EFFECT OF *ANCHOMANES DIFFORMIS* EXTRACT ON BIOCHEMICAL AND HISTOLOGICAL PARAMETERS IN STREPTOZOTOCIN-INDUCED DIABETES AND DIABETIC COMPLICATIONS

by

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DECLARATION

I, Toyin Dorcas Alabi, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

26th November 2019

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ABSTRACT

Diabetes mellitus is one of the major health challenges facing the world today and it is not restricted by age, gender, education or urbanisation. Increased oxidative stress, inflammation and apoptosis are implicated in the pathogenesis of diabetes mellitus. The progression of diabetes mellitus leads to pathological events and alterations in many tissues of the body, thereby causing damage to these tissues and organs. *Anchomanes difformis* is has a strong ethnopharmacological relevance and it is known for its diverse traditional uses against hyperglycemia, kidney damage, pain, wounds, inflammation, onchocerciasis and gastrointestinal pathologies amongst others. Scientific investigations have been performed on some of these ethnobotanical claims on *Anchomanes difformis* using animal models. While some of these claims have been established scientifically, others are yet to be explored. *In vivo* experimental study on the leaves of *Anchomanes difformis* revealed its hypoglycemic effect, however, there is no information on the possible effect of *Anchomanes difformis* on oxidative stress, inflammatory mediators and apoptosis in diabetes mellitus. Therefore, this study investigated the potential benefits of *Anchomanes difformis* in increased oxidative stress, inflammation and apoptosis in a diabetic model. The study also assessed the ameliorative effect of *Anchomanes difformis* in diabetes-induced damage in the organs such as the liver, heart, kidney, testis and epididymis.

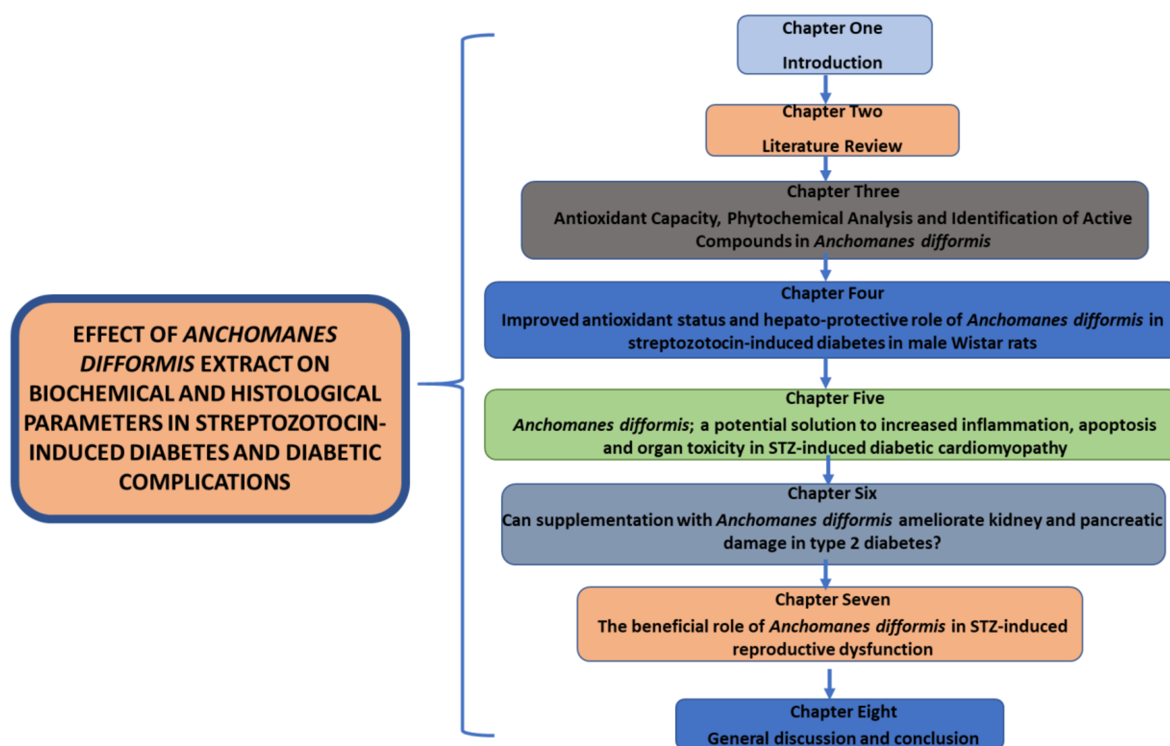
The first phase of the study compared the antioxidant capacity and phytochemical characterisation of three different solvent extracts; aqueous, ethanolic and methanolic from the leaves and rhizome of *Anchomanes difformis*. All these six extracts (3 extracts each from the leaves and rhizome) exhibited antioxidant properties, however aqueous extract demonstrated the highest antioxidant potential, hence it was selected for further experiment in the study. Furthermore, certain bioactive compounds with antioxidant, antidiabetic and anti-inflammatory properties were identified in *Anchomanes difformis.* The second phase of the study involved the induction of diabetes, treatment with AD and standard drug and euthanasia followed by biochemical investigations in male Wistar rats.

Type 2 diabetes was induced with two-weeks administration of 10% fructose, followed by a single intraperitoneal injection of streptozotocin (40mg/kgBW). Dosages of 200 and 400 mg/kgBW of *Anchomanes difformis* leaves extract were administered for six weeks to diabetic and normal rats which served as treatment controls. The effect of *Anchomanes difformis* on glycemic indices, body weights, relative organ weights, organ function markers, antioxidant statuses, inflammatory biomarkers, apoptosis and structural integrity of the liver, kidney, pancreas, testis and the epididymis were conducted. The administration of streptozotocin led to hyperglycemia, hyperlipidemia, body weight loss, increased inflammation, oxidative stress and apoptosis, reduced sperm concentration, viability and distorted sperm morphology. It also induced tissue damage in the liver, kidney, pancreas, testis and epididymis. Treatment with both doses of *Anchomanes difformis* improved organ functions, markedly reduced and repaired tissue damage in a dose-dependent manner and comparable to the standard drug; glibenclamide. Furthermore, *Anchomanes difformis* distinctly lowered blood glucose, abnormal lipid levels, enhanced antioxidant status, modulated inflammation, reduced apoptosis and increased sperm functions better than glibenclamide in diabetic rats.

In conclusion, the protective and ameliorative properties of *Anchomanes difformis* projects it as a potential new, reliable therapeutic agent that should be explored and considered in the management of diabetes mellitus and its associated complications.

PREFACE

This thesis consists of eight chapters, and it is presented in an article-based format, the chapters are written according to the guidelines of the journals where they are published or submitted for review.



Chapter one is a brief introduction of diabetes and its significance, chapter two (literature review) provides an overview of diabetes mellitus, oxidative stress, inflammation, apoptosis and diabetic complications. It also presents background information on *Anchomanes difformis* and its biological relevance. The literature review has been published as a book chapter in the book "Bioactive compounds of medicinal plants: Properties and potentials for human health". Chapters three, four, five, six and seven are research articles which presents the results of the experimental investigations carried out in this study as shown in the chart above. Chapter three has been published in the "Natural Product Journal". Chapter four is under review in the "Journal of Ethnopharmacology". Chapter five has been published in "*Biomedicines*". Chapter six

has been submitted to the “European Journal of Integrative Medicine”. Chapter seven has been submitted to “Andrologia” journal for publication. Chapter eight is a general discussion and conclusion of the study as a whole.

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DEDICATION

This thesis is dedicated firstly to the Almighty God and secondly to my Husband and Prince; Oluwaseun Olayinka Alabi who was my very strong support.

TABLE OF CONTENTS

DECLARATION i
ABSTRACT ii
PREFACE iv
ACKNOWLEDGEMENTS vi
DEDICATION viii
TABLE OF CONTENTS ix
LIST OF TABLES xvi
LIST OF FIGURES xvii
GLOSSARY xx
DEFINITION OF TERMS xxi

CHAPTER ONE 1
1 INTRODUCTION 1
1.1 Background 1
1.2 Rationale for this study 5
1.3 Aim 6
1.4 Objectives 7
1.5 Research Questions 7
1.6 Hypothesis 8
1.7 Ethical consideration 8
References 10

CHAPTER TWO 15
2 LITERATURE REVIEW 15
Medicinal activities of A*nchomanes difformis* and its potentials in the treatment of diabetes mellitus and other disease conditions 15
2.1 Diabetes mellitus 16
2.2 Oxidative stress in Diabetes 17
2.3 Diabetic Complications 19
2.3.1 Classification of Diabetic Complications 20
2.3.2 Diabetic Cardiomyopathy 20
2.3.3 Diabetic Nephropathy 21
2.3.4 Reproductive Complications of Diabetes 21
2.4 Medicinal Plants and the Management of Diabetes Mellitus 22
2.5 *Anchomanes* difformis; its medicinal properties and potentials 23

2.6 Folkloric Uses of *A. difformis* 24
2.7 Nutritive value of A. *difformis* 26
2.8 Phytochemical Constituents of *A. difformis* 27
2.9 Medicinal Benefits of *A. difformis* 28
2.9.1 Anti-diabetic Activities 28
2.9.2 Antioxidant Activities 29
2.9.3 Anti-inflammatory Activities 29
2.9.4 Anti-nociceptive Activities 31
2.9.5 Anti-microbial Activities 31
2.9.6 Gastro-protective Activities 32
2.9.7 Anti-asthmatic Activities 33
2.9.8 Anti-onchocercal activities of *A. difformis* 33
2.10 Future prospective and research opportunities 35
References 36

CHAPTER THREE 48
3 Antioxidant Capacity, Phytochemical Analysis and Identification of Active Compounds in *Anchomanes difformis* 48
ABSTRACT 49
3.1 Introduction 50
3.2 Experimental section 53
3.2.1 General Experimental Procedures 53
3.2.2 Plant Collection and Authentication 53
3.2.3 Preparation of Plant Extracts 53
3.2.4 Phytochemical Analysis 54
3.2.4.1 Determination of Total Polyphenols 54
3.2.4.2 Determination of Flavonol Content 54
3.2.4.3 Determination of Flavanol Content 55
3.2.4.4 Determination of Total Alkaloids 55
3.2.5 Antioxidant capacities 56
3.2.5.1 ORAC Assay 56
3.2.5.2 FRAP Assay 56
3.2.5.3 TEAC Assay 57
3.2.6 Identification of active compounds 57
3.2.6.1 High Performance Liquid Chromatography (HPLC) 57
3.2.6.2 Ultra-Performance Liquid Chromatography and Mass Spectrophotometry 58

3.2.7 Data Analysis ... 59
3.3 Results ... 59
3.3.1 Phytochemical Analysis and Antioxidant capacities ... 59
3.3.2 Relationships between Antioxidant Capacity and Plant Secondary Compound ... 61
3.3.3 Identification of Bioactive compounds ... 62
3.4 Discussion ... 69
3.5 Conclusion ... 72
3.6 Recommendation ... 73
Funding ... 73
Acknowledgements ... 73
References ... 74

CHAPTER FOUR ... 81
4 Improved antioxidant status and hepato-protective role of *Anchomanes difformis* in streptozotocin-induced diabetes in male Wistar rats ... 81
ABSTRACT ... 82
ABBREVIATIONS ... 83
4.1 Introduction ... 84
4.2 Materials and methods ... 87
4.2.1 Plant Preparation ... 87
4.2.1.1 Collection and registration ... 87
4.2.1.2 Extraction ... 88
4.2.2 Ethical Approval ... 88
4.2.3 Animals ... 88
4.2.4 Experimental Design ... 89
4.2.5 Induction of Type 2 Diabetes ... 90
4.2.6 Blood and Tissue Collection ... 90
4.2.7 Measurement of Fasting Blood Glucose and Oral Glucose Tolerance Test ... 90
4.2.8 Determination of Biomarkers for Organ Function ... 90
4.2.9 Tissue Preparation ... 91
4.2.10 Lipid Peroxidation ... 91
4.2.11 ORAC and FRAP ... 92
4.2.12 Superoxide dismutase ... 92
4.2.13 Catalase ... 93
4.2.14 Total Glutathione and Glutathione disulphide (GSH/GSSG) ... 93
4.2.15 Histological Examination of Liver Tissues ... 93

4.2.16 Statistical Analysis 94
4.3 Results 94
4.3.1 Hypoglycemic effect of AD treatment in diabetic rats 94
4.3.2 Treatment with *A. difformis* abated serum levels of hepatic enzymes 95
4.3.3 Regulation/Modulation of lipid Profile by AD treatment in type II diabetes 96
4.3.4 Impact of AD treatment on protein synthesis in STZ-induced type II diabetes 98
4.3.5 AD Intervention ameliorated lipid peroxidation, oxidative stress in type II diabetes 99
4.3.6 AD administration enhanced antioxidant status in the liver of diabetic rats 100
4.3.7 Treatment with AD reversed pathologies and improved the histological structure of the liver in T2D. 102
4.4 Discussion 106
4.5 Conclusion 111
Future prospects 111
Authors contributions 111
Acknowledgments 111
References 113

CHAPTER FIVE 117
5 *Anchomanes difformis*; a potential solution to increased inflammation, apoptosis and organ toxicity in STZ-induced diabetic cardiomyopathy 117
ABSTRACT 118
ABBREVIATIONS 119
5.1 Introduction 120
5.2 Materials and methods 123
5.2.1 Chemicals and reagents 123
5.2.2 Plant Preparation 124
5.2.3 Ethical Approval 124
5.2.4 Animals 124
5.2.5 Experimental Design 125
5.2.6 Blood and Tissue Collection 126
5.2.7 Tissue Preparation 126
5.2.8 Determination of organ function and toxicity markers 126
5.2.9 Analysis of antioxidant status and lipid peroxidation indices 127
5.2.10 Estimation of pro- and anti-inflammatory biomarkers 127
5.2.11 Evaluation of apoptotic and transcriptional proteins expression 127

5.2.11.1 Antibodies ... 128
5.2.11.2 Tissue Preparation and Staining ... 128
5.2.11.3 Imaging ... 129
5.2.12 Data Analysis ... 129
5.3 Results ... 129
5.3.1 AD reduced weight loss and organ toxicity in STZ-induced diabetes ... 129
5.3.2 Effect of AD on antioxidant enzymes and protein synthesis in STZ induced diabetes ... 130
5.3.3 Effect of treatment with AD on antioxidant indices in the heart of normal and diabetic rats ... 131
5.3.4 AD modulated hyperglycaemia-induced immune response in the heart in T2D model ... 132
5.3.5 The effect of AD on lipid peroxidation and heart function markers ... 134
5.3.6 The regulation of transcription factors by AD in T2D model ... 135
5.3.7 Anti-apoptotic effect of AD on T2D model ... 138
5.4 Discussion ... 140
5.5 Conclusion ... 144
5.6 Recommendation ... 144
Acknowledgement ... 144
Funding ... 144
Declaration OF CONFLICT ... 144
References ... 145

CHAPTER SIX ... 152
6 Can supplementation with *Anchomanes difformis* ameliorate kidney and pancreatic damage in type 2 diabetes? ... 152
ABSTRACT ... 152
6.1 Introduction ... 154
6.2 Methodology ... 157
6.2.1 Plant preparation ... 157
6.2.2 Ethical consideration ... 157
6.2.3 Animals ... 157
6.2.4 Modelling and grouping ... 158
6.2.5 Sample collection ... 159
6.2.6 Tissue Preparation ... 160
6.2.7 Estimation of organ function and toxicity markers ... 160
6.2.8 Evaluation of antioxidant status and oxidative stress markers ... 160
6.2.9 Measurement of inflammatory markers ... 161

6.2.10 Quantification of the expression of transcription and apoptotic proteins 161
6.2.11 Histological examination of the kidney and pancreas 161
6.2.12 Statistical analysis 162
6.3 Results 162
6.3.1 Effect of treatment with AD on the relative weight of the kidney and pancreas 162
6.3.2 Effect of AD administration on kidney function markers 163
6.3.3 AD enhanced the antioxidant status in the kidney 164
6.3.4 AD modulated hyperglycaemia-induced immune response in the kidney... 166
6.3.5 Effect of AD supplementation on transcription factors in normal and diabetic rats 168
6.3.6 AD administration upregulated the expressions of Anti-apoptotic proteins in diabetic rats 170
6.3.7 Intervention with AD improved histoarchitecture of the kidney and pancreas in type2 diabetes 172
6.4 Discussion 177
6.5 Conclusion 182
Acknowledgement 182
Funding 183
Declaration of conflict 183
References 184

CHAPTER SEVEN 190
7 The beneficial role of *Anchomanes difformis* in STZ-induced reproductive dysfunction 190
ABSTRACT 191
7.1 Introduction 192
7.2 Methodology 194
7.2.1 Plant collection and extraction 194
7.2.2 Ethical consideration 194
7.2.3 Animal care 194
7.2.4 Induction of diabetes 195
7.2.5 Experimental design 195
7.2.6 Sample collection 196
7.2.7 Sperm isolation (Swim out method) 197
7.2.8 Measurement of Sperm concentration and motility 197
7.2.9 Evaluation of sperm morphology and viability 198
7.2.9.1 Preparation of sperm smears 198

7.2.9.2 Morphology 198
7.2.9.3 Viability 198
7.2.9.4 Sperm deformity index (SDI) 199
7.2.10 Histological analysis of the gonadal tissues 199
7.2.11 Statistical analysis 199
7.3 Results 200
7.3.1 Treatment with AD alleviates organ toxicity in the testis and epididymis 200
7.3.2 AD improved sperm function in normal and diabetic rats 201
7.3.3 AD enhanced sperm velocities and kinematics in STZ-induced diabetes 204
7.3.4 The Effect of AD administration on morphological indices in normal and diabetic rats 207
7.3.5 The effect of intervention with AD on gonadal tissues; testis and epididymis in STZ-induced diabetes 208
7.4 Discussion 211
7.5 Conclusion 214
Acknowledgement 214
Funding 215
Declaration of conflict 215
References 216

CHAPTER EIGHT 220
8 GENERAL DISCUSSION AND CONCLUSION 220
8.1 General discussion 220
8.2 Conclusion 226
8.3 Recommendation 226
ADDENDUM 229

LIST OF TABLES

Table 2.1: Scientific confirmation of some folkloric uses of A. difformis 25

Table 3.1: Phenolic compounds identified in AD leaves and rhizome using HPLC 62

Table 3.2: Further identification of bioactive compounds using UPLC-MS 65

Table 3.3: Characteristics of the unknown compounds 68

Table 4.1: compounds identified in aqueous extract of AD after characterization using UPLC-MS 86

Table 4.2: Effect of treatment on antioxidant enzymes in the liver of diabetic and normal rats 102

Table 4.3: Hepatic injury score in the various treatment groups 103

Table 5.1: the descriptions of the antibodies used for detection of protein expression levels, stating the host, supplier and the optimization factor 128

Table 5.2: Shows the response of TNFα and MCP-1 to treatment with AD in normal and diabetic hearts. 134

Table 5.3: Shows the effect of AD on (A) H-FABP and (B) TBARS levels in the heart of normal and diabetic hearts. 135

