-amylase and human pancreatic alpha-amylase allows its usage in this study as the enzyme for screening inhibitors (Brayer *et. al.*, 1995; Ponnusamy*et. al.*,2015).

3.2.12.3 Docking simulations

All protein preparation and minimization was done using tools and protocols in the Molsoft ICM-pro 3.8-3. A Chemistry at Harvard Macromolecular Mechanics (CHARMm) force field was used to optimize the structure. While using the protein preparation protocol, hydrogen atoms were added to the complex, after which water molecules were removed and the pH of the protein was set to almost neutral value. A sphere binding site with a nine Armstrong A˚ radius was defined around the bonded ligand to identify the binding site of the protein structure. The SD files of the ligands were then imported into icm pro and they were used to dock the prepared receptors. The ligands are scored based on the biased probability Monte Carlo (BPMC) procedure, which randomly selects a conformation in the internal coordinate space and then makes a step to a new random position independent of the previous one but according to a predefined continuous probability distribution. The results of the best scored, binding energy and inhibition constants of all the ligands were reported on a table.

3.2.13Statistical analysis

IC_{50} calculations were performed with Microsoft Excel. Results were presented as mean $\pm$ standard error of mean (SEM). Statistical analyses were performed using two tailed Student's t-test at 95% confidence limit (i.e. $P < 0.05$ being significant); Pearson's Correlations were also run to make comparisons between parameters. SPSS version 17.0 computer software/programme was used to perform the statistical calculations.

CHAPTER FOUR

4.0 RESULTS

4.1 Extractions Yields

Methanol extracts of *G. sylvestre* (GS) and *C. micranthum* (CM) leaves gave significantly highest yields (33.10 ± 0.06% and 26.60± 0.05% respectively) when compared to theother solvents extracts (i.e. hexane, ethyl acetate and water) (Table 4.1).

4.2 Phytochemical Analyses

Tannins and flavonoids were found to be present in the aqueous, n-hexane, ethyl acetate and 60% methanol extracts of GS and CM whereas Terpenoids were not detected in GS ethyl acetate extract (Table 4.2). Other phytochemicals like anthraqinones and alkaloids were detected in the plants' extracts; saponins were found only in the methanol extracts of the plants (Table 4.2).

Quantitatively, the methanol fraction of GS and CM aqueous extracts showed presence of all phytochemicals assayed for (i.e. tannins, saponins flavonoids and alkaloids) with significantly ($P < 0.05$) high levels of flavonoids in both plants – 25.89 ± 0.06 and 14.74 ± 0.21g/100g dry weight respectively (Table 4.3). Moreover, the GS aqueous leaf extract had better total phenolic and total flavonoids contents than the CM counterpart (Figure 4.1).

Table 4.1: Extraction Yield for *G. sylvestre*and*C. micranthum* Leaf Extracts

Extract type	Extraction yield (%)
GSAq	17.07±0.07
GSHex	4.59±0.14
GSEtac	4.28±0.05
GSMeoh	33.10±0.06
CMAq	18.38±0.04
CMHex	13.52±0.03
CMEtac	7.44±0.04
CMMeoh	26.60±0.05

Values expressed as Mean ± SEM of triplicate measures. GSAq= *G. sylvestre* aqueous extract; CMAq = *C. micranthum* aqueous extract; GSHex= *G. sylvestre* n-hexane extract; CMHex = *C. micranthum* n-hexane extract; GSEtac= *G. sylvestre* ethyl acetate extract; CMEtac = *C. micranthum* ethyl acetate extract; GSMeoh= *G. sylvestre* 60% methanolextract; CMMeoh = *C. micranthum* 60% methanolextract.

Table 4.2:Qualitative Phytochemical Composition of Leaf Extracts of *G. sylvestre* and *C. micranthum*

Sample	Type of extract							
	Aqueous		N-hexane		Ethyl acetate		Methanol-water(60:40)	
Phytochemical	GS	CM	GS	CM	GS	CM	GS	CM
Tannins	+	+	+	+	+	+	+	+
Anthraquinones	+	+	-	+	-	+	+	+
Terpenoids	+	+	+	+	-	+	+	+
Saponins	-	-	-	-	-	-	+	+
Flavonoids	+	+	+	+	+	+	+	+
Alkaloids	-	+	+	-	-	-	+	+

Key: (+) means present; (-) means Not detected.GS = *G. sylvestre;* CM = *C. micranthum.*

Table 4.3:Quantitative Phytochemical Contents of Extractsof *G. sylvestre* and*C. micranthum* Leaves

Extract type	Phytochemical Composition (g/100g dry weight)			
	Tannins	Saponins	Flavonoids	Alkaloids
GSAq	1.48±0.02	ND	10.69±0.01	ND
GSHex	0.47±0.01	ND	6.07±0.07	34.00±0.01
GSEtac	0.27±0.02	ND	8.52±0.04	ND
GSMeoh	3.85±0.08	18.53±0.19	25.89±0.06	6.10±0.10
CMAq	1.06±0.07	ND	3.14±0.03	10.03±0.03
CMHex	0.04±0.01	ND	9.63±0.20	ND
CMEtac	0.27±0.01	ND	11.42±0.05	ND
CMMeoh	1.81±0.02	12.10±0.10	14.74±0.21	6.10±0.06

Values expressed as Mean ± SEM of triplicate measures. GSHex= *G. sylvestre* n-hexane; CMHex = *C. micranthum* n-hexane; GSEtac= *G. sylvestre* ethyl acetate; CMEtac = *C. micranthum* ethyl acetate; GSMeoh= *G. sylvestre* 60% methanol; CMMeoh = *C. micranthum* 60% methano. ND = Not Detected.

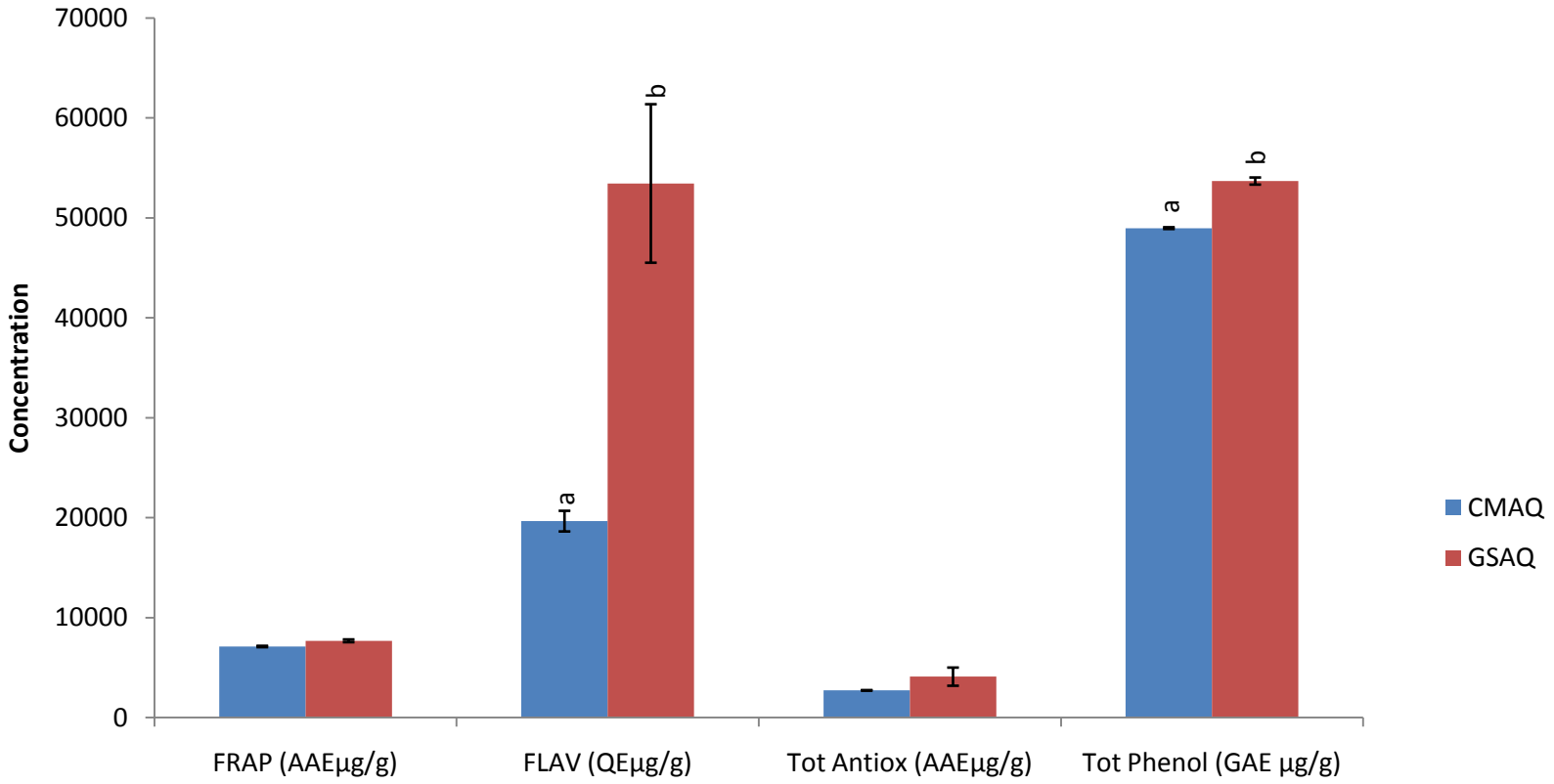


Figure 4.1: Total Flavonoids (FLAV), Total Phenolics (Tot Phenol), Total Antioxidants (Tot Antiox) and Ferric Reducing Antioxidant Power (FRAP) of Aqueous Leaf Extracts of *G. sylvestre* and *C. micranthum*

Values expressed as Mean ± SEM of triplicate measurements, with those bearing different superscripts on the same cluster significantly different ($P < 0.05$).

4.3 Acute Toxicity

LD_{50}:

G. sylvestre and *C. micranthum* leaf aqueous extracts (100-6400 mg kg-1) were found to be safe in mice. Thus, LD_{50} values were found to be >5000 mg kg^{-1}orally in mice, which make the plants to be relatively safe in mice.

4.4Sub-chronic Toxicity

The animals generally did not show much physically observable behavioural toxicity.

4.4.1 Body weight changes

When the rats were treated with 600mg/kg b.w. doses of GS and CM aqueous leaf extracts and their 1:1 combination (GSCM), the GSCM recorded the least percentage increase in weight compared to the separate administration of each plant (Table 4.4).

4.4.2 Feed and water intakes

Only the GS administered group showed significant increase ($P < 0.05$) in feed intake, otherwise all other groups did not yield any change in feed and water intake (Tables 4.5 and 4.6).

4.4.3 Haematological changes

Only the CM and GS 600mg/kg b.w. treated groups showed significant increases ($P < 0.05$) in WBC and MCH respectively when compared with normal untreated control. But there is a general increase in the plasma Hb and PCV levels for the rats given GS, CM and GSCM (Table 4.7).

4.4.4 Relative organsweights

Relative liver weights reduced significantly ($P < 0.05$) in the groups normal + 600mg/kg b.w. GS, (Figure 4.2); normal + 600CM, (Figure 4.3); and normal + 600GSCM (Figure 4.4). The relative kidney weights displayed no significant change (Figures 4.2, 4.3, 4.4).

Table 4.4: Percentage (%) Change in Body Weight of Normal Rats Administered *G.sylvestre*, *C.micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts

Group/Treatment	Percentage (%) Change In Body Weight		
	Week 1	Week 2	Week 3
normal control	6.00±0.90	7.29±1.24[a]	10.69±3.06
normal 600 GS	9.29±2.02	14.49±2.54[b]	13.49±3.38
normal 600 CM	3.22±4.85	10.05±6.95	11.30±6.61
normal 600 GSCM	5.66±0.79	6.88±2.58	5.98±4.36

Values are mean ± standard error of mean with those bearing different superscripts under the same column being significantly ($P< 0.05$) different. N = 6.

Table 4.5: Mean and Percentage (%) Change in Feed Intake of Normal Rats Administered *G. sylvestre* (GS), *C. micranthum* (CM) and 1:1 Combination (GSCM) of Their Aqueous Leaf Extracts for 21 Days.

Group	Mean Feed Intake (g/100g b.w./day)		% change
	Pre-treatment	21st day post-treatment	
Normal control	9.14±0.08	6.41±2.58	-30.06±27.67[a]
Normal+600 GS	10.33±0.36	3.06±1.59	-70.86±14.40[b]
Normal+600 CM	9.37±0.13	4.06±0.79	-56.58±8.96
Normal+600 GSCM	9.12±0.72	3.79±1.68	-59.62±15.26

Values expressed as Mean ± SEM with those bearing different superscripts under the same column significantly different ($P < 0.05$); N = 6; (+) preceding a value means % increase in Feed Intake; (-) preceding a value means % decrease in Feed Intake.

Table 4.6: Mean and Percentage (%) Change in Water Intake of Normal Rats Administered *G. sylvestre* (GS), *C. micranthum* (CM) and 1:1 Combination (GSCM) of Their Aqueous Leaf Extracts for 21 Days.

Group	Mean Water Intake (ml/100g b.w./day)		% Change
	Pre-treatment	21st day post-treatment	
Normal control	12.98±0.62	5.29±4.23	-60.75±30.68
Normal+600 GS	13.71±0.72	4.16±2.97	-68.43±23.32
Normal+600 CM	15.08±0.32	7.27±6.15	-50.90±41.82
Normal+600 GSCM	18.36±0.01	4.89±3.67	-73.37±19.99

Values expressed as Mean ± SEM; N = 6; (+) preceding a value means % increase in Water Intake; (-) preceding a value means % decrease in Water Intake.

Table 4.7: Haematological Indices of Normal Rats Administered *G. sylvestre*, *C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts for 21 days.

Group	WBC ($X10^3$/µL)	RBC ($X10^6$/µL)	Hb (g/dl)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)
normal control	6.65±0.87[a]	6.53±0.28	11.77±0.53	43.97±2.40	67.17±1.21	18.02±0.08[a]	26.85±0.37
normal+ 600 GS	10.75±1.70	6.40±0.47	12.2±0.76	44.75±2.95	70.12±1.28	19.15±0.31[b]	27.3±0.25
normal+ 600 CM	10.33±1.27[b]	6.86±0.08	12.97±0.17	47.08±0.87	68.58±0.57	18.9±0.14	27.57±0.26
normal+ 600 GSCM	10.83±0.43	6.69±0.14	12.73±0.19	45.77±1.07	68.52±1.45	19.08±0.55	27.87±0.50

Values are mean ± standard error of mean with those bearing different superscripts within the same column being significantly ($P< 0.05$) different. N = 6.

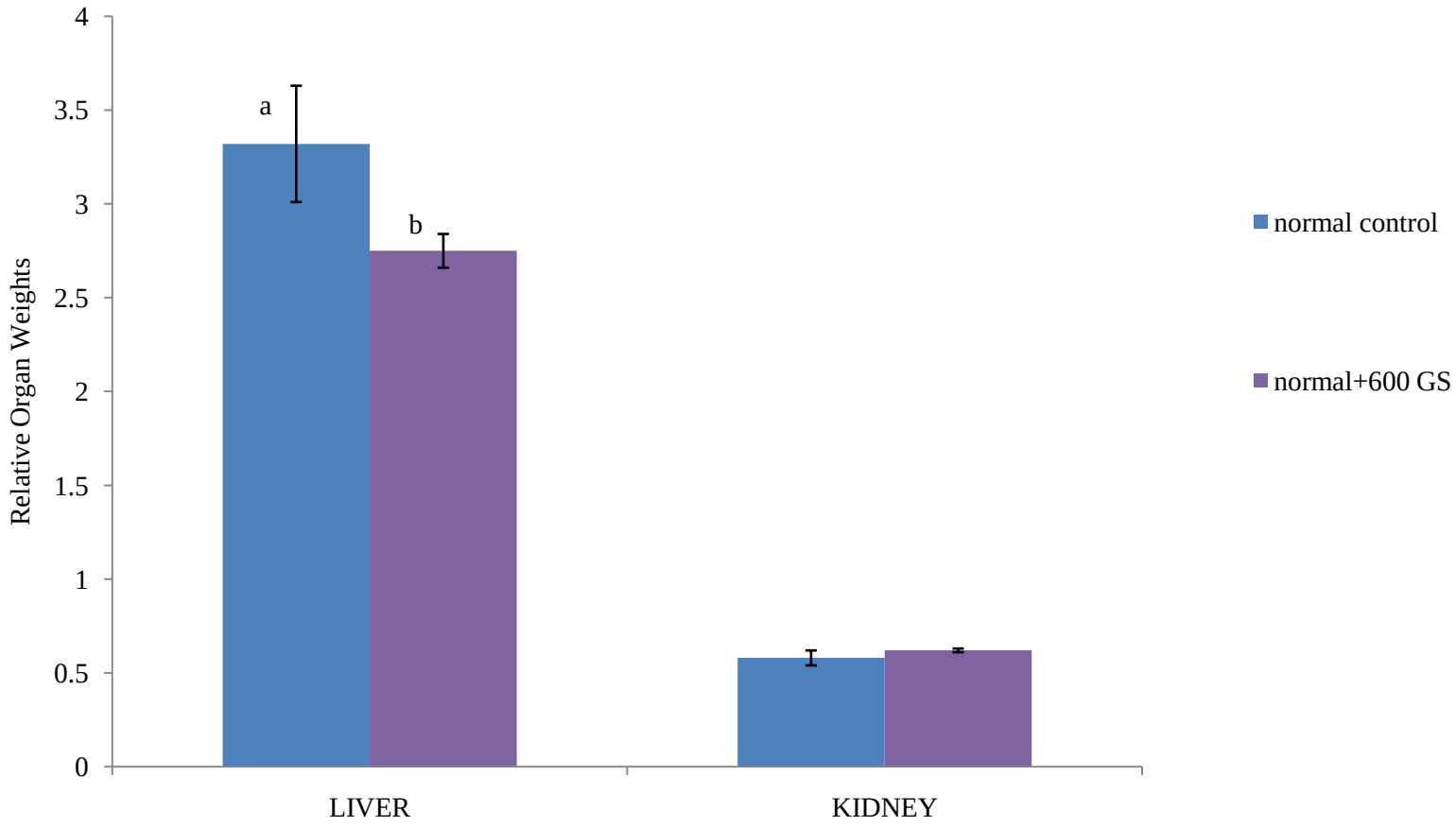


Figure 4.2: Relative Organ Weights of Normal Rats Administered *G. sylvestre* Aqueous Leaf Extract

Values are mean ± standard error of mean (bars) with those bearing different superscripts being significantly ($P < 0.05$) different. N = 6.

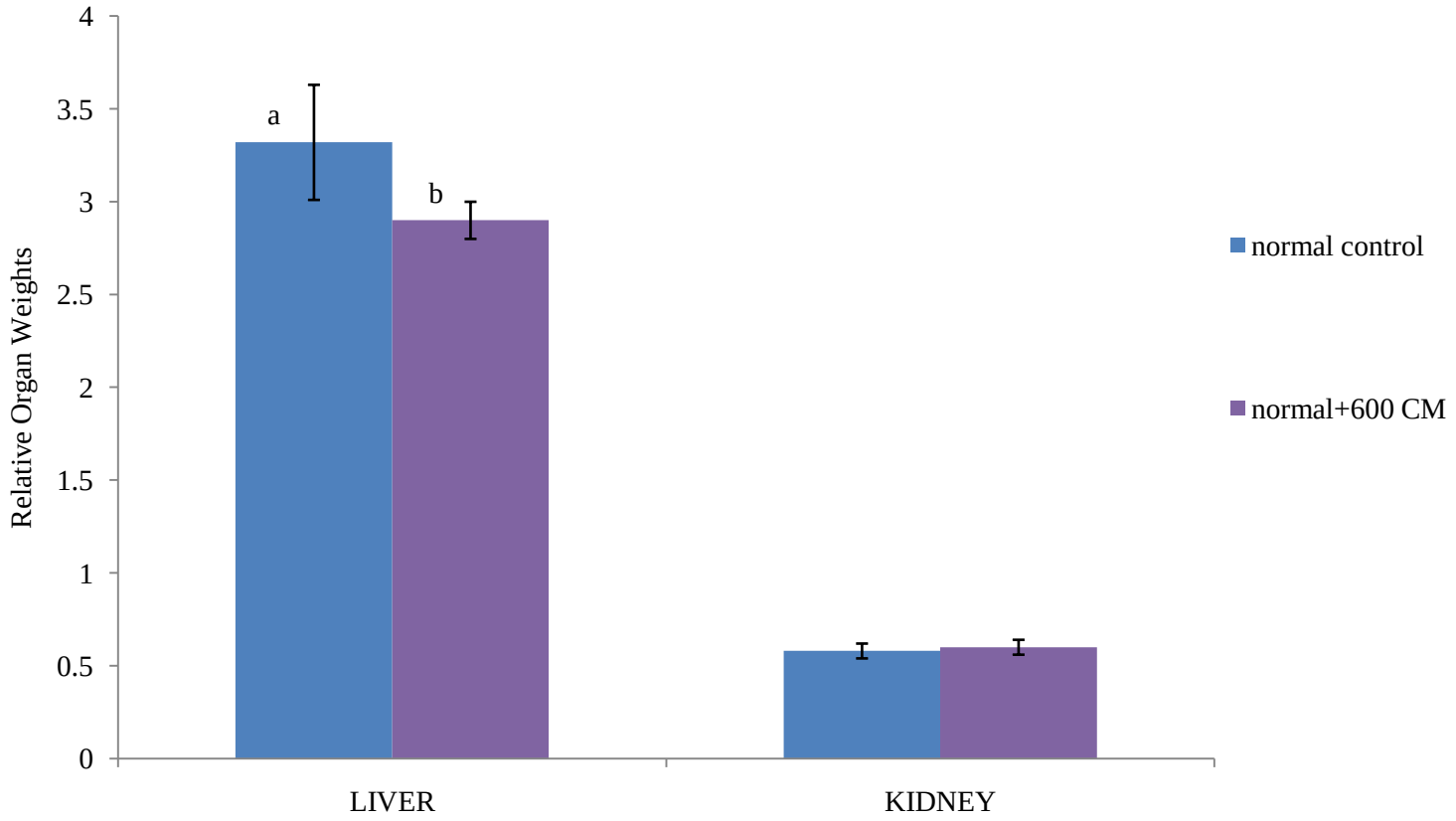


Figure 4.3: Relative Organ Weights of Normal Rats Administered *C. micranthum* Aqueous Leaf Extract

Values are mean ± standard error of mean (bars) with those bearing different superscripts being significantly (P< 0.05) different. N = 6.

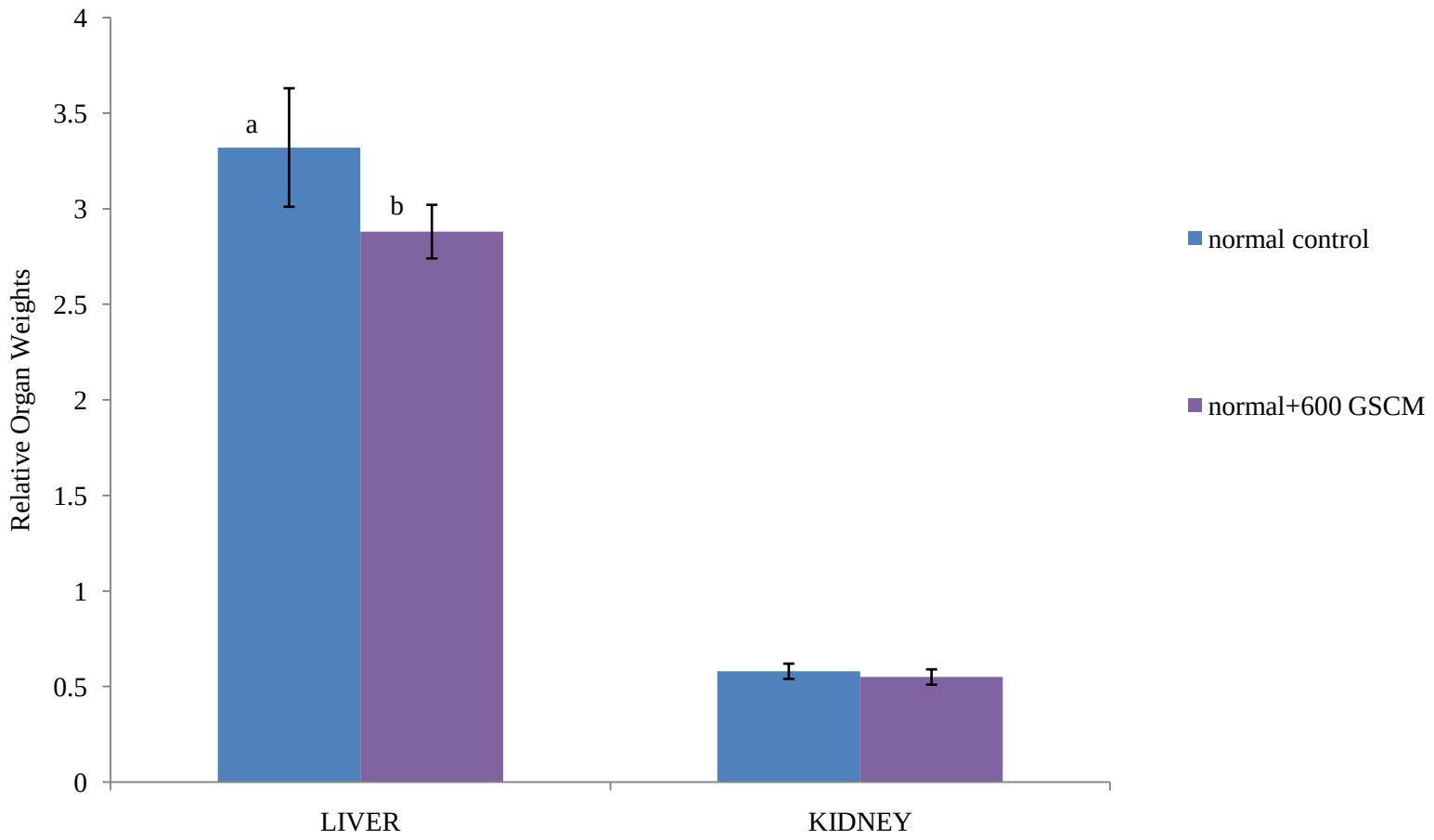


Figure 4.4: Relative Organ Weights of Normal Rats Administered 1:1 Combination of *G. sylvestre* and *C. micranthum* Aqueous Leaf Extracts

Values are mean + standard error of mean (bars) with those bearing different superscripts being significantly (P< 0.05) different. N = 6.

4.4.5 Liver function tests

Except for the 600mg/kg b.w. GSCM treated group which showed significant increase ($P < 0.05$) in serum AST compared with normal, no significant change was observed in levels of AST, ALT, ALP, TP and Total Bilirubin in all the treated groups (Table 4.8).

4.4.6 Renal function tests

Serum urea and creatinine levels in the treated rats did not show any significant change (Table 4.9).

4.4.7*In vitro* Antioxidant (Radical Scavenging) Activity

The aqueous leaf extracts of GS displayed significantly better scavenging activities against ABTS, DPPH and Nitric Oxide when compared to the CM extract (Figure 4.5), with an IC_{50} of between 57.26 and 9.34 µg/ml.

4.4.8 *In vivo* Antioxidant Activity

Catalase, GSH, GSSG and GSH – Px of the liver homogenate did not show any significant changes – there is a general insignificant increases. However, MDA levels in the liver increased significantly ($P < 0.05$) in the group administered 600mg/kg b.w. GS (Table 4.10).

4.5 Preliminary Antidiabetic Screening

Reductions in FBG of the diabetic mice were significant ($P < 0.05$) in groups treated with aqueous GS leaf extracts after 2 and 4 hours sampling time (Table 4.11). Also, CM aqueous extract gave significant decrease in FBG after 2 and 4 hours when compared with the untreated diabetic control. The aqueous extracts of both plants (GS and CM) gave high percentage reductions in FBG (-56.93 ± 18.12 and -58.36 ± 25.30% respectively) hence used for the phase II (long-term treatment/sub-chronic) study. Similarly, different combinations of the aqueous extracts reduced the FBG, with the highest significant ($P < 0.05$) reduction observed in the 1:1 ratio (-57.80 ± 8.45%) (Table 4.11), thus chosen for the next phase of the study too.

Table 4.8: Liver Function Indices of Normal Rats Administered *G. sylvestre*, *C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts for 21 Days.