Table 7.1: Morphology parameters of semen from normal and diabetic rats 207

LIST OF FIGURES

Figure 2.1: The involvement of hyperglycemia, oxidative stress, inflammation and apoptosis in the progression diabetes mellitus and complications. 19
Figure 2.2: Plate 1 is the Leaves of A. difformis and Plate 2 is the Rhizome of A. difformis. 24
Figure 3.1: (A) Total polyphenol and (B) flavonol content of AD leaves and rhizome. .60
Figure 3.2: (A) Flavanol, and (B) alkaloid content of AD leaves and rhizome.. 60
Figure 3.3: Antioxidant capacities; (A) ORAC, (B) FRAP and (C) TEAC of leaves and rhizome extracts of AD. 61
Figure 3.4: Mass spectra showing the overall elution of the compounds in the leaf and rhizome extracts of AD. 68
Figure 3.5: Structure of certain compounds identified in AD leaves and rhizome. 69
Figure 4.1: Experimental design. 89
Figure 4.2: Effect of AD on weekly blood glucose concentrations (A) and oral glucose tolerance test (B) in normal and diabetic rats. 95
Figure 4.3: Effect of AD administration on biomarkers of hepatic injury; (A) ALT (B) AST and (C) ALP in the serum of normal and diabetic rats. 96
Figure 4.4: Effect of treatment with AD on the lipid profile; (A) Total Cholesterol (B) LDL-cholesterol (C) HDL-cholesterol and (D) Triglycerides in the serum of normal and diabetic rats. 97
Figure 4.5: Effect of treatment with AD on (A) Total protein (B) Albumin and (C) Globulin in the serum of normal and diabetic rats. 98
Figure 4.6: Effect of AD administration on biomarkers of lipid peroxidation and antioxidant activity; (A) TBARS, (B) FRAP and (C) ORAC in the serum of normal and diabetic rats. 100
Figure 4.7: Effect of treatment on non-enzymic antioxidant indices; (A) total GSH, (B) GSH-GSSG ratio, (C) total GSH and GSSG, (D) ORAC, and (E) FRAP in the liver of diabetic and normal rats. 102
Figure 4.8: Light micrographs from liver sections of normal and diabetic rats stained with hematoxylin–eosin 106
Figure 4.9: Proposed pathways involved in the antioxidant and hepatoprotective effect of Anchomanes difformis. 110
Figure 5.1: The pathophysiological pathway in the progression of diabetic cardiomyopathy. 120
Figure 5.2: Experimental design. 125

Figure 5.3: Effect of AD administration on (A) Bodyweight change and (B) Heart-body weight ratio. 130
Figure 5.4: Effect of treatment with AD on the activity of (A) Catalase, (B) SOD and (C) Total protein in the heart of normal and diabetic rats.......... 131
Figure 5.5: Effect of intervention with AD on the antioxidant capacities; (A) ORAC and (B) FRAP in the heart of normal and diabetic rats. 132
Figure 5.6: : Effect of AD administration on interleukins (IL) (A) IL-1β, (B) IL-6, (C) IL-10 and (D) IL-18 in the heart of normal and diabetic rats. 133
Figure 5.7: Representatives of Confocal microscopy image showing the effect of AD on the expression of NFkB/p65 (red) and Nrf2 (green) in the heart tissues. 137
Figure 5.8: (A) Fluorescence micrographs showing the effect of AD intervention on apoptotic markers in the hearts of normal and diabetic rats. (B) Quantification of the level of expression of caspase 3 and (C) Bcl2 in the heart tissues. 140
Figure 5.9: Proposed mechanism of action of AD in the management of DCM. 143
Figure 6.1: Pathogenesis of diabetic nephropathy. 156
Figure 6.2: Experimental design. 159
Figure 6.3: Effect of AD administration on the (A) relative kidney weight and (B) relative pancreas weight of normal and diabetic rats. 163
Figure 6.4: Effect of AD administration on the (A) urea and (B) creatinine concentration in the serum of normal and diabetic rats. 164
Figure 6.5: Effect of intervention with AD on the antioxidant capacities; (A) CAT, (B) SOD, (C) ORAC (D) FRAP and (E) Lipid peroxidation in the kidney of normal and diabetic rats (TBARS). 165
Figure 6.6: Effect of AD administration on interleukins (IL) (A) IL-1β, (B) IL-6, (C) IL-10 (D) IL-18 and (E) TNF-alpha in the kidney of normal and diabetic rats.. 167
Figure 6.7: (A) Confocal microscopy image showing the effect of AD on the expression of NFkB/p65 (red) and Nrf2 (green) in the kidney tissues. Quantitative analysis of (B) NFkB/p65 and (C) Nrf2 expression in the Kidney tissues. 170
Figure 6.8: (A) Fluorescence micrographs showing the effect of AD intervention on apoptotic markers in the kidney tissues of normal and diabetic rats. Quantification of the level of expression of (B) caspase 3 and (C) Bcl2 in the kidney tissues. 172
Figure 6.9: Light photomicrographs of haematoxylin and eosin-stained kidney cortex of normal and diabetic rats. 174
Figure 6.10: Light photomicrographs of haematoxylin and eosin-stained pancreatic tissue of normal and diabetic rats. 177
Figure 6.11: Proposed mechanisms by which AD ameliorates diabetic nephropathy and pancreatic damage. 182

Figure 7.1: Experimental design. 196
Figure 7.2: : Effect of AD administration on the (A) testicular weight (B) relative weight of the testis (C) weight of the epididymis and (D) the relative epididymal weight of normal and diabetic rats.. 201
Figure 7.3: Sperm concentration of normal, diabetic and treated diabetic rats 203
Figure 7.4: Effect of AD administration on the sperm function indices....................... 204
Figure 7.5: Effect of AD administration on indices of sperm velocities (A) VCL (B) VSL (C) VAP and (D) BCF of sperm cells in normal and diabetic rats. 205
Figure 7.6: Effect of AD administration on sperm kinematics; (A) LIN (B) STR and (C) WOB of sperm cells in normal and diabetic rats.. 206
Figure 7.7: Effect of AD administration on indices of oscillation index; (A) ALH and (B) BCF of sperm cells in normal and diabetic rats. 207
Figure 7.8: Light micrographs of the testes of normal and diabetic rats stained with hematoxylin–eosin 209
Figure 7.9: Light micrographs of the testes of normal and diabetic rats stained with hematoxylin–eosin 211

GLOSSARY

A. difformis	*Anchomanes difformis*
AGEs	advanced glycation end-products
ALEs	advanced lipoxidation end-products
COX-2	cyclooxygenase-2
IL-1	Interleukin-1
iNOS	inducible nitric oxide synthase
mRNA	messenger RNA (ribonucleic acids)
NFk-B	nuclear factor kappa-light-chain enhancer of activated beta cell
NO	nitric oxide
ppm	part per million
RAGE	receptor for advanced glycation end-products
RNS	reactive nitrogen species
ROS	reactive oxygen species
TNF-α	tumor necrosis factor alpha
TNF-ß	tumor necrosis factor beta

DEFINITION OF TERMS

Ameliorate: to improve or make an unpleasant situation better, more bearable or more satisfactory.

Antioxidant: a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that can produce free radicals, leading to chain reactions that may damage cells. Antioxidantssuch as thiols or ascorbic acid (vitamin C) terminate these chain reactions.

Apoptosis: is a form of cell death where the programmed cascades of events leads to an eventual elimination of the cells without releasing the cell content into the surrounding environment in the system.

Decoction: a concentrated liquor resulting from heating or boiling a substance, especially a medicinal preparation made from a plant and contains the constituents or principles of the substance (plant) soluble in boiling water.

Diuresis: increased or excessive production of urine.

Folklore: the traditional beliefs, myths, tales, and practices of a people which have been disseminated in an informal manner from generations to genrations.

Inflammation: is a part of the complex biological processes involved in the protective response of the tissues to harmful stimuli such as pathogens, damaged cells and irritants in or exposed to the body.

Oxidative stress: An imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage.

Pathogenesis: the origin and development of a disease, it involves the biological mechanism(s) that leads to the diseased state.

Phytochemicals: chemicals compounds occuring in plants and have protective or disease-preventive properties.

CHAPTER ONE

INTRODUCTION

1.1 Statement of Research Problem

Diabetes mellitus is one of the most prevalent pathological conditions worldwide today. Constant hyperglycemia as a result of metabolic abnormalities in diabetic condition contributes to the development of diabetic complications such as cardiomyopathy, retinopathy, nephropathy, hepatic injury and infertility. Furthermore, the enhanced generation of reactive oxygen species in diabetes leads to increased inflammatory response/mediators, lipid and lipoprotein modifications, nitric oxide production and all of these have been implicated in diabetic complications. Conventional drugs such as insulin, metformin, sulfonylureas are being used in the treatment of diabetes, however, the cost and adverse effects as a result of the use of these drugs necessitated the need for alternative medicine. This drive for alternative sources of treatment has led to the exploration of a lot of plant reserves such as *Anchomanes difformis*. This study therefore examined in detail the potential effects of *Anchomanes difformis* in diabetic animal model and the possibility of its contributions as an effective remedy in the treatment of diabetes and its complications.

1.2 Background

Diabetes mellitus (DM) is a metabolic disorder that is characterized by persistent hyperglycemia which is either due to inadequate insulin production or impaired response of body cells to insulin secretion or both (Maritim *et al.,* 2003; Johnson *et al.,* 2019). Diabetes results to over 4 million deaths in a year as reported by the International Diabetes Federation (IDF Diabetes Atlas, 2019). Diabetes has been ranked as the 7th leading cause of death (WHO, 2016). An estimated increase of diabetic patient from 382

million (2013) to 552 million in 2030 and 700 million in 2045 has been predicted (Vos *et al.,* 2012; Melmed *et al.,* 2015; IDF Diabetes Atlas, 2019). The most prevalent types of DM are type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). T1DM accounts for about 10% of all cases of DM with children being diagnosed majorly, while T2DM accounts for about 90% of all diabetes occurrence (IDF Diabetes Atlas, 2019).

Hyperglycemia enhances the production of reactive oxygen species (ROS) which is highly implicated in the development of oxidative stress (Wright *et al.,* 2006). Some of the mechanisms by which hyperglycemia leads to oxidative stress include the increased production of superoxide anion which activates nuclear factor kappa-light-chain enhancer of activated beta cells (NFκB); a transcription factor that leads to the increased expression of inducible nitric oxide synthase (iNOS). This increased iNOS results in enhanced production of nitric oxide (NO). Nitric oxide rapidly reacts with superoxide anion when present in high concentrations to form a strong oxidant; peroxynitrite (Beckman & Koppenol, 1996; Ramdial *et al.,* 2017) which exerts its toxic effects through oxidation of proteins, initiation of lipid peroxidation and nitration of protein. Triggering of the inflammatory response is another mechanism whereby hyperglycemia causes oxidative stress. Hyperglycemia contributes to increased glycation of proteins and lipids to form advanced glycation end products (AGEs) (Lyons & Jenkins, 1997). AGEs bind to their receptors (RAGE) on different cells and macrophages leading to intracellular generation of ROS which in turn activates NFκB causing increased expression of a variety of cytokines such as TNF-α and TNF-ß (tumor necrosis factor alpha and beta), interleukins (IL-1, IL-6, IL-8, IL-18) and interferon-γ. Also, hyperglycemia causes lipid and lipoprotein modification which results in glycation and glyoxidation of proteins, lipid peroxidation and lipoxidation of lipoproteins (Wright *et al.,* 2006; Moldogazieva *et al.,* 2019).

Severe lipid peroxidation, protein oxidation and nitration of proteins leads to oxidative stress which is an important risk factor in the development of diabetic complications such as retinopathy (Pan *et al.,* 2008), nephropathy (Ceriello *et al*., 2016), cardiomyopathy (Miranda-Díaz *et al.,* 2016), and liver injury (Ghanbari et al., 2016). Also, the overproduction of ROS leads to the inactivation of antiatherosclerotic enzymes resulting in atherosclerosis and ultimately cardiomyopathy (Saely *et al.,* 2004; Svensson *et al.,* 2004). Oxidised lipoproteins have pro-atherogenic effects which include increased smooth muscle cell proliferation, increased apoptosis in endothelial cells, activation of protein kinase-C and transforming growth factor-beta (TGF-β), increased non bio-availability of nitric oxide, pro-inflammatory effects and inhibition of antioxidant enzymes (Jenkins *et al.,* 2004).

The increased production of exogenous and endogenous ROS can be detrimental to the biological functions of sperm which is associated with male infertility (Sonmez *et al.,* 2005; Dutta *et al*., 2019) and it is characterized by ATP depletion leading to insufficient axonemal phosphorylation and lipid peroxidation (de Lamirande *et al.,* 1997). The sperm plasma membrane contains a high amount of unsaturated fatty acids which makes it highly susceptible to peroxidative damage (Aitken, 2017). The lipid peroxidation destroys the structure of lipid matrix in the membranes of sperm, and it is associated with loss of motility and the defects of membrane integrity (Sharma & Agarwal, 1996; de Lamirande *et al.,* 1997; Karunakaran *et al.,* 2017). It is hypothesized that insulin is required in the initiation and continuance of spermatogenesis (Maresch et al., 2018). Also, the maintenance of spermatogenic process necessitates the conversion of glucose to lactate by the sertoli cells. Hence, the supply of glucose to the germ cells via the blood is strictly controlled by the blood-testis-barrier. This is however disrupted in diabetic conditions thereby affecting spermatogenesis (Reira *et al.,* 2009; Alves *et al*., 2013). Furthermore, heightened ROS affects sperm viability through dysregulated apoptosis. Apoptosis can occur either in the germ cells during spermatogenesis or in mature cells during sperm

release (spermiation) (Muratori *et al.*, 2015). However, in diabetic conditions which is characterized by increased oxidative stress, apoptosis is poorly regulated especially in the spermiation phase, resulting in the release of non-viable sperm cells and eventual poor sperm quality (Muratori *et al.*, 2015).

Streptozotocin (STZ); a diabetogenic agent is used to induce both IDDM and NIDDM (Szkudelski 2001). It is well known for its cytotoxic action mediated by ROS on beta cells of the rat pancreas and its characteristic alterations in blood insulin and glucose concentration by decreasing insulin biosynthesis and secretion (Bolaffi *et al.,* 1987; Nukatsuka *et al.*, 1990b Szkudelski, 2001) and impairing glucose oxidation (Bedoya *et al.,* 1996). The pancreatic beta cells take up STZ through the glucose transporter (GLUT2), STZ then causes alkylation of DNA which is the main factor of beta cell death (Delaney *et al.*, 1995; Elsner *et al.,* 2000). This alkylating potency of STZ is attributed to its methyl-nitrosourea moiety especially at the O^6 position of guanine (Schnedl *et al.,* 1994; Murata *et al.,* 1999; Lenzen 2008). The transfer of the methyl group from STZ to the DNA molecule causes damage which subsequently results in DNA fragmentation (Pieper *et al.,* 1999) and ultimately destruction of beta cells by necrosis.

Various drugs are used in the treatment of diabetes in addition to insulin; these include sulfonylurea, metformin, alpha-glucose inhibitors, thiazolidinediones, meglitinides, D-PP-4 inhibitors. However, these drugs have limitations due to their side effects such as gastrointestinal disturbances, hypoglycemia, urinary tract infection, dizziness, constipation and certain risks such as increased rate of lactic acidosis (Wang *et al.,* 2003; Fimognari *et al.,* 2006), liver damage (Hinterthuer, 2008) and cardiovascular risk (European Medicines Agency, 2009). These risks and side effects along with other factors such as cost have led to the search for alternative sources especially medicinal plants with natural anti-diabetic potentials.

Anchomanes difformis of the family Araceae is a large herbaceous plant that grows mostly in the West African forests to a height of approximately two metres (Eneojo *et al.,* 2011). *A. difformis* grows from a horizontal tuber that can measure up to 80 cm by 20 cm and produces one huge, much-divided leaf with a stout prickly stem (Afolayan *et al.,* 2012). These two parts have been used for traditional purposes such as in the treatment of river blindness, ulcer, dysentery and diuresis (Okpo *et al.,* 2011). *A. difformis* contains flavonoids, glycoside, tannins (Aliyu *et al.,* 2008b). Flavonoids have anti-inflammatory, anti-tumor, anti-allergy, anti-platelets, antiviral and antioxidant activities (Buhler and Miranda, 2000). *A. difformis* has been shown to possess the following medicinal properties such as antioxidant, anti-inflammatory (Adebayo *et al.,* 2014), hypoglycemic (Adeyemi *et al.*, 2015), gastroprotective (Okpo *et al.,* 2011), antimicrobial (Eneojo *et al.,* 2011), and anti-ulcerative. However, there is very little or no reports on the mechanism by which AD exerts these medicinal properties especially in diabetes. Previous studies carried out on the anti-diabetic properties of *A. difformis* has been very limited and ambiguous, hence the need to examine in detail, its antidiabetic, antioxidant, anti-inflammatory activities as well as the mechanisms of actions in animal model.

1.3 Rationale for this study

Diabetes mellitus is one of the most common chronic diseases in nearly all countries and continues to increase in numbers and significance with it taking about about 10% of the global health expenditure (IDF Diabetes Atlas, 2019). The prevalence of T2DM over T1DM and the associated complications in the management of T2DM is a strong motivation for this study. Also limitations in the use of current anti-hyperglycemic medications due to their high cost, limited action, secondary failure rates and accompanying side effects (Watcher, 2010, Baggio and Drucker, 2008) has necessitated the need for alternative therapies with the aim to discover other therapeutic agents that could be effective in the management and prevention of diabetes and its complications.

Oxidative stress and inflammatory response play a pivotal role in the development of diabetic complications (Pitocco et al., 2013, Ryan et al., 2009). *A. difformis* has been scientifically proven to possess anti-oxidant and anti-inflammatory capabilities (Aliyu et al., 2008 and Adebayo et al., 2014) and other pharmacological potentials such as hypoglycemic (Adeyemi et al., 2015), antimicrobial (Eneojo et al., 2011), anti-nociceptive (Adebayo et al., 2014) and gastroprotective (Okpo et al., 2011). However, these studies are limited in scope; also, to the best of my knowledge, no research has been carried out to demonstrate the effect of this plant in STZ and fructose-induced type 2 diabetes and diabetic complications such as cardiomyopathy, hepatopathy, nephropathy and infertility. These therefore, calls for the need to explore the effects of this plant in diabetes and its complications.

This study wil investigate how *Anchomanes difformis* could ameliorate and prevent the development of type 2 diabetes melitus and its complications due to oxidative damage, inflammation, apoptosis and lipid peroxidation. It is envisaged that the findings would provide a better understanding and insight into the possible mechanisms of action of the plant extract in diabetic aninmal model.

1.4 Aim

This research study was conducted to investigate the antidiabetic properties of *A. difformis* administration in fructose and streptozotocin-induced type 2 diabetes mellitus. It also examined the potential ability of AD to prevent or ameliorate resultant diabetic complications such as cardiomyopathy, nephropathy, hepatopathy and reproductive dysfunctions as evaluated through the following:

1. Antioxidant status
2. Inflammatory response
3. Apoptotic protein

4. Glycemic and lipidemic index
5. Biomarkers of organ function
6. Reproductive parameters
7. Histological alterations in the organs

1.5 Objectives

In order to pragmatically achieve the above-mentioned aim, the following specified objectives were performed:

1. Evaluation of the phytochemical characterization and antioxidant capacities of three solvent extracts from the leaves and rhizome of *A. difformis*, for optimization.
2. Investigation of the hypoglycemic and hypolipidemic potential of *A. difformis* in normal and diabetic rats.
3. Assessment of the impact of *A. difformis* administration on the liver and kidney functions in normal and diabetic rats.
4. Measurement of the antioxidant ability of *A. difformis* in the serum, liver, kidney and heart of diabetic and normal rats.
5. Determination of the influence of *A. difformis* on the inflammatory response and apoptosis in the liver, kidney and heart of diabetic and normal rats.
6. Estimation of the effect of *A. difformis* on sperm concentration, motility, viability and morphology in diabetic and non-diabetic rats.

1.6 Research Questions

- What are the potential effects of *A. difformis* on serum lipid profile and glycemic parameters in diabetic and non-diabetic male Wistar rats?
- Does *A. difformis have* potential effects on the oxidative status and antioxidant systems in diabetic and non-diabetic male Wistar rats?

- What are the potential effects of *A. difformis* on inflammatory biomarkers in the heart and kidney of diabetic and non-diabetic male Wistar rats?
- Does *A. difformis* provide any potential effects on expression of apoptotic proteins in diabetic rats and non-diabetic male Wistar rats?
- What are the potential effects of *A. difformis* on liver and kidney function in diabetic rats and non-diabetic male Wistar rats?
- What are the potential effects of A. *difformis* on the reproductive system of diabetic and non-diabetic rats?
- What are the concentrations of total polyphenol content and the individual phytochemicals present in *A. difformis* plant extract?

1.7 Hypothesis

H_0: Aqueous leaf extract of *Anchomanes difformis* (200mg and 400mg) will not ameliorate diabetes mellitus type 2 and will not prevent the development of diabetic complications and infertility

H_1: Aqueous leaf extract of *Anchomanes difformis* (200mg and 400mg) will ameliorate diabetes mellitus type 2 and will prevent development of diabetic complications.

1.8 Significance of the study

This study provided insight into the pharmacological effects of A. difformis on inflammatory, oxidative and apoptotic parameters in diabetic cardiomyopathy, nephropathy and liver injury resulting from streptozotocin-induced damage in diabetic male Wistar rats. Findings of this research could contribute to the treatment and management of diabetes and male infertility. The research outputs from this study include published manuscripts in various reputable, peer-reviewed journals of high impact factors. Outcomes from this research was presented at local and international conferences, workshops and seminars which contributed immensely to the pool of scientific knowledge in diabetic research.

1.9 Ethical consideration

Ethical approval for this study was obtained from the Research Ethics Committee of the Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, South Africa (CPUT/HW-REC 2016/A4) and from the Ethics Committee for Research on Animals from South African Medical Research Council where the animal study was carried out (REF. 04/17).

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CHAPTER TWO

LITERATURE REVIEW

Medicinal activities of A*nchomanes difformis* and its potentials in the treatment of diabetes mellitus and other disease conditions

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2.1 Diabetes mellitus

Diabetes mellitus is strongly associated with persistent hyperglycemia and other metabolic disorders including insulin resistance, impaired glucose tolerance dyslipidemia, obesity and hypertension (American Diabetes Association, 2017). Diabetes mellitus is associated with the partial or complete destruction of pancreatic β-cells which consequently leads to insulin deficiency which may further result in insulin resistance (American Diabetes Association, 2010).

2.2 Types of Diabetes mellitus

There are two major types of diabetes mellitus; type-1 which occurs when the body does not produce insulin and it is referred to as insulin-dependent diabetes mellitus (IDDM), type-2 which occurs when the body does not secret enough insulin or the cells does not respond properly to insulin, this is referred to as non-insulin dependent diabetes mellitus (NIDDM) or insulin resistance diabetes (DeFronzo *et al.*, 1997; Zimmet *et al.*, 2004). There are other classes of diabetes mellitus which are less prevalent, these include gestational diabetes, monogenic diabetes and secondary diabetes (Hu & Jia, 2018).

2.2.1 Type 1 diabetes

T1DM is an autoimmune disease where the beta cells of the pancreas are being attacked and destroyed by the body's immune system, leading to little or no production of the insulin. The mechanisms underlying the destruction of the beta cells are not fully understood, however, genetic susceptibility and environmental factors have been implicated (Maahs *et* al., 2010; Atkinson *et al.*, 2014). Although, T1DM is prevalent in children and young people, it can occur in any age group or be diagnosed in adults. T1DM can be effectively managed with daily dose of insulin which delays and prevent the development of diabetic complications (Alberti et al., 1998). Due to the involvement

of auto-immune reactions in the pathogenesis of T1DM, a preventive control is yet to be unraveled.

2.2.2 Type 2 diabetes

T2DM is uniquely characterized with insulin resistance, unlike T1DM which is majorly triggered by autoimmune responses, T2DM has multifactorial aetiology ranging from obesity, increasing age, heredity, sedentary lifestyle and environmental factors (Basu *et al*., 2013). The increased production of hepatic glucose, reduced secretion of insulin and or impaired response to insulin secreted (also known as insulin resistance) are fundamental defects in the development of T2DM (Stumvoll *et al*., 2005). In the course of insulin resistance, the insulin secreted is ineffective and the beta cells make efforts to overcome it by increased production of insulin (Kumar, 2020). A prolonged demand for increased insulin secretion in turns weakens the beta cells of the pancreas and eventually leads to decreased production of insulin. Insulin resistance is highly implicated in the development of the metabolic, cardiovascular, and endocrine disorders in T2DM (Kumar, 2020).

2.3 Oxidative stress in Diabetes

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in the body, during normal metabolism and energy production (Mayi *et al.,* 2004). They are produced to help the normal healthy tissues perform physiological roles such as signaling, regulation of signal transduction and gene expression, activation of receptor and nuclear transduction (Valko *et al*., 2007). Oxidative stress occurs as a result of imbalance between the systemic production of these free radicals; (ROS and RNS) and the antioxidant capacity of the system to readily detoxify and eliminate the reactive intermediates or to repair the resulting damage (Aliyu *et al.,* 2013). Constant hyperglycemia is one of the major factors leading to oxidative stress. Hyperglycemia

enhances the production of reactive oxygen species which is highly implicated in the development of oxidative stress (Wright *et al.,* 2006).

Some of the molecular mechanisms by which hyperglycemia leads to oxidative stress and subsequently diabetes includes the increased activation of the hexosamine pathway, polyol pathway, and the protein kinase C (PKC) pathway and the formation of AGEs (Rolo & Palmeira, 2006). During persistent hyperglycemia, overproduction of free radicals occurs, and this leads to DNA damage (Styskal *et al*., 2012). A DNA repair enzyme: Poly-ADP-ribose polymerase 1 (PARP-1) is subsequently activated and impedes the activity of glyceraldehyde-3-phophate dehydrogenase (GAPDH). GAPDH is an important enzyme in the breakdown of glucose and it catalyzes the oxidation of glyceraldehyde-3-phosphate (GAP) (Giacco *et al.,* 2010; Ighodaro, 2018). The inhibition of GAPDH leads to the accumulation of GAP which further activates the PKC, hexosamine, polyol and AGEs pathways (Rolo and Palmeira, 2006).

Furthermore, hyperglycemia is strongly associated with glycation and glycoxidation of lipoproteins, ROS resulting from hyperglycemia contribute to initiation of lipid peroxidation (Cosentino *et al*., 1997) and ultimately lipoxidation to yield advanced lipoxidation end products (ALEs) (Esterbauer *et al.,* 1992; Esterbauer *et al.,* 1991; Spiteller, 1998). Severe lipid peroxidation, protein oxidation and nitration of proteins leads to oxidative stress, increased inflammation and apoptosis which are important factors in the development of diabetic complications such as retinopathy (Pan *et al.,* 2008), nephropathy, liver injury, and cardiovascular diseases (Januszewski *et al*., 2003). Figure 2.1 shows the relationship between hyperglycemia, oxidative stress, inflammation and apoptosis and their importance in the progression of diabetes.

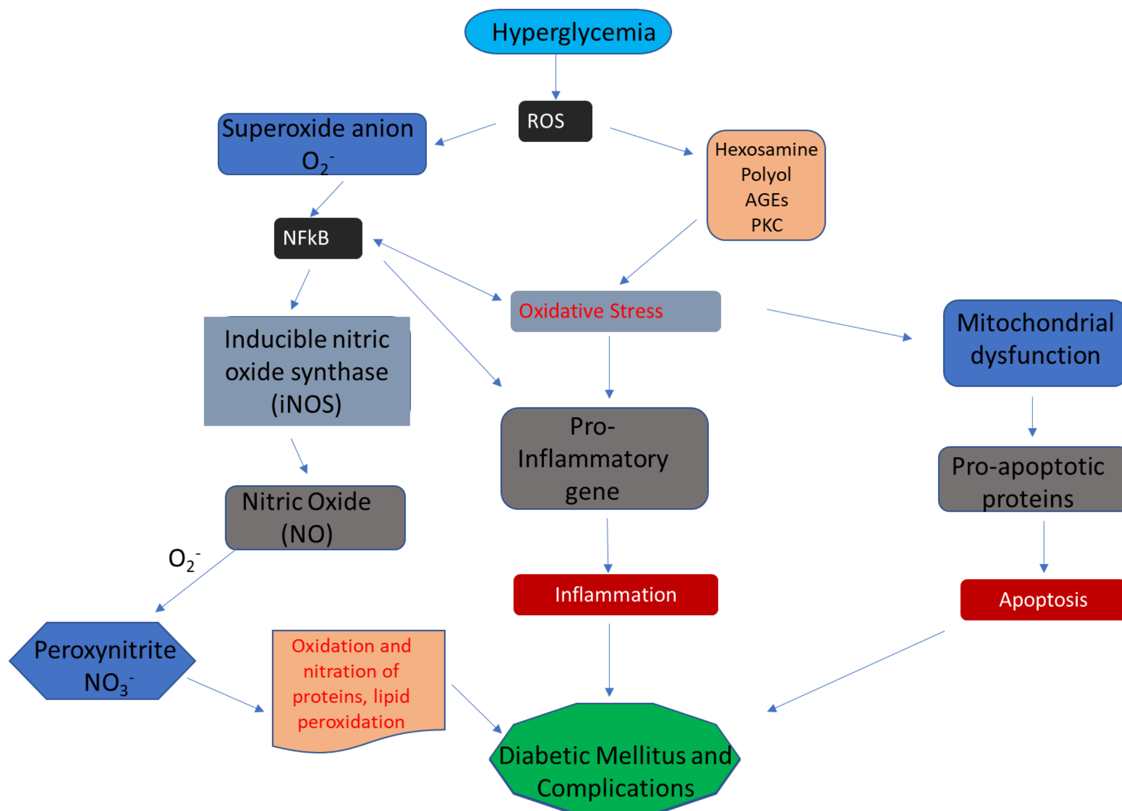

Figure 0.1: The involvement of hyperglycemia, oxidative stress, inflammation and apoptosis in the progression diabetes mellitus and complications.

2.4 Diabetic Complications

Chronic hyperglycemia affects almost all organs of the body. This is probably due to its presence in the blood which is supplied to almost all parts of the body (Dang, 2012). Scientific evidences show that complications result later after the onset of diabetes. These complications with etiology linked to diabetes are referred to as diabetic complications (DCM) (Resnick and Howard 2002; Singh *et al.*, 2013). DCM present structural pathologies in the organs such as kidney, retina, nerves, heart and blood vessels. The severity of DCM has been strongly correlated with the duration of the disease and the degree of glycemic control (Resnick and Howard 2002). DCM is the principal cause of morbidity and mortality in diabetic patients (Nicholson 2006; Koulis *et al.*, 2015), with etiology linked to multifaceted mechanisms (Pop-Busui *et al.,* 2006).

2.4.1 Classification of Diabetic Complications

DCM can be broadly categorized into two, microvascular and macrovascular complications. Microvascular complications stem from alterations in the small vessels and capillaries and are generally reported in people living with diabetes (Kulkarni *et al.,* 2016; Gæde *et al.*, 2003;). It could be caused by several risk factors such as hyperlipidemia, hyperglycemia, hypertension. However, hyperglycemia seems to be the chief cause of microvascular complications (Solini *et al.*, 2012). Microvascular complications are significant in diabetes mellitus as its progression can lead to visual impairment (retinopathy), kidney damage (nephropathy), nerves dysfunctions (neuropathy) and dementia among others (Avogaro & Fadini, 2019). These microvascular modifications are strongly connected to macrovascular complications as they are observed in other organs including the heart (Paulus & Tschöpe, 2013; Avogaro & Fadini, 2019).

Macrovascular complications arise from defects in the large vessels such as the arteries and the veins (Kulkarni *et al.,* 2016). Atherosclerosis is the major mechanism involved in the development of macrovascular complications, and it is a resultant effect of chronic inflammation and injuries to the walls of the arteries (Fowler, 2008). Macrovascular pathologies usually end in cardiovascular diseases and related conditions, which is highly associated with diabetes (Laing *et al.,* 2003; Fowler, 2008).

2.4.2 Diabetic Cardiomyopathy

Constant hyperglycemia is a key factor in the progression of diabetic cardiomyopathy (Fuentes-Antras *et al.,* 2015); a pathological condition that alters structure and function of the heart muscle in diabetic subjects (Wang & Hill, 2015). Persistent high glucose levels in the blood can lead to mitochondrial impairment, hypertrophy and degradation in the cardiac muscle cell, as well as myocardial dilatation and eventual dysfunction of

the systolic and diastolic function of the left ventricle (Yue *et al.,* 2017). Other factors implicated in the pathogenesis of diabetic cardiomyopathy include oxidative stress and inflammation. Oxidative stress caused by constant hyperglycemia triggers the production of AGEs which affects the structural functions of proteins in the heart (Nishikawa *et al*., 2000). Also, increased recruitment of cytokines disrupts homeostasis of the myocytes thereby affecting the contractility and remodeling of the heart muscle (Shi *et al.,* 2018).

2.4.3 Diabetic Nephropathy

Diabetic nephropathy (DN); also referred to as diabetic kidney disease is an heterogenous condition in which many pathologies may exist alongside and it has been majorly implicated in end-stage renal disease (Rivero-González *et al*., 2017; Avogaro and Fadini, 2019). The presence of hyperglycemia with inflammation affects the structure of the glomerular endothelium. This leads to alteration in the permeability and selectivity of the glomeruli and subsequently apoptosis of the glomerular cells. Abnormalities in angiogenesis are also observed in DN (Jeansson *et* al., 2011). Maladaptive expression of growth factors such as vascular endothelial growth factor (VEGF) as a result of irregular angiogenesis further leads to albuminuria (Avogaro and Fadini, 2019). Pathological features seen in DN include thickening of the glomerular basement membrane, albuminuria and loss of podocytes (Wharram *et al.,* 2005). DN can be managed by effective glycemic control and amelioration of dyslipidemia (John, 2016).

2.4.4 Reproductive Complications of Diabetes

Diabetes mellitus adversely affects spermatogenesis and lead to male infertility through oxidative stress and endocrine disorder (Johnson *et al.,* 2019). Also, impaired spermatogenesis and DNA damage are linked to insulin resistance; a typical

characteristics of diabetes mellitus (Glazer *et al.,* 2017). Oxidative stress has been highly implicated in male infertility, it leads to altered protein expression in the seminal plasma which is a source of antioxidant defense of the spermatozoa (Agarwal *et al.,* 2014). Increased oxidative stress also leads to lipid peroxidation of the poly unsaturated fatty acids of the sperm membrane (Tremellen, 2008), mitochondrial DNA mutations and damage (Agarwal *et al*., 2014) resulting in apoptosis (Wang *et* al., 2003). Nuclear DNA fragmentation is also a resultant effect of oxidative stress in the spermatozoa (Henkel *et* al., 2005). Diabetes also have impact on the sexual functions due to its influence on the endocrine system, which in turn affects spermatogenic processes (Sexton & Jarrow, 1997). Glycolysis and oxidative phosphorylation are major supplies of energy in the spermatozoa. The deficiency of insulin causes endocrine disturbances (Omolaoye & du Plessis, 2018), thereby altering glucose homeostasis in the gonads. Reports show that Leydig cell function and reproductive hormones such as testosterone, follicle stimulating hormone, luteinizing hormone are reduced in diabetes mellitus (Condorelli *et al.,* 2018).

2.5 Medicinal Plants and the Management of Diabetes Mellitus

Medicinal plants are plants with potency to prevent and ameliorate specific ailment and pathological conditions (Nwachukwu *et al.,* 2011; Nwachukwu *et al.,* 2012). Apart from the well-known fact that plants serve as a source of food, it is also used for medicinal purposes (Obi, 2011). Many of these plants are used as spices and local remedies and sometimes added to foods for supplementation or as treatment regimens (Okwu and Ibeawuchi, 2005).

Medicinal plants are of great importance to the health of individuals and communities (Edeoga *et al.,* 2005). The medicinal value of plants is attributed to certain bioactive chemical substances present in those plants, which produce definite physiological

actions in the human body (Edeoga *et al.,* 2005). Some of the bioactive constituents in plants that are of high importance include alkaloids, tannins, flavonoids and phenolic compounds (Soetan and Oyewole, 2009) and they elicit antioxidant, anticancer, anti-inflammatory, antimalarial properties amongst many others (Buhler and Miranda, 2000). These bioactive constituents are often referred to as phytochemicals.

2.6 *Anchomanes* difformis; its medicinal properties and potentials

Anchomanes difformis is a plant with many reported therapeutic properties (Adebayo *et al.,* 2014; Oghale and Idu, 2015b; Okpo *et al.,* 2011; Osho and Adetunji, 2010) and it is commonly used in traditional medicine to treat diseases with pathogenesis linked to oxidative stress among other factors (Oyetayo, 2007). *A. difformis* (Blume) is a specie of flowering plants in the family Araceae, an herbaceous plant with prickly stem (up to 2 cm high) having huge divided leaf and spathe that arise from a horizontal tuber (which could be up to 80cm long and 20cm wide) growing as wild yam in the moist and shady places of tropical African forest (Afolayan *et al*., 2012). It is prevalent in West African countries such as Ghana, Ivory Coast, Nigeria, Togo, Sierra Leone, Senegal and Guinea (Adebayo *et al*., 2014) and also found in southern-tropical Africa; Zambia, and Angola, (Boudet *et al.,*1986) Tanzania and Uganda.

A. Difformis have a wide range of local names which is based on the location; in Nigeria it is called *ìgo lángbòdó, ògìrìòsákó* (*YORUBA*)*, oje, olumahi* (*IGBO*)*, chakara, hántsàr gàdaá, hántsàr giawaá* (*HAUSA*)*, eba enàŋ* (*EFIK*)*;* in Zambia*, Kabaka-kachulu* (*LUNDA*)*;* in Ivory Coast*, niamé kwanba* (*BAULE*)*, eupé, niamatimi* (*AKAN-ASANTE*)*, kohodié* (*ABURE*)*, alomé* (*AKYE*)*, tupain* (*ANYI*)*;* in Sierra Leone*, a-thoŋbothigba* (*TEMNE*)*, kalilugbo* (*MENDE-KPA*)*, alatala-kunde-na (SUSU-DYALONKE),* in Ghana*, atõe, nyame kyin* (*FANTE*)*, lukpogu* (*DAGBANI*)*;* in Senegal*, éken* (*DIOLA*)*;* in Togo, *nau* (*TEM*) (Eneojo *et al*., 2011).

Plate 2

Figure 0.2: Plate 1 is the Leaves of A. difformis and Plate 2 is the Rhizome of A. difformis.

2.7 Folkloric Uses of *A. difformis*

Medicinal plants have been used for various traditional purposes varying from one community to another. Majority of people especially from the underdeveloped and developing countries, rely on traditional plant usage for their day-to-day health care needs (Eneojo *et al*., 2011; Gurib-Fakim, 2006), mostly due to the high cost of conventional health care or side effects of drugs used. A. *difformis* has been reported for its wide range of traditional uses, some of which have been scientifically proven and confirmed. Different parts of the plants (leaves, stem and tuber) are used to treat diverse complications and ailments (Fongod *et al*., 2014; Olawale *et al.,* 2013).

The decoction from the leaves of A. *difformis* is traditionally used as an anti-bacterial agent particularly against *Staphylococcus aureus* in northern Nigeria (Aliyu *et al*., 2008a); to ameliorate pain and inflammation (Adebayo *et al*., 2014), in the treatment of cough, ulcer and asthma in southern Nigeria, (Idu *et al*., 2003). The rhizome has been used for the treatment of many disease conditions in various parts of the world, commonly Africa. In Ivory Coast, it is considered to be a powerful purgative and it is used to treat oedema, control complications during child delivery, as an antidote to poison as well as a strong diuretic for treating urethral discharge, jaundice and kidney pains (Akah and Njike, 1990). In Nigeria, the peeled tuber soaked in water is used in treating cases

of dysentery and diarrhea (Eneojo *et al.,* 2011). In some part of Africa such as Tanzania, the juice from the root tuber is used as eye drop in the treatment of river blindness (Gills, 1992). The decoction from the tuber is used to treat cough, diabetes, dysentery and throat-related problems (Oyetayo, 2007). Most of the ethno-medicinal uses of *A. difformis* has been scientifically proven and established (Table 2.1).