Group	AST(U/l)	ALT(U/l)	ALP(U/l)	TP(g/dl)	TBIL(mg/dl)
normal control	56.56±10.55[a]	23.33±0.88	158.15±70.28	2970.2±948.31	1.78±0.12
Normal+ 600 GS	73.71±8.26	21±0.01	363.91±87.91	4669.53±285.45	1.68±0.08
Normal+ 600 CM	81.21±6.73	16.5±4.50	305.33±62.17	2613.93±1101.62	1.64±0.15
Normal+ 600 GSCM	91.33±8.2[b]	31.5±14.50	256.82±13.8	4814.37±152.09	1.45±0.12

Values are mean + standard error of mean with those bearing different superscripts within the same column being significantly ($P< 0.05$) different. N = 6.

Table 4.9: Renal Function Indices of Normal Rats Administered *G. sylvestre*, *C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts for 21 Days.

Group	Serum Urea (mg/dl)	Serum Creatinine (mg/dl)
normal control	42.16±4.21	0.63±0.11
normal 600 GS	37.08±1.57	0.75±0.06
normal 600 CM	41.39±14.98	0.75±0.04
normal 600 GSCM	52.3±1.88	1.67±0.55

Values are mean + standard error of mean. N = 6.

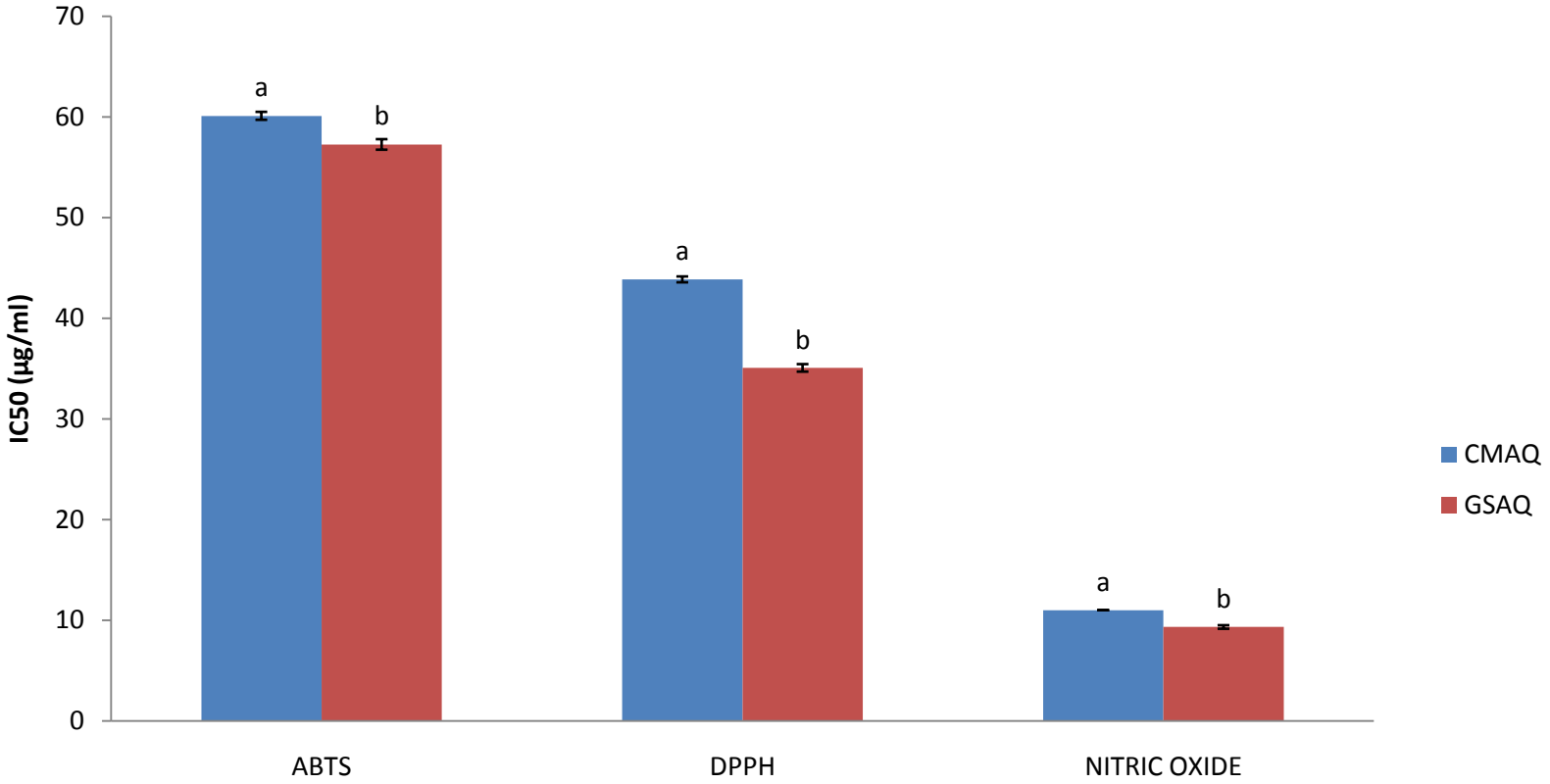


Figure 4.5: Radical Scavenging Activities of Aqueous Leaf Extracts of *G.sylvestre* and *C. micranthum*

Values expressed as Mean ± SEM of triplicate measurements, with those bearing different superscripts on the same cluster significantly different ($P < 0.05$).

Table 4.10: Some Liver Antioxidant Indices of Normal Rats Administered *G. Sylvestre, C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts

Group	Mean Antioxidant Parameters						
	CAT (µmol/mg protein)	TPP/ROS (µmol/min/mg protein)	GSH (U/mg protein)	GSSG (U/mg protein)	SOD (U/mg protein)	GSH-PX (U/mg protein)	MDA(µmolTBARS/mg protein)
normal control	10±2.19	14.87±3.37	6.37±1.58	6.17±0.41	4.67±0.35	5.87±0.74	47.23±3.69[a]
normal 600 GS	9.83±1.12	23.1±4.38	5.53±0.78	5.77±1.12	5.67±0.63	7.37±0.52	87.49±6.56[b]
normal 600 CM	8.03±1.35	19.33±1.10	5.47±0.69	5.07±0.87	5.07±1.2	6.63±0.69	78.17±11.82
normal 600 GSCM	8±1.70	19.9±2.60	6.73±0.90	7.1±1.37	5.43±0.52	4.97±0.46	61.67±15.07

Values are mean ± standard error of mean with those bearing different superscripts within the same column being significantly ($P < 0.05$) different. N = 6.

Table 4.11: Effect of Administration ofAqueous Leaf Extracts of *G. sylvestre*, *C. micranthum* and their Combinations (GSCM) onMean Fasting Blood Glucose Level (mg/dl) and the Percentage (%) Reductions in FBG of Alloxan-induced Diabetic Mice for Four Hours

Extract Type/Combination	Fasting Blood Glucose Level				
	0hr(mg/dl)	2hr(mg/dl)	% Reduction (2hr)	4hr(mg/dl)	% Reduction (4hr)
Diabetic+GSaq	327.50±193.50	272.00±171.00	-19.80 ± 4.83	106.00±24.00	-56.93 ± 18.12
Diabetic+CMaq	320.50±199.50	301.00±208.00	-12.63 ± 10.51	83.00±2.00	-58.36 ± 25.30
Diabetic+GSCM(1:1)	195.50±41.50	139.00±26.00	-29 .00± 1.88[a]	79.00±1.00	-57.80 ± 8.45[a]
Diabetic+GSCM(1:2)	337.50±197.50	304.50±196.50	-15.00 ± 8.25	226.50±138.50	-34.46 ± 2.68
Diabetic+GSCM(2:1)	498.50±68.50	479.50±87.50	-04.00 ± 4.42	253.50±73.50	-50.23 ± 7.91[a]
Normal	131.00±9.54	139.67±6.49	+07.00 ± 5.06[b]	100.33±5.61	-22.61 ± 6.67[b]
Diabetic control	536.50±35.50	579.00±33.00	+7.99 ± 0.99[b]	470.00±58.00	-11.29 ± 16.68[b]
Diabetic+Glibenclamide	301.50±112.50	258.00±79.00	-12.00 ± 6.65	92.50±10.50	-65.87± 9.25[a]

Values expressed as Mean ± SEM with those bearing different superscripts under the same column significantly different ($P < 0.05$); N = 3. (+) preceding a value means % increase in Fasting Blood Glucose; (-)preceding a value means % decrease in Fasting Blood Glucose; GSAq= *G. sylvestre* aqueous extract; CMAq = *C. micranthum* aqueous extract.

4.6 Antidiabetic Action of GS and CM

4.6.1 Feed and water intakes

Induction of diabetes caused significant increase in food intake (polyphagia) in the rats which was reversed significantly when the rats were treated with different doses of the aqueous extracts of the leaves of GS and CM; the highest reductions seen in groups given 600mg/kg b.w. GS and CM and 200mg/kg b.w. CM. Dose - dependent reduction in food intake was observed in the groups given 1:1 combination of the two extracts (Table 4.12). Increase in water intake (polydipsia) was also observed in the diabetic untreated group and this was reversed when the rats were administered the extracts – the highest reductions recorded in 600mg/kg b.w. and 200mg/kg b.w. GS groups. Dose-depency in the 1:1 combination group was also observed (Table 4.13).

4.6.2 Fasting blood glucose

Table 4.14 show the percentage changes in FBG after three weeks of extract administration. Significant percentage reductions in FBG of the diabetic rats were seen in those groups treated with 600mg/kg b.w. CM, 400mg/kg b.w. GS, 400mg/kg b.w. CM, 400mg/kg b.w. GSCM, 200mg/kg b. w. CM and glibenclamide (6mg/kg b.w.) when compared with untreated diabetic group.

4.6.3 Lipid profile

In the diabetic group, TC, TG, LDL and VLDL were significantly increased while HDL decreased. Serum levels of these parameters were reversed in the groups treated with different doses of GS, CM and their 1:1 combination for 21 days (Table 4.15).

Table 4.12: Mean and Percentage (%) Change in Feed Intake of Streptozotocin-Induced Diabetic Rats Administered Different Doses of *G. sylvestre* (GS), *C. micranthum* (CM) and 1:1 Combination (GSCM) of Their Aqueous Leaf Extracts For Twenty-One (21) Days

Group	Mean Feed Intake (g/100g b.w./day)		% change post-treatment
	Pre-treatment	21st day post-treatment	
Normal control	9.14±0.08	6.41±2.58	-30.06±27.67
Diabetic control	11.62±1.31	12.57±1.11	+10.69±21.99[a]
Diabetic+600 GS	7.31±1.04	3.89±0.06	-45.54±8.54[b]
Diabetic+600 CM	8.21±1.50	5.98±1.54	-28.15±5.68[c]
Diabetic+600 GSCM	9.41±0.88	3.77±1.95	-57.69±24.63
Diabetic+400 GS	7.63±1.03	3.98±0.72	-45.57±16.78
Diabetic+400 CM	8.55±0.56	4.48±1.46	-48.52±13.68
Diabetic+400 GSCM	7.61±0.77	4.48±1.51	-38.54±25.97
Diabetic+200 GS	8.21±1.48	4.35±0.65	-43.84±17.98
Diabetic+200 CM	9.92±1.16	4.41±0.45	-54.43±9.80[b]
Diabetic+200 GSCM	7.19±0.23	5.97±3.13	-18.34±40.85
Diabetic+Glibenclamide	5.93±0.70	3.74±1.23	-33.65±28.49

Values expressed as Mean ± SEM with those bearing different superscript under the same column significantly different ($P < 0.05$); N = 6; (+) preceding a value means % increase in Feed Intake; (-) preceding a value means % decrease in Feed Intake.

Table 4.13: Mean and Percentage (%) Change in Water Intake of Streptozotocin-Induced Diabetic Rats Administered Different Doses of *G. sylvestre* (GS), *C. micranthum* (CM) and 1:1 Combination (GSCM) of Their Aqueous Leaf Extracts for 21 Days

Group	Mean Water Intake (ml/100g b.w./day)		% Change
	0th day	21st day	
Normal control	12.98±0.62	5.29±4.23	-60.75±30.68[a]
Diabetic control	13.24±0.70	23.03±3.26	+75.79±33.86[b]
Diabetic+600 GS	14.59±1.51	10.15±1.13	-30.52±0.52[a]
Diabetic+600 CM	14.33±1.31	8.35±2.79	-39.47±24.96
Diabetic+600 GSCM	18.90±1.46	12.13±6.53	-32.74±39.74
Diabetic+400 GS	11.83±0.01	14.75±6.32	+24.68±53.42
Diabetic+400 CM	17.59±1.26	11.32±5.15	-33.23±34.02
Diabetic+400 GSCM	16.59±0.66	14.36±10.98	-15.90±62.88
Diabetic+200 GS	14.03±1.87	12.43±5.12	-14.79±25.10
Diabetic+200 CM	24.39±0.01	15.42±3.67	-36.78±15.05[a]
Diabetic+200 GSCM	14.33±0.50	15.43±7.31	+6.04±47.33
Diabetic+Glibenclamide	15.61±0.01	13.87±6.57	-11.15±42.09

Values expressed as Mean ± SEM with those bearing different superscripts under the same column significantly different ($P < 0.05$); N = 6; (+) preceding a value means % increase in Water Intake; (-) preceding a value means % decrease in Water Intake.

Table 4.14: Percentage (%) Changes in Mean Fasting Blood Glucose of Streptozotocin-Diabetic Rats Administered Different Doses of *G.sylvestre*, *C.micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts for 21 Days

Group	Percentage (%) change in Fasting Blood Glucose (FBG)		
	Week 1	Week 2	Week 3
Normal control	-0.15±4.46[a]	-7.39±4.00	-20.04±3.60[a]
Diabetic + control	-14.68±7.20[b]	8.77±5.59[a]	-19.54±5.10[b]
Diabetic + 600 GS	+29.03±17.21[c]	-33.87±13.79[b]	+3.32±11.30[c]
Diabetic + 600 CM	+8.68±10.24[a, c]	-68.26±4.01[b]	-63.53±2.48[d]
Diabetic + 600 GSCM	+10.34±8.37[a, c]	-34.97±15.80[b]	-34.81±10.32[a]
Diabetic + 400 GS	+29.82±18.05[c]	-59.38±4.84[b]	-65.02±5.97[d]
Diabetic + 400 CM	+6.88±7.27[a, c]	-51.33±17.19[b]	-56.20±15.25[d]
Diabetic + 400 GSCM	+39.30±3.41[c]	-32.95±15.71[b]	-5.84±20.52
Diabetic + 200 GS	+8.51±5.72[a, c]	+26.82±10.63[a]	-13.02±16.08
Diabetic + 200 CM	+43.61±11.06[c]	-8.14±8.52[a]	-42.45±16.56
Diabetic + 200 GSCM	+24.78±7.89[c]	+0.02±4.99[a]	-22.83±15.26
Diabetic+Glibenclamide	+12.76±7.08[a, c]	-38.10±10.35[b]	-35.60±11.99

Values Mean ± SEM with those bearing different superscripts under the same column significantly different ($P < 0.05$); N = 6; (+) preceding a value means % increase in FBG; (-) preceding a value means % decrease in FBG.

Table 4.15: Lipid Profile of Streptozotocin-Diabetic Rats Administered Different Doses of *G.sylvestre*, *C.micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts for 21 Days

Parameter / Group	Lipid Levels (mg/dl)				
	TC	HDL	TG	LDL	VLDL
Normal control	83.00±20.19[a]	66.11±5.11[a]	104.15±35.04[a]	40.38±11.74[a]	13.15±12.26[a]
Diabetic control	133.35±40.92	41.64±35.15[b]	239.80±73.23[b]	104.46±13.96[b]	40.28±20.04[b]
Diabetic + 600 GS	93.25±22.81	12.43±2.60[c]	65.12±13.14[c]	131.27±36.90[c]	12.42±0.64[c]
Diabetic + 600 CM	74.71±10.13	31.80±5.46[b]	76.48±22.77	7.89±5.19	13.92±6.30
Diabetic + 600 GSCM	71.95±13.51	59.33±10.11	86.50±28.12[c]	21.72±8.19[d]	11.08±0.12[c]
Diabetic + 400 GS	104.20±22.11	47.20±8.49	163.23±66.43	51.26±25.67	31.17±19.39
Diabetic + 400 CM	104.97±32.11	31.50±21.07	80.43±49.98	59.97±59.26	18.87±16.63
Diabetic + 400 GSCM	87.07±7.27	54.02±6.76	34.48±4.81	18.61±11.47	7.95±0.75[c]
Diabetic + 200 GS	110.17±24.81	36.50±16.50	63.33±27.51	41.71±19.78	7.19±0.93[c]
Diabetic + 200 CM	86.21±20.37	36.54±10.23	29.02±6.97[d]	15.84±3.19	6.27±0.74[c]
Diabetic + 200 GSCM	81.01±17.23	43.97±1.21[d]	27.70±15.00	31.50±15.44	5.54±3.00
Diabetic +Glibenclamide	91.54±12.52[b]	40.89±7.04	20.32±8.67[d]	23.48±4.20[d]	5.76±4.28

Values are mean + standard error of mean with those bearing different superscripts under the same column being significantly ($P< 0.05$) different. N = 6.

4.6.4 Serum insulin and liver hexokinase

In Figures4.6 and 4.7, diabetes induction caused decreases in serum insulin and liver hexokinase levels. These, upon administration of different doses of the aqueous GS, CM and 1:1 combinations were increased significantly.

4.6.5 *In vivo* antioxidant activity in STZ diabetic rats

Total plasma peroxides (TPP)/reactive oxygen species (ROS) levels in the rats increased significantly in diabetes and these were reversed in the treated groups with significant decreases observed in the diabetic groups administrated 600mg/kg b.w. CM and GSCM, 400mg/kg b.w. GS and CM, 200mg/kg b.w. CM and GSCM, and glibenclamide (6mg/kg b.w.) (Table 4.16). Catalase, GSH, GSSG and GSH – Px of the liver homogenate did not show any significant changes – there were general insignificant increases. However, SOD and MDA levels in the liver increased in diabetes which was later reversed with the administration of extracts and standard drug (Table 4.16). Percentage decrease in FBG correlates negatively with α-amylase % inhibition (i.e. an increase in % inhibitory activity of α-amylase leads to a more negative % decrease in FBG) (Table 4.17). Also, correlation analyses established a significant ($p < 0.05$) positive relationship between phenolic / flavonoid contents of the GS and CM leaves and radical scavenging capacity of ABTS, DPPH and NO (Table 4.18). Moreover, % decrease in FBG showed significantly ($p < 0.05$) positive correlation with levels of liver MDA and CAT; MDA also correlates well with ROS (Table 4.19).

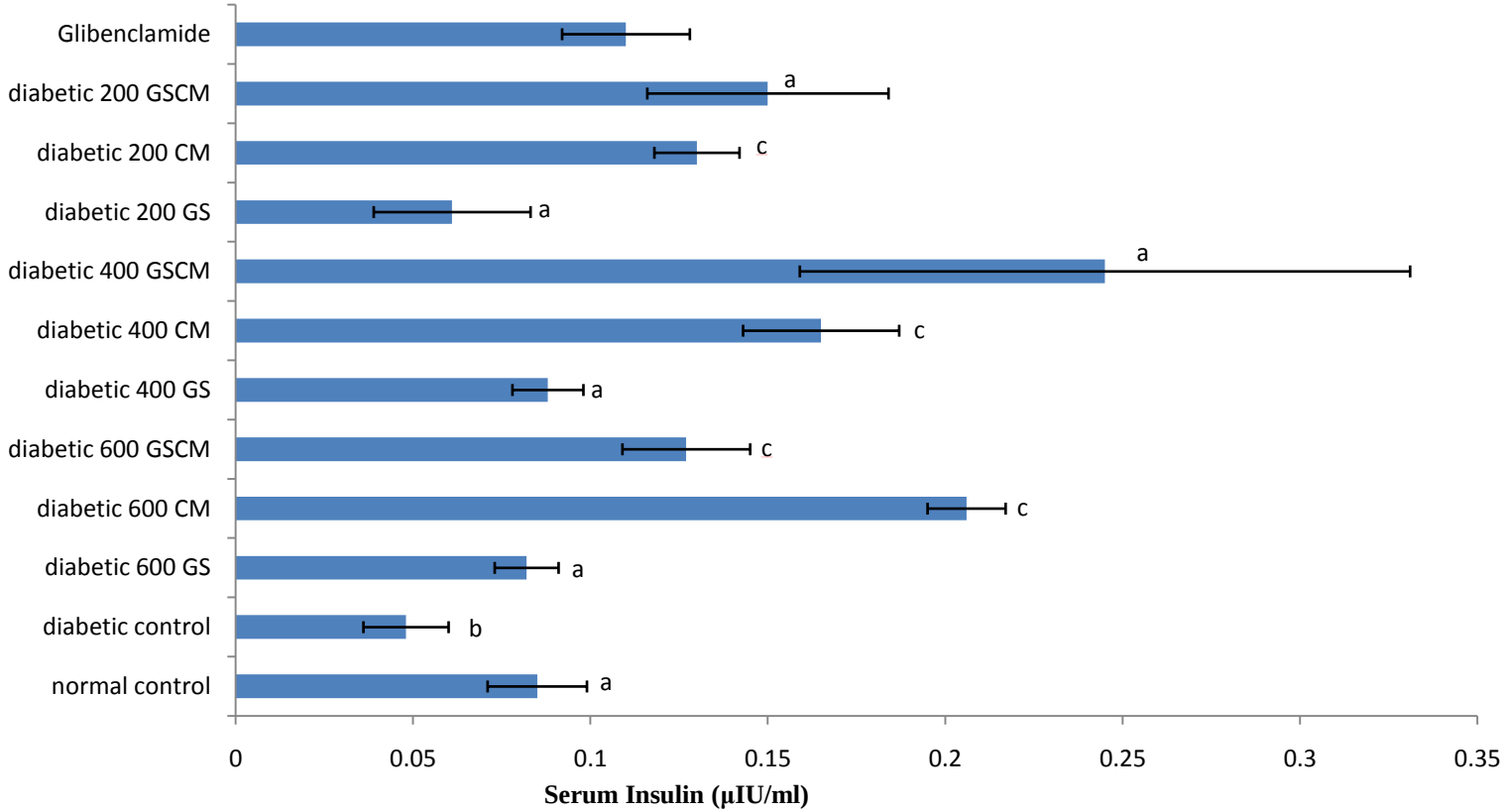


Figure 4.6: Serum Insulin Level (µIU/ml) In Streptozotocin-induced Diabetic Rats Administered *G. sylvestre*, *C. micranthum*, and their (1:1) Combination for 21 Days

Values are expressed as mean ± standard error of mean (bars) with those bearing different superscripts being significantly ($P < 0.05$) different. N= 6.

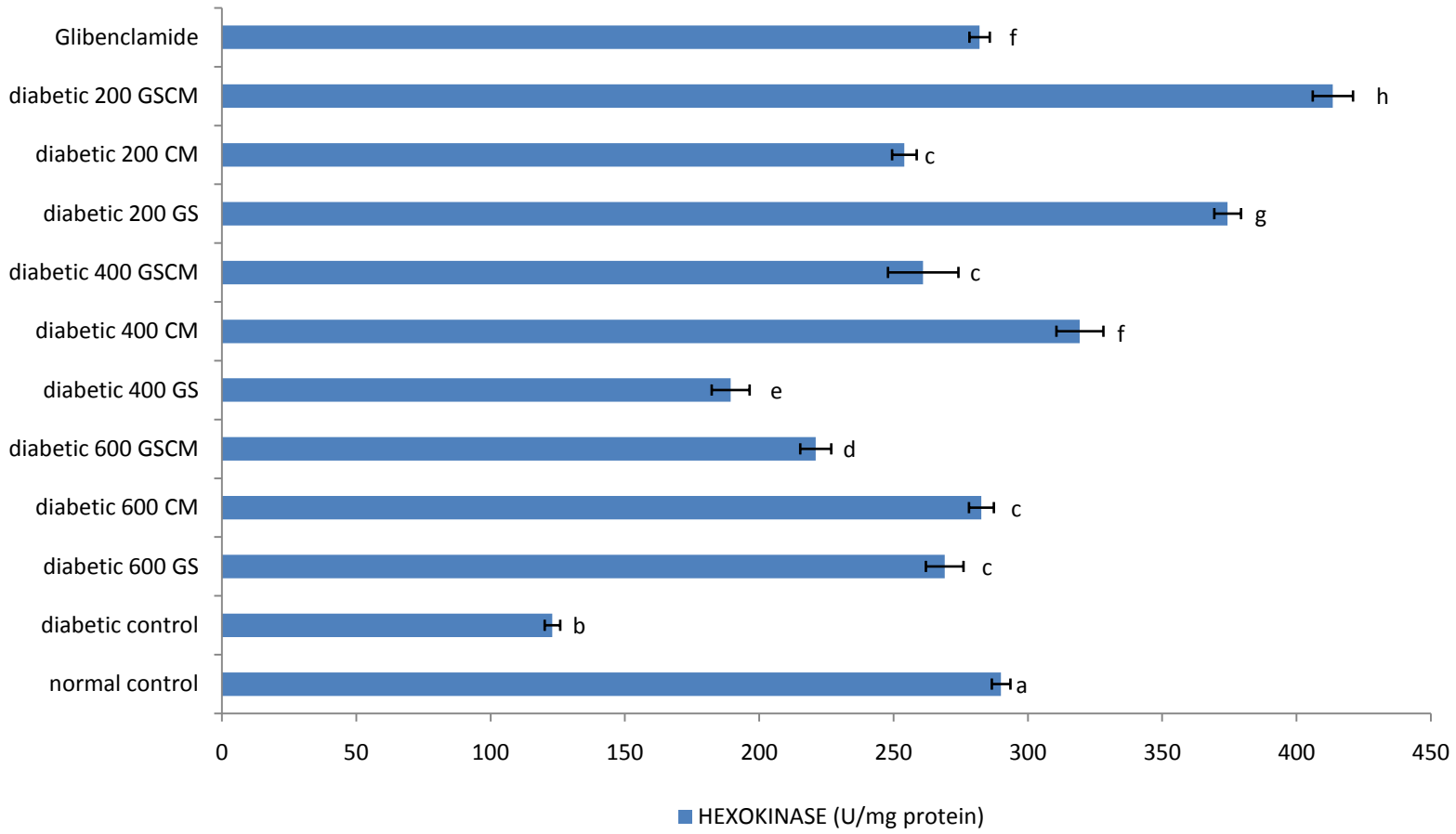


Figure 4.7: Liver Hexokinase Level (U/mg protein) In Streptozotocin-Induced Diabetic Rats Administered *G. sylvestre*, *C. micranthum*, and their (1:1) Combination for 21 Days

Values are expressed as mean ± standard error of mean (bars) with those bearing different superscripts being significantly ($P < 0.05$) different. N = 6.

Table 4.16: Some Liver Antioxidant Indices of Streptozotocin-Diabetic Rats Administered *G. sylvestre, C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts

Group	Mean Antioxidant Parameters						
	CAT (µmol/mg protein)	TPP/ROS (µmol/min/mg protein)	GSH (U/mg protein)	GSSG (U/mg protein)	SOD (U/mg protein)	GSH-PX (U/mg protein)	MDA(µmolTBARS/mg protein)
Normal control	10.00±2.19	14.87±3.37^{a}	6.37±1.58	6.17±0.41^{a}	4.67±0.35^{a}	5.87±0.74^{a}	47.23±3.69^{a}
Diabetic control	9.23±1.71	25.80±4.14^{b}	5.93±0.64	7.10±1.11	7.97±0.15^{b}	5.83±1.24b^{a}	96.33±17.33^{b}
Diabetic + 600 GS	10.30±1.18	16.60±1.97	5.33±0.49	9.23±1.00^{b}	4.93±0.33^{a}	6.23±0.71	69.87±10.18
Diabetic + 600 CM	13.90±1.42	5.70±0.21^{c}	7.73±1.26	7.90±0.97	5.47±0.69^{a}	6.83±1.27	60.00±5.31
Diabetic + 600 GSCM	8.63±1.26	11.70±0.70^{d}	4.77±0.58	6.23±1.36	5.13±0.42^{a}	6.53±1.25	78.63±2.00^{b}
Diabetic + 400 GS	7.30±0.99	10.70±1.93^{d}	7.07±1.34	7.73±1.09	5.77±1.08	4.43±0.20	82.57±5.24^{b}
Diabetic + 400 CM	5.47±0.50	11.67±0.71^{d}	6.93±1.25	7.07±0.75	7.33±0.99	8.53±0.65^{b}	71.87±11.14
Diabetic + 400 GSCM	11.07±1.63	22.43±2.03^{e}	8.93±1.56	7.23±0.85	5.83±1.66	5.90±0.29	75.30±12.68
Diabetic + 200 GS	8.43±2.04	16.73±4.03	5.77±0.26	4.53±0.71	3.40±0.70^{a}	10.43±1.04^{b}	82.80±3.84^{b}
Diabetic + 200 CM	6.23±1.08	9.40±1.83^{d}	5.20±0.55	5.00±1.04	3.90±0.75^{a}	6.57±0.50	65.57±16.57
Diabetic + 200 GSCM	6.15±0.55	8.20±0.90^{d}	5.10±0.90	5.30±2.00	6.00±0.20^{a}	4.70±0.20	30.85±7.45^{c}
Diabetic + Glibenclamide	8.17±1.89	11.53±0.63^{d}	4.73±0.77	6.77±1.07	4.37±1.30^{a}	5.53±1.74	64.27±4.77^{d}

Values are mean ± standard error of mean with those bearing different superscripts within the same column being significantly ($P< 0.05$) different. N = 6.

Table 4.17: Relationship between % Decrease in FBG, Insulin, Alpha-Amylase/Alpha-Glucosidase and Hexokinase in Rats Administered *Gymnema sylvestre* and *Combretum micranthum* Aqueous Leaf Extracts

Pearson Correlations		% FBG	insulin	Alpha-amylase	Alpha-glucosidase	Hexokinase
% FBG	r	1	.152	**-.548**	.224	-.291
	p		.260	.101	.535	.073
insulin	r	.152	1	-.263	.461	-.097
	p	.260		.494	.212	.611
Alpha-amylase	r	**-.548**	-.263	1	.334	.280
	p	.101	.494		.346	.433
Alpha-glucosidase	r	.224	.461	.334	1	-.022
	p	.535	.212	.346		.952
Hexokinase	r	-.291	-.097	.280	-.022	1
	p	.073	.611	.433	.952	

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). r = correlation coefficient; p = significance level.

Table 4.18: Relationship between % Decrease in FBG of Rats and *In vitro* Antioxidant Capacity of *Gymnema sylvestre* and *Combretum micranthum* Leaves

Pearson Correlations		% FBG	FRAP	TOTFLAV	TOTANTIOX	TOTPHENOL	ABTS	DPPH	NOXIDE
% FBG	r	1	.385	.343	.089	.375	**.847****	**.719***	**.810***
	p		.615	.657	.911	.625	.008	.044	.015
FRAP	r	.385	1	.775	.900	.897	-.741	-.875	-.874
	p	.615		.225	.100	.103	.259	.125	.126
FLAV	r	.343	.775	1	.474	**.975***	**-.999****	**-.977***	**-.984***
	p	.657	.225		.526	.025	.001	.023	.016
TOTANTIOX	r	.089	.900	.474	1	.650	-.432	-.647	-.619
	p	.911	.100	.526		.350	.568	.353	.381
TOTPHENOL	r	.375	.897	**.975***	.650	1	**-.962***	**-.994****	**-.999****
	p	.625	.103	.025	.350		.038	.006	.001
ABTS	r	**.847****	-.741	**-.999****	-.432	**-.962***	1	**.922****	**.940****
	p	.008	.259	.001	.568	.038		.001	.001
DPPH	r	**.719***	-.875	**-.977***	-.647	**-.994****	**.922****	1	**.845****
	p	.044	.125	.023	.353	.006	.001		.008
NOXIDE	r	**.810***	-.874	**-.984***	-.619	**-.999****	**.940****	**.845****	1
	p	.015	.126	.016	.381	.001	.001	.008	

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). r = correlation coefficient; p = significance level.

Table 4.19: Relationship between % Decrease in FBG, *In vivo* Antioxidant/Peroxidation Status in Rats Administered *Gymnema sylvestre* and *Combretum micranthum* Aqueous Leaf Extracts

Pearson Correlations		% FBG	MDA	ROS	GSH-Px	GSH	SOD	GSSG	CAT
% FBG	r	1	**.337***	.282	.029	.153	.002	.189	**.393***
	p		.036	.082	.863	.354	.991	.250	.013
MDA	r	**.337***	1	**.530****	.040	-.152	.001	-.194	.220
	p	.036		.000	.794	.325	.994	.207	.151
ROS	r	.282	**.530****	1	.006	.005	.078	-.224	.221
	p	.082	.000		.967	.975	.616	.144	.150
GSH-Px	r	.029	.040	.006	1	.010	-.084	-.060	-.244
	p	.863	.794	.967		.947	.589	.697	.111
GSH	r	.153	-.152	.005	.010	1	**.330***	**.350***	.048
	p	.354	.325	.975	.947		.029	.020	.758
SOD	r	.002	.001	.078	-.084	**.330***	1	**.531****	**-.316***
	p	.991	.994	.616	.589	.029		.000	.036
GSSG	r	.189	-.194	-.224	-.060	.350*	**.531****	1	-.114
	p	.250	.207	.144	.697	.020	.000		.460
CAT	r	**.393***	.220	.221	-.244	.048	**-.316***	-.114	1
	p	.013	.151	.150	.111	.758	.036	.460	

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). r = correlation coefficient; p = significance level.

4.6.6 Body weight changes in STZ diabetic rats

The diabetic rats generally lost weight when compared with the normal ones. However, this was reversed significantly ($P < 0.05$) when the rats were treated with different doses of GS and CM aqueous leaf extracts and their 1:1 combination (GSCM). The rats treated with 600mg/kg b.w. GSCM and 400mg/kg b.w. GS recorded the highest reduction in weight loss (Table 4.20).

4.6.7 Haematological changes in STZ diabetic rats

Some significant ($P < 0.05$) increases in the PCV was seen in those diabetic rats treated with 600mg/kg b.w. CM and 400mg/kg b.w. CM when compared with normal untreated control (Table 4.21).

4.6.8 Relative organs weights in STZ diabetic rats

Relative liver and kidney weights increased significantly ($P < 0.05$) in untreated diabetic control which were ameliorated when the rats were given different dose treatments with GS, CM and GSCM. Relative liver weights reduced significantly in the groups diabetic + glibenchamide (6mg/kg b.w.) (Figures4.8, 4.9, 4.10), normal + 600mg/kg b.w. GS, diabetic + 600mg/kg b.w. GS (Figure 4.8); normal + 600CM, diabetic + 600CM (Figure 4.9); and normal + 600GSCM and diabetic + 600GSCM (Figure 4.10). The relative kidney weights displayed a similar pattern in addition to diabetic + 400mg/kg b.w. GS (Figure 4.8); diabetic + 200mg/kg b.w. CM (Figure 4.9) and diabetic + 200mg/kg b.w. GSCM (Figure 4.10) also significantly remedied the increase in kidney weight.

Table 4.20: Percentage (%) Change in Body Weight of Streptozotocin-Diabetic Rats Administered Different Doses of *G.sylvestre*, *C.micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts

Group/Treatment	Percentage (%) Change In Body Weight		
	Week 1	Week 2	Week 3
Normal + control	6.00±0.90^{a}	7.29±1.24^{a}	10.69±3.06^{a}
Diabetic + control	7.35±3.06^{a}	1.11±6.13^{b}	-14.67±7.53^{b}
Diabetic + 600 GS	-15.11±2.06^{b}	-14.99±2.81^{c}	-8.95±4.36^{b}
Diabetic + 600 CM	-7.48±1.25^{b}	-11.01±1.48^{c}	-8.16±2.13^{b}
Diabetic + 600 GSCM	-7.22±6.35^{b}	-11.16±5.51^{c}	-5.95±6.34^{b}
Diabetic + 400 GS	-2.92±3.54^{b}	-10.90±4.66^{c}	-5.67±5.55^{b}
Diabetic + 400 CM	-8.92±3.67^{b}	-17.12±2.69^{c}	-16.11±5.56^{b}
Diabetic + 400 GSCM	-7.06±1.42^{b}	-12.66±2.13^{c}	-12.26±2.10^{b}
Diabetic + 200 GS	-18.87±2.16^{b}	-21.60±1.95^{d}	-19.84±3.11^{b}
Diabetic + 200 CM	-18.16±1.15^{b}	-15.74±1.49^{c}	-15.83±3.05^{b}
Diabetic + 200 GSCM	-9.74±2.17^{b}	-17.99±3.08^{d}	-10.78±2.89^{b}
Diabetic + Glibenclamide	-13.41±1.68^{b}	-19.94±2.28^{d}	-12.19±3.04^{b}

Values are mean + standard error of mean with those bearing different superscripts under the same column being significantly ($P< 0.05$) different. N = 6. (-) preceding a value means % decrease.

Table 4.21: Haematological Indices of Streptozotocin-Diabetic Rats Administered Different Doses of *G.sylvestre*, *C.micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts

Group	Mean Haematological Indices						
	WBC ($X10^3/\mu L$)	RBC ($X10^6/\mu L$)	Hb (g/dl)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)
normal control	6.65 ± 0.87^{a}	6.53 ± 0.28^{a}	11.77 ± 0.53^{a}	43.97 ± 2.40^{a}	67.17 ± 1.21^{a}	18.02 ± 0.08^{a}	26.85 ± 0.37^{a}
diabetic control	7.62 ± 1.14^{a}	9.25 ± 1.65	13.14 ± 0.30^{b}	55.66 ± 11.37	60.30 ± 2.17^{b}	15.42 ± 1.74	26.08 ± 3.45
diabetic+ 600 GS	8.47 ± 0.68^{b}	7.72 ± 0.29^{b}	14.30 ± 0.40^{c}	48.15 ± 1.76	62.48 ± 1.58^{b}	18.57 ± 0.29^{b}	29.77 ± 0.64^{b}
diabetic+ 600 CM	17.72 ± 1.91^{c}	8.52 ± 0.21^{b}	15.55 ± 0.17^{c}	50.57 ± 1.40^{b}	59.37 ± 1.05^{b}	18.28 ± 0.29^{b}	30.83 ± 0.64^{b}
diabetic+ 600 GSCM	$15.76\pm3.52^{b,c}$	7.96 ± 0.52^{b}	13.86 ± 0.82^{c}	48.08 ± 2.51	60.66 ± 1.61^{b}	17.44 ± 0.33	$28.82\pm0.82^{b,d}$
diabetic+ 400 GS	16.12 ± 4.45	8.36 ± 0.41^{b}	14.93 ± 0.51^{c}	48.30 ± 1.84	57.98 ± 1.11^{b}	17.95 ± 0.37	30.97 ± 0.29^{b}
diabetic+ 400 CM	$13.10\pm3.72^{b,c}$	8.05 ± 0.16^{b}	14.77 ± 0.23^{c}	47.70 ± 1.92	59.27 ± 2.40^{b}	18.33 ± 0.18^{b}	31.03 ± 1.00^{b}
Diabetic+ 400 GSCM	$17.25\pm3.26^{b,c}$	9.32 ± 0.50^{b}	16.35 ± 0.47^{c}	53.80 ± 2.07^{b}	57.88 ± 1.20^{b}	17.63 ± 0.55	30.43 ± 0.57^{b}
diabetic+ 200 GS	$13.03\pm0.82^{b,c}$	8.29 ± 0.27^{b}	14.97 ± 0.41^{c}	46.93 ± 1.79	56.57 ± 0.50^{b}	18.07 ± 0.18	31.90 ± 0.38^{b}
diabetic+ 200 CM	$13.40\pm2.13^{b,c}$	8.84 ± 0.10^{b}	15.32 ± 0.23^{c}	50.88 ± 0.75^{b}	57.58 ± 0.62^{b}	17.35 ± 0.21^{b}	$30.12\pm0.28^{b,d}$
diabetic+ 200 GSCM	10.85 ± 1.65^{b}	8.70 ± 0.16^{b}	15.60 ± 0.01^{c}	49.9 ± 0.30	57.40 ± 1.40^{b}	17.95 ± 0.35	31.30 ± 0.20^{b}
diabetic +Glibenclamide	$18.86\pm4.29^{b,c}$	9.09 ± 0.17^{b}	15.56 ± 0.20^{c}	49.82 ± 0.61^{b}	54.82 ± 0.69^{c}	17.14 ± 0.35^{c}	$31.22\pm0.39^{b,e}$

Values are mean $\pm$ standard error of mean with those bearing different superscripts within the same column being significantly ($P< 0.05$) different. N = 6.

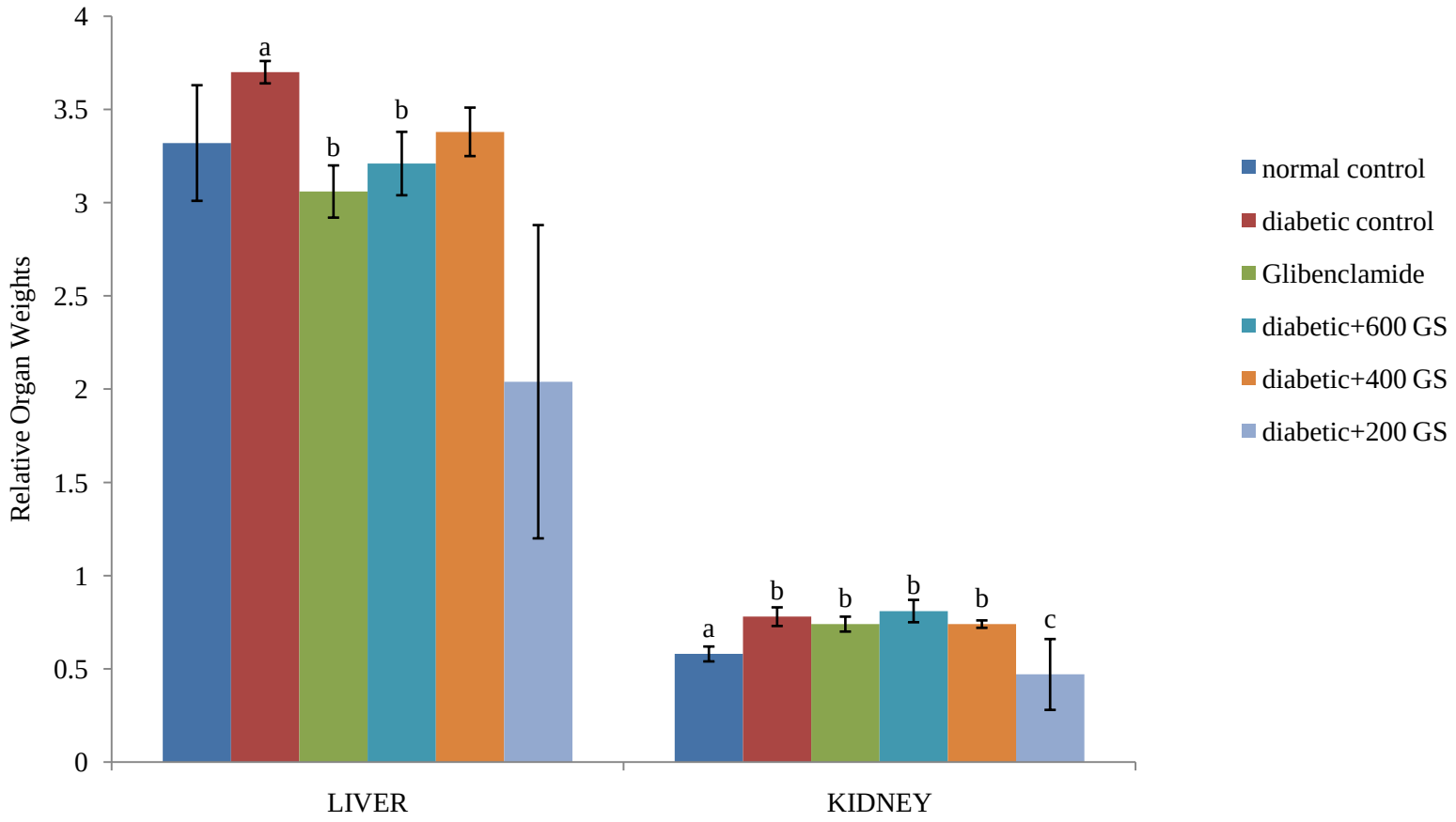


Figure 4.8: Relative Organ Weights of Streptozotocin-Diabetic Rats Administered Different Doses of *G. sylvestre* Aqueous Leaf Extract

Values are mean ± standard error of mean (bars) with those bearing different superscripts on same cluster being significantly ($P < 0.05$) different. N = 6.

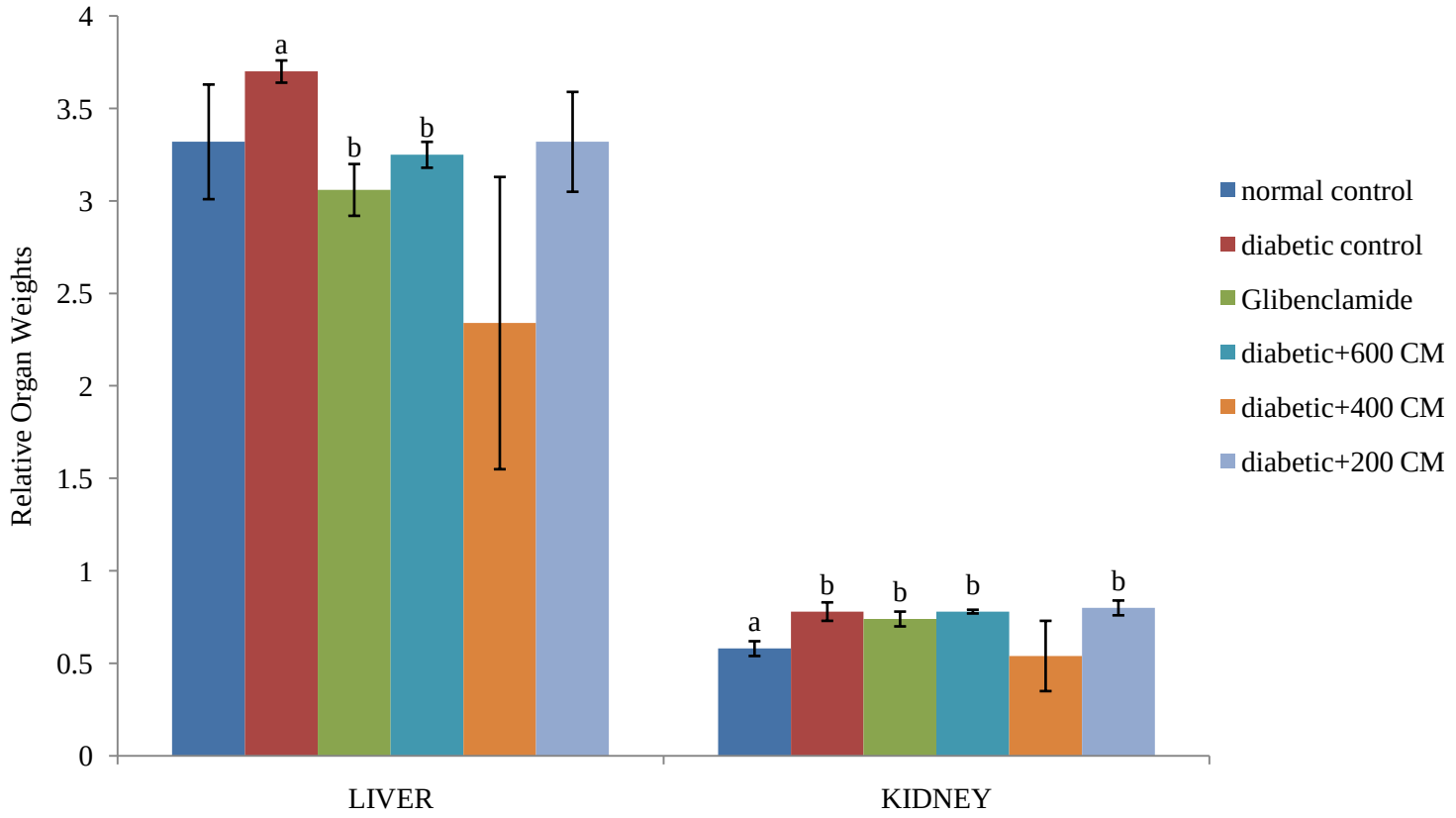


Figure 4.9: Relative Organ Weights of Streptozotocin-Diabetic Rats Administered Different Doses of *C. micranthum* Aqueous Leaf Extract

Values are mean ± standard error of mean (bars) with those bearing different superscripts on same cluster being significantly ($P < 0.05$) different. N = 6.

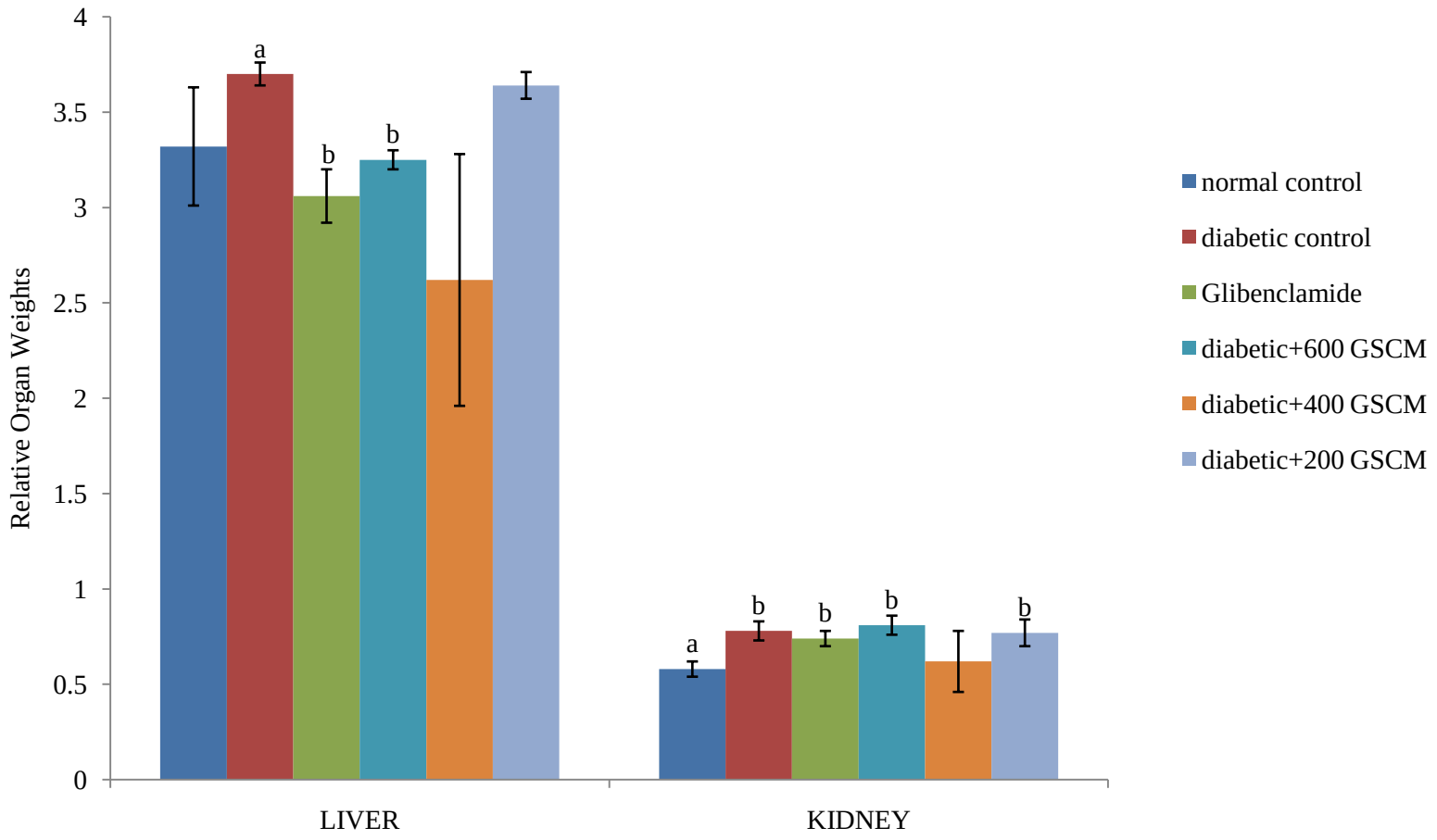


Figure 4.10: Relative Organ Weights of Streptozotocin-Diabetic Rats Administered Different Doses of 1:1 Combination of *G. sylvestre* and *C. micranthum* Aqueous Leaf Extracts

Values are mean + standard error of mean (bars) with those bearing different superscripts on same cluster being significantly ($P< 0.05$) different. N = 6.

4.6.9 Liver function tests in STZ diabetic rats

Levels of serum ALT and ALP increased significantly in the diabetes state and these were reversed with significant decreases observed in those groups administered 600mg/kg b.w. CM, GSCM, 400mg/kg b.w. CM, GSCM (in case of ALT); serum AST also increased in diabetes but this was not ameliorated with the administration GS, CM and GSCM. Similarly, serum total bilirubin did not show any significant change. However, total protein levels in the serum showed significant increases in the diabetic + 400mg/kg b.w. CM, diabetic + 400mg/kg b.w. GSCM, diabetic + 200mg/kg b.w. GS and CM and diabetic + 200mg/kg b.w. GSCM groups (Table 4.22).

4.6.10 Renal function tests in STZ diabetic rats

Serum urea and creatinine levels increased in diabetes control but these were not reversed in the treated groups, with rather significant increases ($P < 0.05$) seen in the higher dose levels compared with the lower ones (Table 4.23).

4.7 Alpha-amylase and Alpha-glucosidase Inhibition

Both GS and CM aqueous leaf extracts showed inhibitory activities against α-amylase and α-glucosidase. GS and CM gave an IC_{50} of 81.49μg/ml and 104.63μg/ml respectively for α-glucosidase inhibition which were significant when compared to that of the standard acarbose (124.48μg/ml). When the methanol fraction was compared with the aqueous, the former gave significantly better α-amylase and α-glucosidase inhibitory activities for both GS and CM (Figure 4.11). The methanol fraction also gave an IC_{50} significantly lower than that of the standard acarbose.

Table 4.22: Liver Function Indices of Streptozotocin-Diabetic Rats Administered *G. sylvestre*, *C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts

Group	Liver Function Parameters				
	AST(U/l)	ALT(U/l)	ALP(U/l)	TP(mg/dl)	TBIL(mg/dl)
normal control	56.56±10.55[a]	23.33±0.88[a]	158.15±70.28[a]	2.97±9.48[a]	1.78±0.12[a]
diabetic control	74.77±12.94[a]	33.50±0.50[b]	1078.93±283.29[b]	4.36±0.20[a]	1.55±0.31
Diabetic+ 600 GS	105.84±9.76[b]	23.50±3.50	690.00±66.10[b]	3.78±1.27	1.68±0.22
Diabetic+ 600 CM	125.58±14.95[b]	21.00±10.00[c]	282.00±23.00[c]	4.31±1.37	2.03±0.36
Diabetic+ 600 GSCM	96.44±15.58[b]	19.50±2.50[c]	414.07±116.13	4.79±1.27	1.50±0.27
Diabetic+ 400 GS	110.01±13.08[b]	41.50±24.50	462.76±125.63	4.19±1.23	1.41±0.09[b]
Diabetic+ 400 CM	180.69±24.30[b]	23.50±0.50[c]	305.00±5.00[c]	6.13±0.33[a,b]	2.64±0.84
Diabetic+ 400 GSCM	105.03±11.95[b]	24.00±2.00[c]	403.72±8.63	6.00±0.19[a,b]	1.60±0.49
Diabetic+ 200 GS	136.80±9.27[b]	29.50±4.50	5341.70±4306.70	5.60±0.23[a,,b]	2.17±0.36
Diabetic+ 200 CM	140.72±12.55[b]	26.50±8.50	681.86±115.37[b]	5.59±0.24[a,b]	1.50±0.18
Diabetic+ 200 GSCM	136.27±29.74[b]	24.00±20.00	5803.80±4993.46	5.39±0.12[a,b]	1.60±0.25
Diabetic+Glibenclamide	113.90±25.48[b]	41.50±11.50	1073.43±164.70[b]	3.60±1.43	1.92±0.19[a]

Values are mean + standard error of mean with those bearing different superscripts within the same column being significantly ($P< 0.05$) different. N = 6.