Table 0.1: Scientific confirmation of some folkloric uses of A. difformis

Folklore (scientifically proven)	**Parts Used**	**Reference**
Anti-diabetes	leaf (ethanolic extract) rhizome (ethanolic extract)	Adeyemi *et al.,* 2015 Aderonke and Ezinwanne, 2015
Anti-inflammation	leaf (ethanolic extract)	Adebayo *et al*., 2014
Pain killer/analgesic	leaf (ethanolic fraction) rhizome (ethanolic fraction)	Adebayo *et al*., 2014 Gabriel *et al*., 2013
Anti-ulcer & gastro-protective	rhizome (ethyl acetate extract) leaf and rhizome (ethanol:methanol:water)	Okpo, 2011 Eneojo *et al*., 2011
Anti-asthma	rhizome (aqueous fraction)	Oghale and Idu, 2015b
Dysentery and diarrhea	leaf and rhizome (ethanol:methanol:water)	Eneojo *et al*., 2011
Anti-malarial	rhizome (methanolic, aqueous and dichloromethane fractions)	Bero *et al.,* 2009
Anti-onchocercal (river blindness)	rhizome (methanolic extract)	Nkoh *et al.,* 2015
Anti-trypanosomal	rhizome (dichloromethane extract) aqueous and methanolic fractions	Bero *et al*., 2011 Atawodi, 2005
Anti-microbial	leaf and rhizome (ethanol:methanol:water) leaf and rhizome extracts	Eneojo *et al*., 2011 Agyare *et al.,* 2016

2.8 Nutritive value of A. *difformis*

The leaves of *A. difformis* are consumed as vegetables in some states in Nigeria (Obi, 2011; Olawale *et al*., 2013 and some other West African countries. Proximate analysis carried out on the leaves revealed that it contains carbohydrate 58.63%, crude protein 30.55%, crude fiber 14.77%, fat and oil 0.49%, ash 11.71% and moisture content 12.39% (Oyeyemi and Tadela, 2014). The crude protein content (30.55%) of *A. difformis* leaves when compared with some other vegetables such as *Momordica balsamina* (11.29%), *Moringa oleifera* (20.72%), *Lesianthera africana* leaves (13.10-14.90%), *Ocimum gratissimum* (8.00%) and *Hibiscus esculentus* (8.00%) is found to be relatively higher (Akindahunsi and Salawu, 2005; Oduro *et al*., 2008). The high protein value in the leaves of *A. difformis* suggests a potential source of plant protein and therefore, can be used as protein supplement in diet.

A. difformis is also considered to be rich in essential minerals (Aliyu *et al.,* 2008b). Essential mineral elements play significant roles in human nutrition and metabolism (Soetan *et al.,* 2010), and their deficiencies could lead to anemia (iron), low glucose tolerance and liver necrosis (selenium), hypersensitivity (chloride) and reduced fertility (lithium) (Turan *et al*., 2003). Certain macro elements play physiological roles such as structural (calcium in bones, iron in heme, phosphorus in phospholipids and nucleic acids), catalytic (magnesium) and signal transduction; calcium in nerve and muscular cells (O'Dell and Sunde, 1997). Potassium, Sodium and chlorine are essential for the maintenance of osmotic balance between cells and the interstitial fluid (Turan *et al*., 2003; Soetan *et al.,* 2010).

Other micro elements such as manganese and copper serve as co-factors of certain enzymes and are indispensable in numerous biochemical pathways that are essential for the normal functioning of the cell (Soetan *et al.,* 2010). These include protein

synthesis, carbohydrate metabolism, cell growth and cell division. The mineral analysis of *A. difformis* (leaves) showed the presence of essential minerals; potassium 1.74%, calcium 0.26%, phosphorus 157.11 parts per million (ppm), magnesium 24525 ppm, iron 81.5 ppm, manganese 95.5 ppm, sodium 0.026% and copper 8.0 ppm (Oyeyemi and Tadela, 2014). This was supported by the output of mineral analysis carried out on the same plant by (Aliyu, 2008b). The appreciable concentrations of minerals such as sodium, potassium, calcium and phosphorus obtained from the plant showed that the plant has the potential of providing various secondary metabolites and mineral supply that could enhance the curative process of ill- health (Aliyu, 2008b; Oyeyemi and Tadela, 2014). Another study (Arigbede *et al.*, 2010) compared the nutritive value of the leaves and tuber of *A. difformis* and also used it as a feed supplement to basal diet of West African dwarf sheep. The proximate analysis conducted, indicates that the leaves contain more crude protein than the tuber, and non-fiber carbohydrate is higher in the tuber than in the leaves. The results illustrated that 10% inclusion of *A. difformis* into ruminant animal concentrate diet, increased nutrient digestibility coefficient in the animals (Arigbede *et al.*, 2010).

2.9 Phytochemical Constituents of *A. difformis*

Phytochemicals are bioactive, non-nutrients compounds found in plants (Liu, 2004). Medicinal plants contain numerous phytochemicals which are responsible for the plants' therapeutic potentials. Phytochemicals have been associated with amelioration of diseases and pathological conditions given that they possess several properties such as antioxidant, antidiabetic, anti-cancer, antimicrobial, anti-inflammatory and gastro-protective (Surh *et* al., 2001; Loew and Kaszkin, 2002; Liu, 2004; Hausteen, 2005; Aggarwal *et al.*, 2008; Nandakuma *et al.*, 2008; Li-Weber, 2009). The knowledge of phytochemical constituents of plants helps in the discovery of therapeutic agents and exploring new resources of such active chemical agents (Mojab *et al.,* 2003).

Ethanolic and methanolic leaf extracts of *A. difformis* contain phytochemicals such as flavonoids, tannins, phlebotannins, cardiac glycosides, saponins and reducing sugars (Aliyu *et al.,* 2008b; Aderonke and Ezinwanne, 2015). Conversely, aqueous extract from leaves indicates the presence of flavonoids, alkaloids, phenolics, and terpenoids while cardiac glycosides and anthraquinones are absent (Oghale and Idu, 2015b). A comparative study on the total phenolics content of root extract using three different solvents was done by Aliyu et al (2013). The results showed that n-butanol extract has significantly higher phenolics content than the methanol and acetone extracts. Other studies compared the presence of phytochemicals in the tuber and leaves of *A. difformis* (Eneojo *et al.*, 2011; Agyare *et al.,* 2016).

2.10 Medicinal Benefits of *A. difformis*

2.10.1 Anti-diabetic Activities

Diabetes mellitus (Type I and II) is an endocrinological and metabolic disorder arising from insulin deficiency or impaired response of the body cells to insulin production (Maritim *et al.,* 2003; Rahmatullah *et al.*, 2012). Diabetes mellitus is characterized by persistent hyperglycemia. Anti-hyperglycemic and anti-diabetic properties of numerous plants have been explored, among which is *Anchomanes difformis* (Saxena and Vikram, 2004; Aderonke and Ezinwanne, 2015). Ethanolic extract of *A. difformis* tuber significantly reduced blood glucose concentration in alloxan monohydrate-induced diabetes in Wistar rats (Adeyemi *et al.,* 2015). Similar result was found when the ethanolic extract of the leaves were used (Aderonke and Ezinwanne, 2015). Conversely, a study carried out on aqueous extract of leaves of *A. difformis* revealed that it does not reduce blood glucose in normoglycemic rats (Lustigman *et al.,* 2007). This was supported by (Adeyemi *et al.,* 2015). The mechanism underlying the anti-hyperglycemic and anti-diabetic effect of *A. difformis* has not been reported.

2.10.2 Antioxidant Activities

Antioxidants and other bioactive components in plants, have received much attention especially in the medicinal field and food industry in recent years (Reddy and Grace, 2016). Natural antioxidants may function as reducing agents, free radical scavengers, complexers of pro-oxidant metals, or as quenchers of the formation of singlet oxygen (Hernández *et al*., 2009). The most common natural antioxidants are flavonoids (flavanols, isoflavones, flavones, catechins, flavanones), cinnamic acid derivatives, coumarins, tocopherols, and polyfunctional organic acids (Lee *et al*., 2000).

The free radical scavenging activity, total antioxidant capacity, and reducing power assay were measured in the acetone, n-butanol and methanolic extracts of *A. difformis* tuber (Aliyu *et al*., 2013). From the report, all the extracts displayed strong radical scavenging activity, but n-butanol extract has the strongest reducing ability which is comparable to that of gallic acid at all the concentrations tested (Aliyu *et al*., 2013). Similar antioxidant studies were carried out on methanolic leaf and root extracts of *A. difformis* by Agyare *et al.* (2016). The report of the study revealed that the methanolic root extract has more antioxidant capacity than the leaves and this is directly proportional to the total phenolic content. A study on the *in vivo* antioxidant capacity of *A. difformis* as reported by Oghale and Idu (2015b) confirmed the plant as a potent inhibitor of free radicals and lipid peroxidation in guinea pigs. Aqueous fraction of *A. difformis* leaves was used for the study.

2.10.3 Anti-inflammatory Activities

Inflammation plays a key role in the pathogenesis of certain disease-conditions such as asthma, diabetes, arthritis and cardiovascular diseases (Adebayo *et al*., 2014). Elevated markers of inflammation are implicative of oxidative stress (Wright *et al*., 2006). During oxidative stress, resident T-lymphocytes and activated macrophages around the β-cells

releases cytokines; particularly interleukin-1 (IL-1) which have been tagged as immunological molecules that inhibit secretion of insulin by the pancreatic β-cells and causes destruction of the β-cells (Hohmeier *et al.*, 2003; Meares *et al.*, 2013). Many inflammatory diseases are associated with the synthesis of prostaglandins, which are responsible for a sensation of pain (Loew and Kaszkin, 2002). The primary enzyme responsible for prostaglandins synthesis is the membrane-associated cyclooxygenase (COX), which occurs in two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed while COX-2 is induced in the inflamed tissue. Modulation of the activity of the enzyme implies that the inflammation process can be modified.

Adebayo *et al* (2014) conducted a study to demonstrate the anti-inflammatory activity of *A. difformis*. This was done by inducing oedema; injecting raw egg albumin (0.1ml) on the left paw of the rats after thirty minutes of administration of *A. difformis* leaves (ethanolic extract). Oedema size was measured and evaluated at intervals using a digital letica plethysmometer. The results showed that administration of *A. difformis* showed significant inhibition of oedema in the paw.

The anti-inflammatory studies performed by Agyare *et al* (2016) on leaf and tuber extracts of *A. difformis* in chicks revealed that it possesses significant anti-inflammatory activity and inhibitory effects on preformed mediators such as histamine and serotonin which are involved in the initial phase of the acute inflammatory process. Both extracts at all the doses tested, demonstrated higher anti-inflammatory activity than aspirin. The anti-inflammatory activity could be due to the presence of steroids in the extracts which exert their effects through switching off multiple activated inflammatory genes and repressing NF-κB-regulated inflammatory genes, it may also activate several anti-inflammatory genes and increases the degradation of mRNA encoding certain inflammatory proteins (Barnes, 2005).

2.10.4 Anti-nociceptive Activities

Nociception which is the causing of pain and injury or reacting to pain stimuli (Cammack *et al.*, 2006), can be induced by various methods such as tail-flick, hot plate, writhing, injection of formalin among others depending on the nociceptors to be investigated (Björkman, 1994). In this study, 0.25% injection of formalin on the hind paw was used to induce pain in Wistar rats following the administration of ethanolic extract of *A. difformis* leaves and a standard drug; aspirin (Adebayo *et al.*, 2014). The result indicated that formalin-induced paw nociception was significantly reduced, and this was dose-dependent. Therefore, *A. difformis* possesses anti-nociceptive ability as displayed by its inhibition of formalin-induced pain.

2.10.5 Anti-microbial Activities

Microorganisms especially pathogens are of great medical and economical value (Postgate and Abdollahi, 1982; Mc Neil and Brown, 1994). Due to the side effects of antibiotics and the resistance built up by pathogenic microbes (Monroe and Polk, 2000; Parekh *et al.*, 2006) against these drugs, much attention has been shifted to plant extracts and the biological active components in them (Cordell, 2000; Essawi and Srour 2000). Anti-microbial of plant origin show great therapeutic potentials (Parekh *et al.*, 2006). Medicinal plants have been explored for their anti-microbial properties (Atawodi and Atawodi, 2009; Bero *et al.*, 2009; Eneojo *et al.*, 2011; Nkoh *et al.*, 2015).

Reports on investigations of anti-microbial activities of *A. difformis* vividly showed that it is potent against common and drug-resistant microorganisms (Agyare *et al.*, 2016; Atawodi, 2005; Eneojo *et al.*, 2011). A comparative study on anti-microbial activities of the leaves and rhizome extracts of *A. difformis* using a ratio of different solvent was reported by Eneojo and his colleagues (2011). The microorganisms tested against were of public health importance; *Salmonella paratyphi, Salmonella typhi, Candida*

albican, Proteus vulgaris, Staphylococcus aureus, Shigella flexneri, Shigella dysenterae, Pseudomonas aeniginosa and four different strains of *Escherichia coli.* The result revealed that *A. difformis* leaf and rhizome extracts demonstrated inhibition against all the microorganisms except for *Shigella flexneri* and *Pseudomonas aeniginosa* which conferred resistance against leaf and rhizome extracts respectively (Eneojo *et al*., 2011; Agyare *et al*., 2016). Another assessment of aqueous and methanolic extract of *A. difformis* against *trypanosoma brucei* presents trypanocidal activities of the plant (Atawodi, 2005).

In vitro antiplasmodial activity of *A. difformis* rhizome was tested against chloroquinne-sensitive strain of *plasmodium falciparum* (3D7). Roots of *A. difformis* exhibited moderate antiplasmodial activities when compared with other plants used in the study (Bero *et al*., 2009). I*n vivo* study of antiplasmodial activities of *A. difformis* has been recommended giving that inhibition or other mechanisms of action may take place at other stages of plasmodium cycle such as the pre-erythrocytic development in the liver (Bero *et al*., 2009).

2.10.6 Gastro-protective Activities

Experimental peptic ulcer models can be induced using the following; acetic acid, ethanol, indomethacin, histamine, reserprine, pylorus ligation, ischemia-reperfusion, stress (hypothermic restraint, water-immersion) and diethyldithiocarbamate among others (Rodríguez *et al*., 2003; Zayachkivska *et al*., 2005; Adinortey *et al*., 2013). Investigations carried out on Sprague-dowley rats to assess the gastro-protective effect of ethyl acetate extract of *A. difformis* rhizome using different gastric ulcer models showed a positive result (Okpo *et al*., 2011). Ulcer conditions were induced using ethanol, indomethacin and pylorus ligation in the animals after pre-treatment with ranitidine. Although the mechanism through which it exerts its effect is not clear, the

ethylacetate fraction of *A. difformis* extract was shown to possess a clear anti-ulcer activity in all three, tested models of ulcer (Okpo *et al*., 2011). The gastro-protective ability of *A. difformis* can be associated with the presence of flavonoids which have been proven to be gastro-protective (Zayachkivska *et al*., 2005). It was discovered that flavonoids obtained from different plants were gastro-protective against ethanol-induced damage by causing increase in gastric microcirculation.

2.10.7 Anti-asthmatic Activities

Bronchial asthma is one of the common syndromes of the several respiratory diseases affecting humans (Parmar *et al*., 2010). Anti-asthmatic evaluation was carried out on the leaves (aqueous extract) of *A. difformis* using guinea pigs as experimental animals (Oghale and Idu, 2015b). The experimental groups were first sensitized with ovalbumin intraperitoneal administration; 100mg/kg body weight, intramuscular administration; 50mg/kg body weight, 24 hours later. *A. difformis* leaf extract was administered for seven days after which the animals were exposed to 0.2% histamine aerosol (bronchioconstrictor). The effect of *A. difformis* was assessed on the tracheal fluid volume and the tracheal fluid viscosity against salbutamol; a standard drug for treating asthma (Oghale and Idu, 2015b). The results of the study showed that *A. difformis* is effective against asthma as revealed by its bronchodilator properties similar to the effect of the standard drug used. Similar results were reported by Boskabady and his colleagues (Boskabady *et al*., 2010), who assessed the effect of *Nigella sativa* on airways of asthmatic patients.

2.10.8 Anti-onchocercal activities of *A. difformis*

Onchocerciasis or subcutaneous filariasis, popularly known as river blindness, is caused by the filarial worm *Onchocerca volvulus* and transmitted by the black fly, *Simulium damnosum* (Lustigman and McCarter, 2007). Onchocerciasis is one of the

leading causes of blindness due to infections. It is prevalent in 37 countries of the world among which 30 of these countries are in Africa (Boatin and Richards, 2006) and these account for 99% of the global burden of onchocerciasis and its related maladies while the other 1% is confined to Yemen and some countries of Central and South America (Tanya *et al.*, 2011).

Mass drug administration (MDA) and vector control programs have been the major approaches to control the disease (Wanji *et al.*, 2012). Although onchocerciasis control in Africa has lasted more than forty years, the disease is still a public health concern in many African countries. Ivermectin which is the main chemotherapeutic agent currently used has limited microfilaricidal efficacy (Richards *et al.*, 1998). Also, the fear of side effects and the duration of treatment (15-18 years) in order to terminate parasite transmission have led to a reduction in the ivermectin intake with serious epidemiological consequences (Tanya *et al.*, 2011). These factors and the potential development of resistance to Ivermectin have necessitated the need for new drugs in the treatment of onchocerciasis to achieve elimination of transmission. The search for an efficient microfilaricide has led to exploration of certain plants such as *Cyperus articulates Craterispermum laurinum, Morinda lucida and Anchomanes difformis* (Cho-Ngwa *et al.*, 2010; Metuge *et al.*, 2014). A study on the anti-onchocercal ability of *A. difformis* was performed on the methanolic extracts and on different fractionated extracts obtained at varying polarity using column chromatography (Nkoh *et al.*, 2015). The extracts and fractions were tested against *O. ochengi;* a strain very similar to *O. volvulus.* The methanolic extract and the four fractions obtained exhibited 100% inhibition against the microfiliariae (Nkoh *et al.*, 2015) and this lends credence to its ethno-botanical use in the treatment of river blindness.

A. difformis has been clearly shown to possess antioxidant and anti-inflammatory properties (Adebayo *et al.*, 2014); essential factors that minimize oxidative stress and

ultimately ameliorate diabetes (Aderonke and Ezinwanne, 2015; Adeyemi *et al.*, 2015) and possibly diabetic complications.

2.11 Future prospective and research opportunities

Further investigations should be performed extensively on *A. difformis* to be able to understand the molecular mechanisms involved in its therapeutic abilities against diabetes and associated factors. It will be of importance to identify the bioactive constituents present in the leaves and rhizome, and to investigate comprehensively the antioxidant potentials of *A. difformis*.

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CHAPTER THREE

Antioxidant Capacity, Phytochemical Analysis and Identification of Active Compounds in *Anchomanes difformis*

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ABSTRACT

Background: *Anchomanes difformis* (ENGL: Blume) is specie of flowering plants in the family Araceae. *Anchomanes difformis* is commonly reported for ameliorating hyperglycemia, inflammation, ulcer, malaria, and inhibiting microbial growth.

Objective: This study evaluated total yields of phytochemicals present, measured antioxidant capacities and identified bioactive compounds in the leaves and rhizome extracts of *A. difformis* using solvents of different polarity (ethyl acetate, ethanol and water).

Methods: Total polyphenolic, flavonoid content and alkaloids were measured, ORAC, TEAC and FRAP were performed as antioxidant capacity indices, and identification of bioactive compounds was done using UPLC-MS and HPLC.

Results: All extracts contained polyphenols, flavonols, flavanols, and alkaloids in varying concentrations. All extracts exhibited antioxidant properties. However, aqueous leaves extract had highest antioxidant properties and polyphenols with significance ($p<0.05$). Thirty-four compounds were identified altogether in the leaves and rhizome.

Conclusion: *A. difformis* leaves and rhizome are potential sources of natural antioxidants and can serve as potential therapeutic agents against diseases linked with oxidative stress. Presence of health-promoting compounds indicates possible ameliorative potentials of *A. difformis.*

Keywords: *Anchomanes difformis*, antioxidant, bioactive compounds, medicinal plant, phytochemicals

3.1 Introduction

Free radicals are generated in the body during normal biological processes such as oxidative phosphorylation, and respiratory burst; phagocytosis[1]. The body has its own antioxidant mechanisms[2], but there is the need for dietary supplementation of antioxidants. This is because overproduction of free radicals leading to imbalance in free radical production and antioxidant levels results in oxidative stress[1]. Oxidative stress has been implicated in several disease conditions such as diabetes mellitus, arthritis, cardiovascular diseases, cancer, cognitive impairment and gastrointestinal dysfunctions[3,4]. Antioxidants have multiple biological effects such as anti-inflammatory, anticarcinogenic and anti-atherosclerotic properties[5]. This is due to their capacity to protect cells and bio-macromolecules from oxidative damage by neutralizing free radicals or upregulating antioxidant enzymes thereby preventing oxidative degradation and neoplastic processes[4].

Phytochemicals are groups of non-nutrient substances, mostly present in fruits, vegetables, nuts and teas. They are biologically active and have high health-promoting impacts[5]. Phytochemicals are secondary plant metabolites that elicit biological properties such as antioxidant activity, modulation of detoxification enzymes, antimicrobial effect, stimulation of the immune system, modulation of hormone metabolism, decrease of platelet aggregation, and anticancer property[6]. Phytochemicals have also been widely promoted for the prevention and treatment of diabetes, muscular degeneration, hypertension and other pathological conditions. Several compounds in this group of phytochemicals are antioxidants and scavengers of free radicals. Examples of phytochemicals that act as antioxidants are polyphenolic compounds, alkaloids, tocopherols and carotenoids[4]. These groups of phytochemicals also act against lipid peroxidation[4,8].

Polyphenols are the largest group of phytochemicals; they have gained more attention in recent years especially flavonoids, in reference to their anti-diabetic properties[9]. Polyphenols possess free radical scavenging potentials[10]. Polyphenols are strong antioxidants that can independently or in synergy with other antioxidant enzymes and vitamins, to act as a defense against oxidative stress[4]. Polyphenols also elicit their antioxidant ability by inducing antioxidant enzymes such as catalase, glutathione-S-transferase, superoxide dismutase, and glutathione reductase[11]. Polyphenols further protect cells from apoptosis and other oxidative damage induced by xanthine oxidase. This is revealed by the effect of catechin and proanthocyanidin on cardiomyocytes exposed to xanthine oxidase[11].

Flavonoids are naturally occurring polyphenolic compounds. They comprise of flavonols, flavanols, flavones, flavanones isoflavonoids and anthocyanidins. Examples of flavonols include; quercetin, kaempferol, myricetin, galangin and fisetin, while catechin, epicatechin and gallate are examples of flavanols[12]. The study conducted by Wang and his colleagues,[13] revealed that intake of foods with high content of flavonoids reduced the risk of cardiovascular diseases in human. Flavonols protect against neuronal excitotoxicity and mitochondrial dysfunction[14].

Alkaloids act as antibacterial and antifungal in plants, against insects and herbivores[15,16]. Alkaloids have many pharmacological activities including, antiarrhythmic effect, antihypertensive effects, antimalarial activity, antioxidant activity and anticancer actions[7,17]. Berberine; an alkaloid has been shown to be effective as an analgesic and antimalarial therapeutic agent[17]. Medicinal plants have shown to contain lots of phytochemicals; hence they possess antioxidant activities. They play important roles in the management of diseases especially those with aetiology linked to oxidative stress[18,19].

Anchomanes difformis (Blume)*;* a tropical plant found mostly in African forests, is rich in phytochemicals[20,26] and have been reported in ameliorating pathological conditions. *A. difformis* (AD) reduced blood glucose in alloxan-induced diabetes[21], pain and inflammation in rats and chicks[22], ameliorates ulcer and asthma[20,23]. AD has also shown to have a positive effect on female sex hormones and activity against microbes[24]. Studies on the qualitative phytochemical analysis of AD reported the presence of flavonoids, tannins, saponins, phlebotannins, cardiac glycosides, and reducing sugars[21,25]. Investigations on the phytochemical contents of AD have mostly been limited to qualitative studies[20,25], which were carried out to determine the presence or absence of certain groups of phytochemicals in AD leaves and rhizome[26]. The current study carried out the quantification of the polyphenols, flavonols, flavanols and alkaloids present in the leaves and rhizome of AD. Furthermore, literature review on AD shows that no study has reported the determination and identification of active compounds in the leaves.

Different methods have been developed and recommended to standardize the measurement of antioxidant capacities in food, dietary supplements and nutraceuticals[27]. These include; oxygen radical antioxidant capacity (ORAC), trolox equivalent antioxidant capacity (TEAC), and Folin-Ciocalteu (for polyphenols) methods. Ferric reducing antioxidant power (FRAP) have been widely used to investigate the total antioxidant power of biological samples. These assays have underlying principles that are based on either single electron transfer (SET) reaction or hydrogen atom transfer (HAT) reaction between an antioxidant and a free radical.

This study measured total polyphenols, flavonols, flavanols and alkaloids of aqueous, ethanol and ethyl acetate fractions of AD leaves and rhizome. It also evaluated antioxidant capacities on these six extracts and identified some of the compounds present in them.

3.2 Experimental section

3.2.1 General Experimental Procedures

All chemicals and reagents used were of grade quality. The reagents were supplied by Merck, South Africa, while chemicals were purchased from Sigma Aldrich, South Africa. The following equipment were used: rotary evaporator and vacuum filter to concentrate extracts, Spectrophotometric readings were taken using a computer-assisted UV-VIS Multiskan Spectrum (Thermoscan Electron Corporation, USA) and Fluoroscan Ascent plate reader (Thermo Fischer Scientific, Waltham, MA, USA) was used for ORAC assay. HPLC analysis was performed using Agilent Technology (1200 series, Bellefonte, USA). UPLC-MS was carried out in a Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) and a Waters Acquity ultra performance liquid.

3.2.2 Plant Collection and Authentication

A. difformis leaves and rhizome were collected from a farm in Abeokuta, Ogun state, Nigeria, between August and December 2015. This was authenticated at the Herbarium, University of Lagos, Nigeria with herbarium number: LUH6623. A specimen voucher was deposited at the herbarium.

3.2.3 Preparation of Plant Extracts

Six different extracts of AD were extracted using cold-stirred extraction method. The extracts investigated were aqueous, ethanol and ethyl acetate extracts of the leaves and rhizome. The harvested plant parts were dried under shade and blended to increase the surface area. The blended leaves and rhizome were soaked in the solvent (water, ethanol and ethyl acetate separately) in the ratio 1:10 of plant material and solvent. The mixtures were then filtered using vacuum filtration method and the solvent evaporated. Ethanol and ethyl acetate were evaporated under reduced pressure using rotary

evaporator and later left to dry in the fume extraction hood. Aqueous mixture was evaporated to dryness using the lyophilizer.

3.2.4 Phytochemical Analysis

Quantitative measurement of polyphenols, flavonols, flavanols, alkaloids and carotenoids were performed using spectrophotometry and colorimetry techniques.

3.2.4.1 Determination of Total Polyphenols

Total polyphenols were determined by adapting the method of Waterhouse[55]. This involved the use of Folin-Ciocalteu reagent and sodium carbonate. 25µl of samples followed by 125µl of 200mM Folin reagent was dispensed into each well. After 5 minutes, 100µl of 7.5% aqueous sodium carbonate was added. A blue colour complex was formed from the reaction between phenolic compounds and Folin's reagent. The plates were incubated at room temperature for 2 hours. The absorbance was measured at 765 nm. The intensity of this colour-complex measured is directly proportional to the concentration of phenolic compounds present in the sample. Gallic acid (0, 20, 50, 100, 250 and 500mg/L) in 10% ethanol was used to prepare the standard curve. Results were expressed as mg gallic acid equivalents per gram dry mass of the plant (mgGAE/g DM).

3.2.4.2 Determination of Flavonol Content

Flavonol content was determined using quercetin (0, 5, 10, 20, 40, and 80mg/L) in distilled water as a standard. 50mg of the extracts were weighed and dissolved in water (for the aqueous extract) or 95% ethanol (for ethanolic and ethyl acetate extracts). 12.5µl of samples were pipetted into the 96-well plates. 12.5µl of 0.1% HCl was added to each well, followed by 225µl of 2% HCl. This was left to incubate for 30 minutes at room temperature[56]. Readings were taken at 360nm and results expressed as mg quercetin equivalent per gram dry mass of the plant (mgQE/g DM).

3.2.4.3 Determination of Flavanol Content

Spectrophotometric determination of flavanols was done using 4-(dimethylamino)-cinnamaldehyde (DMACA) reagent. DMACA reacts with flavanols to form a characteristic light blue colour. 1mM Catechin hydrate was used to prepare caliberation curve at the following concentrations; 0, 5, 10, 25, 50, 100µM. DMACA reagent was prepared by dissolving DMACA in a solvent-mixture of methanol-HCl (in the ratio 3:1 respectively) to a final concentration of 10µg/ml. 25µl of sample was dispensed into each well, 275µl of DMACA reagent was added to each well. Plates were incubated for 30 minutes at room temperature. Readings were taken at 640 nm. The intensity of the coloration is directly proportional to the concentration of flavonols in the sample. Results were expressed as mg catechin equivalent per gram dry mass of the plant (mgCatechin/g DM).

3.2.4.4 Determination of Total Alkaloids

Total alkaloids was determined using spectrophotometric method described by Fadhil, *et al*[57]. The assay is based on the reaction of Bromocresol green (BCG) with alkaloids which produces a yellow-coloured complex. 5mls of 2M sodium phosphate buffer (pH 4.7) was added to 500µl of AD extracts, followed by 5mls of BCG solution. The mixture was vortexed, and the yellow complex formed was extracted with 12mls of chloroform and strongly vortexed. Two layers were formed; the lower layer which contains the alkaloid (yellow complex in chloroform) is pipetted (300µl) into 96 wells-plate, absorbance read at 470nm against blank. 0.1mg/ml Atropine of varying concentrations (8, 12, 16, 20, and 24µg/ml) in 2M sodium phosphate buffer (pH 4.7) was used as a standard reference.

3.2.5 Antioxidant capacities

3.2.5.1 ORAC Assay

The ORAC assay kinetically measured the peroxyl-radical absorbing potential of the antioxidants present in the plant extracts according to the method of Ou *et al*[58]. Trolox; a water-soluble analogue of vitamin E, was used as the antioxidant standard reference. The change in fluorescence of the reaction mixture was monitored over a period of 2 hours and recorded every minute (excitation = 485 nm and emission = 535 nm). Results were determined with a regression equation, that relates trolox concentrations with the net area under the kinetic fluorescein decay curve ($y = ax2 + bx + c$). The ORAC values were expressed in micromoles of trolox equivalents per gram of sample (µmol TE/g sample).

Working solutions were 1.2mM fluorescein solution, 75mM phosphate buffer (pH 7.4) and 2,2'-Azobis (2-methylpropionamidine) dihydrocholide (AAPH) solution. Samples were diluted (x10) using phosphate buffer. 12µl of samples were pipetted into the wells, followed by 138µl of fluorescein solution. 50µl of AAPH was added and readings were taken. 12µl of samples, followed by 138µl of fluorescein solution and 50µl of AAPH were pipetted into the wells, readings were taken.

3.2.5.2 FRAP Assay

The ferric-reducing power of the plant extracts was determined using spectrophotometry according to the method of Benzie and Strain[59]. The stock solution (FRAP reagent) was prepared with acetate buffer (300mM, pH 3.6), TPTZ solution, Iron (III) chloride hexahydrate solution and distilled water. The solution mix was in a ratio of 10:1:1:2 respectively. 10µl of sample followed by 300µl of FRAP reagent was pipetted into the microplate. After 30 min of incubation at room temperature, the absorbance was determined at a wavelength of 593 nm. Ascorbic acid was used as the reference

standard and results expressed as milligram of ascorbic acid per gram of extract (mgAAE/g sample).

3.2.5.3 TEAC Assay

TEAC was used to measure the antioxidant capacity of the plant samples using ABTS (2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulphonic acid)) decolorization assay. This was carried out by assessing the ability of the sample to scavenge/reduce ABTS radical, converting the solution (blue-green) to a colourless product. The extent of decolorization by the sample is proportional to the concentration of the antioxidant present and it is compared to that of trolox; thereby giving TEAC value. The TEAC value was expressed as milligram of trolox equivalent per gram of sample (mgTE/mg sample). 25µl of sample was pipetted into the 96-well plate, and 275µl of ABTS reagent (prepared with 150mM potassium persulfate ($K_2S_2O_8$) and ABTS in distilled water and placed in the dark room overnight) was added. This was incubated for 30 minutes at room temperature and the readings were taken at 734nm.

3.2.6 Identification of active compounds

Bioactive compounds preseent in the leaves and rhizome were determined using liquid chromatographic methods, as discussed below.

3.2.6.1 High Performance Liquid Chromatography (HPLC)

HPLC analysis was performed on the six extracts. Water (solvent A) and methanol (solvent B) were used for the mobile phase. The gradient program ran from 100% water at 0 minute to 100% methanol at 35 minutes for each sample, after which the column was washed and reconditioned. The sample injection volume was 20 µl, a flow rate of 1 ml/min and a temperature of 25°C was maintained throughout the analytical run. The diode array detector was controlled at a wavelength between 200nm and 700nm. The

detection wavelengths were 260, 320, 360 and 520nm. Individual compounds were identified by comparing their retention time and UV spectrum with those obtained from their respective standards injected under the same HPLC conditions. Concentration of each compound was calculated using the area of standard, area of sample, injection volume, weight of sample (mg) and extraction volume. Results were expressed as µg/mg of sample.

3.2.6.2 Ultra-Performance Liquid Chromatography and Mass Spectrophotometry

Ultra-performance liquid chromatography and mass spectrophotometry (UPLC-MS) was carried out by connecting a Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) to a Waters Acquity ultra performance liquid chromatograph to achieve high resolution. Electrospray ionization was used in negative mode with a cone voltage of 15 V, desolvation gas at 650 L/hr and desolvation temperature of 275°C. The rest of the MS settings were optimized for best resolution and sensitivity. Scanning from *m/z* 150 to 1500 in resolution mode as well as in MS^E mode was done for data acquisition. The data acquired in the MS^E mode were in two channels, one at a low collision energy (4 V) and the second using a collision energy ramp (40 to 100 V) to obtain fragmentation data. Sodium forbate was used to calibrate the instrument and leucine enkaphalin was used as a lock mass (reference mass) for accurate mass determination. The mobile phase consisted of 0.1% formic acid (Solvent A) and acetonitrile containing 0.1 % formic acid as solvent B. The gradient started at 100% solvent A for 1 minute and changed to 28 % B over 22 minutes in a linear way. This further changed to 40% B over 50 seconds, a wash step of 1.5 minutes at 100% B followed and re-equilibration to initial conditions for 4 minutes. An injection volume of 2 µL was used and a flow rate of 0.3 mL/min. The column (Waters HSS T3, 2.1 x 100 mm, 1.7 µm) temperature was maintained at 55 ºC.

3.2.7 Data Analysis

All data were expressed as mean ± standard deviation (SD) of triplicate determinations. The data were analyzed by ANOVA using GraphPad Prism 5 software and SPSS. Differences were considered significant at 5% level of significance. Multiple correlation analysis was done on secondary plant compounds vis a vis their antioxidant capacity using bivariate correlation to estimate the Pearson correlation coefficient.

3.3 Results

3.3.1 Phytochemical Analysis and Antioxidant capacities

Polyphenols, flavonols and alkaloids were present in all the extracts of AD leaves and rhizome used in this study. The aqueous and ethanol extracts of *A. difformis* leaves had higher concentrations of polyphenols, flavonols, flavanols and alkaloids than their respective rhizome extracts (Figures 3.1 & 3.2). Consequently, this is expressed in the higher antioxidant capacity of ethanol and aqueous leaves extract when compared to their respective rhizome extracts (Figure 3.3). Conversely, ethyl acetate of the rhizome contained higher concentration of polyphenols and flavonols and lower concentrations of alkaloids and flavanols when compared with the ethyl acetate leaf extract (Figures 3.1 & 3.2). This may be related to its higher antioxidant capacity for FRAP and TEAC assays (Figure 3.3). AD does not contain high amount of flavanols (Figure 3.2), when compared with other phytochemicals investigated. Very low concentrations of flavanols were obtained in the leaves and rhizome extracts of AD with exception to ethanol leaf extract.

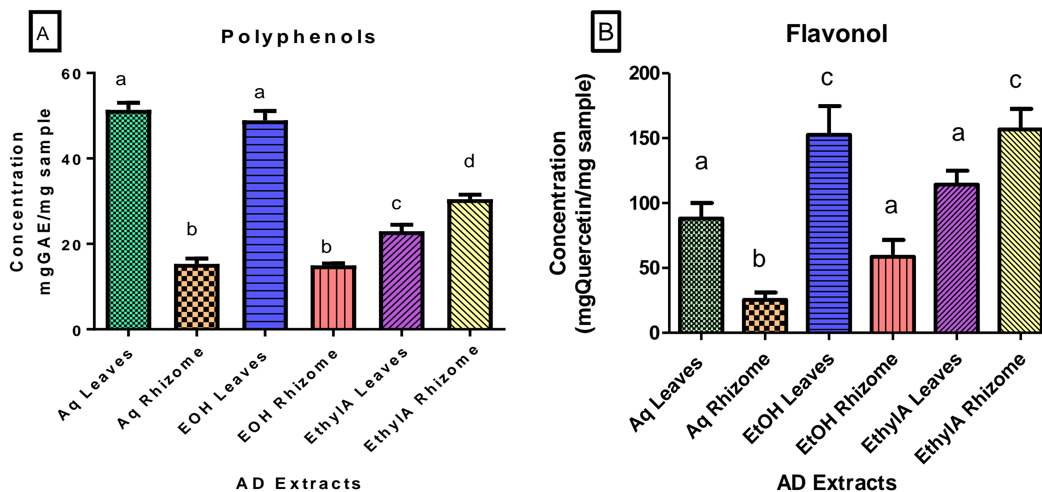

Figure 0.1: **(A)** Total polyphenol and **(B)** flavonol content of AD leaves and rhizome. Bars with different letters are significantly ($p<0.05$) different from each other. Aq- aqueous, EtOH- ethanol, Ethyl A- ethyl acetate.