Table 4.23: Renal Function Indices of Streptozotocin-Diabetic Rats Administered *G. sylvestre, C. micranthum* and 1:1 Combination of Their Aqueous Leaf Extracts

Group	Kidney Function Parameters	
	Serum Urea (mg/dl)	Serum Creatinine (mg/dl)
normal control	42.16±4.21[a]	0.63±0.11[a]
diabetic control	50.48±20.99[a]	0.83±0.09[a]
diabetic 600 GS	219.91±10.55[b]	1.30±0.08[b]
diabetic 600 CM	87.34±1.67[b]	1.14±0.15[b]
diabetic 600 GSCM	107.65±39.25	1.16±0.03[b]
diabetic 400 GS	157.76±63.12	1.26±0.40
diabetic 400 CM	158.44±1.57[b]	1.03±0.06[b]
diabetic 400 GSCM	94.09±5.38[b]	0.73±0.01
diabetic 200 GS	241.00±7.00[b]	0.87±0.03
diabetic 200 CM	215.71±27.22[b]	0.95±0.09
diabetic 200 GSCM	159.48±19.27[b]	1.09±0.02[b]
Glibenclamide	146.82±21.68[b]	1.04±0.12

Values are mean + standard error of mean with those bearing different superscripts within the same column being significantly ($P< 0.05$) different. N = 6.

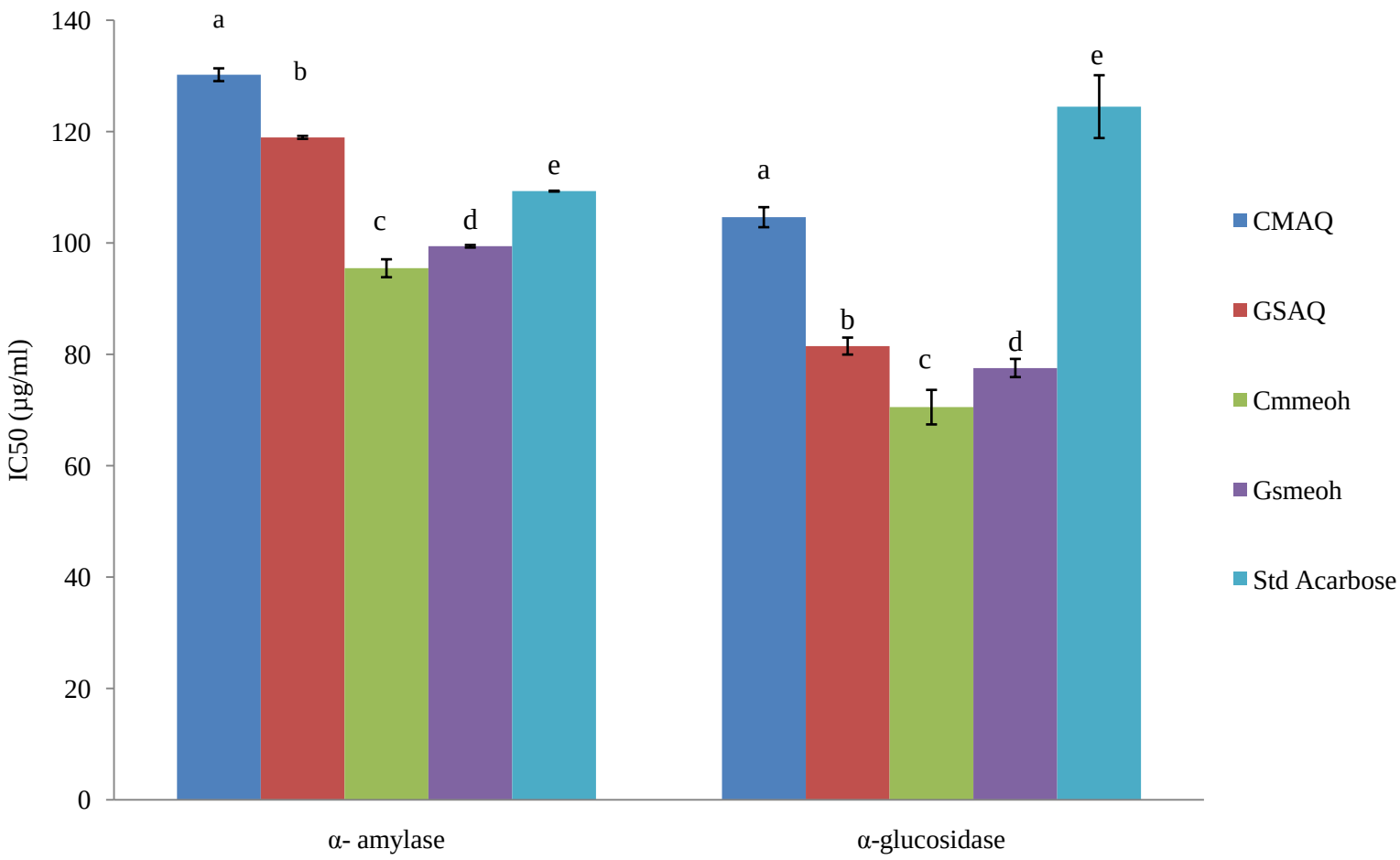


Figure 4.11: *In vitro* α- amylase and α-glucosidase Inhibition Activities of *G.sylvestre* and *C. micranthum* aqueous and methanolic Leaf Extracts

Values expressed as Mean SEM of triplicate measurements, with those bearing different superscripts for the same cluster significantly different ($P < 0.05$).

4.8 Bioassay – Guided Studies

4.8.1 Fractionation by column chromatography guided by TLC

Both GS and CM aqueous extracts wereinitially partitionedbertweenn-hexane, ethyl acetate and aqueous-methanol (60%).The methanol partition/fraction of both GS and CM aqueous extracts gave the best reductions in FBG compared to the other solvents' partitions (Table 4.24). Also the methanol fractions' ability to inhibit the carbohydrate hydrolyzing enzymes was found to be better than that of the aqueous extracts (Figure 4.11); moreover, the methanol fractions showed appreciable quantities of all the phytochemicals assayed for (Table 4.3). Hence they were chosen for the column chromatography studies.

Twenty five (25) grams each of the GS and CM aqueous/methanol fractions were subjected to column chromatography. One hundred and thirty (130) fractions were collected for GS which were pooled,based on their TLC profile, into 16 sub-fractions. The total recovery (i.e. eluted amount) of the GS extract was 12.04g (i.e. 48.17%). For the CM, 171 fractions collected, pooled into 7 sub-fractions with a recovery of 6.28g (25.12%).

When the sub-fractions were administered to the diabetic mice, fraction D (n-hexane : ethyl acetate – 20:80) for GS and fraction G (ethyl acetate : methanol – 30:70) for CM gave the best percentage reduction in FBG between 2 and 4 hours of administration (Tables 4.25 and 4.26); they were chosen for further fractionation and isolation/characterization of the compounds. Table 4.27compared the 1:1 combination of the fractions (D and G) and an additive effect was established since the combination index for both fractions was approximately 1.

Table 4.24: Mean Fasting Blood Glucose and their Percentage (%) Changes for Alloxan-induced Diabetic Mice Administered Different Solvent Partitioned Fractions of *G. sylvestre* and *C. micranthum* Aqueous Leaf Extracts for Four Hours

Fraction	Mean Fasting Blood Glucose (mg/dl)			% Change in Fasting Blood Glucose	
	0hrs	2hrs	4hrs	2hr	4hr
GShex	554.51±121.67	600.50±104.79	435.00±9.82	8.29±25.39	-21.55±21.26
GSetac	354.00±9.54	315.00±7.00	295.50±13.00	-11.02±1.00	-16.52±4.54
GSmeoh	287.25±87.50	241.50±76.00	141.75±25.50	-15.93±1.60	-50.65±11.66[a]
CMhex	483.00±74.14	587.00±64.68	453.00±74.07	21.53±11.58	-6.21±6.11
CMetac	334.01±17.07	331.50±6.00	304.99±4.33	-0.75±14.74	-8.68±13.34
CMmeoh	298.50±19.50	267.50±35.37	255.00±32.91	-10.38±10.88	-14.57±7.35
Normal	131.00±9.54	139.67±6.49	100.33±5.61	6.62±5.06	-23.41±6.67[b]
Diabetic control	536.50±35.50	579.00±33.00	470.00±58.00	7.92±1.37	-12.39±20.58[b]
Glibenclamide	301.50±112.50	258.00±79.00	92.50±10.50	-14.42±6.65	-69.32±9.25[c]

Values expressed as Mean ± SEM with those bearing different superscripts under the same column significantly different ($P < 0.05$); N = 3. (+) preceding a value means % increase in Fasting Blood Glucose; (-)preceding a value means % decrease in Fasting Blood Glucose; GSHex= *G. sylvestre* n-hexane fraction; CMHex = *C. micranthum* n-hexane fraction; GSEtac= *G. sylvestre* ethyl acetate fraction; CMEtac = *C. micranthum* ethyl acetate fraction; GSMeoh= *G. sylvestre* 60% methanolic fraction; CMMeoh = *C. micranthum* 60% methanolic fraction.

Table 4.25: Mean Fasting Blood Glucose and Their Percentage (%) Changes of Alloxan-Induced Diabetic Mice Administered Different Fractions (40mg/kg body weight) of Aqueous/Methanolic Leaf Extracts of *G. sylvestre* for Four Hours

Fraction	Mean Fasting Blood Glucose (mg/dl)			% Change in Fasting Blood Glucose	
	0hrs	2hrs	4hrs	2hrs	4hrs
A	271.33±74.05	169.00±27.18	190.33±15.86	-27.80±21.27	-09.91±38.00
B	259.00±133.00	312.00±182.00	325.50±19.50	14.60±11.42	65.43±77.42
C	224.00±95.69	122.67±15.84	121.00±11.93	-31.92±15.64	-29.54±21.47
D	213.33±67.66	132.33±25.10	129.33±19.55	-32.75±08.31[a]	-32.18±12.44[a]
E	232.67±107.74	112.00±31.32	84.67±18.41	-22.84±33.63	-41.23±24.37[a]
F	417.67±116.75	340.00±113.32	315.67±80.35	-22.49±10.10	-21.98±07.12
G	500.67±4.10	424.00±82.89	370.33±70.94	-15.03±17.28	-25.80±14.73
H	416.33±139.45	321.00±216.00	342.00±208.00	-10.12±16.46	01.31±07.60
I	256.33±63.85	242.00±112.07	195.00±55.43	-14.35±18.83	-24.98±04.69[a]
J	445.33±155.67	335.33±112.09	374.33±132.53	-21.01±07.40	-15.10±05.42
K	326.67±139.1	297.00±151.9	257.33±112.2	-15.70±07.7	-18.31±15.5

	5	7	9	8	4
L	145.50±15.50	169.00±20.00	149.00±23.00	16.00±01.39	01.88±04.94
M	458.33±142.67	448.33±161.67	448.33±161.67	-08.25±09.75	01.00±0.01
N	596.33±4.67	512.67±68.86	453.00±32.62	-14.20±10.97	-24.03±05.45[a]
O	305.00±148.36	298.67±155.77	143.00±1.00	-04.14±10.23	-07.78±09.94
Normal	87.33±2.91	95.33±10.33	89.67±12.68	08.96±09.89	02.80±14.40
Diabetic control	210.33±62.59	196.00±78.23	205.33±77.65	-11.78±08.79[b]	-06.46±07.36[b]
Glibenclamide	260.00±103.68	170.67±28.67	130.00±20.11	-22.93±14.65	-40.90±11.46[a]

Values are expressed as mean + standard error of mean with those bearing different superscripts being significantly ($P< 0.05$) different. N = 4.

Table 4.26: Mean Fasting Blood Glucose and Their Percentage (%) Changes of Alloxan-Induced Diabetic Mice Administered Different Fractions (40mg/kg body weight) of Aqueous/Methanolic Leaf Extracts of *C. micranthum* For Four Hours

FRACTION	Mean Fasting Blood Glucose (mg/dl)			% Change in Fasting Blood Glucose	
	0hrs	2hrs	4hrs	2hrs	4hrs
B’	408.50±171.50	368.50±231.50	332.50±210.50	-19.37±22.82	-27.45±21.07
C’	319.00±159.00	206.00±80.00	154.40±44.50	-30.71±09.46	-44.81±13.56[a,b]
D’	305.50±116.50	229.00±90.00	210.50±88.50	-25.43±01.02[a]	-32.30±03.15[a]
E’	453.00±31.00	259.00±18.00	238.00±15.00	-42.28±07.92[b]	-47.44±00.29[b]
F’	290.00±62.00	185.50±27.50	186.00±16.00	-35.10±04.39[b]	-34.03±08.59[b]
G’	433.50±167.50	148.00±6.00	120.00± 11.00	-60.50±13.88[c]	-66.31± 15.56[b]
Normal	87.33±2.91	95.33±10.33	89.67±12.68	08.96±09.89	02.80±14.40
Diabetic control	210.33±62.59	196.00±78.23	205.33±77.65	-11.78±08.79[d]	-06.46±07.36[c]
Glibenclamide	260.00±103.68	170.67±28.67	130.00±20.11	-22.93±14.65	-40.90±11.46

Values are expressed as mean + standard error of mean with those bearing different superscripts being significantly (P< 0.05) different. N = 4.

Table 4.27: Mean Fasting Blood Glucose and Their Percentage (%) Changes of Alloxan-Induced Diabetic Mice Administered Fractions D and G (and their 1:1 Combinations) at 40mg/kg body weight of Aqueous/Methanolic Leaf Extracts of *G. sylvestre* and *C. micranthum* Respectively for Four Hours

Fraction	Mean Fasting Blood Glucose (mg/dl)			% Change in Fasting Blood Glucose	
	0hrs	2hrs	4hrs	2hrs	4hrs
D	213.33±67.66	132.33±25.10	129.33±19.55	-32.75±08.31	-32.18±12.44 **(0.95)**
G’	433.50±167.50	148.00±6.00	120.00± 11.00	-60.50±13.88[a]	-66.31± 15.56[a] **(1.18)**
D:G’ (1:1)	227.33±9.82	211.00±77.16	153.00±15.13	-09.04±29.74	-32.81±05.08[b]
Normal	87.33±2.91	95.33±10.33	89.67±12.68	08.96±09.89[b]	02.80±14.40[c]
Diabetic control	210.33±62.59	196.00±78.23	205.33±77.65	-11.78±08.79[c]	-06.46±07.36[c]
Glibenclamide	260.00±103.68	170.67±28.67	130.00±20.11	-22.93±14.65	-40.90±11.46

Values are expressed as mean + standard error of mean with those bearing different superscripts being significantly ($P< 0.05$) different. N = 4. Values in parentheses represent Combination Index (CI).

4.8.2 Further fractionation (preparative TLC)

Preparative TLC of GS – D was used to isolate 5 compounds whereas CM – G revealed 6 compounds. Fasting Blood Glucose analyses showed subfractions D4 for GS and G4 for CM to have the highest percentage reductions (Table 4.28). When 1:1 combination of D4:G4 was also administered, a synergistic effect was observed with combination index of 0.23 for GS - D4 and 0.29 for CM – G4 (Table 4.28). Thin Layer chromatograms of GS – D4 revealed more co-eluting compounds which are mostly phenolics whilst that of CM – G4 showed a single spot of a glycoside.

4.9 Alpha-amylase and Alpha-glucosidase Inhibition

Moreover, the subfractions D4 (for GS) and G4 (for CM) gave significantly better α-amylase and α-glucosidase inhibitory activities when compared to standard acarbose (Figure 4.12). However, acarbose was found to have better IC_{50} (137.13µg/ml for α-amylase and 83.47µg/ml for α-glucosidase) than the 1:1 combination of D4:G4 with IC_{50} of 258.75µg/ml (for α-amylase) and 636.28µg/ml (for α-glucosidase) (Figure 4.13). Significantly ($p < 0.01$) negative correlations were established between % decrease in FBG and inhibitory activities of both alpha-amylase/alpha-glucosidase for sub-fraction D4 of GS. In case of sub-fraction G4 of CM and the 1:1 combination of both plants, a positive correlation was observed between the % decrease in FBG and the inhibitory activities of both carbohydrate-hydrolyzing enzymes under study (Table 4.29).

Table 4.28: Mean Fasting Blood Glucose and their Percentage (%) Changes of Alloxan-induced Diabetic Mice Administered Subfractions (10mg/kg body weight) of *G. sylvestre* (Fractions D1-D5), *C. micranthum* (Fractions G0-G5) and 1:1 Combinations of Most Active Subfractions (D4:G4) Leaf Extracts for Four Hours

SubFraction	Mean Fasting Blood Glucose (mg/dl)			% Change in Fasting Blood Glucose	
	0hrs	2hrs	4hrs	2hr	4hr
D1	438.25±44.51	301.75±51.68	525.00±35.71	-31.56±7.44	22.80±13.44
D2	569.50±10.46	412.25±82.58	550.00±0.01	-27.97±13.82	-3.33±1.72
D3	331.25±23.44	262.75±16.47	271.00±23.28	-20.48±2.56	-18.07±5.47
D4	311.50±6.46	254.75±19.73	239.25±14.15	-18.48±4.92[a]	-23.34±3.38[a] **(0.23)**
D5	316.00±18.49	261.00±18.07	265.75±18.29	-17.06±5.78	-15.53±5.93
G0	321.67±32.30	161.33±65.26	266.33±34.81	-48.68±22.39	-17.33±7.28
G1	381.33±46.74	330.33±129.19	361.67±109.33	-19.17±24.45	-8.90±17.77
G2	373.67±86.73	325.00±99.59	404.33±145.89	-16.13±7.90	-1.09±21.37
G3	283.00±63.69	227.67±93.85	148.67±34.57	-25.50±16.72	-39.17±20.51
G4	453.67±71.49	273.33±77.82	154.00±68.13	-32.52±22.88	-64.65±13.71[b] **(0.29)**
G5	406.33±30.02	395.33±73.84	404.00±58.56	-4.17±10.74	-1.25±8.45
D4:G4 (1:1)	350.50±73.14	130.25±5.96	94.50±6.59	-58.25±7.54[b]	-69.11±6.78[b]
Normal	87.33±2.91	95.33±10.33	89.67±12.68	8.96±9.89[c]	2.80±14.40[c]
Diabetic control	210.33±62.59	196.00±78.23	205.33±77.65	-11.78±8.79[d]	-6.46±7.36[c]
Glibenclamide	260.00±103.68	170.67±28.67	130.00±20.11	-22.93±14.65	-40.90±11.46[d]

Values expressed as Mean ± SEM with those bearing different superscripts under the same column significantly different ($P < 0.05$); N = 4. (-)preceding a value means % decrease in Fasting Blood Glucose. Values in parentheses represent Combination Index (CI).

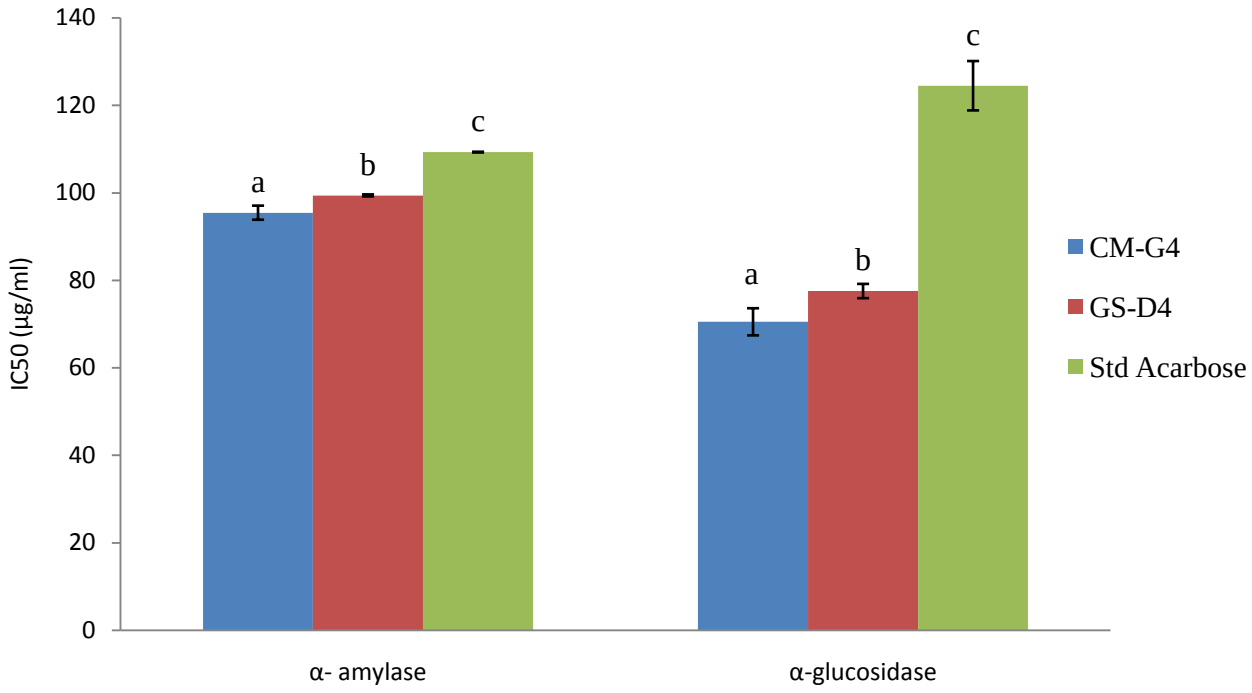


Figure 4.12: *In vitro* α- amylase and α-glucosidase Inhibition Activities of *G.sylvestre* (Subfraction D4) and *C. micranthum* (Subfraction G4) methanolic Leaf Extracts

Values are expressed as mean ± standard error of mean with those bearing different superscripts on same cluster being significantly (P< 0.05) different.

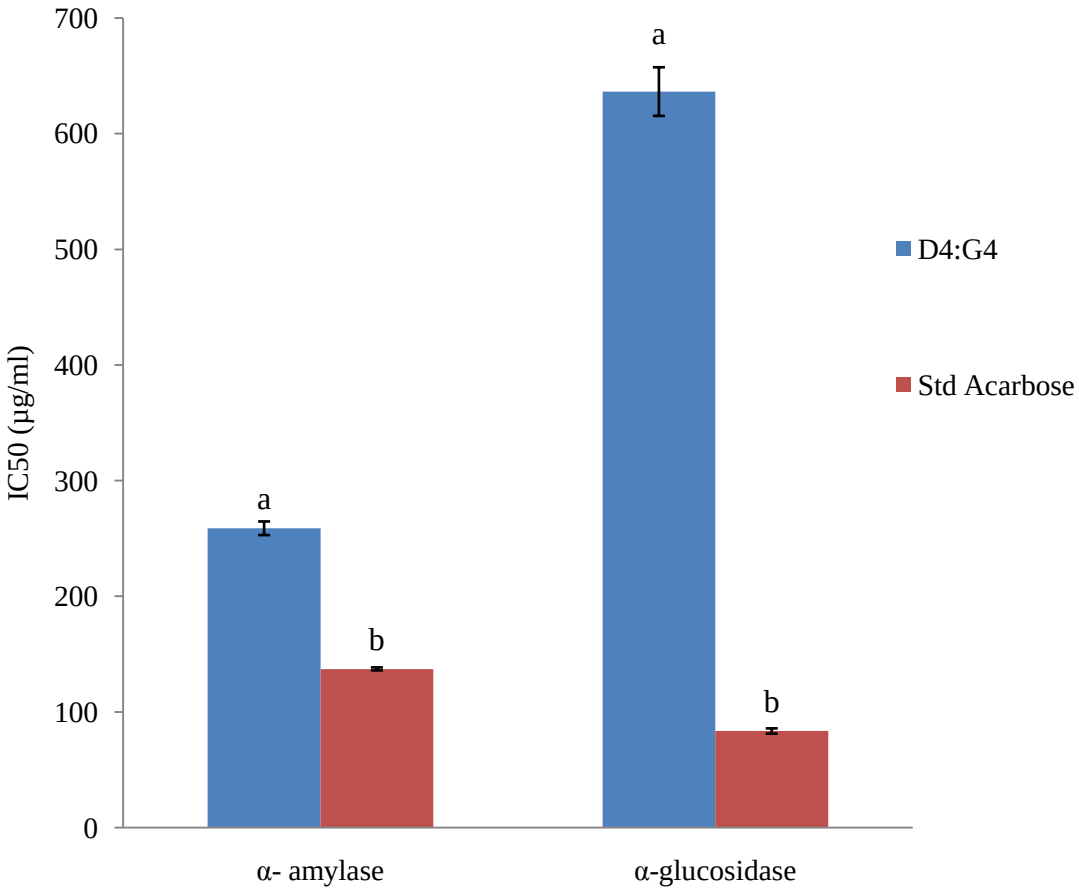


Figure 4.13: *In vitro* α- amylase and α-glucosidase inhibitory activity of 1:1 combination (Subfractions D4:G4) of *G. sylvestre* and *C. micranthum* Methanolic Leaf Extracts

Values expressed as Mean ± SEM of triplicate measurements, with those bearing different superscripts on the same cluster significantly different ($P < 0.05$).

Table 4.29: Relationship between % Decrease in FBG and Inhibitory Activities of Alpha-Amylase/Alpha-Glucosidase in Rats Administered *Gymnema sylvestre* (sub-fraction D4), *Combretum micranthum*(sub-fraction G4)and their 1:1 combination (D4:G4)Methanol Leaf Extracts.

		D4 FBG	G4 FBG	D4 amylase	D4 glucosidase	G4 amylase	G4 glucosidase	D4:G4 amylase	D4:G4 glucosidase	D4:G4 FBG
D4FBG	r			**-1.000****	**-1.000****					
G4FBG	r					**1.000****	**1.000****			
D4amylase	r	**-1.000****								
D4glucosidase	r	**-1.000****								
G4amylase	r		**1.000****							
G4glucosidase	r		**1.000****							
D4:G4amylase	r									**1.000****
D4:G4glucosidase	r									**1.000****
D4:G4FBG	r							**1.000****	**1.000****	1

**. Correlation is significant at the 0.01 level (2-tailed). r = correlation coefficient.

4.10 Isolation and Characterization of Compounds

4.10.1 Ultra-high-performance liquid chromatography – mass spectroscopy (UHPLC-MS) analyses:

The eluted fractions (from preparative TLC) which gave the highest reduction in FBG were analyzed by LC – MS (UHPLC-ESI-QTOF/MS – MS); GS – D4 gave four major separate peaks and about five minor ones. The mass spectra of the peaks identified some phenoliccompounds and a few other ones using the ESI – Molecular Formula Extractor (MFE) Algorithm. The compounds were identified base on their retention times and mass-to-charge ratio (m/z) and listed in Table 4.30. Chromatogram of CM – G4 by LC – MS gave a single major peak at a retention time of 2.003 and a mass – to – charge ratio (m/z) of 329.1392 from the mass spectrum. The compound was identified as a trimethyl ether (Table 4.31).

4.10.2 Fourier-transform infra-red spectroscopy (FTIR)

The FTIR spectra were used to identify principal functional groups in GS – D4 and CM – G4 to further support the compounds identified by LC – MS and NMR.

4.10.3 Nuclear magnetic resonance spectroscopy (NMR)

Giving the purity level of CM – G4 from the preparative TLC and LC – MS analyses, sample of the sub – fraction was subjected to ^{1}H and ^{13}C NMR studies and the spectral data also compared with previous works to elucidate the structure and identify the compound as a glycoside (Figure 4.14).

4.11 Molecular Docking Studies

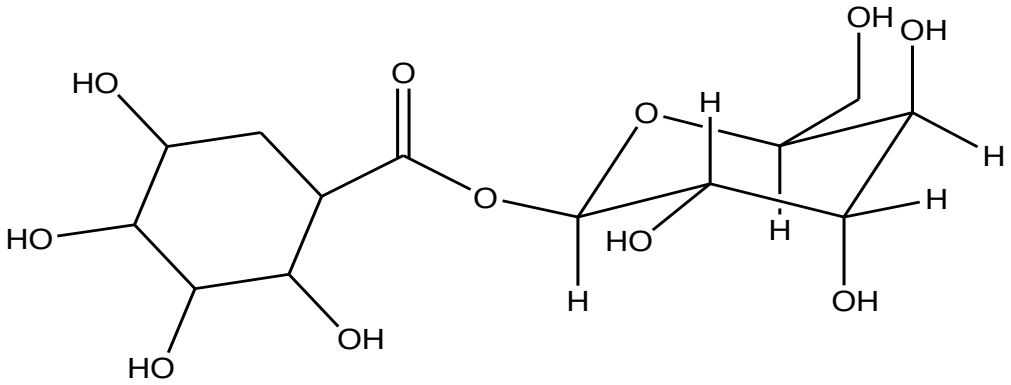
Docking studies showed the compounds isolated from GS to have inhibitory activities against human pancreatic α-amylase (PDB ID = 4W93) with 2,2 –Dimethyl -3-[-4-(acetyloxy) phenyl] - 4 – ethyl -2H-1- benzopyran -7-0l acetate having the best ranking of -24.11Kcal/mol (Table 4.32).