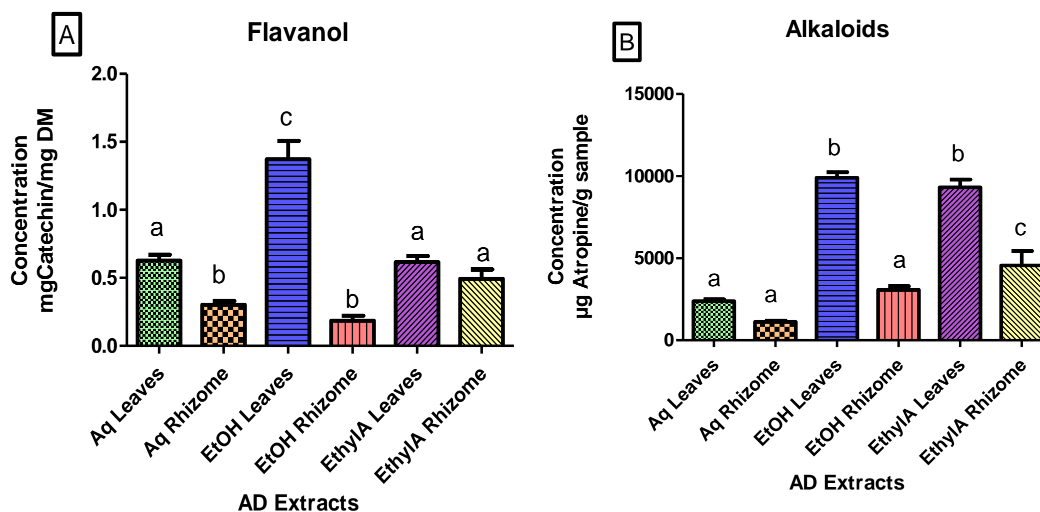

Figure 0.2: **(A)** Flavanol, and **(B)** alkaloid content of AD leaves and rhizome. Bars with different letters are significantly ($p<0.05$) different from each other. Aq- aqueous, EtOH- ethanol, Ethyl A- ethyl acetate.

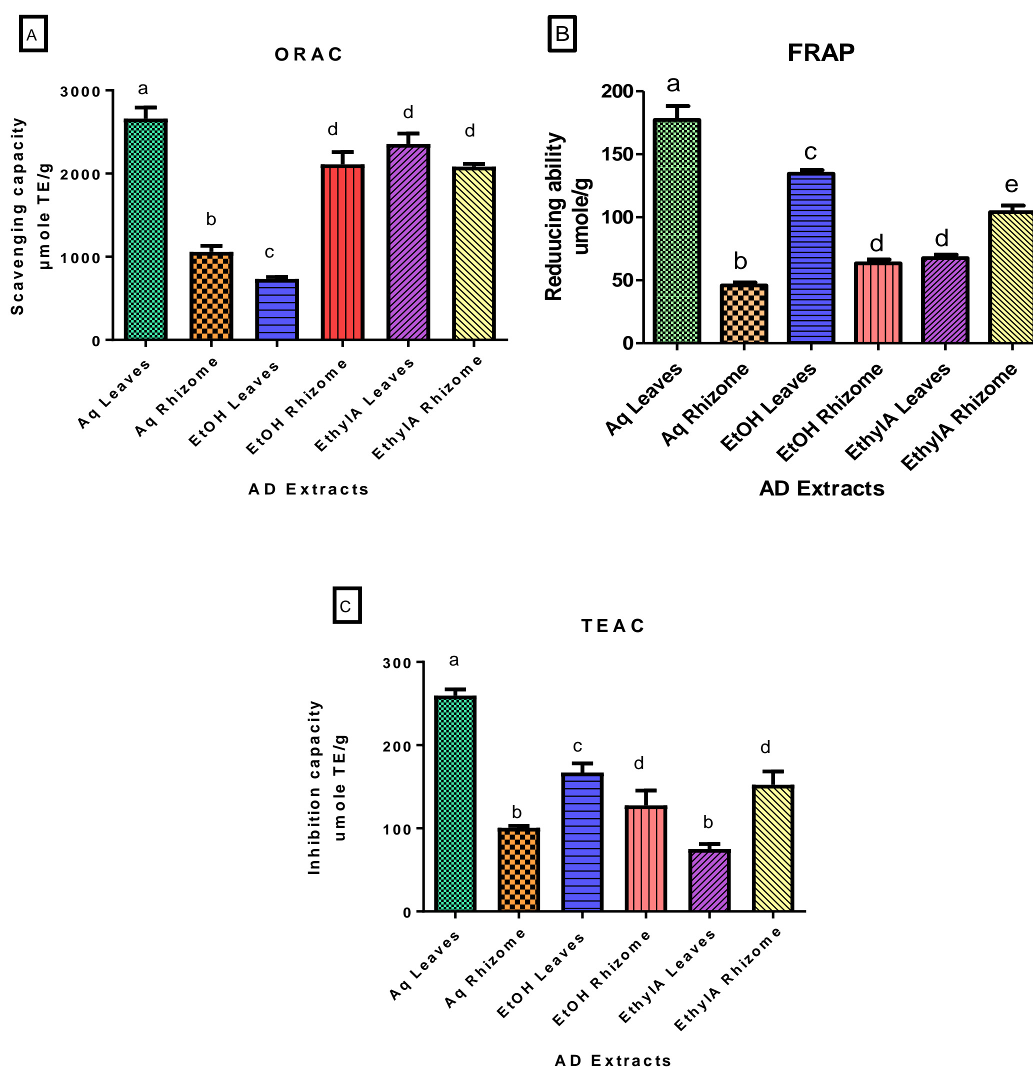

Figure 0.3: Antioxidant capacities; **(A)** ORAC, **(B)** FRAP and **(C)** TEAC of leaves and rhizome extracts of AD. Bars with different letters are significantly (p<0.05) different from each other. Aq- aqueous, EtOH- ethanol, Ethyl A- ethyl acetate.

3.3.2 Relationships between Antioxidant Capacity and Plant Secondary Compound

The plant parts and extraction-solvent type influenced the relationship between the secondary compounds and antioxidant capacity of AD. Aqueous leaves extract showed a highly significant correlation between ORAC and total alkaloids (r = 1.0, p<0.009). A significant, positive correlation was observed between total polyphenols and flavanols (r = 0.998, p<0.044), and with alkaloids (r = 0.999, p<0.022) in aqueous extract of AD rhizome. Ethanol extraction of AD rhizome resulted in a significant correlation between

FRAP and total polyphenols (*r* = 0.997, *p*<0.048). A significant, inverse relationship (*r* = -0.998, *p*<0.044) is observed between TEAC and total polyphenols, in the ethyl acetate extract of AD rhizome.

3.3.3 Identification of Bioactive compounds

The HPLC analysis revealed the presence of phenolic acids; cinnamic acid, ferulic acid, chlorogenic acid. Flavonols present include quercetin, kaempferol, and rutin. Catechin was the only flavanol identified, and it was present in the aqueous leaves extract only (Table 3.1). There are other polyphenols present in the extracts as revealed by the wavelength scan spectra of the peaks in the chromatogram, these could not be identified in this study due to unavailability of their standards.

Table 0.1: Phenolic compounds identified in AD leaves and rhizome using HPLC

Compounds investigated	AD Extracts					
	Aqueous (µg/mg sample)		Ethanol (µg/mg sample)		Ethyl acetate (µg/mg sample)	
	Leaves	Rhizome	Leaves	Rhizome	Leaves	Rhizome
Chlorogenic acid	0.1626	-	-		-	-
Rutin	0.3652	0.0004	0.9062	0.0004	-	0.0009
Kaempferol	-	0.0023	0.0602	0.0013	-	0.0031
Quercetin	-	-	0.0215	0.0080	-	-
Cinnamic acid	0.0220	-	0.3064	0.0340	0.3374	0.0010
Ferulic acid	0.0865	-	0.0680	-	-	-
Catechin	1.7797	0.0072	-	-	-	-
Coumaric acid	-	0.0007	-	0.0980	-	0.0030
Epigallo-catechin gallate	-	-	-	-	-	-
Hesperidin	-	-	-	-	-	-
Hesperitin	-	-	-	-	-	-

Myricetin	-	-	-	-	-	-
Epicatechin	-	-	-	-	-	-
Mangiferin	-	-	-	-	-	-
Luteolin	-	-	-	-	-	-
Protocatechuic acid	-	-	-	-	-	-
Apigenin	-	-	-	-	-	-
Sinapic acid	-	-	-	-	-	-
Isorhannetin	-	-	-	-	-	-
Caffeic acid	-	-	-	-	-	-
Syringic acid	-	-	-	-	-	-
Naringenin	-	-	-	-	-	-

*compound not detectable in the extract (-)

Thirty-five (34) phenolic compounds were identified (22 known and 12 unknown compounds), when the extracts were subjected to UPLC-MS. It is important to note that no research has been conducted before now to the best of our knowledge, on identification and quantification of compounds in AD leaves. Our study is therefore the first to identify and quantify active compounds in AD leaves, while report showed that 3 compounds were recently isolated from AD rhizome[28]. Compounds identified in in the leaves and rhizome of AD are shown in Table 3.2 while Table 3.3 shows the characteristics of the unknown compounds identified Tentative assignment of names was given to each peak (each peak depicts the elution of a compound at characteristic wavelength and time), based on Single Mass Analysis (SMA), fragmentation of parent ion. SMA revealed the elemental composition, the calculated mass, the chemical formula, and the PPM error. The choice of name is also based on the compound with the lowest PPM error. Certain peaks have more than one compound with the same above-mentioned features on the SMA; these are referred to as isomers (Table 3.2).

The result obtained when HPLC was used, showed that some compounds were not detectable in the extracts (Table 3.1). This is probably due to their low concentrations in the extracts. However, some of these compounds were further identified when the extracts were analysed using UPLC-MS (Table 3.2). These include; apigenin, luteolin, and hesperidin. In addition, due to unavailability of standards, some phenolic compounds could not be identified using HPLC; however, UPLC-MS revealed the presence of these compounds in the extracts.

Table 0.2: Further identification of bioactive compounds using UPLC-MS

Compounds (mg/mg dry mass)		Extracts					
		Aqueous Leaves	**Ethanol Leaves**	**Ethyl acetate Leaves**	**Aqueous Rhizome**	**Ethanol Rhizome**	**Ethyl acetate Rhizome**
1.	Luteolin Scutellarein Kaempferol	0.008267	0.096017	0.095664	0.009789	0.004862	0.003153
2.	Vicenin_2	0.165466	0.008687	-	0.003526	0.007592	-
3.	Chryosoeriol	0.102627	0.968536	1.247479	0.083549	0.035016	0.041105
4.	Vitexin	0.043549	0.062865	0.078313	0.053169	0.06839	0.065452
5.	Phloridzin	0.539154	0.444256	0.412962	0.174422	0.115476	0.087471
6.	Rutin	0.019947	0.000625	0.000338	0.000424	0.000289	-
7.	Orientin	-	0.003004	-	0.002949	0.01439	-
8.	Luteolin-7-O-glucoside	-	0.005031	0.002332	0.002295	0.009057	-
9.	Catechin	0.000521	-	-	-	-	-
10.	Hesperedin	0.003414	0.004686	0.003728	0.003759	0.004769	0.002225
11	p-Coumaric acid m-Coumaric acid o-Coumaric acid	0.012879	0.018938	0.019564	0.004058	0.012482	0.027696

12.	4-HBA	0.003496	-	0.00896	0.00338	0.056935	0.080544
13.	4-HBA_2	-	0.021755	0.02005	0.003369	0.041509	0.07447
14.	Luteolin-6,8-di-C-hexoside	0.012534	-	-	-	-	-
15.	4-Vinylsyringol	0.180939	0.254919	0.121674	0.031925	0.068379	0.026668
16.	Chrysoeriol-7-O-glucoside Or Isorhamnetin-3-O-rutinoside	0.146249	0.357013	0.308613	0.011774	0.012807	0.038917
17.	Apigenin	0.272873	0.011578	0.000807	0.009025	0.024645	0.000713
18.	Apigenin-6,8-di-C-glucoside, Chrysoeriol-7-O-apiosyl-glucoside, Luteolin-7-O-rutinoside, Kaempferol 3-O-rutinoside,	0.103761	0.00703	-	-	0.001743	0.000996

	Kaempferol 3-O-galactoside 7-O-rhamnoside						
19.	Hispidulin	0.104893	0.953434	1.248529	0.087445	0.035437	0.040733
20.	Hispidulin_2	0.104792	0.923351	1.236589	0.085448	0.036455	0.040839
21.	Kaempferol	0.008554	0.00421	-	0.007822	0.001313	-
22.	Quercetin	0.000635	0.008628	0.014729	0.000141	0.022195	0.012004
23.	AD-A	0.051546	0.022319	0.019931	0.011598	0.008724	0.002833
24.	AD-B	2.399537	2.933177	3.904022	2.71322	2.007623	2.392479
25.	AD-C	0.03819	-	0.044156	0.01228	0.01553	0.03332
26.	AD-D	0.897766	1.643279	1.003963	0.648098	0.480378	0.798512
27.	AD-E	0.865262	1.653133	2.140938	0.989202	0.837193	0.919322
28.	AD-F	-	-	0.013349	0.034143	0.051478	-
29.	AD-G	-	0.008238	0.021818	0.007299	0.284961	0.532973
30.	AD-H	0.443833	1.618828	1.848603	0.023327	0.037872	0.509491
31.	AD-I	0.111219	0.47024	0.567598	0.217556	1.069945	0.829917
32.	AD-J	0.015344	0.054942	0.03207	0.034796	0.085547	0.027386
33.	AD-K	-	0.002255	0.003026	0.007617	0.036329	0.005365
34.	AD-L	0.007748	0.016227	0.015851	-	-	-

Table 0.3: Characteristics of the unknown compounds

S/N	Compound	Measured mass (grams)	Molecular formula	Retention time (minutes)
1.	**AD-A**	265.1440	$C_{15}H_{22}O_4$	25.69
2.	**AD-B**	267.0716	$C_9H_{16}O_9$	6.19
3.	**AD-C**	309.1913	$C_{14}H_{30}O_7$	25.96
4.	**AD-D**	311.2222	$C_{18}H_{32}O_4$	24.72
5.	**AD-E**	313.2379	$C_{18}H_{34}O_4$	24.85
6.	**AD-F**	326.2120	$C_{21}H_{29}O_2$	24.16
7.	**AD-G**	327.2171	$C_{18}H_{32}O_5$	24.32
8.	**AD-H**	329.2328	$C_{18}H_{34}O_5$	24.37
9.	**AD-I**	431.1917	$C_{20}H_{32}O_{10}$	14.32
10.	**AD-J**	447.1863	$C_{20}H_{32}O_{11}$	10.12
11.	**AD-K**	451.2543	$C_{21}H_{40}O_{10}$	12.86
12.	**AD-L**	737.2293	$C_{34}H_{42}O_{18}$	5.99

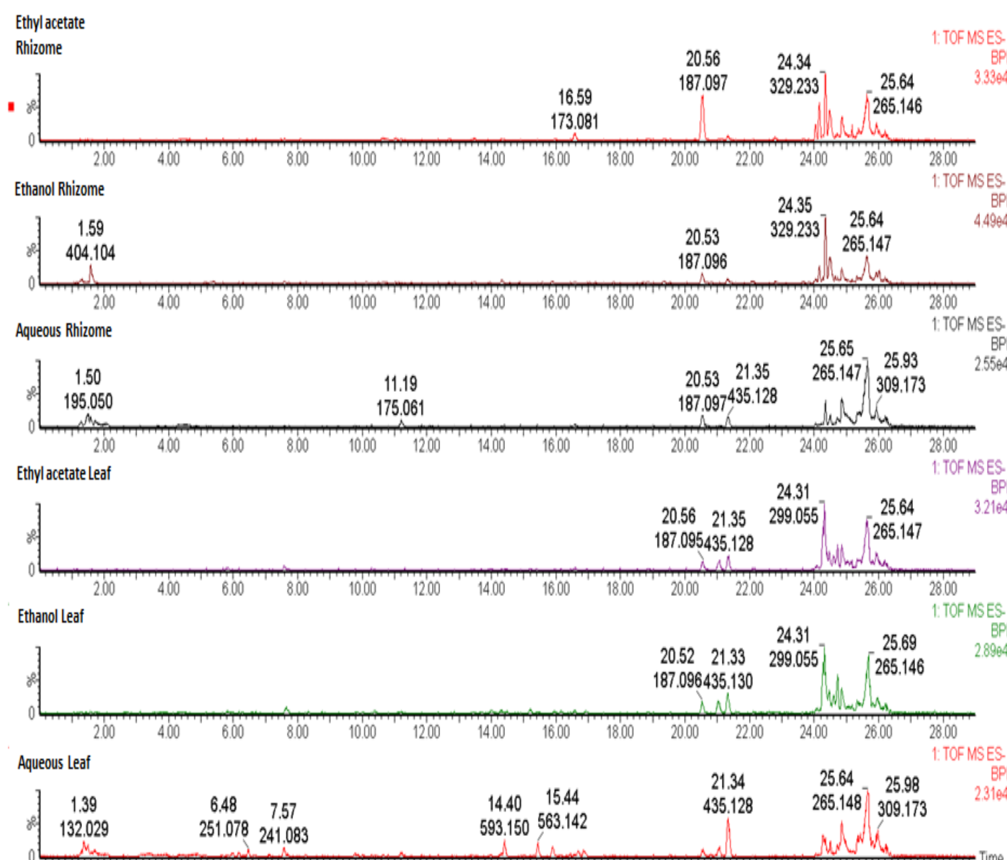

Figure 0.4: Mass spectra showing the overall elution of the compounds in the leaf and rhizome extracts of AD, with the mass/charge and retention time on the major peaks.

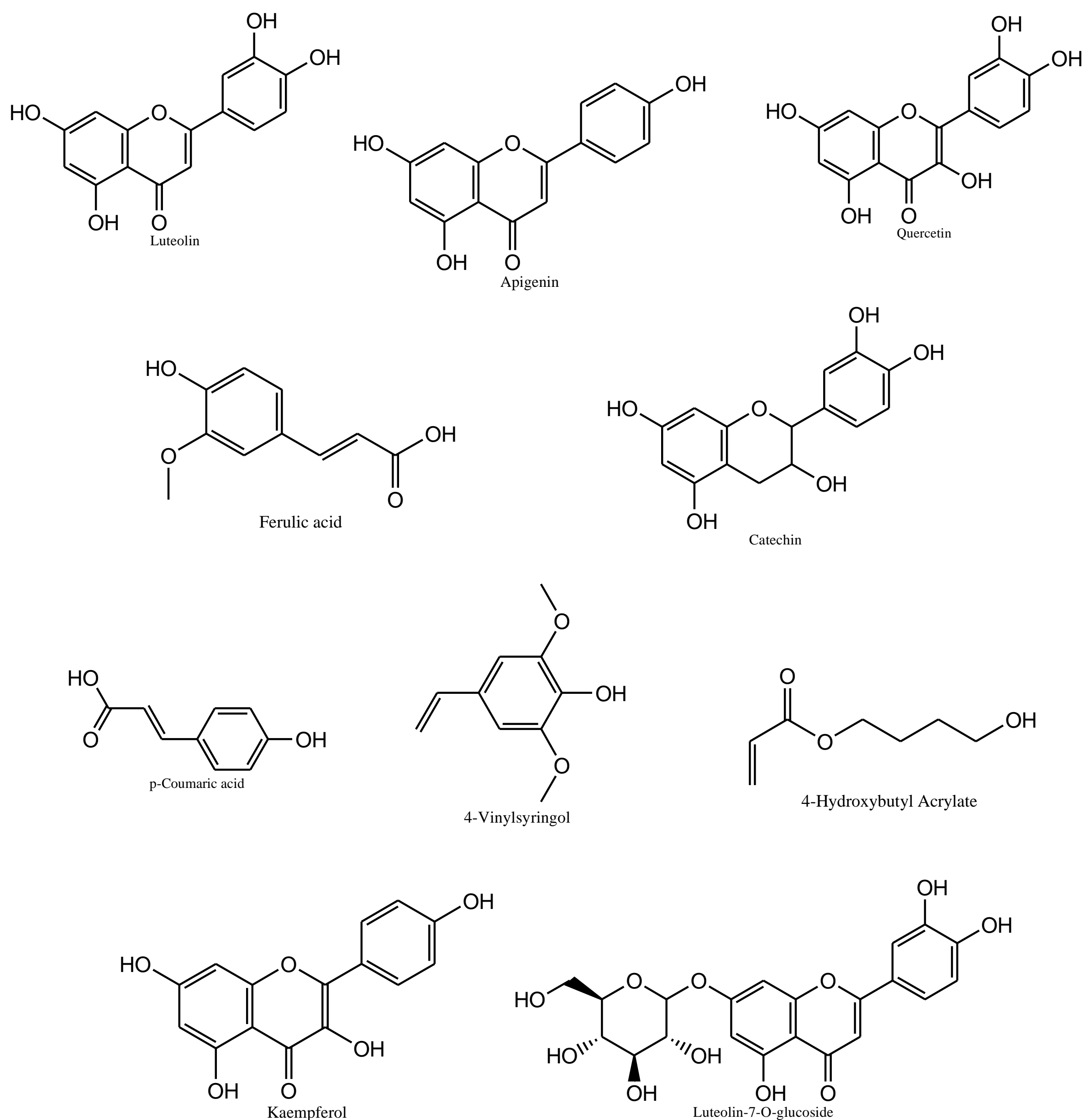

Figure 0.5: Structure of certain compounds identified in AD leaves and rhizome.