Table 4.30: Compound Composition of Subfraction 4 of Fraction D [N-Hexane:Ethylacetate (20:80)] for *G. sylvestre* Aqueous/Methanolic Leaf Extract Using LC/MS in the Negative Ion Mode

S/N	RT	Mass	Name	Formula	DB Formula	DB Diff (ppm)	Hits (DB)
1	1.993	164.0475	4-Hydroxycinnamic acid	$C_9 H_8 O_3$	$C_9 H_8 O_3$	-0.75	10
2	2.514	186.125	(3S)-6-Hydroxy-3-isopropenyl-heptanoate	$C_{10} H_{18} O_3$	$C_{10} H_{18} O_3$	3.31	10
3	2.558	228.1362	(-)-11-hydroxy-9,10-dihydrojasmonic acid	$C_{12} H_{20} O_4$	$C_{12} H_{20} O_4$	-0.16	10
4	19.962	200.1777	4-Methyl-undecanoic acid	$C_{12} H_{24} O_2$	$C_{12} H_{24} O_2$	-0.39	10
5	21.063	380.162	2,2-Dimethyl-3-[4-(acetyloxy)phenyl]-4-ethyl-2H-1-benzopyran-7-ol acetate	$C_{23} H_{24} O_5$	$C_{23} H_{24} O_5$	0.87	6
6	21.704	292.2036	Colnelenic acid	$C_{18} H_{28} O_3$	$C_{18} H_{28} O_3$	0.94	10
7	23.724	586.349	6,8a-Seco-6,8a-deoxy-5-oxoavermectin "2a" aglycone	$C_{34} H_{50} O_8$	$C_{34} H_{50} O_8$	2.73	2
8	24.049	300.2083	9,13-di-cis-retinoic acid	$C_{20} H_{28} O_2$	$C_{20} H_{28} O_2$	2.01	10
9	24.83	408.1934	Glyinflanin A	$C_{25} H_{28} O_5$	$C_{25} H_{28} O_5$	0.79	10

Table 4.31: Compound Composition of Sub-fraction 4 of Fraction G [Ethylacetate:Methanol (30:70)] For *C. micranthum*Aqueous/Methanolic Leaf Extract Using LC/MS In The Negative Ion Mode

S/N	RT	Mass	Name	Formula	DB Formula	DB Diff (ppm)	Hits (DB)
1	2.242	330.1464	3-Deshydroxysappanol Trimethyl Ether	C_{19} H_{22} O_5	C_{19} H_{22} O_5	1.01	10



2,3,4,5-Tetrahydroxy-cyclohexanecarboxylic acid 3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yl ester

Figure 4.14: Structure/Name of Compound Isolated from Sub-fraction G4 of *Combretum micranthum* Leaf Extract.

Table 4.32: ICM docking scores for compounds (ligands) from sub-fractions D4 and G4 of *G. sylvestre* and *C. micranthum* respectively into the active site of Human Pancreatic alpha-amylase (PDB ID= 4W93)

Name	Score	Ki	Nflex	Hbond	Hphob	VwInt	Dsolv	SolEl	mfScore
2,2-Dimethyl-3-[4-(acetyloxy)phenyl]-4-ethyl-2H-1-benzopyran-7-ol acetate	-24.1091	0.9601	1	-3.97931	-6.54736	-29.9252	11.6462	16.5694	-158.944
(3S)-6-Hydroxy-3-isopropenyl-heptanoate	-4.09438	0.9931	7	-6.6967	-3.96589	-15.1177	9.53999	29.1942	-77.3967
4-Methyl-undecanoic acid	-10.209	0.9829	9	-2.12437	-4.08753	-17.4588	7.0578	7.07529	-83.764
9,13-di-cis-retinoic acid	-21.0646	0.9651	1	-4.49151	-7.1597	-28.5303	11.1612	22.2052	-91.4803
4-Hydroxycinnamic acid	-19.5765	0.9675	1	-4.90917	-3.0141	-14.9481	8.44892	4.35545	-60.0558
(-)-11-hydroxy-9,10-dihydrojasmonic acid	-2.7457	0.9954	7	-5.75156	-4.04947	-16.4217	13.2739	25.432	-59.1592
Colnelenic acid	-3.4008	0.9943	10	-5.25285	-6.93565	-24.6692	11.459	37.2889	-91.8358
Glyinflanin A	-19.9828	0.9668	6	-3.17519	-6.14159	-35.4483	17.4252	16.5828	-110.361
6,8a-Seco-6,8a-deoxy-5-oxoavermectin "2a" aglycone	-5.84437	0.9902	5	-3.7146	-8.7439	-31.6492	20.5337	34.3347	-179.911
3-Deshydroxysappanol Trimethyl Ether	2.978678	1.0050	3	-3.31129	-6.33893	-23.8185	15.72104	39.13788	-128.126
2,3,4,5-Tetrahydroxy-cyclohexane ester glucoside	-9.74556	0.9837	11	-6.85643	-3.48439	-22.0646	17.86277	18.18684	-139.149

SCORE is the overall docking score; Ki is the Inhibition Constant; NFLEX is the number of rotatable torsions; HBOND is the hydrogen bond energy; HPHOB is hydrophobic energy of a water-exposed surface; VWINT is the van der Waals interaction energy; DSOLV is desolvation of hydrogen bond donors/acceptors; SOLEL is the solvation electrostatics energy change from binding; MFSCORE is the mean force score; (ICM User's Guide).

In ICM, a lower (i.e. more negative score) indicates better predicted binding ability for the ligand screened. Also, 2,3,4,5 – tetrahydroxy – cyclohexane ester glucoside isolated from CM gave the best ranking (-9.75 Kcal/mol) in terms of human pancreatic α-amylase inhibition (Table 4.32).

Moreover, docking with the protein α-glucosidase (PDB ID= 3WY2) revealed the compound Glyinflanin A from GS and the ester glucoside from CM to have the best rankings (total binding energies of – 27.33Kal/mol and – 13.92Kal/mol respectively) (Table 4.33). Table 4.34 showed the compounds (-)-11-hydroxy-9, 10-dihydrojasmonic acid (from GS) and 3 – Deshydroxysappanol Trimethyl Ether (from CM) to have the best docking results with total score of their respective binding energies to be -15.72Kcal/mol and -11.43Kcal/mol when docked with Phosphorylated-insulin Receptor Tyrosine Kinase (PDB ID = 1IR3).

Table 4.33: ICM docking scores for compounds (ligands) from sub-fractions D4 and G4 of *G. sylvestre* and *C. micranthum* respectively into the active site of Alpha-glucosidase (PDB ID= 3WY2)

Name	Score	KI	Nflex	Hbond	Hphob	VwInt	Dsolv	SolEl	mfScore
2,2-Dimethyl-3-[4-(acetyloxy)phenyl]-4-ethyl-2H-1-benzopyran-7-ol acetate	-21.1	0.9650	1	-2.6434	-6.31409	-26.6227	14.4807	7.54609	-18.6129
(3S)-6-Hydroxy-3-isopropenyl-heptanoate	-10.7105	0.9821	7	-4.19992	-3.75877	-12.4242	11.7564	2.78867	-25.0895
4-Methyl-undecanoic acid	-13.2168	0.9779	9	-3.44385	-5.07038	-17.5755	10.1828	5.19939	-17.4952
9,13-di-cis-retinoic acid	-9.91661	0.9834	1	0	-6.31909	-27.7872	15.6176	14.8351	-45.7043
4-Hydroxycinnamic acid	-22.5491	0.9626	1	-6.22209	-2.79642	-14.4287	10.2174	1.86665	-5.76411
(-)-11-hydroxy-9,10-dihydrojasmonic acid	-16.7132	0.9722	7	-4.39561	-4.16815	-16.8683	12.3332	0.93766	-30.1484
Colnelenic acid	-15.223	0.9746	10	-2.95346	-7.02483	-25.8541	14.3109	9.20718	-44.9127
Glyinflanin A	-27.3314	0.9549	6	-6.87869	-4.69134	-34.7556	20.8795	13.1983	-72.1797
6,8a-Seco-6,8a-deoxy-5-oxoavermectin "2a" aglycone	-6.79478	0.9886	5	-2.80688	-7.07512	-24.6958	18.9985	18.9301	-76.2757
3-Deshydroxysappanol Trimethyl Ether	1.69633	1.0029	3	-4.67603	-7.19857	-28.0862	14.8269	50.7814	-100.254
2,3,4,5-Tetrahydroxy-cyclohexane ester glucoside	-13.9238	0.9768	11	-6.60623	-4.37827	-25.2595	19.1776	15.1510	-87.0844

SCORE is the overall docking score ; Ki is the Inhibition Constant; NFLEX is the number of rotatable torsions; HBOND is the hydrogen bond energy; HPHOB is hydrophobic energy of a water-exposed surface; VWINT is the van der Waals interaction energy; DSOLV is desolvation of hydrogen bond donors/acceptors; SOLEL is the solvation electrostatics energy change from binding; MFSCORE is the mean force score; (ICM User's Guide).

Table 4.34: ICM docking scores for compounds (ligands) from sub-fractions D4 and G4 of *G. sylvestre* and *C. micranthum* respectively into the active site of Phosphorylated-insulin receptor tyrosine kinase (PDB ID= 1IR3)

Name	Score	KI	Nflex	Hbond	Hphob	VwInt	Dsolv	SolEl	mfScore
2,2-Dimethyl-3-[4-(acetyloxy)phenyl]-4-ethyl-2H-1-benzopyran-7-ol acetate	4.94066	1.0084	1	-1.90016	-7.79289	-24.6254	13.1031	44.193	-127.881
(3S)-6-Hydroxy-3-isopropenyl-heptanoate	0.449632	1.0008	7	-1.16564	-4.26391	-13.4876	7.48931	15.0396	-45.8979
4-Methyl-undecanoic acid	-5.59844	0.9906	9	-1.80951	-5.69234	-18.1516	6.63242	16.0164	-84.1439
9,13-di-cis-retinoic acid	13.9569	1.0239	1	-2.25036	-7.87612	-19.065	10.5208	53.7903	-140.93
4-Hydroxycinnamic acid	-14.6704	0.9755	1	-5.62094	-3.77424	-13.4841	8.06541	13.5065	-90.1667
(-)-11-hydroxy-9,10-dihydrojasmonic acid	-15.7246	0.9738	7	-8.70608	-5.00685	-14.8258	11.8693	17.2428	-104.914
Colnelenic acid	-14.3012	0.9761	10	-1.14552	-7.70307	-25.9435	10.5422	8.78001	-88.3716
Glyinflanin A	-4.69367	0.9921	6	-2.60923	-6.69369	-30.6507	17.1751	31.1542	-124.133
6,8a-Seco-6,8a-deoxy-5-oxoavermectin "2a" aglycone	-12.4151	0.9793	5	-1.1753	-8.22455	-29.3579	19.9668	11.2905	-118.716
3-Deshydroxysappanol Trimethyl Ether	-11.4294	0.9809	3	-1.42995	-4.71538	-23.4043	14.83896	8.822393	-37.2552
2,3,4,5-Tetrahydroxy-cyclohexane ester glucoside	-1.61333	0.9973	11	-5.10691	-3.05328	-17.8773	23.9074	10.15093	-39.1967

SCORE is the overall docking score ; Ki is the Inhibition Constant; NFLEX is the number of rotatable torsions; HBOND is the hydrogen bond energy; HPHOB is hydrophobic energy of a water-exposed surface; VWINT is the van der Waals interaction energy; DSOLV is desolvation of hydrogen bond donors/acceptors; SOLEL is the solvation electrostatics energy change from binding; MFSCORE is the mean force score; (ICM User's Guide).

CHAPTER FIVE

5.0 DISCUSSION

Acutetoxicitytestandsub-chronictoxicitystudy that lastedfor 21dayswasconductedtoevaluatethesafetyprofile of theleafextracts of GS and CM in experimental animals. Safetyprofile onmedicinalplantsisimportantassafetyof herbal medicineusehasbeenquestionedduetoreports of illnessesandfatalities(Park*et. al.*,2010).

General behavior and body weights are one of the critical parameters for the evaluation of first signs of toxicity (Sireatawong *et. al.,* 2008). In the acute toxicity study(OECD, 1996; 2006), there were no observable signs of morbidity or mortality throughout the duration of the experiment at all the doses used, which is an indication that the extracts were well tolerated by the mice.The low acute toxicity (LD_{50}> 5000mg/kg b.w.) observed might be associated with the low amount/concentration of some toxic phytochemicals like alkaloids (Pengelly, 2004; Aiyegoro and Okoh 2010; Kuete *et. al.*, 2010).

This concurs with toxicity studies of *Gymnema sylvestre* extract which had shown its safety when taken in recommended doses – 1% powder in the diet (Ogawa *et. al.,* 2004).Moreso, in an acute toxicity assay, it was demonstrated that treatment with *Combretum lanceolatum*ethanol extract and *Combretum micranthum* aqueous extract in limit dose of 5000 mg/kg orally did not promote any behavioral changes or mortality in animals (Chika and Bello, 2010; Dechandt *et. al.,* 2013).However, acute toxicity alone does not suffice to qualify a drug as being safe. As a result, long-term (sub-chronic) toxicity was carried out to further ascertain the toxicity risk of GS and CM. There are a number of markers of toxicity, e.g. changes in body weight, food and water intake, urine output, and organ weight may all be sensitive indicators of either general or specific toxicity. Thus, animals often consume less food and lose weight after exposure to toxic compounds, and increased organ weight

may be due to a tumor, fluid, or triglyceride accumulation, hypertrophy, or enzyme induction. These changes may of course be confirmed by chemical, biochemical, or histopathological measurements.

Streptozotocin(STZ) is one of the most commonly used substances to induce diabetes in experimental animals. This toxin causes the death of pancreatic - cell by alkylation of DNA resulting in reduced synthesis and release of insulin. Furthermore, it has been shown to be involved in the fragmentation of DNA by means of production of reactive oxygen species (Ezeja *et. al.,* 2014). The diabetic animals induced by STZ are always associated with polydipsia and loss in body weight. In the present study, the body weights of the diabetic rats were significantly lower ($P < 0.05$) than those of the normal control after 21 days of experiment and the reversal of this condition in the groups treated with GS, CM and their 1:1 combination (GSCM) might be associated with a correction in abnormalities due to glucose intolerance, resulting from insulin resistance/inadequate secretion or hyperlipidemia in diabetes mellitus (Wang *et. al.,* 2010). Therefore, it meant GS, CM and GSCM extracts helped regain body weight, which is similar to (Tiwari *et. al.,* 2014) or even better thanglibenclamide.

The tendency for microcytic hypochromic anemia following a fall in the packed cell volume (PCV), haemoglobin (Hb) concentration and red blood cell (RBC) count (Azeez et.al., 2010; Song *et. al.,* 2010) could be allayed as GS, CM and GSCM extracts improved plasma levels of these parameters during the 21 days administration when compared with normal and diabetic controls. The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were also improved in the diabetic/normal treated rats than in their respectivecontrols.

In the present study, exposure to STZ caused a significantincrease in therelative liver and kidney weights of the rats, thusindicating organ toxicity in the rats.Moreover, relative organ weight is an important index ofswelling, atrophy, or hypertrophy. An increase in thisparameter indicates inflammation while a decrease may beassociated with cellular constriction (Adedara *et. al.,* 2014; Kim *et. al.,* 2014). Hence, the increase in the relative liver and kidney weights of animals exposedto STZ may suggest swelling/inflammationof the organs. Moreso, the liver integrity of control and STZtreatedrats was determined bymeasuring biochemicalmarkerssuch as the levels of serum transaminases (AST and ALT), alkalinephosphatase, total protein and bilirubin. Aminotransferases are localized inperiportal hepatocytes where they are involved in amino acidmetabolism; transamination reactions and their serumactivitiespresumably increase as a result of cellular membranedamage and leakage (Timbrell, 2009; Shah *et. al.,* 2016). The increases in the serum ALTandASTobserved Inthe STZ-diabetic rats indicate hepaticdamage which may be associated with altered membranepermeability – this agrees with a study conducted by Adeyemi and Orekoya (2014). Alkaline phosphatase (ALP) is a marker enzyme for theintegrity of the hepatobiliary system and the flow of bileinto the small intestine. The increase in serum ALP activityindicates obstructive event or cholestatic effect following STZinduction.However, the elevation in total bilirubin levels observed in the present study, which is an indication ofposthepatic toxicity possibly caused by an interruption tothe drainage of bile in the biliary system following exposureto STZ, was not ameliorated by the extracts. Total protein measurements can reflect nutritional status, kidney disease, liver disease, and many other conditions. Hypoproteinemia, a total protein level less than the reference interval, occurs in any condition where a negative nitrogen balance exists. One cause of a low level of plasma proteins is excessive loss. Plasma proteins can be lost by

excretion in the urine in renal disease; leakage into the gastrointestinal tract in inflammation of the digestive system; and the loss of blood in open wounds, internal bleeding, or extensive burns. Another circumstance producing hypoproteinemia is decreased intake either because of malnutrition or through intestinal malabsorption as seen in sprue.Hyperproteinemia, an increase in total plasma proteins, is not an actual disease state but is the result of the underlying cause, dehydration. When excess water is lost from the vascular system, the proteins, because of their size, remain within the blood vessels. Although the absolute quantity of proteins remains unchanged, the concentration is elevated due to a decreased volume of solvent water. Dehydration results from a variety of conditions including, vomiting, diarrhea, excessive sweating, diabetic acidosis, and hypoaldosteronism (Bishop*et. al.,* 2010). The increased effect on the serum total protein levels observedin the experimental animals in the present study might be as a result of dehydration due to the diabetes induced by STZ.Moreover, alteration in the levels of major lipids such as LDL,HDL, cholesterol, and triacylglycerol could provide usefulinformation on the predisposition to atherosclerosis.Triacylglycerol, LDL, and HDL are associated with lipolysis,transport of plasma cholesterol, and atherosclerotic tendency,respectively. The increases in serum levels of triacylglycerol, LDL (and VLDL), and cholesterolwith a significant decrease in the serumHDL level observedin the STZ-diabetic rats in the present investigation suggest thatthe animals may be predisposed to dyslipidaemiaand other traits of metabolic syndrome associatedwithcardiovascular diseases (a complication of diabetes mellitus) (Ezeja *et. al.,* 2014). Thus, reversal of the hyperlipidaemic traits by the plants is an indication of the ameliorative effects of the diabetic complication. This result was in agreement withDaisy *et. al.* (2009) and Aralelimath and Bhise (2012) who reported

that increasing insulin secretion after administrationof *G. sylvestre* extract led to a decrease of cholesterogenesisand fatty acid synthesis.This result was supported also by Mall *et. al.* (2009) whoreported that *G. sylvestre* decreases total cholesterol, LDL-cholesterol,VLDL-cholesterol and triglyceride levels in diabeticrats and that could be due to the presence of hypolipidemicagent such as sitosterol in the aqueous leaf extract. *G. sylvestre*inhibited the intestinal absorption of oleic acid in rats, whichsuggested the possibility of *G. sylvestre* to inhibit lipid absorption (Wang *et. al.*, 1998). Shigematsu *et. al.* (2001) reported that administration of *G. sylvestre* leaves extract decreased the total cholesterol, LDL-cholesterol, VLDL-cholesterol and triglyceride levels in the serum of rats fed with a high fat diet or normal fat diet and that might be due to the effect of *G. sylvestre* in increasing neutral sterols and acid steroids excretion into faeces. Luo *et. al.* (2007) discovered the ability of *G. sylvestre* water extract to prevent obesity by improving the cholesterol metabolism and inhibiting polyphagia. Ishijima *et. al.* (2008) suggested that gymnemic acid might have some pharmacological activities including antidiabetic activity and lipid lowering effects via the inhibition of glycerol-3-phosphate dehydrogenase activity *in vitro*.

Kidney damage is associated with decline in renal functionwhich could lead to renal failure. The decrease in renalfunction evidenced by significant increase in plasma levels ofurea and creatinine in rats with STZ-diabetes was established.Also, although, administration of 1.00% basal powder *Gymnema sylvestre* extract (GSE) in the diet in Wistar rats for 52 weeks has shown no toxic effects and no animal died during an experiment (Ogawa *et. al.*, 2004), treatment of diabetic patients with *Gymnema sylvestre* has been shown to cause toxic hepatitis or drug-induced liver injury (DILI) (Shiyovich *et.al.*, 2010). So the plant should be used with care.

Normally the deleterious effects of oxidative stress arecounteracted by the natural antioxidant defense mechanismsto protect the biological system against reactive oxygenspecies. Antioxidant statuses are regulated by multiple factors.The oxidative status of the cell is the primary factorregulating gene expression and the activity of antioxidantenzymes (Adedara *et. al.,* 2014). Superoxide dismutase (SOD), catalase (CAT),glutathione peroxidase (GPx), and glutathione-S-transferase(GST) are endogenous antioxidant enzymes responsible forthe detoxification of deleterious oxygen radicals and theiractivities are used to assess oxidative stress in cells.Thefirst line of defense to the cells is provided by the existence ofa mutually supportive relationship between metalloenzymeSOD, which accelerates the dismutation of endogenous cytotoxicsuperoxide radicals to H_2O_2, and CAT, which convertsthe deleterious peroxide radicals into water and oxygen. In the present study, the activities of hepatic CAT, GSH, GSSG and GSH-Px were markedly increased inthe extract treated rats. According to a study (Vasi and Austin, 2009),administration of *G. sylvestre* extract to diabetic rats increased superoxide dismutase activity and decreased lipid peroxide by either directly scavenging the reactive oxygen species, due to the presence of various antioxidant compounds, or by increasing the synthesis of antioxidant molecules . Moreso, some poly-herbal ayurvedic formulations like Hyponidd and Dihar containing *G. sylvestre* extract had shown antioxidant activity by increasing superoxide dismutase, glutathione and catalase levels in rats (Mahajan *et. al*., 2011). The induction of theseantioxidant enzymes may indicate an adaptive response tocounter the damaging effect of oxidative stress possiblygenerated during STZ metabolism. Glutathione playsa pivotal role in the scavenging of hydroxyl radical andsinglet oxygen directly as well as in the detoxification ofhydrogen peroxides and lipid hydroperoxides by the activityof GSH-Px. Further, GST is involved in the biochemical

conjugationof electrophilic oxidants with GSH to form watersolublecompound products that are readily excreted fromthe system. The deleterious chemical effects of H_2O_2molecules canbe divided into the categories of direct activity, originatingfromtheir oxidizing properties, and indirect activity inwhichthey serve as a source for more deleterious species, such ashydroxyl radicals and hypochlorous acid. Reactive oxygenspecies attack cellular components containing polyunsaturatedfatty acid residues to produce peroxyl radicals whichundergo a cyclization reaction to form endoperoxides and eventually trans-4-hydroxy-2-nonenal and MDA. Theincreases in the hepatic MDA levels observed in this study clearly indicate a stateof stress in the tissues possibly induced by STZ or itsmetabolites. Thus by reducing the hepatic TPP/ROS and MDA levels in the treated-rats compared with diabetic control is a promising sign that GS, CM and their 1:1 combination have the tendency of reversing oxidative stress associated with diabetes mellitus (Table 4.16).

Ability of the plants to combat oxidative stress is further supported by *in vitro* antioxidants (radical scavenging) activities where they were found to have scavenging activities against DPPH, ABTS and Nitric Oxide with a very good FRAP – all the *in vitro* antioxidant parameters correlate well with the *in vivo* ones(Touré *et. al.*2011.). The radical scavenging activities of the plants could be attributed to the total phenolics and total flavonoids contents (Aiyegoro and Okoh 2010; Djeridane *et. al.,* 2010; Prathapan *et. al.,* 2012), compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical's reactivity. The scavenging activity of $ABTS^+$ radical by the plants extract was found to be appreciable; this implies that the plant extract may be useful for treating radical related pathological damage.

Nitric oxide (NO) is a reactive free radical produced by phagocytes and endothelial cells, to yield more reactive species such as peroxynitrite which can be decomposed to form OH radical. The level of nitric oxide was significantly reduced in this study by the crude extracts. Since NO plays a crucial role in the pathogenesis of inflammation, these plants(GS and CM) might be useful in curbing ulcerations asssociated with diabetes mellitus. Plants with antioxidant activities have been reported to possess free radical scavenging activity. Free radicals are known as major contributors to several clinical disorders such as diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases as a result of deficient natural antioxidant defence mechanism (Aiyegoro and Okoh, 2010; Alexiou and Demopoulos, 2010; Yasir *et. al.,* 2012; Mistry and Mona, 2015).

The hypoglycaemic/antidiabetic activity of the plants might be due to the presence of some active components like flavonoids (Evans, 1999) and may be due to regeneration of β-cells in the pancreas (Mhasker and Caius, 1930; Shanmugasundaram *et. al.,* 1990; Ahmed *et. al.,* 2010). Hyperglycaemia-induced impairments in redox balance are considered a key trigger of diabetic complications,through upregulated generation of reactive oxygen species (ROS) , together with an impaired ability of the endogenous antioxidant defense system to remove them (Huynh *et. al.,* 2013). The current study has displayed the ability of the plants, GS and CM to have glucose lowering effect which correlates well with reduced ROS in rats and improved antioxidant parameters like CAT, GSH, GSSG, GSH-Pxand MDA, hence suggesting that the plants improved ability of the endogenous antioxidant defense system through their hypoglycaemic activity (Tables 4.14, 4.16, 4.18 and 4.19). Phytochemical compounds have been shown to possess significant antidiabetic properties as obtained in the current study. This agreed with some studies where LC/MS analysis revealed the presence of antihyperglycemic compounds like

gymnemagenin and gymnemic acids in *G. sylvestre* extract and the level of lipid peroxidation reduced by 31.7% in serum, 9.9% in liver, and 9.1% in kidney in diabetic rats fed with the ethanolic extract. Antihyperglycemic effect of crude saponin fraction and five triterpene glycosides (gymnemic acids I–IV and gymnemasaponinV), isolated from the methanolic extract of the leaves, was also reported. It was found that gymnemic acid IV (3.4/13.4mg/kg) decreased blood glucose levels by 14.0–60.0% within 6 hours of administration as compared to glibenclamide. It has been reported that gymnemic acid IV increased plasma insulin levels in STZ-diabetic mice at a concentration of 13.4mg/kg (Tiwari *et. al.*, 2014). Also, polyphenolic compounds have been shown to have effects on a number of diseases including diabetes mellitus. Resveratrol, anthocyanins and other condensed tannins have all demonstrated antihyperglycemic activity, whether by reducing obesity or other proposed mechanisms. Three theaflavins, from black tea or fermented *Camellia sinensis*, as well as two more flavonoids from *Artemisia dranunculus* L., exhibit glucose-lowering activity via downregulation of hepatic gluconeogenesis. In another study, ethyl acetate and n-butanol fractions of crude leaf extract of *C. micranthum*revealed a number of polyphenolic compounds including catechins, glycosylflavones, flavans, galloylated C-glycosylflavone derivatives and a flavan-piperidine alkaloid. The catechins and flavans were the active compounds by antioxidant capacity and epicatechin was identified as a glucose-lowering compound by phosphoenolpyruvate carboxykinase (PEPCK) inhibition (Welch, 2010).

The plants might be good hypoglycaemic/antidiabetic agent for type 2 diabetics because of their effect on weight control/reduction, especially as seen in the normal rats treated with 1:1 GSCM. The weight reduction properties of the plants might be due to the presence of saponins and terpenoids in the leaves since previous studies had shown that

these phytochemicals have weight reduction properties (Sotaniemi *et al.*, 1995; Nyarkoa *et. al.*, 2002; Alexiou and Demopoulos, 2010)Weight reduction property of the plants agrees with the work of Preuss *et. al.* (2004).

The leaf extracts also showed some hypolipidaemic activity after 21 days of administration. This finding is in agreement with the works of Bishayee and Chatterjee (1994) and Atiku *et. al.* (2005).The hypolipidaemic and antidiabetic activities of the plants might be due to the presence of flavonoids (Li *et. al*., 2009)as the current study has shown high levels of these phytochemicals in both plants.

Pancreatic and intestinal glucosidases are the key enzymes in dietary carbohydratedigestion and inhibitors of these enzymes are found to be effective in retardingglucose absorption to suppress hyperglycaemia. Inhibition of these enzymesreportedly decreased blood glucose levels in diabetic patients.Findings of this work correlates well with those of earlier studiesconducted using *G. sylvestre*, which reported reduced blood glucose and increasedplasma insulin levels in both type 1 and type 2 diabetic rats (Shanmugasundaram*et. al.*, 1990;Ramkumar *et. al*.. 2010; El Shafey *et. al.*, 2013). Hexokinase plays an important role in the maintenance of glucose homeostasis. The activity of hexokinase significantly decreased in the liver of diabetic rats whileadministration of GS and CM to diabetic rats resulted in significant reversal in the activity of hexokinase. The increased activity of hexokinase causes an increase in glycolysis and utilization of glucose for energy production. Administration of the plants' extracts to diabetic rats also resulted in a significant increase in the level of serum insulin which will favour glycolysis(Adewole and Ojewole 2009; Ramkumar *et. al.*. 2011; Patience *et. al.*,2014).

Several plants were known to have hypoglyceamic property via the inhibition of carbohydrates hydrolyzing enzymes. The result of this study indicates that the extracts possess significant inhibitors of α–amylase and α-glucosidase and may thus be effective in reducing postprandial hyperglyceamia which may not be unconnected with the polyphenolic contents of the plants under study (Bello *et. al.*,2011; Etxeberria *et. al.*, 2012; Sama *et. al.*, 2012).

Medicinal plants have beneficial multiple activitiesincluding manipulating the carbohydrate metabolism by variousmechanisms, preventing and restoring integrity and functioningof β-cells, controlling insulin release, improving glucose uptakeand utilization, and antioxidant properties (Prabhakar *et. al.,* 2014).Drug/herbal combinations when used together may sometimes produce enhanced, same or diminished effect. In this interaction there might be three different types of behaviors namely synergy, antagonistic and additive/indifferent. In the current study, GS and CM, in a 1:1 combination synergistically caused a significant reduction in FBG(Table 4.28), as well as improved lipid profile and antioxidant status of the rats (in the semi-purified fractions), which might be as a result of combined effect of the phytochemicals present in them, especially flavonoids (phenolics) (Table 4.30). This could be attributed to the restoration of integrity and functioning of β-cells, insulin mimetic potential of the compounds which modulate membrane receptors to enhance peripheral glucose entry into tissues or insulin sensitivity, and antioxidant properties(Steve *et. al.*, 2008; Ramkumar *et. al.,* 2010; Atangwho *et. al.*, 2012; Okoro *et. al.,* 2014; Prabhakar*et. al.,* 2013; Prabhakar *et. al.*, 2014) (Tables 4.32, 4.33 and 4.34).

In the current study, the compoundswere automatically profiled by a combination ofin-house database as well as UHPLC-Q-TOF-MS analysis and further identified by their retention times and MS/MS spectra including characteristic MS/MS fragmentation ions.

The mass differences between the calculated (theoretical) and observed values were less than 4 ppm for the molecular ions. Thus, nine (9) main compounds were identified, for GS,majority of which are phenols (Table 4.30). Plant phenolics, including flavonoids have been implicated in the management/treatment of diabetes mellitus and other diseases (Satheeshkumar *et. al.,* 2014; Gu*et. al.,* 2015; Qiao *et. al.,* 2015). Of the compounds identified in the GS subfraction, 2,2-Dimethyl-3-[4-(acetyloxy)phenyl]-4-ethyl-2H-1-benzopyran-7-ol acetate, Glyinflanin A and (-)-11-hydroxy-9,10-dihydrojasmonic acid gave good lower energy binding predictions for alpha-amylase, alpha-glucosidase inhibitions and Phosphorylated-insulin receptor tyrosine kinase activation respectively. The Molecular docking study radically corroborates the binding affinity and inhibition of α-glucosidase and α-amylase enzymes (Ahmed *et. al.,* 2014) and further substantiates the insulin-mimicking ability of compounds present in GS (Tables 4.32, 4.33 and 4.34). Also, 2,3,4,5-Tetrahydroxy-cyclohexane ester glucosideisolated from CM subfraction and identified by ^{1}H and ^{13}C NMR displayed very good binding affinity for and inhibition of both alpha-amylase and alpha-glucosidase enzymes, thereby, further supporting the postprandial glucose lowering ability of the plant, CM seen in this study. This could be attributed to the similarity in structure of the conventional α-amylase/α-glucosidase's substrate (glucose) and that of the glycoside isolated in the CM plant. Thus, the synergism observed upon combination of the plants could be associated with combined antioxidants property of the phenols in GS(Alexiou and Demopoulos, 2010; Azeez *et. al.,* 2010; Yadav *et. al.,* 2010; Okoro *et. al.,* 2014; Emami and Ghanbarimasir, 2015) (which reverses the oxidative stress due to STZ) and the inhibition of the carbohydrate-hydrolyzing enzymes by the glycoside present in CM (Lee*et. al.,* 2008; Akkarachiyasit *et. al.,* 2010;Ramkumar *et. al.,* 2010; Upendra Rao *et. al.,* 2010; Patience *et. al.,*2014; Tiwari *et. al.,* 2014).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

G. sylvestre and *C. micranthum* leaf extracts were found to be relatively safe with an LD_{50}>5000mg/kg b. w. and also showed reduction in ROS, MDA, serum ALT and ALP. Both plants also displayed radical scavenging activities against ABTS, DPPHand nitric oxide which is likely connected to the high total flavonoidsand total phenolic contents.The plants also caused increases in total body weight, serum total protein, serum Hb, liver GSH, GSSG and GSH – Px. Leaf extracts of the plants caused significantly better *in vitro* α – glucosidase inhibitory activities when compared to standard acarbose which could be attributed to some phytochemicals like glycosidesand flavonoids. GS and CM also reduced, significantly, *in vivo*FBG both in the acute and long-term studies with ameliorating effects on the increased feed and water intakes, reduction in TC, LDL, VLDL and TG and a concomitant increase in HDL; both plants also increased seruminsulin and liver hexokinase levels. Preparative – TLC with n–hexane:ethyl acetate (30 : 70) revealed **GS–D4 and CM-G4** sub-fractions to have the best % reduction in FBG and also displayed inhibitory activities towards α – glucosidase and α – amylase. The LC-MS (QTOF – MS/MS) in the negative ion mode coupled with FTIR revealed that GS – D4 is a phenolic rich fraction while CM – G4 is a glycoside.^{1}HNMR, ^{13}CNMR and IR spectra were used to identify the isolated compound in CM-G4 as2,3,4,5-Tetrahydroxy-cyclohexane ester glucoside with

antihyperglycaemic effects.The 1:1 combination of GS – D4: CM – G4 yielded synergistic reduction in FBG.

Molecular docking studies (ICM) indicates best predicted binding ability (i.e. more negative values) of the ligands **2,2-Dimethyl-3-[4-(acetyloxy)phenyl]-4-ethyl-2H-1-benzopyran-7-ol acetate and 2,3,4,5-Tetrahydroxy-cyclohexane ester glucoside** to the protein human pancreatic alpha-amylase (PDB ID=4W93).Also, the ligands **Glyinflanin A and 2,3,4,5-Tetrahydroxy-cyclohexane ester glucoside** displayed best predicted binding ability to alpha-glucosidase (PDB ID=3WY2).However, **(-)-11-hydroxy-9,10-dihydrojasmonic acid and 3-Deshydroxysappanol** Trimethyl Ether gave the best predicted binding ability to phosphorylated insulin receptor tyrosine kinase (PDB ID=1IR3).

6.2Conclusion

- A 1:1 combination of the aqueous leaf extracts of both plants at a doses of 400 to 600 mg/kg body weight administered to STZ-induced diabetic rats showed a dose-dependent decrease in polyphagia, polydipsia, hyperlipidaemia and hyperglycaemia.
- At a dose of 40 mg/kg body weight, a 1:1 combination of GS-D and CM-G fractions significantly ($P < 0.05$) reduced FBG from 9.04 – 32.81±5.08% in an additive manner.
- At a dose of 10 mg/kg body weight, a 1:1 combination of GS-D4 and CM-G4 sub-fractions significantly ($P < 0.05$) reduced FBG to58.25±7.54% in 2 hoursand69.11±6.78% in 4 hours in a synergistic manner.
- 2,3,4,5-Tetrahydroxy-cyclohexane ester glucoside with antihyperglycaemic activity was isolated from G4 sub-fraction of *Combretum micranthum* methanol leaf extract; identified by ^{1}H and ^{13}C NMR.

6.3 Recommendations

i. The *G. sylvestre* sub-fraction D4 should be further fractionated by preparative-HPLC and run bioassay-guided study to confirm the alpha-amylase and alpha-glucosidase inhibitory activities as well as insulin-mimicking properties (hypoglycaemic potential) of the compounds as predicted by the ICM Molecular docking analyses.

ii. NMR studies should also be carried out to elucidate the possible structure(s) of the compounds that might be identified in (1) above.

iii. Other fractions (E and F) of *G. sylvestre* from the column chromatography stage of the study should also be subjected to further studies as in 1 and 2 above.

iv. ADMET studies should also be carried out on the compounds.

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APPENDICES

Appendix I: Reagent Composition of Commercial Kits

Appendix Ia: Reagent Composition for Insulin

a. Insulin Calibrators – 2.0ml/vial (dried)

Six (6) vials of references for insulin antigen at levels of 0(A).5(B), 25(C). 50(D), 100(E), and 300(F) µIU/ml. Reconstitute each vial with 2ml of distilled or deionized water. The reconstituted calibrators are stable for sixty (60) days at 2-8°C. A preservative has been added.

Note: The calibrators, human serum based, were calibrated using a reference preparation, which was assayed against the WHO 1st IRP 66/304.

b. Insulin Enzyme Reagent – 13ml/vial

One (1) vial containing enzyme labelled affinity purified monoclonal mouse x-insulin lgG, biotinylated monoclonal mouse x-insulin 1gG in buffer, dye, and preservative. Store at 2 – 8°C.

c. Streptavidin Coated Plate – 96 wells

One 96-well microplate coated with streptavidin and packaged in an aluminium bag with a drying agent. Store at 2-8°C.

d. Wash Solution Concentrate – 20 ml

One (1) vial containing a surfactant in buffered saline. A preservative has been added. Stored at 2-8°C.

e. Substrate A- 7.0ml/vial

One (1) bottle containing tetramethylbenzidine (TMB) in buffer. Store at 2-8°C.

f. Substrate B- 7.0ml/vial

One (1) bottle containing hydrogen peroxide (H_2O_2) in buffer. Store at 2-8°C.

g. Stop Solution – 8.0ml/vial

One (1) bottle containing a strong acid (1N HCl). Store at 2-30°C.

Appendix Ib: Reagent Composition for Serum Total Cholesterol

Contents	Concentrations in the Test
R1. Reagent	

Pipes buffer	80mmol/l, pH 6.8
4 – Aminoantipyrine	0.25mmol/l
Phenol	6mmol/l
Peroxidase (E.C.l.ll.l.7. Horse Radish, + 25°C)	≥0.5U/ml
Cholesterol esterase (E.C.3.l.l.13. Pseudomonas, 37°C)	≥15Uml
Cholesterol oxidase (E.C. 1.1.3.6. Nocardia, 37°C)	≥ 0.10U/ml

Appendix Ic: Reagent Composition for Triglycerides

Contents	**Concentrations in the Test**
R1a. Buffer	
Pipes Buffer	40mmol/l, pH 7.6
4 – chloro – phenol	5.5 mmol/l
Magnesium – ions	17.5mmol.l
R1b. Enzyme Reagent	
4 – aminophenazone	0.5mmol/l
ATP	1.0mmol/l
Lipases	≥150U/ml
Glycerol – kinase	≥0.4U/ml
Glycerol – 3-phosphate oxidase	≥1.5 U/ml
Peroxidase	≥0.5U/ml
CAL. Standard	192mg/dl

Appendix Id: Reagent Composition for HDL - Cholesterol

Contents	**Initial Concentrations of Solution**
R1. Phosphotungstic Acid	0.55mmol/l

Magnesium Chloride	25mmol/l

Appendix Ie: Reagent Composition for AST

Contents		**Initial Concentration of Solutions**
R1. Buffer		
	Phosphate buffer	100 mmol/l, pH 7.4
	L – aspartate	100mmol/l
	α-oxoglutarate	2mmol/l
R2.	2,4 – dinitrophenylhydrazine	2mmol/l

Appendix If: Reagent Composition for ALT

Contents		**Initial Concentrations of Solutions**
R1. Buffer		
	Phosphate buffer	100 mmol/l, pH 7.4
	L – alanine	200 mmol/l
	α-oxoglutarate	2.0 mmol/l
R2.	2,4-dinitrophenylhydrazine	2.0 mmol/l

Appendix Ig: Reagent Composition for ALP

Contents	**Concentrations in the Test**
R1a. Buffer	

Diethanolamine buffer	1mol/l, pH 9.8
$MgCl_2$	0.5mmol/l
R1b. Substrate	
p-nitrophenylphosphate	10mmol/l

Appendix Ih: Reagent Composition for Total Protein

Contents	**Concentration of Solutions**
R1. Biuret Reagent	
Sodium hydroxide	100mmol/l
Na-K-tartrate	16mmol/l
Potassium iodine	15mmol/l
Cupric Suphate	6mmol/l
R2. Blank reagent	
Sodium hydroxide	100mmol/l
Na-K-tartrate	16mmol/l
CAL. Standard	
Protein	5.99g/dl

Appendix Ii: Reagent Composition for Total Bilirubin

Contents	**Initial Concentration of Solutions**
R1. Sulphanilic acid	29mmol/l

Hydrochloric acid	0.17N
R2. Sodium Nitrite	38.5mmol/l
R3. Caffeine	0.26mol/l
Sodium benzoate	0.52 mol/l
R4. Tartrate	0.93 mol/l
Sodium Hydroxide	1.9N

Appendix Ij: Reagent Composition for Serum Urea

Contents	**Initial Concentration of Solutions**
R1. EDTA	116mmol/l
Sodium nitroprusside	6mmol/l
Urease	1g/l
R2. Phenol (diluted)	120mmol/l
R3. Sodium Hypochlorite (diluted)	27mmol/l
Sodium Hydroxide	0.14N
CAL Standard	80.77mg/dl

Appendix Ik: Reagent Composition for Serum Creatinine

Contents	**Initial Concentration of Solutions**
CAL. Standard	2.08mg/dl
R1a. Picric Acid	35mmol/l
R1b. Sodium Hydroxide	0.32mol/l

APPENDIX II: Computation tables for enzyme activities

APPENDIX II a: Computation table for AST activities

Absorbance	**U/I**	**Absorbance**	**U/I**
0.020	7	0.100	36

0.030	10	0.110	41
0.040	13	0.120	47
0.050	16	0.130	52
0.060	19	0.140	59
0.070	23	0.150	67
0.080	27	0.160	76
0.090	31	0.170	89

APPENDIX II b: Computation table for ALT activities

Absorbance	**U/I**	**Absorbance**	**U/I**
0.025	4	0.275	48
0.050	8	0.300	52
0.075	12	0.325	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77
0.200	34	0.450	83
0.225	39	0.475	88
0.250	43	0.500	94

APPENDIX III: Details of Fractionations of GS and CM Leaf Extracts/Fractions

Table III a: Fractionation and Pooling of Fractions for *G. sylvestre* Aqueous/Methanolic Leaf Extract

S/N	Fraction Label	Pooled Fractions (column chromatography)	Solvent System (column chromatography)	Amount Recovered (mg)	Rf Values (TLC)	Appearance
1	A^0	001-026	N-hexane:Ethylacetate	0	0.00	Plain

			(90:10)			
2	A	027-036	N-hexane:Ethylacetate (60:40)	343	0.65	Light yellow
3	B	037-040	N-hexane:Ethylacetate (40:60)	109	0.61	Yellow
4	C	041-045	N-hexane:Ethylacetate (30:70)	90	0.63	Yellowish green
5	D	046-053	N-hexane:Ethylacetate (20:80)	169	0.66	Green
6	E	054-059	N-hexane:Ethylacetate (20:80)	110	0.60	Greenish brown
7	F	060-069	N-hexane:Ethylacetate (20:80)	135	0.09	Yellowish brown
8	G	070-091	N-hexane:Ethylacetate (10:90)	134	0.14	Yellow
9	H	092-094	Ethylacetate:Methanol (70:30)	25	0.80	Light yellow
10	I	095-098	Ethylacetate:Methanol (70:30)	43	0.69	Light yellow
11	J	099-101	Ethylacetate:Methanol (50:50)	37	0.83	Light yellow
12	K	102-106	Ethylacetate:Methanol (30:70)	651	0.98	Yellowish brown
13	L	107	Ethylacetate:Methanol (30:70)	578	0.90	Brown
14	M	108-120	Methanol (100%)	7218	0.93	Brown
15	N	121-122	Methanol (100%)	726	0.71	Brown
16	O	123-130	Methanol (100%)	1697	0.85	Brown

NB: Pooling was done based on Physical appearance and UV-Fluorescent visibility (at 254nm and 364nm wavelengths); some of the TLC Chromatograms were also Daylight visible.

Table III b: Fractionation and Pooling of Fractions for *C.micranthum* Aqueous/Methanolic Leaf Extract

S/N	Fraction Label	Pooled Fractions (column chromatography)	Solvent System (column chromatography)	Amount Recovered (mg)	Rf Values (TLC)	Appearance
1	A	001-026	N-hexane:Ethylacetate (80:20)	0	0.00	Plain

2	B	027-037	N-hexane:Ethylacetate (60:40)	300	0.63	Light yellow
3	C	038-046	N-hexane:Ethylacetate (30:70)	160	0.71	Light yellow
4	D	047-053	N-hexane:Ethylacetate (20:80)	100	0.57	Light yellowish
5	E	054-078	N-hexane:Ethylacetate (10:90)	260	0.49	Yellowish brown
6	F	079-096	Ethylacetate:Methanol (70:30)	2580	0.75	Brown
7	G	097-171	Ethylacetate:Methanol (30:70)	2880	0.83	Dark brown

NB: Pooling was done based on Physical appearance and UV-Fluorescent visibility (at 254nm and 364nm wavelengths) of the TLC Chromatograms.

Table III c: Further Fractionation (Preparative-TLC) of Fraction D [N-hexane:Ethylacetate (20:80)] for *G.sylvestre* Aqueous/Methanolic Leaf Extract

S/N	Sub-Fraction Label	Rf Values	Appearance
1	D1	0.62	Green
2	D2	0.49	Yellow
3	D3	0.40	Light yellow
4	D4	0.26	Yellowish grey
5	D5	0.11	Yellowish green

Table III d: Further Fractionation (Preparative-TLC) of Fraction G [Ethylacetate:Methanol (30:70)] for *C. micranthum* Aqueous/Methanolic Leaf Extract

S/N	Sub-Fraction Label	Rf Values	Appearance
1	G0	0.95	Pale yellow

2	G1	0.80	Sandy
3	G2	0.62	Light yellow
4	G3	0.47	Yellowish brown
5	G4	0.36	Light brown
6	G5	0.24	Brown

APPENDIX IV: TLC Plates of Fractions/Sub-fractions of GS and CM Leaf Extracts

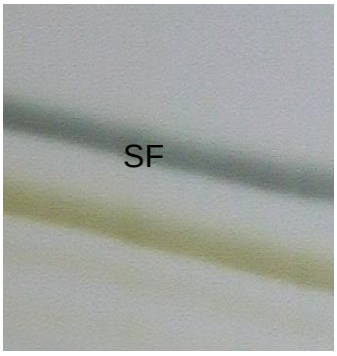

Plate IV a: TLC Plate of Methanolic Extract of Leaves of *Gymnema sylvestre* (Fraction D) Developed Using N–Hexane:Ethyl Acetate (30 : 70) and Viewed In Daylight/White Light. SF = Solvent front, D1 –D5 = Subfractions of D, B = Baseline.

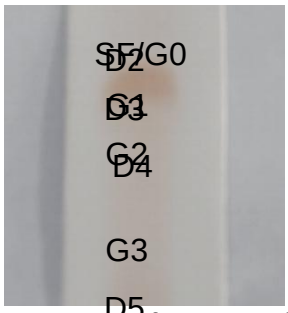

Plate IV b: TLC Plate of Methanolic Extract of Leaves of *Combretum micranthum* (Fraction G) Developed Using N–Hexane:Ethyl Acetate (30 : 70) and Viewed In Iodine Vapour. SF = Solvent Front, G0 –G5 = Subfractions of G, B = Baseline.



Plate IV c: TLC Plate of Methanolic Extract of Leaves of *Gymnema sylvestre* (Sub-Fraction D4) Developed Using N–Hexane:Ethyl Acetate (30 : 70) and Viewed Using P-Anisaldehyde – Sulfuric Acid Reagent. SF = Solvent Front, 1 –9 = Sub-Subfractions of G4, B = Baseline.



Plate IV d: TLC Plate of Methanolic Extract of Leaves of *Combretum micranthum* (Sub-Fraction G4) Developed Using N–Hexane: Ethyl Acetate (30: 70) and Viewed Using P-Anisaldehyde – Sulfuric Acid Reagent. SF = Solvent Front, G4 = Subfraction G4, B = Baseline.

G4

APPENDIX V: LCMSChromatograms and Mass Spectra of Sub-fractions of GS and CM Leaf Extracts

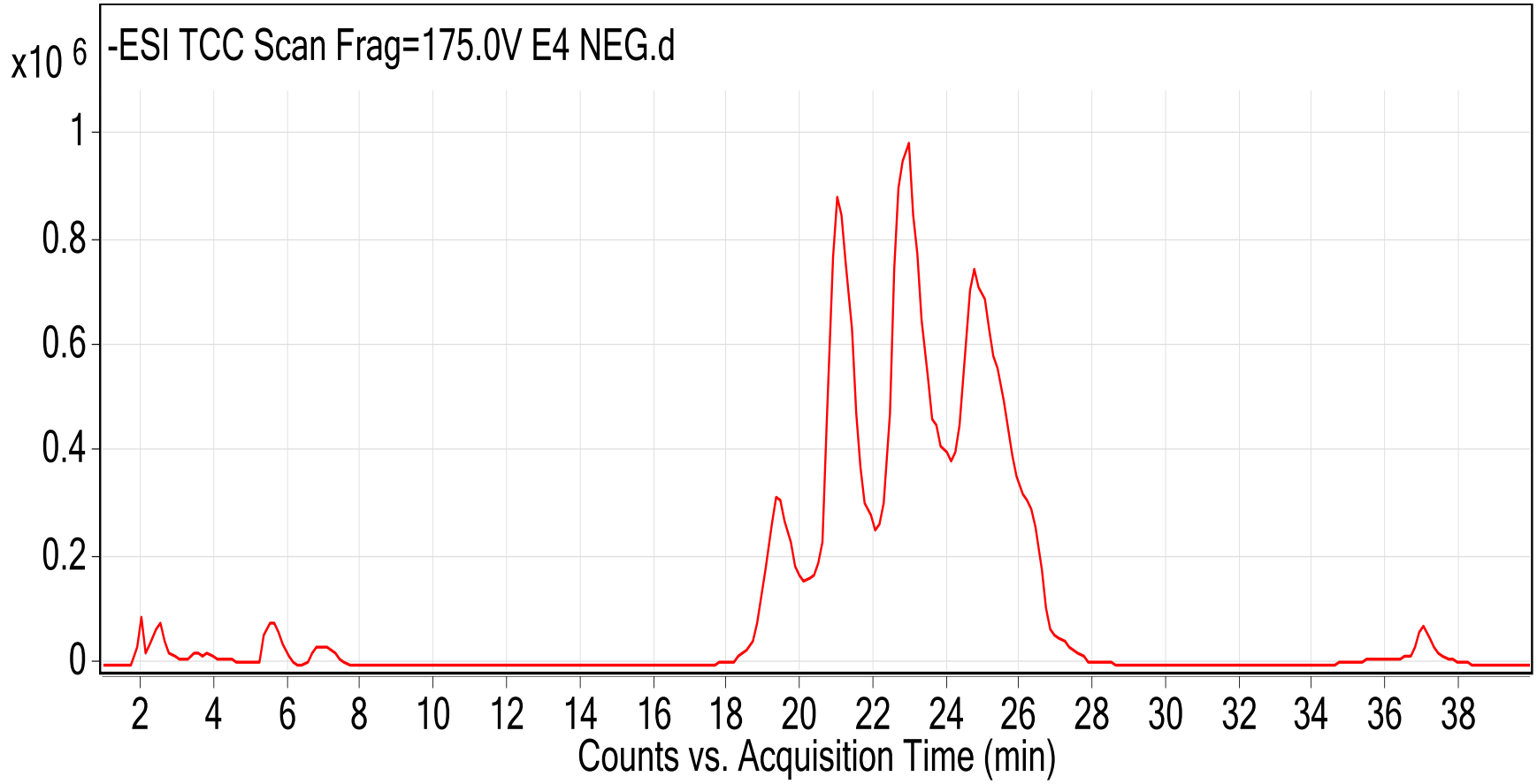

Figure V a: Chromatogram of Sub-fraction D4 [N-Hexane:Ethylacetate (20:80)] for *G. sylvestre* Aqueous/Methanolic Leaf Extract Using LC/MS in the Negative Ion Mode

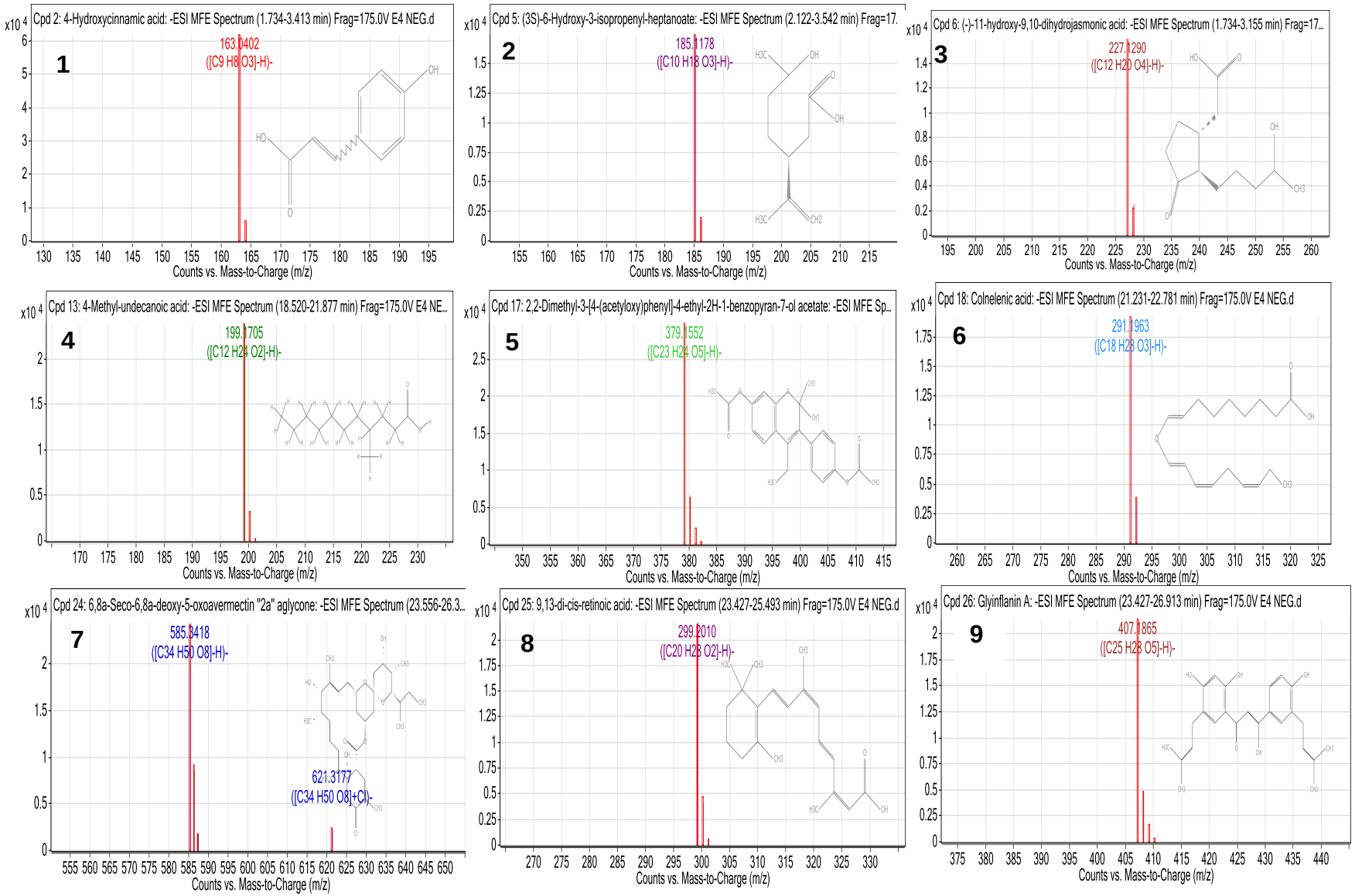

Figure V b: Mass Spectra for Sub-fraction D4 of *G. sylvestre* Aqueous/Methanolic Leaf Extract with Structures of the Compounds (1 to 9) by LCMS (Q-TOF/MS-MS) in the Negative Ion Mode.