3.4 Discussion

Quantitative phytochemical analysis performed on the plant extracts revealed the presence of health promoting phytoconstituents; polyphenols, flavonols and alkaloids. This is in line with previous findings which documented the presence of alkaloids, flavonoids, phenolics and other groups of phytochemicals in the leaves of AD[20]. Similarly, qualitative screening of AD plant for phytochemicals by Aliyu and his team,[25] showed that flavonoids are present in AD, this corroborates with findings in this study.

The groups of phytochemicals contained in the leaves and rhizome of AD have been shown to possess medicinal abilities. Flavonols are well researched for their activity against hypertension, insulin resistance, inflammation, obesity, cancer, and atherosclerosis[29,30] in animal models. This suggests that all the three extracts of leaves and rhizome have medicinal potentials. Aqueous and ethanolic extracts of *A. difformis* leaves may have more medicinal potentials than that of the rhizome. This is because aqueous and ethanolic extracts contains higher concentrations of phytochemicals and showed more antioxidant capacity. Results from HPLC analysis showed that chlorogenic acid is present only in the aqueous leaves extract, while catechin is present in the aqueous leaves and rhizome extract only, with a very significant concentration in the leaves (Table 3.1). This is like the LCMS output, but with further identification of other compounds such as luteolin-6,8-di-C-hexoside present only in the aqueous leaves extract (Table 3.2). This suggests that chlorogenic acid, catechin and luteolin-6,8-di-C-hexoside may have conferred antioxidative properties on aqueous extract of AD leaves. It is challenging to specify which compounds are responsible for the antioxidant ability of ethanolic leaves extract, has it contained high concentration of polyphenols (especially flavonoids) and alkaloids. However, HPLC results revealed that ferulic acid which is present in ethanolic and aqueous leaves extract only (Table 3.1), might have enhanced the antioxidant capacity of ethanolic leaves extract when compared with its rhizome or ethyl acetate extracts. Conversely, ethyl acetate extracts of AD rhizome may show more medicinal potency than that of the leaves seeing that it demonstrated higher antioxidant power and contained more phytochemicals. Rutin, kaempferol and coumaric acid was detected in the ethyl acetate extract of the rhizome while they were lacking in the corresponding leaves extract (Table 3.1). These compounds may have increased the antioxidant quality of ethyl acetate rhizome extract over the leave extract.

Secondary compounds that were identified in the leaves and rhizome of AD are pharmacologically active and have broad spectrum of biological activities and health-promoting abilities. Studies revealed that quercetin is a strong antioxidant and it possesses anti-

inflammatory, anti-hypertensive, anti-cancer, and anti-thrombotic potentials[29,30,31]. Kaempferol has anti-bacterial, antioxidant, neuro-protective abilities[32]. Investigations on rutin revealed its strong antioxidant power and ameliorative capacity when treated against cancer, diabetes, hypertension and pathogens[13]. Studies suggest that cinnamic acids may be potent against diabetes[33]. Chlorogenic acids are cinnamic acid derivatives with antioxidant and anti-inflammatory effects. Caffeoylquinic acids are naturally occurring chlorogenic acids and exhibits bioactivities against neuronal damage and obesity[34,35]. They also act as analgesic through inhibition of histamine[36,37]. Caffeoylquinic acids are potential agents of transferrin-mediated drug delivery for targeted cancer therapy[38]. Ferulic acid is a derivative of curcumin, it possesses the unique pharmacokinetic properties which makes it retained in the body circulation for several hours. Ferulic acid is neuroprotective; inhibits neuro-inflammation and attenuates damage of macrovascular endothelia cells of the brain[39]. It demonstrated cardio-protection against ischaemia reperfusion through up-regulation of HSP70 via NO-ERK1/2 pathway[40,41]. It has been used as a therapeutic agent against epilepsy and it was effective[42]. Ferulic acid also exhibit other biological activities such as anti-apoptotic, cardioprotective, anti-diabetic, and anti-inflammatory properties[43,44,45]. Catechins possess strong antioxidant properties, they have been reported to reduce incidence of cancer[46,47]. Low concentrations of catechins have demonstrated the ability to increase the motility and viability of sperm cells thereby improving the semen quality[48]. Catechins also lower the risk of type II diabetes by regulating insulin resistance[49]. Treatment with catechins minimizes pro-inflammatory cytokines[50]. In addition, catechins ameliorate cardiac failure by improving the endothelial function via up-regulation of Akt-eNOS pathway[51]. The active principles present in the leaves and rhizome of AD may be responsible for its medicinal abilities as treatment with AD leaves and rhizome have also shown to exhibit most of these properties[26].

The biological properties of natural antioxidants have been widely attributed to phenolic compounds[52,53]. Interestingly, there is a strong correlation between alkaloids and ORAC in the aqueous leaf extract; which exhibited the highest antioxidant ability. This suggests that

alkaloids present in the aqueous leaf extract, may be more responsible for its high oxygen scavenging ability when compared to other secondary compounds present in the extract. This is supported by antioxidant analysis carried out by Koolen and colleagues[7]. From his research, certain isolated alkaloids indicated high scavenging ability for oxygen radical relative to trolox. Total polyphenolic compounds present in the ethanol extract of AD rhizome showed a strong and positive correlation with the ferric reducing power of the extract. This reveals that phenolic acids appear to have more antioxidant potential in the ethanol extract of AD rhizome than other compounds present. In contrast, total polyphenols inversely and strongly correlated with TEAC revealing that polyphenols in AD were not involved in the trolox-reducing activity of the plant. This is similar to the findings of Locatelli and his colleagues[53], where the phenolic compounds from garlic samples showed a strong and negative correlation with its antioxidant activity. Correspondingly, some polyphenolic compounds have redox potentials[4]. This agrees with Block's finding[54] who reported that depending on specific conditions, compounds containing sulphur may act as antioxidants or pro-oxidants. The significant correlation between total polyphenols, flavanols, and alkaloids in the aqueous extract of AD rhizome shows that there could be a synergetic effect of these phytochemicals in conferring antioxidant ability on the extract.

3.5 Conclusion

The leaf and the rhizome extracts demonstrated free radical scavenging activity on highly reactive hydroxyl radical, oxygen radical and ABTS radical. Hence, leaves and tuber of *A. difformis* are potential sources of natural antioxidants. *A. difformis* may be a therapeutic agent targeted at treatment of diseases linked with oxidative stress, and prevention of complications that might arise from such diseases. Thirty-four (34) compounds were identified in the leaves and rhizome extracts of *A. difformis*, which is the first report on identification of compounds in the leaves of *A. difformis*. Presence of active compounds in the leaf and rhizome extracts of *A. difformis,* may confer possible preventive and ameliorative potentials during pathological conditions.

3.6 Recommendation

Further research work could be done to isolate and characterize the identified compounds for structural elucidation. Furthermore, these isolated active compounds with little or no scientific knowledge, present in *A. difformis* can be extensively investigated for their medicinal potentials.

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CHAPTER FOUR

Improved antioxidant status and hepato-protective role of *Anchomanes difformis* in streptozotocin-induced diabetes in male Wistar rats

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ABSTRACT

Ethnopharmacological relevance: The liver is an important organ involved in metabolism of xenobiotics and their metabolites and it is vulnerable to oxidative damage. Hyperglycaemia is highly implicated in the progression of diabetes mellitus, and adversely affects the liver. Though conventional hypoglycaemic drugs may be effective in reducing blood glucose, they do not appear to be effective in attenuating the progression of diabetes and its complications. *Anchomanes difformis* have been used traditionally to treat diabetes, pain and other related conditions.

Aim of the study: This study evaluated the ameliorative effects of *Anchomanes difformis* on hyperglycaemia and hepatic injuries in type 2 diabetes.

Materials and methods: Type 2 diabetes was induced in male Wistar rats with a single intraperitoneal injection of streptozotocin (40mg/kgBW) after two weeks of fructose (10%) administration. Aqueous extract of *A. difformis* (200 and 400mg/kgBW) and gilbenclamide (5mg/kgBW) were administered orally for six weeks. Blood glucose concentrations were measured. Serum levels of liver dysfunction markers (ALT, AST, and ALP), total cholesterol, triglycerides, HDL- and LDL-cholesterol were investigated. Total protein, albumin, and globulin were also assessed. Antioxidant parameters: ORAC, GSH, GSSG, SOD, CAT and FRAP were evaluated in the liver while ORAC, FRAP and lipid peroxidation was determined in the serum. Histological examination of the liver tissue was carried out.

Results: Treatment with aqueous extract of *A. difformis* significantly ($p<0.05$) reduced the blood glucose and reversed steatosis in the diabetic-treated rats. The antioxidant status of diabetic-treated rats was significantly ($p<0.05$) improved. Serum levels of liver dysfunction markers were significantly ($p<0.05$) reduced in diabetic-treated rats.

Conclusion: *Anchomanes difformis* ameliorated hyperglycaemia and hyperlipidaemia, improved antioxidant status, and protected against hyperglycaemia-induced hepatopathy in a diabetic animal model.

Keywords: anchomanes difformis, antioxidants, diabetes, hyperglycaemia, hyperlipidaemia, liver

ABBREVIATIONS

T2D	Type 2 diabetes
TP	Total protein
ROS	Reactive oxygen species
SOD	Superoxide dismutase
CAT	Catalase
GSH	Glutathione
GSSG	Glutathione disulphide/oxidized glutathione
ORAC	Oxygen radical absorbing capacity
FRAP	Ferric reducing antioxidant power
AD	*Anchomanes difformis*
STZ	Streptozotocin
SRC	Standard rat chow
FBG	Fasting blood glucose
ALT	Alanine transaminase
ALP	Alkaline phosphatase
AST	Aspartate transaminase
TBA	Thiobarbaturic acid
TBARS	Thiobarbaturic acid reactive substances
MDA	Malondialdehyde
FAS	Fatty acid synthetase
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
H&E	Hematoxylin and eosin

4.1 Introduction

Sedentary lifestyle and nutritional overload have been directly implicated in the increased prevalence of type 2 diabetes (T2D) (Anhê et al., 2013). T2D which is shown by constant hyperglycaemia and insulin resistance leads to various organs dysfunction, such as the kidneys, livers, heart, blood vessels, reproductive system and the eyes (Lüscher et al., 2003). Persistent hyperglycaemia results in an increased production of free radicals and ultimately oxidative stress. Oxidative stress is an important factor in most pathological conditions. Oxidative damage in the structure and functions of cellular biomolecules including lipids, nucleic acids, and proteins, is highly implicative in the progression of diabetes and the development of its complications (Ghanbari et al., 2016).

The liver is highly essential in glucose and lipid homeostasis (Bora et al., 2016; Sivajothi et al., 2007). Diabetes mellitus leads to severe damage in the liver such as necrosis, cirrhosis, hepatic steatosis, inflammation (Guven et al., 2006). This is usually depicted by abnormal levels of serum hepatic enzymes due to leakage from the damaged hepatocytes into the bloodstream. Increase in the serum levels of the hepatic enzymes may lead to reduced concentrations of total protein (TP) and albumin in the serum (Kaysen et al., 2002). Poor glycaemic control during diabetes mellitus causes hyperlipidaemia, which is associated with an increased flux of free fatty acid due to insulin resistance (Biradar et al., 2018; Sirsikar et al., 2016).

The body has its own antioxidant defence mechanisms by which it combats reactive oxygen species (ROS) generated from metabolic and environmental sources. The antioxidant defence mechanisms prevent the initiation of free radical chain reactions (Turk et al., 2002). These defence mechanisms may be enzymic or non-enzymic (Vertuani et al., 2005). The antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase and they act as free radical scavengers by donating electrons to react with the ROS (Ghanbari et al., 2016). Glutathione (GSH) and glutathione disulphide (GSSG) are small

non-enzymic molecules used in fighting oxidants in the body. Other factors used to determine antioxidant status in the body are oxygen radical absorbing capacity (ORAC) and ferric reducing antioxidant power (FRAP). These antioxidant mechanisms that safeguard the body against oxidative damage is compromised in diabetic conditions (Ghanbari et al., 2016; Valko et al., 2006).

Fructose is majorly metabolised in the liver and its consumption disturbs glucose metabolism and uptake. This results in a significantly increased rate of *de* novo lipogenesis and triglyceride synthesis, which contributes to decreased insulin sensitivity and hepatic insulin resistance. This appears to be the underlying mechanism of insulin resistance. Furthermore, fructose, is preferentially metabolized to lipids in the liver compared with glucose (Basciano et al., 2005). Fructose consumption therefore induces insulin resistance, impairs glucose tolerance, causes hyperinsulinemia and hypercholesterolemia in animal models (ter Horst et al., 2016). A study on commonly consumed fruit juices revealed that natural fructose can alter lipid and protein oxidation biomarkers in the blood, and mediate oxidative stress responses *in vivo* (Breinholt et al., 2003). Also, the short-term consumption of fructose was demonstrated to promote insulin resistance in the liver of non-diabetic adults (ter Horst et al., 2016). Administration of 10% fructose for two weeks, followed by a single intraperitoneal or intravenous injection of streptozotocin (40 mg/kg body weight) has been shown to result in a persistent diseased state characterized by hyperglycaemia (high blood glucose) with major clinical signs of T2D such as insulin resistance (Wilson and Islam, 2012).

Anchomanes difformis (AD)*;* a tropical plant found mostly in African forests, is rich in phytochemicals and has been reported for its biological activities (Ahmed, 2018). Folkloric uses of AD include the intake of the its decoction to treat coughs, diabetes, dysentery and throat related problems (Oyetayo, 2007). Some of these ethno-medicinal uses of A. *difformis* has been established through scientific evidences (Udje et al., 2018). Previous *in vitro* studies carried out in our research group on the six different extracts of AD revealed its free radical

scavenging property and antioxidant ability, while the aqueous leaf extract demonstrated the highest antioxidant (Alabi et al., 2019). We further investigated the phytochemical characterization of AD extracts using HPLC and LC-MS (Table 4.1), and we identified nineteen bioactive compounds from the aqueous extracts of AD (Alabi et al., 2019) (Alabi et al., 2019). Three compounds were also isolated from the rhizome of AD by Nkoh and colleagues (Nkoh et al., 2015).

Table 0.1: compounds identified in aqueous extract of AD after characterization using UPLC-MS (Alabi et al., 2019)

	Compounds	Concentration (mg/mg dry mass of sample)
1.	Luteolin Scutellarein Kaempferol	0.008267
2.	Vicenin_2	0.165466
3.	Chryosoeriol	0.102627
4.	Vitexin	0.043549
5.	Phloridzin	0.539154
6.	Rutin	0.019947
7.	Catechin	0.000521
8.	Hesperedin	0.003414
9.	p-Coumaric acid m-Coumaric acid o-Coumaric acid	0.012879
10.	4-HBA	0.003496
11.	Luteolin-6,8-di-C-hexoside	0.012534
12.	4-Vinylsyringol	0.180939
13.	Chrysoeriol-7-O-glucoside Or Isorhamnetin-3-O-rutinoside	0.146249
14.	Apigenin	0.272873
15.	Apigenin-6,8-di-C-glucoside,	0.103761

	Chrysoeriol-7-O-apiosyl- glucoside, Luteolin-7-O-rutinoside, Kaempferol 3-O-rutinoside, Kaempferol 3-O-galactoside 7-O- rhamnoside	
16.	Hispidulin	0.104893
17.	Hispidulin_2	0.104792
18.	Kaempferol	0.008554
19.	Quercetin	0.000635

A study reported only the hypoglycemic effect of ethanolic extract of AD rhizome in alloxan-induced diabetes using Wistar rats (Adeyemi et al., 2015), however the hepatoprotective ability and antioxidative properties of AD extracts *in vivo* diabetic model has not been investigated. This study therefore explored the hypoglycaemic property of aqueous leaves extract of AD in fructose-streptozotocin induced T2D in male Wistar rats, focusing on its role in ameliorating liver injury, hyperlipidaemia and restoring structural liver architecture. This is the first study to evaluate the antioxidant effect of aqueous leaf extract of AD in diabetes and diabetic complications. The potentials of AD were measured in comparison to a standard anti-diabetic drug; glibenclamide.

4.2 Materials and methods

4.2.1 Plant Preparation

4.2.1.1 Collection and Registration

The leaves of *Anchomanes difformis* (*Engl*: blume) were harvested from a farm in Abeokuta, Ogun state, Nigeria. The authentication was conducted at the Herbarium, University of Lagos, Nigeria (LUH6623) and a specimen voucher was deposited at the herbarium. The plant's name has been checked with http://www.theplantlist.org. (ID: kew-8734). Some of the local names of *A. difformis* include *Ògìrìòsákó (Yoruba); Kabaka-kachulu (Lunda); Niamé kwanba (Baule).*

4.2.1.2 Extraction

Aqueous extraction of AD leaves was done using cold-stirred extraction method. The leaves were dried under shade and blended to increase the surface area. The blended leaves were defatted using n-hexane ($10\%^{W}/_{V}$) for 48 hours. The leaves were further soaked in water for 48 hours in the ratio 1:10 of plant material and solvent at 2-4°C. A vacuum filtration method was used to filter off the debris and the filtrate was lyophilized. The pulverized extract was stored at -20°C for further analysis.

4.2.2 Ethical Approval

The experimental protocols described in this study was approved by the Faculty of Health and Wellness Sciences, Research Ethics Committee (REC) of Cape Peninsula University of Technology (CPUT), Bellville, South Africa. Ethical approval was granted for this study with REC approval reference number: CPUT/HW-REC 2016/A4. Also, ethical clearance was obtained (REF. 04/17) from the Ethics Committee for Research on Animals from South African Medical Research Council where the animal study from acclimatization to euthanasia was carried out.

4.2.3 Animals

Male Wistar rats (180 ± 10g, 8 weeks old) were obtained from the Animal facility, Stellenbosch University, South Africa. This study was carried out at the Primate Unit & Delft Animal Centre (PUDAC), South African Medical Research Council (SAMRC), Cape Town, South Africa. Animal handling, care and other procedures were done in accordance to the standard operating procedure of SAMRC PUDAC (SOP No: 2016-R01) which conforms to the internationally accepted, revised, South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008). The animals were acclimatized for 3 weeks. The rats were housed under controlled, standard, laboratory conditions; humidity between 45% to 55%, and an ambient temperature between

22°C to 26°C. Standard rat chow (SRC) and water was fed to all the rats *ad libitum* and they were exposed to the normal photo period (12hour dark/12hour light).