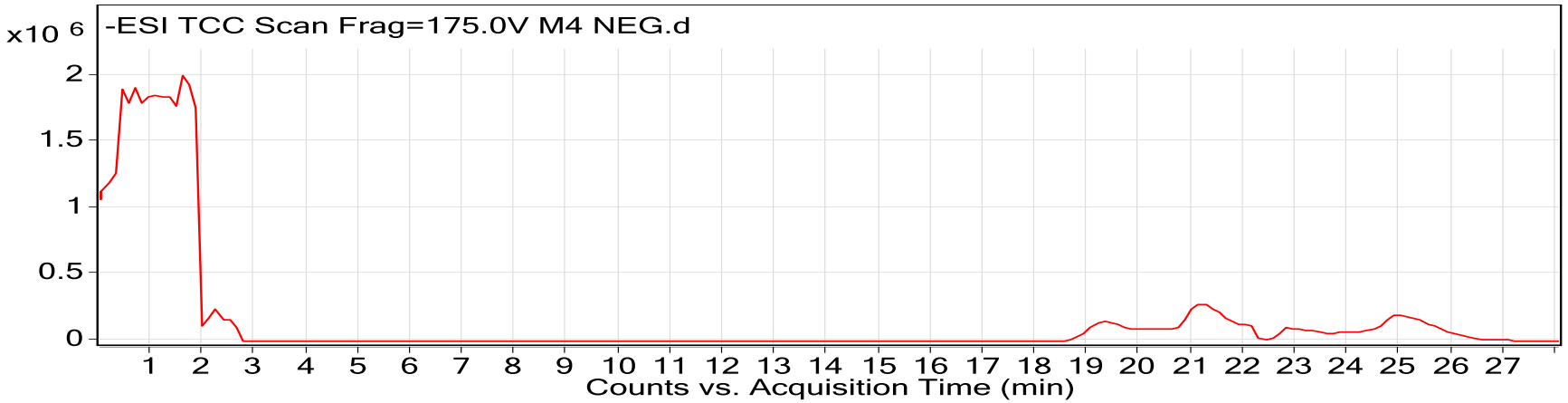

Figure V c: Chromatogram of Sub-fraction 4 of Fraction G of *C. micranthum*Aqueous/Methanolic Leaf Extract by LCMS (Q-TOF/MS-MS) in the Negative Ion Mode

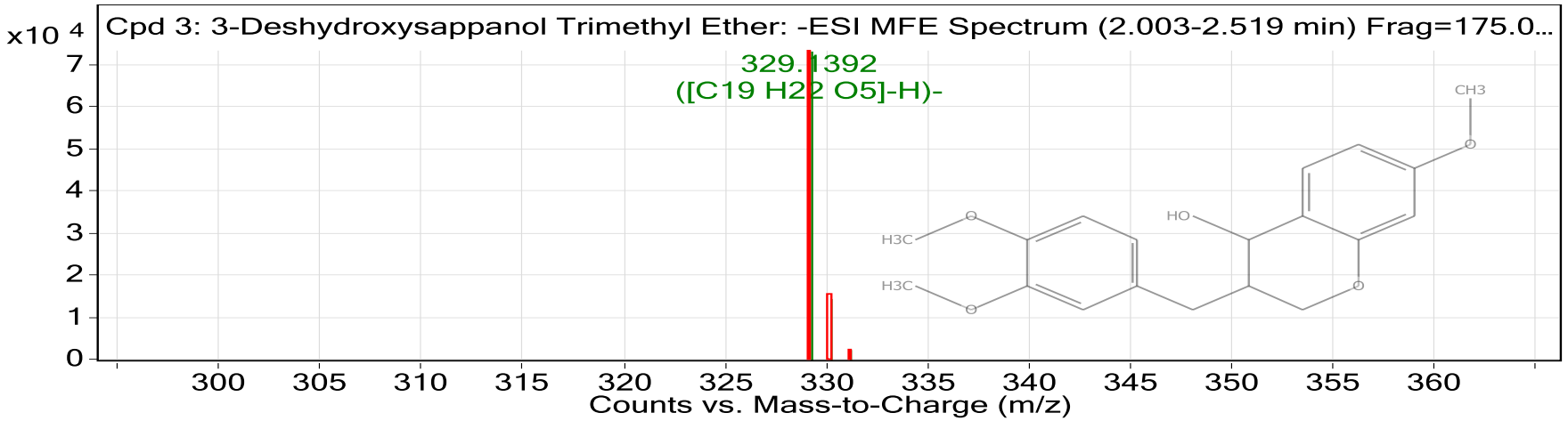

Figure Vd: Mass Spectrum for Sub-fraction 4 of Fraction G of *C. micranthum*Aqueous/Methanolic Leaf Extract with Structure of the Compound by LCMS (Q-TOF/MS-MS) in the Negative Ion Mode

APPENDIX VI: FTIRSpectra and Principal Functional Groups of Sub-fractions of GS and CM Leaf Extracts

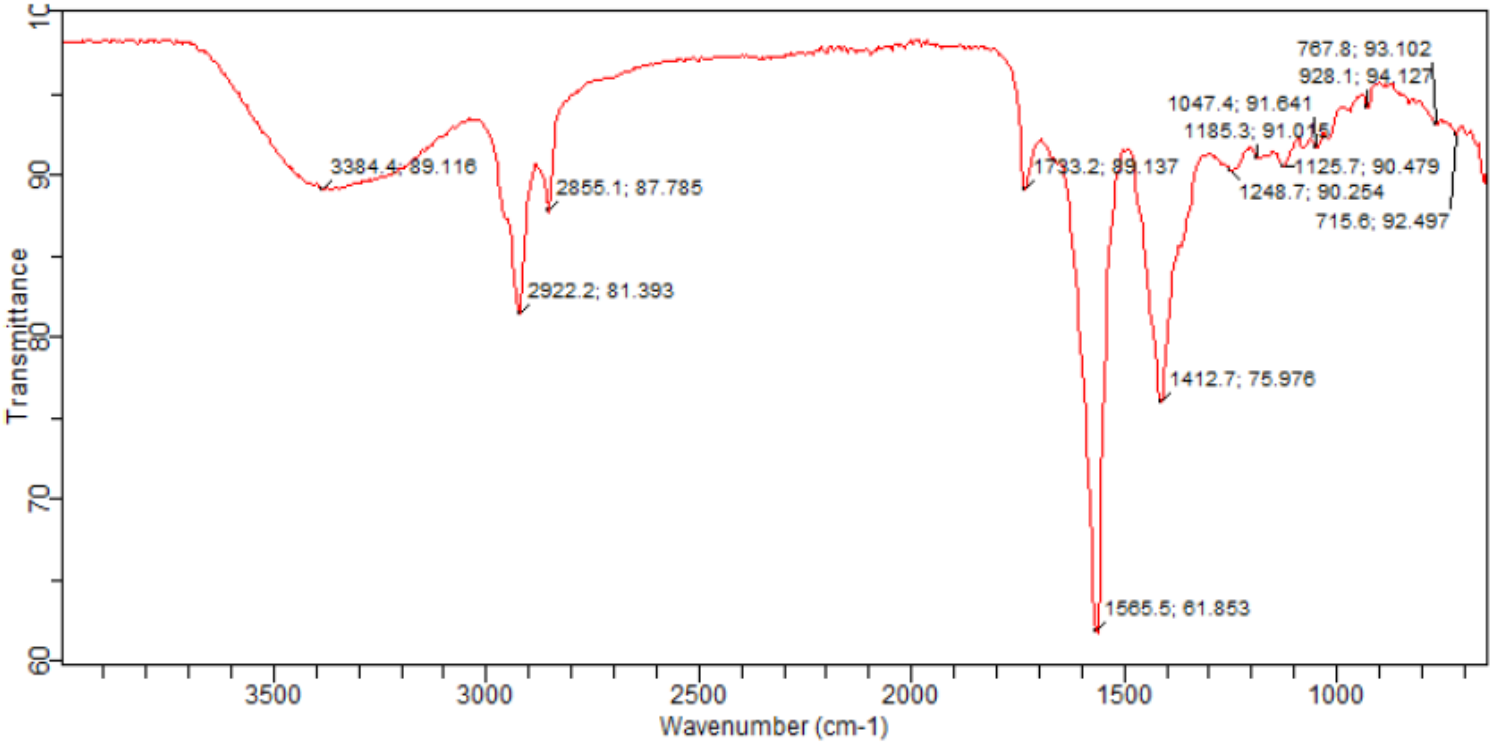

Figure VI a: FTIR Spectra for Subfraction D4 of *G. sylvestre* Methanolic Leaf Extract

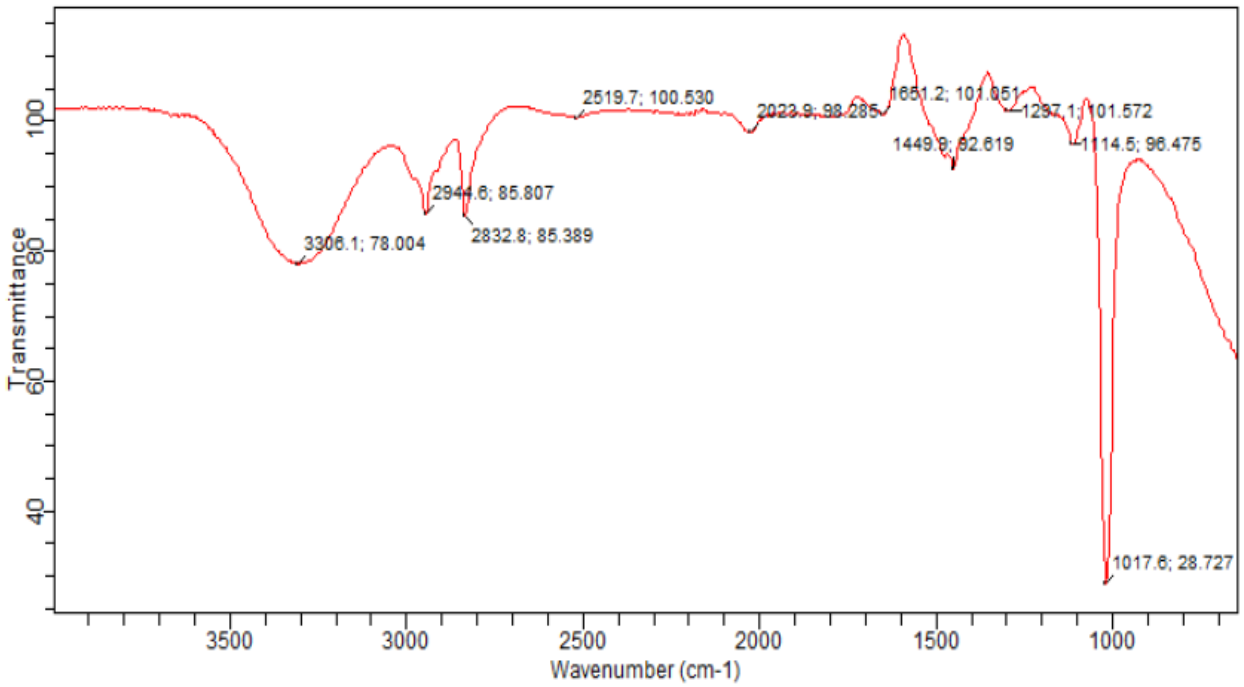

Figure VI b: FTIR Spectra for Subfraction 4 of Fraction G of *C. micranthum* Methanol Leaf Extract

Table VI a: Principal Infra-Red Transmittance for Functional Groups in Sub-Fraction D4 of *G. sylvestre* Methanol Leaf Extract

S/N	Wavenumber (cm^{-1})	Peak Intensity	Principal Functional Group Assigned
1	3384.4	Broad	(O-H) Stretch
2	2922.2	Strong	(C-H) Stretch
3	2855.1	Medium	(C-H) Stretch
4	1733.2	Medium	C=O Stretch
5	1565.5	Very strong	(C=C) Stretch
6	1412.7	Strong	C-O Stretch
7	1248.7	Weak	C-O Stretch
8	1185.3	Weak	C-O Stretch
9	1125.7	Weak	C-O Stretch

Table VI b: Principal Infra-Red Transmittance for Functional Groups in Sub-fraction G4 of *C. micranthum* Methanol Leaf Extract

S/N	Wavenumber (cm^{-1})	Peak Intensity	Principal Functional Group Assigned
1	3306.1	Broad	(O-H) Stretch
2	2944.6	Medium	(C-H) Stretch
3	2832.8	Medium	(C-H) Stretch
4	1651.2	Weak	C=O Stretch
5	1449.9	Medium/strong	(C=C) Stretch
6	1297.1	Weak	(C-O) Stretch
7	1114.5	Weak	(C-O) Stretch
8	1017.6	Very strong	C-O Stretch

APPENDIX VII: ^{1}H NMR and ^{13}C NMR Spectra and the Spectral Data of Sub-fractions of CM Leaf Extracts

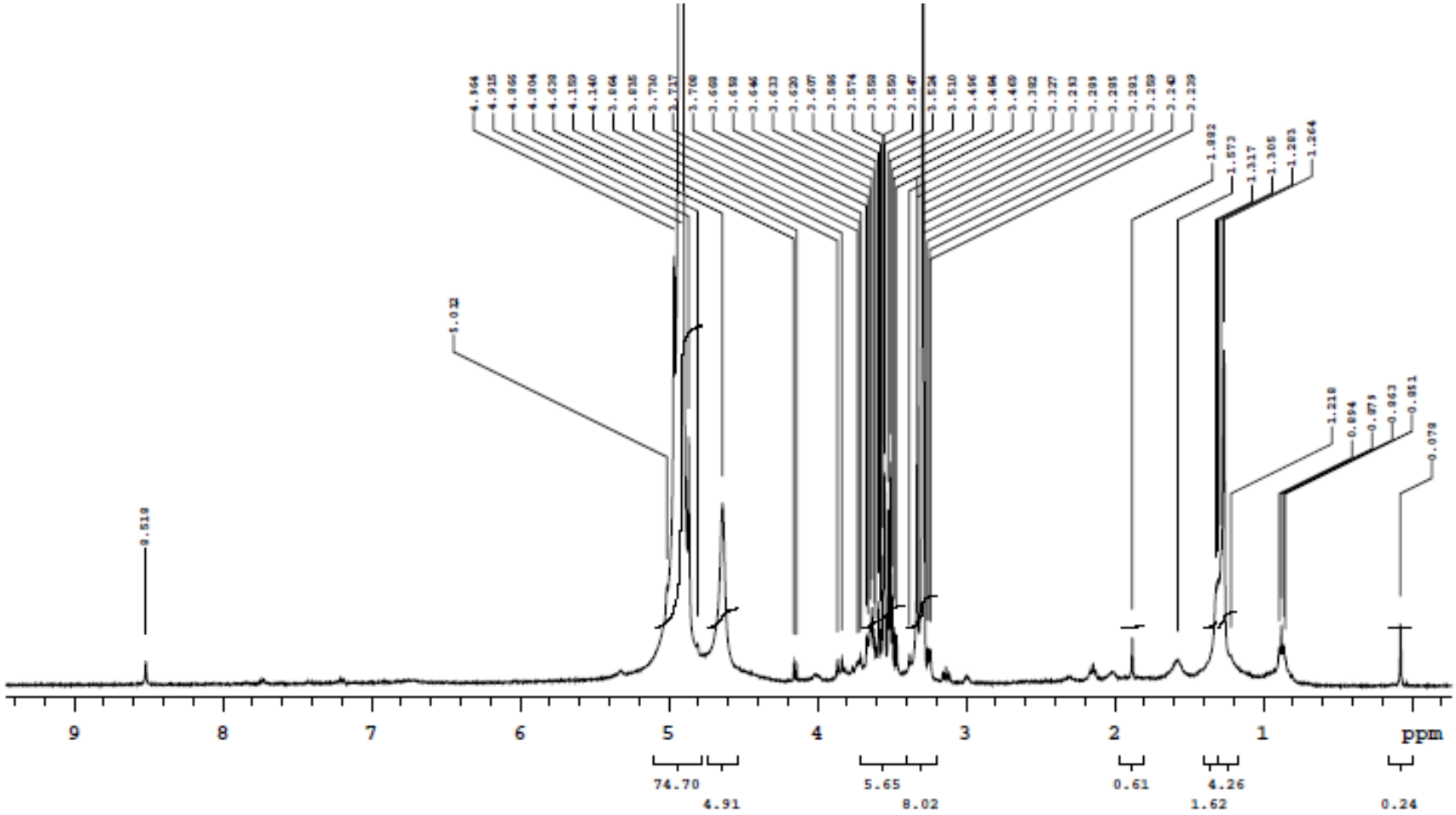

Figure VII a: ^{1}H NMR of Sub-fraction 4 of Fraction G for *C. micranthum* Aqueous/Methanolic Leaf Extract using Agilent NMR Vnmrs4000

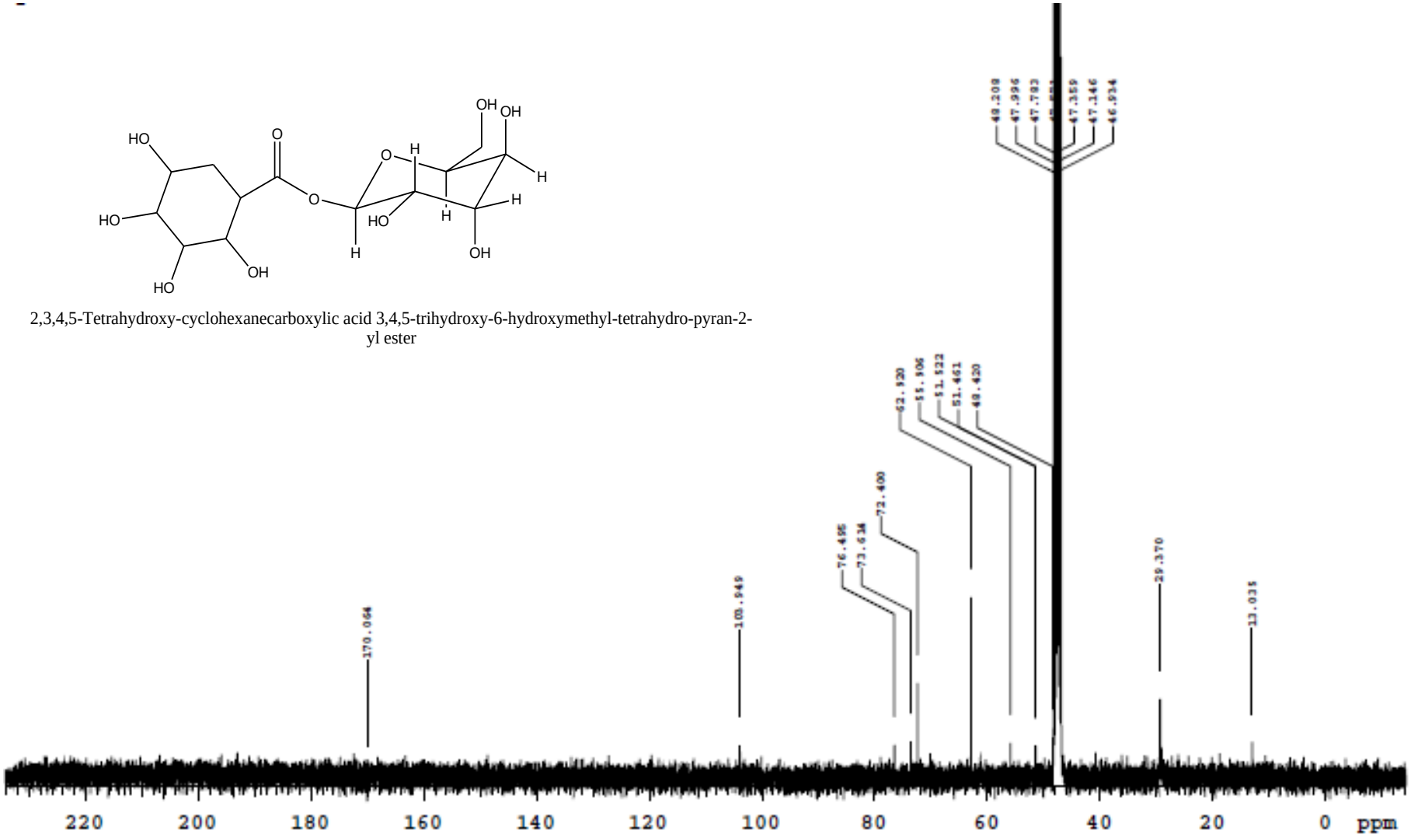

Figure VII b: ^{13}C NMR of Sub-fraction 4 of Fraction G for *C. micranthum* Aqueous/Methanolic Leaf Extract using Agilent NMR Vnmrs4000 and elucidated structure of the isolated compound

Table VII a: ^{13}C NMR Spectral Data of The Compound 2,3,4,5-Tetrahydroxy-Cyclohexane Ester Glucoside

S/N	^{13}C NMR δ (ppm) G4	^{13}C NMR δ (ppm) Litt.	Assigned Carbon
1	170.1	171.3	C=O (Ester)
2	103.9	103.15	C1 (Anomeric) of the sugar moeity.
3	76.5	78.58	C4 of the sugar moeity.
4	73.6	75.61	C5 of the sugar moeity.
5	72.4	71.67	C3 of the sugar moeity.
6	62.9	61.3	C2, C6 of the sugar moeity.
7	55.9*	60*	C7' (two bonds away from the carbonyl and has C-O bond).
8	51.5	51.5	C5' (in between C-OH of C7' & C4' and has C-O bond).
9	51.4	51.5	C6' (in between C-OH of C7' & C4' and has C-O bond).
10	48.4	49.4	C4'(three bonds away from the carbonyl and has C-O bond).
11	29.3	27.6	C2' (adjacent to C=O & C-OH).
12	13.0	13.0	C3'

(Pretsch *et.al.*, 2009; Bubb, 2003) * Points of slight difference

APPENDIX VIII: Docking Poses of Isolated/Predicted Compounds from Sub-fractions of GS and CM Leaf Extracts

The ligand-protein interactions between the benzopyran-7-01 acetate and α-amylase is stabilized by hydrogen bonds formed by amino-acid residues Arg A:389; van der Waals forces by residues Phe A: 315, Thr A: 377, Asp A:375, Gln A: 390 and Lys A:322; a pi-cation bond by Arg A:343; a carbon-hydrogen bond involving Trp A:316; a pi-pi stacked bond with Trp A:388; and some alkyl (pi-alkyl) bonds by the residue Ala A: 318. The ligand-protein interactions between the ester glucoside and α-amylase is stabilized by five hydrogen bonds formed by the amino acid residues Thr A:6, Asp A:402, Arg A:10, Arg A: 398 and Pro A:332; eight van der Waals forces formed by the amino acids Pro A:4, Gly A:9, Phe A:335, Gly A:334, Tyr A:333, Ser A:289, Asp A: 290 and Arg A:252; and one carbon – hydrogen bond by the residue Thr A:11.Both the benzopyran-7-01 acetate and the ester glucoside from GS and CM were also found to have docked well in the binding pocket of α-amylase (4W93) with good hydrophobicity.

Glyinflanin A and α-glucosidase interaction is stabilized by four hydrogen bonds involving Asn B:4, Trp B:7, Gly B:94 and His B:459; the van der Waals interactions involved the residues Trp B:8, Asp B:464, Lys B: 483, Leu B:462 and Arg B:456; other bonds include a pi-anion with the residue Asp B:48 and five alkyl (pi – alkyl) formed by the amino acid residues Lys B:96, Phe B:463, Pro B:460 and Arg B: 457.Interactions between ester glucoside and α-glucosidase is stabilized by hydrogen bonds formed by amino acid residues Asn B:301, Asp B:333, Ala B:229 and Leu B:227; van der Waals forces formed by residues Ala B: 232, Pro B: 230, Val B:334, Val B:335, Phe B: 397, Gly B: 228, Phe B:297, Leu B:300 and Met B: 302. Glyinflanin A and the ester glucoside were also found to dock well into the binding pocket of the enzyme (α-glucosidase) with good hydrophobicity.

Interactions of the Jasmonic acid with 1IR3 is stabilized by amino acid residues Glu A:1043, Arg A:1155, Arg A:1131, Arg A:1039 and PTR A:1163 forming hydrogen bonds (Figure 4.26). The Sappanol:1IR3 interaction is stabilized by amino acid residues Arg A: 1136 (hydrogen bond) Glu A:1043 (carbon-hydrogen bond), and a metal pi-cation bond with MG A: 302. The compounds (Jasmonic acid and Sappanol) also showed good hydrophobic interactions with the 1IR3.

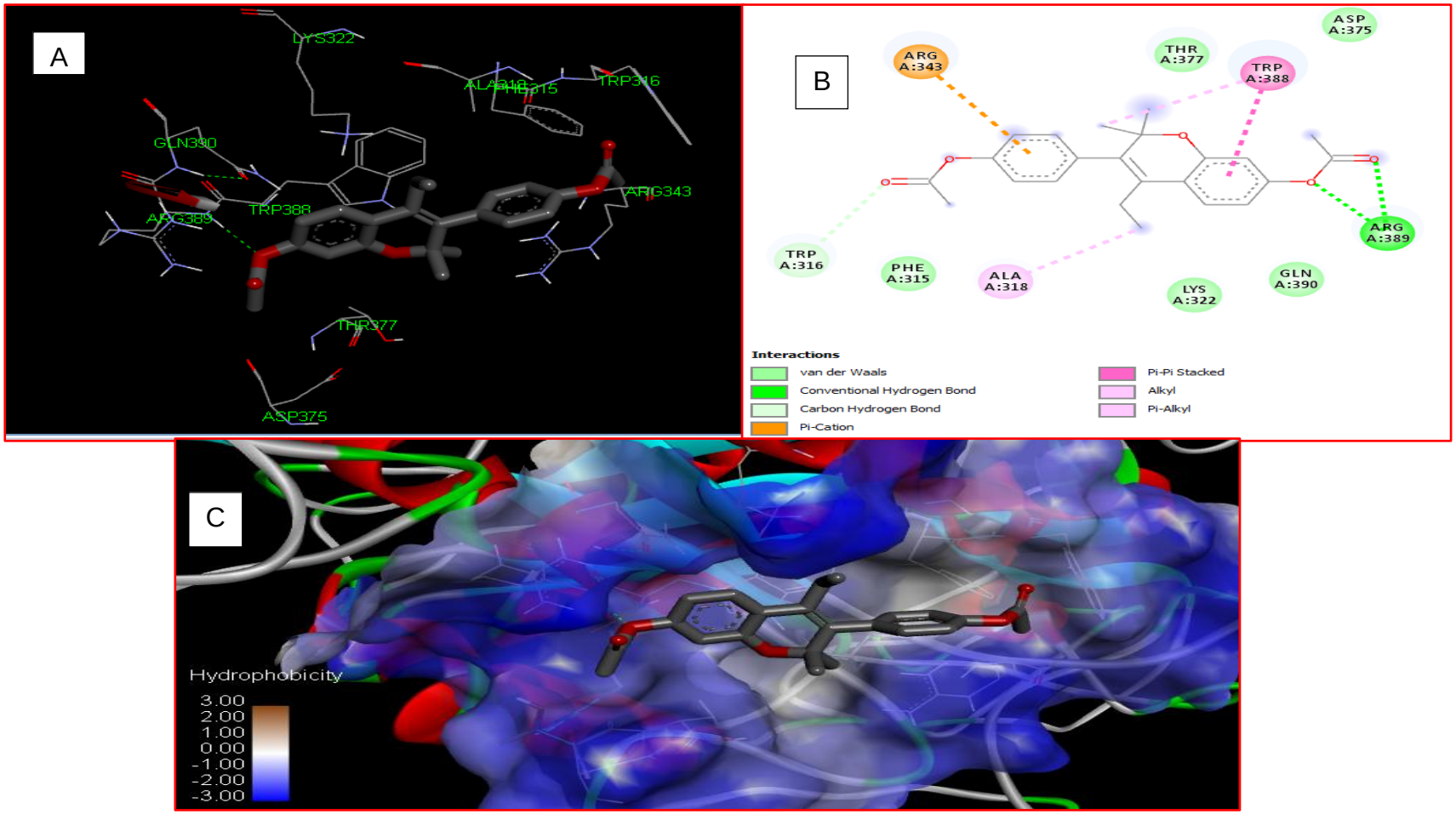

Figure VIII a: ICM Docking Models for "2,2-Dimethyl-3-[4-(acetyloxy)phenyl]-4-ethyl-2H-1-benzopyran-7-ol acetate" into the active site of Human Pancreatic alpha-amylase (PDB ID= 4W93) showing bonds with amino acids (Panels A and B) and hydrophobic interactions with the compound docked into the binding pocket of the protein (Panel C).

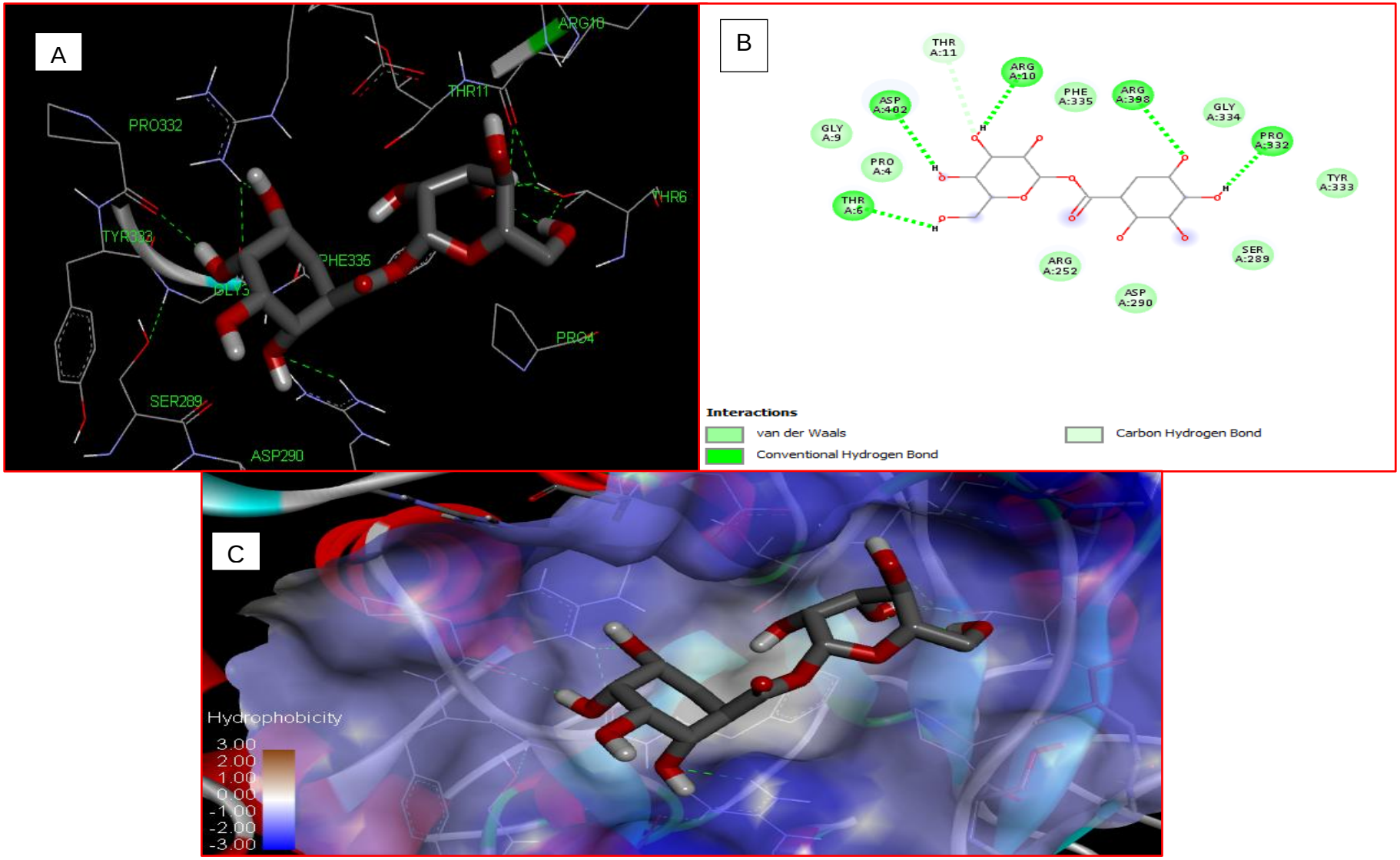

Figure VIII b: ICM Docking Models for "2,3,4,5-Tetrahydroxy-cyclohexane ester glucoside" into the active site of Human Pancreatic alpha-amylase (PDB ID= 4W93) showing bonds with amino acids (Panels A and B) and hydrophobic interactions with the compound docked into the binding pocket of the protein (Panel C).

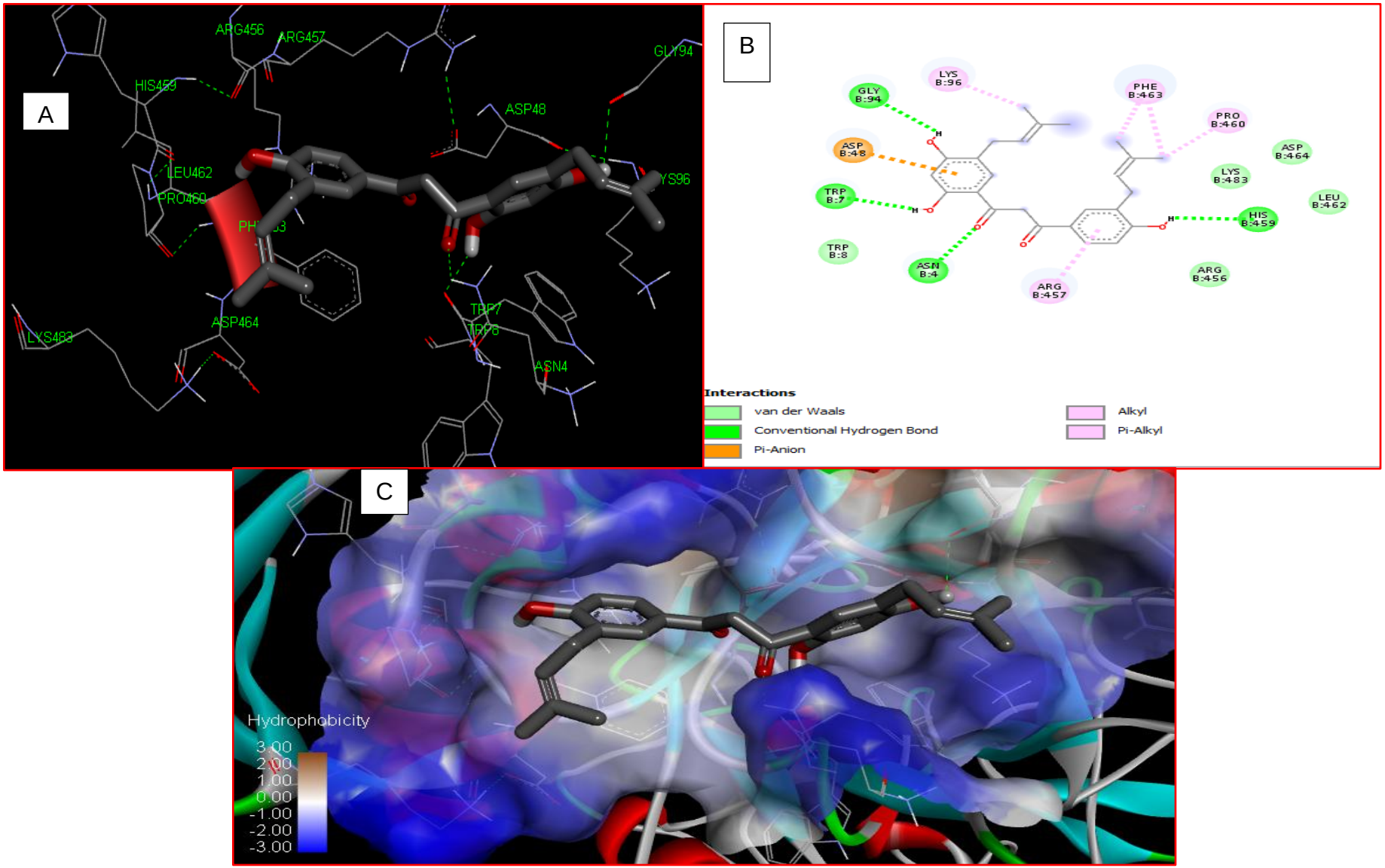

Figure VIII c: ICM Docking Models for "Glyinflanin A" into the active site of Alpha-glucosidase (PDB ID= 3WY2) showing bonds with amino acids (Panels A and B) and hydrophobic interactions with the compound docked into the binding pocket of the protein (Panel C).

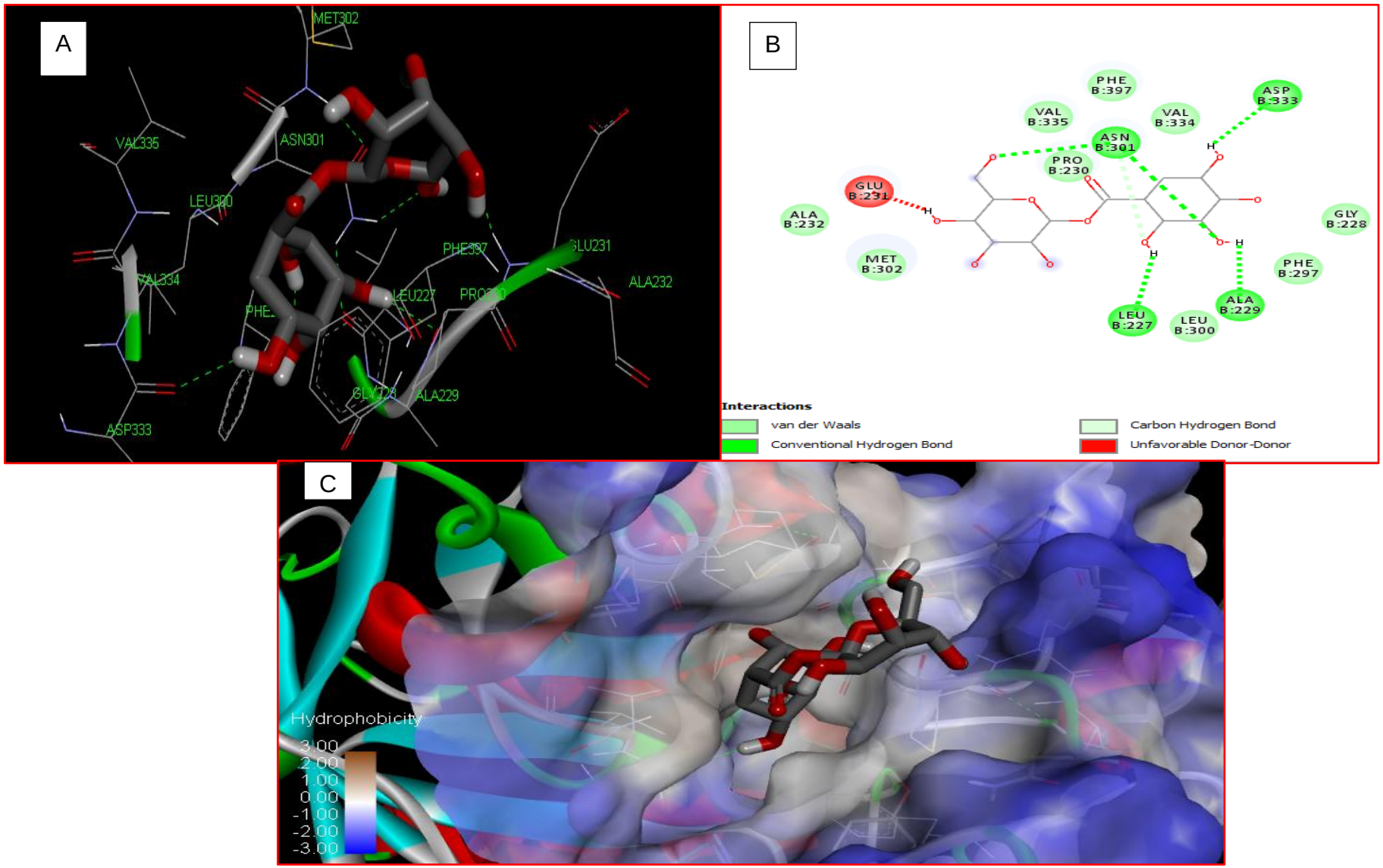

Figure VIII d: ICM Docking Models for "2,3,4,5-Tetrahydroxy-cyclohexane ester glucoside" into the active site of Alpha-glucosidase (PDB ID= 3WY2) showing bonds with amino acids (Panels A and B) and hydrophobic interactions with the compound docked into the binding pocket of the protein (Panel C).

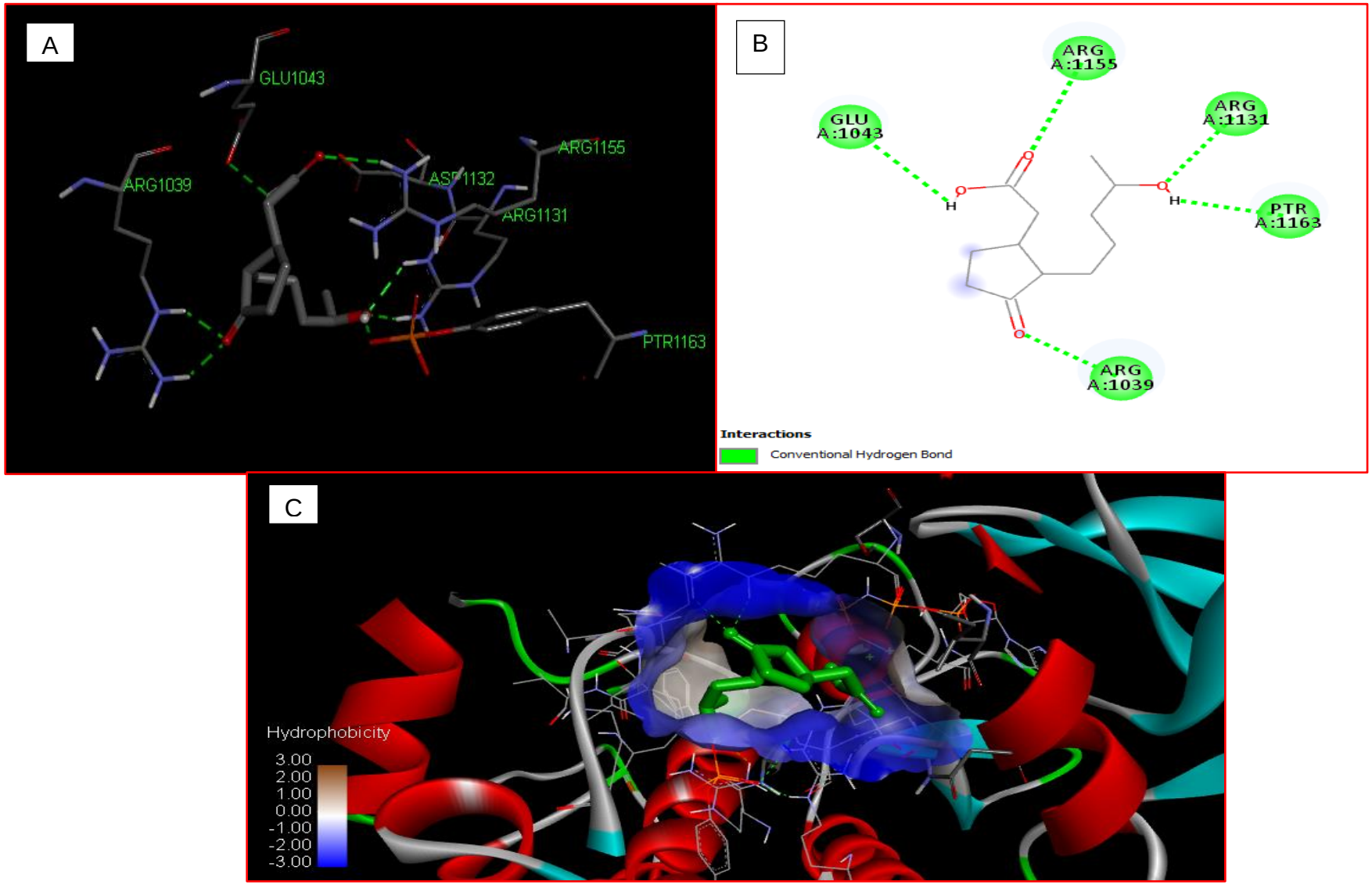

Figure VIII e: ICM Docking Models for "(-)-11-hydroxy-9,10-dihydrojasmonic acid" into the active site of phosphorylated insulin receptor tyrosine kinase (PDB ID: 1IR3) showing bonds with amino acids (Panels A and B) and hydrophobic interactions with the compound docked into the binding pocket of the protein (Panel C).

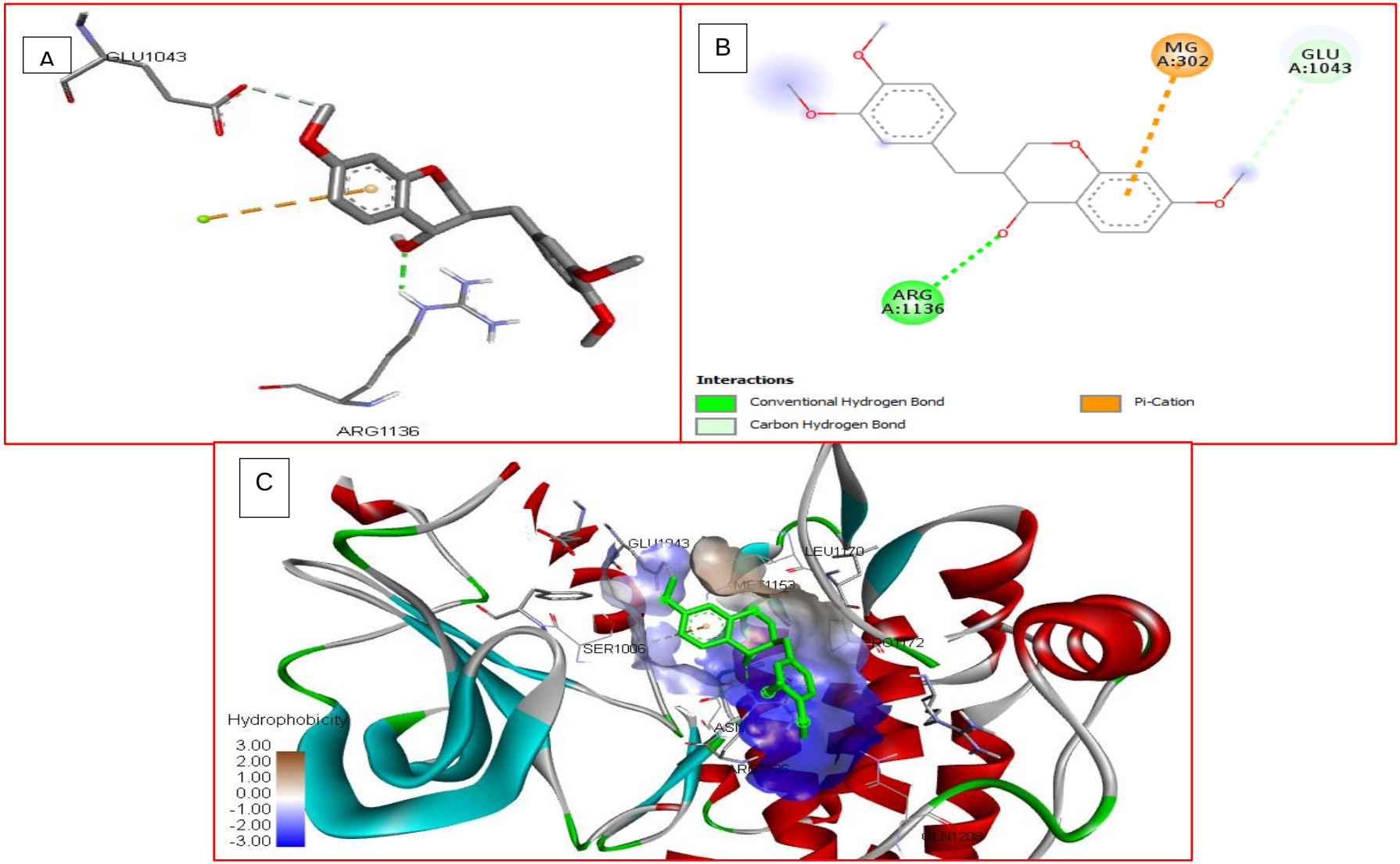

Figure VIII f: ICM Docking Models for "3-Deshydroxysappanol Trimethyl Ether" into the active site of phosphorylated insulin receptor tyrosine kinase (PDB ID: 1IR3) showing bonds with amino acids (Panels A and B) and hydrophobic interactions with the compound docked into the binding pocket of the protein (Panel C).

APPENDIX IX: Publications and Papers Presented At Conferences

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HPLC PROFILE, *IN-VITRO* ALPHA-AMYLASE, ALPHA-GLUCOSIDASE INHIBITORY AND ANTIOXIDANT ACTIVITIES OF *GYMNEMA SYLVESTRE* ETHYL ACETATE LEAF EXTRACT

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ABSTRACT
Gymnema sylvestre (GS) has been used locally as antidiabetic, antioxidant, antihyperlipidemic and anticancer medicinal plant. Oxidative stress has been implicated in pathogenesis of various diseases including diabetes and antioxidants play a leading role in preventing the progression of the disease. This study aimed to determine antidiabetic potential relative to carbohydrate hydrolyzing enzymes (alpha-amylase and alpha-glucosidase) and antioxidant activities towards oxidative stress and lipid peroxidation (MDA) of Gymnema sylvestre ethyl acetate leaves extract (GSEALE). The extract was found to inhibit α-glucosidase than α-amylase with Inhibitory concentration (IC_{50}) values of 170.45±13.61 and 130.77±3.15 respectively. The extract was also found to scavenge DPPH (53.87±17.12%), hydroxyl (28.10±14.35%) and hydrogen peroxide (34.26±20.12%) radicals. The activities of superoxide dismutase (SOD) was found to be significantly lower ($p<0.05$) when the extract-treated and extract+H_2O_2-treated were compared with the normal control (untreated group). Levels of reduced glutathione (GSH) in the extract-treated was found to be significantly lower ($p<0.05$) when compared with both normal and positive controls while the extract+H_2O_2-treated was significantly lower than the positive control but higher than extract-treated group. Levels of MDA was significantly higher in extract-treated and extract+H_2O_2-treated than the positive control ($p<0.05$). The total flavonoids (9.29±0.76) and total phenolics (6.18±0.52) and saponins (62.98±0.31) were found to be present in the leaves in high amount with tannins, steroids, soluble starch and carbohydrates also present. High Performance Liquid Chromatography profile revealed Resorcinol/Catechol to be one of the phenolics detected and may be responsible for GSEALE's inhibition of α-amylase and α-glucosidase and antioxidant properties. This shows that GS can probably be used for antidiabetic purpose due to significant antioxidant and carbohydrate hydrolyzing enzymes inhibitory properties.
Keywords: Antioxidants, HPLC, Gymnema sylvestre, carbohydrate hydrolyzing enzymes, lipid peroxidation, Diabetes mellitus

INTRODUCTION

Diabetes mellitus, (DM), is a chronic hyperglycaemia that may be caused by one or more of numerous underlying processes (WHO, 2014). Symptoms of high blood sugar include frequent urination, increased thirst, and increased hunger. If left untreated, diabetes can cause many complications (WHO, 2014). Acute complications include diabetic ketoacidosis and non-ketotic hyperosmolar coma (Kitabchi *et al.*, and is unable to protect against free radical damage (Bonnefont-Rousselot *et al.*, 2000). It was documented that diabetes is associated with increased oxidative stress as evidenced by the increased accumulation of lipid peroxides (Maritim *et al.*, 2003). Under physiological conditions, antioxidant defense system protects the body against adverse effects of free radical generation. In diabetes mellitus hyperglycemia may depress the natural antioxidant

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7th July, 2017.

Dear Authors,

ACCEPTANCE LETTER

We are glad to inform you that your manuscript:

Title: TOXICOLOGICAL STUDY OF COMBINED AQUEOUS LEAF EXTRACTS OF GYMNEMA SYLVESTRE AND COMBRETUM MICRANTHUM IN RATS

.

Paper ID: BJBS/17/062

Authors: Ibrahim A, Onyike E, Nok AJ and Umar IA

is accepted by Bayero Journal of Biomedical Sciences for the June, 2017 issue (Volume 2, issue 1).

Toxicological Studyof CombinedAqueous Leaf Extracts of*Gymnema sylvestre*and*Combretum micranthum*In Rats

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ABSTRACT

Gymnema sylvestre R.Br. ("periploca of the woods" in English) (Asclepiadaceae) and Combretum micranthum, Fam. (Combretaceae)) are used in combination for the management of DM in the North-Eastern part of Nigeria with little or no scientific basis for their safety. It is thus the aim of this research to determine the acute and sub-chronic toxicity risk of G. sylvestre (GS) and C. micranthum (CM) leaves, individually and in a 1:1 combined form (GSCM). The study assayed for the LD_{50} (modified Lorke method) as well as some markers of toxicity, Haematology and histopathology of liver and kidney in the sub-chronic phase. Body and Relative Organ Weights were calculated. Qualitative/quantitative phytochemicals were also assayed for. Both plants were found to be relatively safe (LD_{50}> 5000mg/kg) in mice. Sub-chronic studies in rats revealed that GS, CM and GSCM (1:1) showed slight increases in rats' body weight and a decrease in serum TC, TG, LDL/VLDL at 600mg/kg b.w. (P< 0.05) when compared with the control after 3 weeks of administration. Relative liver weight reduced significantly only in the GS administered rats whereas GSCM reversed it; relative kidney weight, haematology indices, liver and kidney function parameters and feed/water intake were not significantly affected. Both plants also showed good total flavonoids and total phenolic contents. But histopathology of liver and kidney showed some fibrous tissue formation which was milder in the GSCM group. It can be concluded that GS, CM and GSCM are safe but should be used with caution at high doses (600mg/kg b. w. and above).

Keywords: Herbalism, Combination Therapy, Acute and Sub-chronic Toxicity.

TOXICITY RISK ASSESSMENT OF COMBINED USE OF GYMNEMA SYLVESTRE AND COMBRETUM MICRANTHUM AQUEOUS LEAF EXTRACTS IN NORMAL WISTAR RATS

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ABSTRACT

Background: Latest surveys have indicated that many medicinal plants used as traditional medicine showed undesirable effects. Therefore emphasis should be given to elucidate the safe use of any plant for medicinal purposes. Hypoglycaemic plants including (*Gymnema sylvestre* (GS) R.Br. ("periploca of the woods" in English; "Kafi suga" in Hausa) (Asclepiadaceae) and *Combretum micranthum* (CM) Fam. (Géézà in Hausa) (Combretaceae)) are used in combination for the management of diabetes mellitus (DM) in the North-Eastern part of Nigeria with little or no scientific basis for their safety.

Objectives: It is thus the aim of this research to determine the acute and sub-chronic toxicity risk of *G. sylvestre* (GS) and *C. micranthum* (CM) leaves, individually and in a 1:1 combined form (GSCM).

Methodology: The study carried out phytochemicals, mineral elements and *in vitro* radical scavenging analyses of the plants. The LD_{50} was determined in mice and rats were used for analyses of some serum biochemical markers of toxicity on major organs like the liver and kidney sub-chronically. Also, *in vivo* antioxidant activities (in the liver homogenate), lipid profile/peroxidation, haematology, body and relative organ weights and histopathology (liver and kidney) were assayed for in the sub-chronic phase. Feed and water intake were also recorded in the 21 days experiment.

Results: Both plants were found to be relatively safe (LD_{50} > 5000mg/kg) in mice. Sub-chronic toxicity studies in rats revealed that GS, CM and GSCM (1:1) showed slight increases in rats' body weight, malondialdehyde (MDA) and a decrease in serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein/very low-density lipoprotein (LDL/VLDL) at 600mg/kg b.w. ($p< 0.05$) when compared with the control after 3 weeks of administration. Relative liver weight reduced significantly only in the GS administered rats whereas GSCM reversed it; relative kidney weight, haematology indices, liver and kidney function parameters, *in vivo* antioxidants level and feed/water intake were not significantly affected. Moreover, GS and CM revealed good ferric reducing antioxidant power (FRAP) and radical scavenging activities against 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Nitric Oxide (NO) with *G. sylvestre* being significantly better. Both plants possessed significant flavonoids and phenolic contents. However, histopathology of liver and kidney showed some fibrous tissue formation which was milder in the GSCM group.

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Conclusion: It can be concluded that GS, CM and GSCM are safe but should be used with caution at high doses (600mg/kg b. w. and above).

Keywords: Combination Therapy, Toxicity, *Gymnema sylvestre*, *Combretum micranthum*.