4.2.4 Experimental Design

Sixty-four (64) male, Wistar rats with weights ranging from 270-300g were used for this study. The rats were randomly grouped into one of seven (7) groups with a minimum of eight rats in each group (8 rats in normal groups and 10 in diabetic groups) as summarised in Figure 4.1. Water served as the vehicle for fructose and AD administration, while citrate buffer was the vehicle for streptozotocin. Animals in group 1 served as the normal control (NC) and received vehicle only. Animals in group 2 and 3 are normal rats who received only 200 and 400mg/kgBW of AD aqueous extract respectively (N+AD 200 and N+AD 400), these served as the treated control. Groups 4 to 7 consisted of animals that were placed on 10% fructose for 2 weeks followed by streptozotocin (STZ). Group 4 received vehicle only (DC), group 5 and 6 were given 200 and 400mg/kgBW of AD aqueous extract (D+AD 200 and D+AD 400) respectively while group 7 received 5mg/kgBW of gilbenclamide; an antidiabetic drug (D+G).

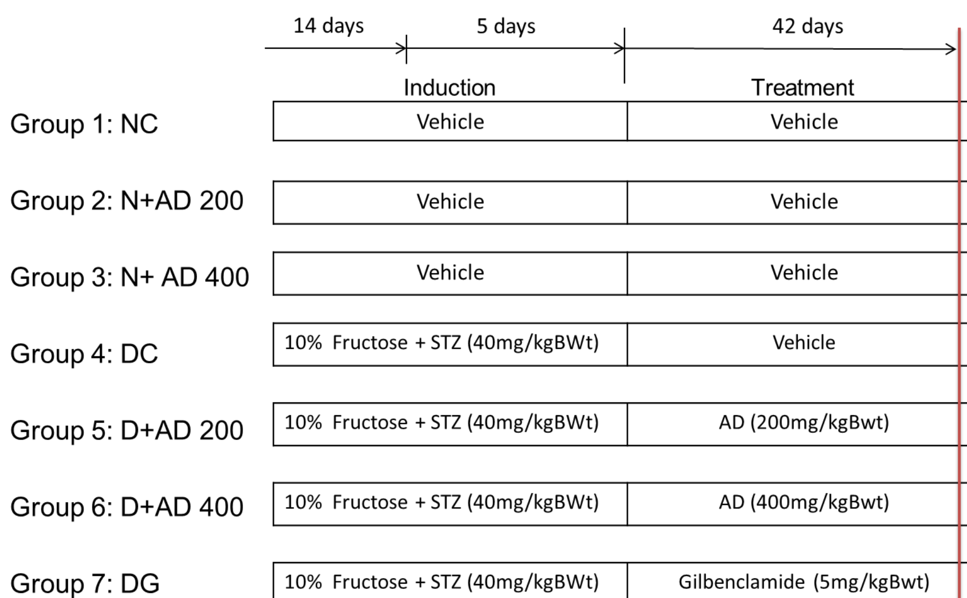


Figure 0.1: Experimental design. Animals were randomly assigned into 7 groups (n≥8). 14 days administration of 10% fructose preceded a single-dose injection of STZ (40mg/kg). Animals confirmed diabetic after 5 days. Normal rats were administered the vehicle; water.

4.2.5 Induction of Type 2 Diabetes

A 10% fructose solution was administered to the rats *ad libitum* (150mls/per day) for 2 weeks followed by a single dose intraperitoneal injection of STZ; 40 mg/kg body weight (Wilson and Islam, 2012). Diabetes was confirmed after 5 days of STZ administration, rats with fasting (overnight; 16 hours) blood glucose level greater than 15mmol/l were considered diabetic. Treatment commenced immediately with aqueous extract of AD which was delivered via oral gavage.

4.2.6 Blood and Tissue Collection

After the treatment, the rats were weighed and anaesthetized with 2% isoflurane per oxygen (1L/min flow rate) via inhalation. Blood was collected from the portal vein into Z-serum clot activator tubes. Blood samples were centrifuged at 4,000g for 10 min at 4°C. Aliquots of the supernatant were stored at -80^{0}C for biochemical analysis. The liver was excised immediately, washed in ice-cold phosphate buffered saline (PBS), dabbed and weighed. The liver was then frozen using liquid nitrogen and later stored at -80^{0}C for further analysis.

4.2.7 Measurement of Fasting Blood Glucose and Oral Glucose Tolerance Test

Fasting blood glucose (FBG) levels were measured weekly for 10 weeks, this period spans from fructose administration, induction of diabetes, treatment with AD to euthanasia. Rats were fasted overnight (16 hours), blood glucose concentrations were taken using ACCU-CHEK glucometer (Roche, South Africa). Oral glucose tolerance test was done over a period of 2 hours, readings were taken at 0, 30, 60, 90 and 120 minutes immediately after an oral administration of 0.5g/kg body weight of glucose.

4.2.8 Determination of Biomarkers for Organ Function

In this study, biochemical and histological parameters were assessed for organ integrity. Alanine transaminase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), albumin, total protein and lipid profile; total cholesterol, HDL-cholesterol, LDL-cholesterol and

triglycerides were measured in the serum. Serum AST and ALT levels were estimated using optimized ultraviolet test (340 nm), ALP was measured in the serum at 405 nm by a kinetic photometric procedure. HDL cholesterol was determined using an enzymatic colorimetric test; accelerator selective detergent method, while LDL cholesterol was estimated with Friedewald equation (Friedewald et al., 1972). Total cholesterol and triglycerides were measured by an enzymatic photometric assay. The analyses were carried out using Horiba kits (Montpellier, France) and performed on an ABX Pentra 400 Chemistry Analyzer (Horiba) according to manufacturer's instructions.

4.2.9 Tissue Preparation

A 200mg sample of the liver was homogenized on ice in 2mls of 50mM phosphate buffer with 0.5% triton and centrifuged at 10,000x gravitational force for 15 minutes at 4^0C. The supernatants were aliquoted and stored at -80 ˚C.

Enzymic and non-enzymic antioxidant indices; superoxide dismutase (SOD), catalase (CAT), oxygen radical absorbing capacity (ORAC), Ferric reducing antioxidant power, (FRAP), total glutathione (GSH), oxidized glutathione (GSSG) were measured in the liver homogenates. Lipid peroxidation (TBARS), ORAC and FRAP were also measured in the serum to estimate the antioxidant levels of the serum.

4.2.10 Lipid Peroxidation

Lipid peroxidation, a major indicator of oxidative stress was measured using thiobarbituric acid (TBA) reaction according to the modified methods of Matsunami et al. (2010) and Wasowicz et al. (1993). A 100 µL of sample was pipetted into a 2mL Eppendorf tube followed by the addition of 12.5 µL of 4mM BHT (butylated hydroxy toluene) in ethanol. Approximately 100 µL of 0.2M *O*-phosphoric acid was added and the solution vortexed for 10 seconds. This was followed by the addition of 12.5 µL of 0.11M TBA reagent and the mixture was then vortexed. The solution was incubated at 90^0C for 45 minutes, left on ice for 2 minutes, and further allowed to cool at room temperature for 5 minutes. The reaction of malondialdehyde

(MDA) present in the sample with TBA forms a pink colored chromophore. The thiobarbituric reactive substances (TBARS) were extracted with *n*-butanol, followed by the addition of 100 µL of saturated NaCl for better separation and the samples were then vortexed. The solution was centrifuged at 15000x g at $4^{O}C$ for 2 minutes and the solution separated into two layers. The butanol solution formed the top layer of which 300 µL of the butanol-TBARS layer was pipetted into the microplate and absorbance read at 532nm.

4.2.11 ORAC and FRAP

ORAC and FRAP were used to evaluate the antioxidant capacity of the serum and liver using a fluorescence Multiskan Spectrum plate reader (Thermoscan Electron Corporation, USA). The ORAC assay was performed following a modified method of Prior *et al*., (2003) which measured the potential of the antioxidants present in the sample to quench peroxyl radicals in comparison to Trolox; the standard reference. FRAP was assayed according to the method described by Benzie and Strain (1999), where the ability of a sample to reduce Iron (III) to Iron (II) was measured at a wavelength of 593nm using L-Ascorbic acid as the standard reference.

4.2.12 Superoxide Dismutase

The activity of SOD was determined by measuring the auto-oxidation of 6-hdroxydopamine (6-HD) and the amount of the enzyme needed to exhibit dismutation of the superoxide radicals (Ellerby and Bredesen, 2000). This assay quantifies all the three types of SOD (CU/Zn-, Mn-, and Fe-SOD) that could be present in the sample. A 15 µL sample was placed into wells containing 170 µL of 0.1mM DETAPAC (Diethylenetriaminepentaacetic acid), followed by the addition of 15 µL of 6-HD (1.6mM) to kick off the reaction. The absorbance was read at 490nm for 4 minutes in 1-minute intervals. The activity of SOD present in the sample was calculated from the equation obtained from the linear regression of the SOD standard curve.

4.2.13 Catalase

The activity of catalase was assessed by the method of Ellerby and Bredesen (2000) where the rate of conversion of hydrogen peroxide to water and oxygen by catalase was measured at 240nm. A sample of 10 µL was added to 170 µL sodium phosphate buffer (50mM, pH 7.0) and 75 µL of hydrogen peroxide (30% $^{V}/_{V}$) was added to initiate the reaction. The absorbance was read over a minute at 15 seconds intervals. The activity of catalase present in the sample was extrapolated from the activity of the standard, the catalase enzyme.

4.2.14 Total Glutathione and Glutathione disulphide (GSH/GSSG)

This assay is based on the enzymatic recycling process of GSSG to GSH catalysed by glutathione reductase (GR) in the presence of DTNB (5,5'-dithiobis-2-nitrobenzoic acid). The sulfhydryl group of GSH reacts with DTNB to form a yellow coloured compound; TNB and GS-TNB. GS-TNB is further reduced by GR and more TNB is produced. The assay measured the rate of TNB production which is directly proportional to the recycling reaction and the concentration of GSH. A sample of 200mg of the liver was homogenized in 2mls of ice-cold, 50mM sodium phosphate buffer containing 1mM EDTA. For GSSG, liver samples were homogenized in the same phosphate buffer containing 1mM EDTA and 3mM M2VP. Homogenates were then centrifuged at 15,000x g for 5 minutes, the supernatant was used for the analysis. A sample of 50 µL of the supernatant, 50 µL of GR (0.02U/µL) and 50 µL of 0.3mM DNTB were added to each of the wells of the microplate. To this, 50 µL of GSH (3µM) was added, the reaction was initiated by the addition of 50 µL of 1mM NADPH. A change in absorbance over 5 minutes was measured at 412nm.

4.2.15 Histological Examination of Liver Tissues

Liver tissues were harvested from all the animals and fixed in 10% buffered formalin solution for 24 hours. Tissues were placed in small cassettes, dehydrated using ethyl alcohol series ranging from 50% to 100% and cleared in xylene. Embedding of tissues in paraffin was done at 56°C and paraffin blocks were sectioned at 5µm using a rotatory ultra-microtome. Sections

were deparaffinised, rehydrated and stained with haematoxylin and eosin (H&E) dyes and mounted. The slides were examined under a light microscope at 10x and 40x magnification. Observations of any changes in the structural architecture, portal or lobular inflammation, sinusoidal dilatation and congestion, oedema, degeneration, necrosis and fatty change were noted, and photomicrographs taken with Motic camera (MOTICAM BTU10) using a Moticonnect Image Plus 2.0 software.

4.2.16 Statistical Analysis

Values were expressed as mean ± standard error of mean (SEM). Statistical analysis of results was performed using one-way or two-way analysis of variance (ANOVA) to find differences between groups. Bonferroni test was used for all pair-wise comparisons. Differences (F values) were considered statistically significant at p values less than 0.05. All statistical calculations were done using GraphPad Prism Version 5.00 for Windows, GraphPad Inc., San Diego, California USA.

4.3 Results

4.3.1 Hypoglycemic effect of AD in diabetic rats

The administration of 10% fructose for two weeks and 40 mg/kgBW of STZ led to a significant ($p<0.05$) increase in the blood glucose concentration in the diabetic rats when compared to normal rats (Figure 4.2A). However, treatment with 200mg and 400mg/kgBW of AD brought about a significant ($p<0.05$) reduction in the glucose levels of treated diabetic rats when compared to the untreated diabetic rats. Interestingly, both concentrations of AD were able to lower glucose concentrations more than the standard drug; gilbenclamide throughout the treatment period. The oral glucose tolerance test (OGTT) is a derived measure of insulin resistance and this was done to confirm constant hyperglycemia and insulin resistance in the rats administered with 10% fructose and injected with STZ in comparison with normal rats. The result revealed a constant significant ($p<0.05$) increase in the blood glucose

concentration of rats administered fructose and STZ when compared to normal rats. There was no significant (p<0.05) reduction in the glucose concentrations for 2 hours of observation.

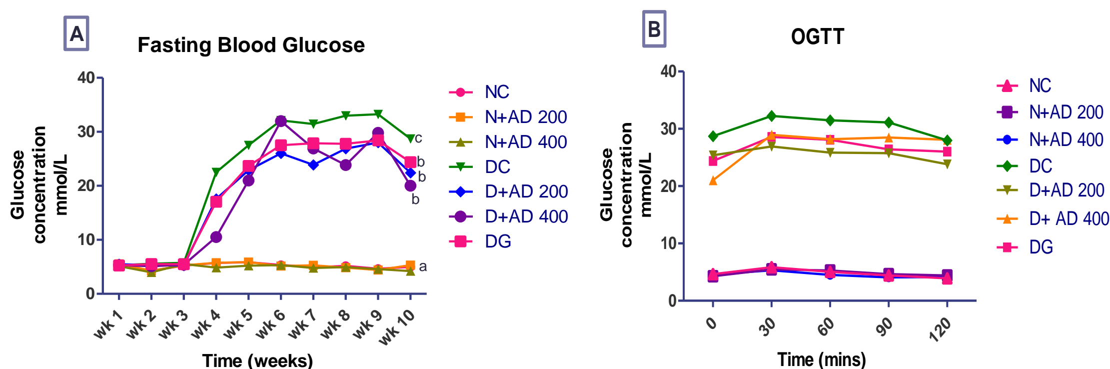


Figure 0.2: Effect of AD on weekly blood glucose concentrations **(A)** and oral glucose tolerance test **(B)** in normal and diabetic rats. Points are indicative of mean values ± SEM of blood glucose concentrations. Bars with different letters are significantly (p<0.05) different from each other.

4.3.2 Treatment with *A. difformis* abated serum levels of hepatic enzymes

The administration of AD for six (6) weeks significantly (p<0.05) lowered biomarkers of hepatic injury in treated diabetic rats as illustrated in Figure 4.3. Induction of type II diabetes with STZ caused a significant (p<0.05) increase in the serum levels of ALT, AST and ALP. Treatment with 400mg/kgBW of AD extract and Gilbenclamide significantly reduced ALT levels in diabetic rats to normal levels (Figure 4.3A), while treatment with the 200mg/kgBW AD extract showed a non-significant decrease (p<0.05). A similar trend was observed in the levels of AST (Figure 4.3B), however, treatment with both 200mg/kgBW and 400mg/kgBW AD extract significantly (p<0.05) lowered AST levels in diabetic treated rats when compared to normal rats. It is interesting to note that AST levels in diabetic rats treated with gilbenclamide were significantly reduced (p<0.05) when compared with normal rats. ALP activity declined significantly (p<0.05) in diabetic rats treated with 400mg/kgBW AD extracts when compared with non-treated diabetic rats, while an observable decrease was observed in the diabetic rats treated with 200mg/kgBW of AD extract and gilbenclamide (Figure 4.3C).

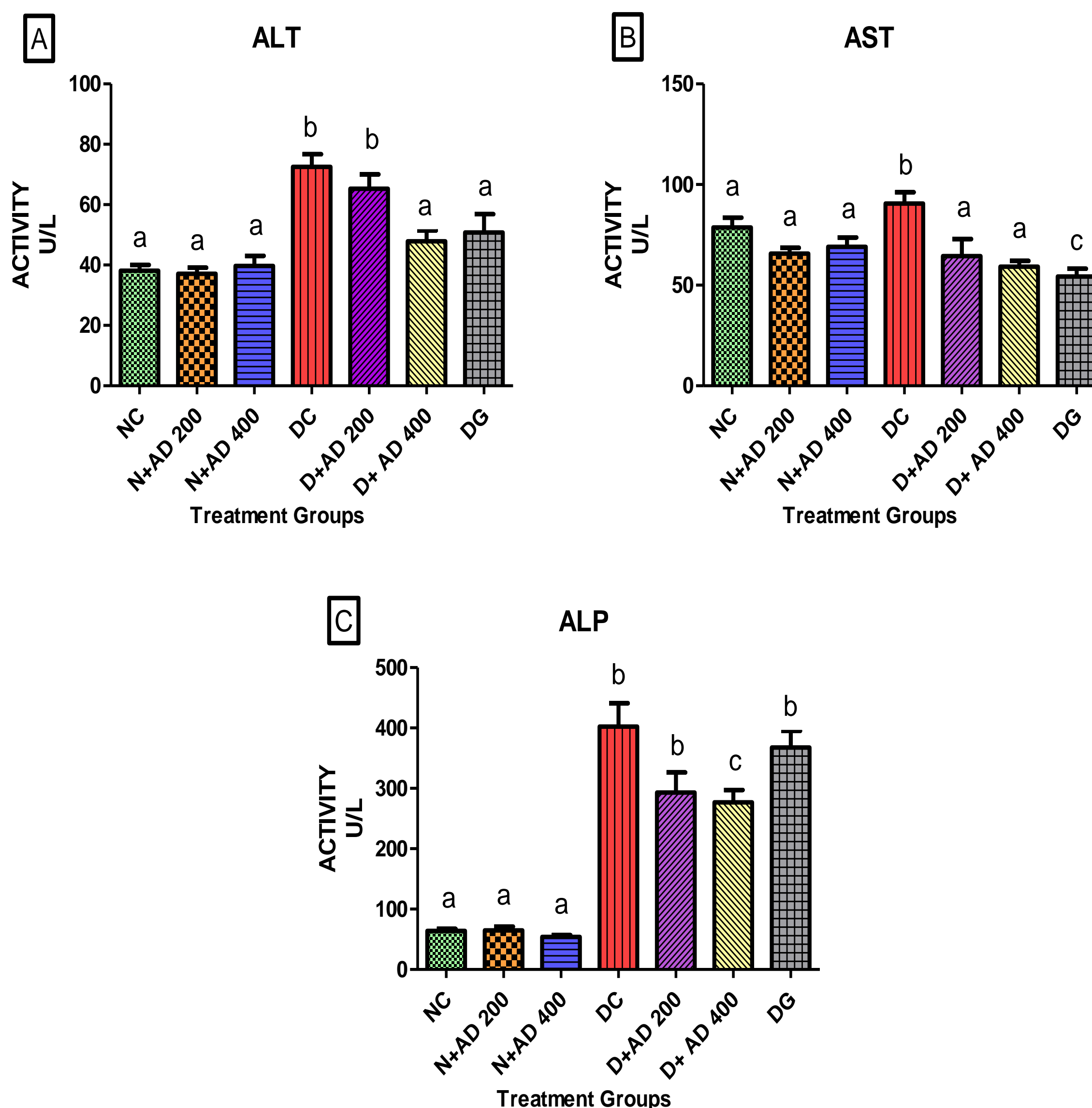


Figure 0.3: Effect of AD administration on biomarkers of hepatic injury; **(A)** ALT **(B)** AST and **(C)** ALP in the serum of normal and diabetic rats. Bars with different letters are significantly (p<0.05) different from each other.

4.3.3 Regulation/Modulation of lipid Profile by AD treatment in type II diabetes

The concentrations of total cholesterol, HDL-cholesterol and triglycerides increased significantly (*p*<0.05) in the serum of diabetic rats (Figure 4.4(A, C and D)). Likewise, the serum concentration of LDL-cholesterol also increased in diabetic rats, but not significantly (Figure 4.4B). Treatment with 200mg/kgBW and 400mg/kg BW of AD extracts significantly (*p*<0.05) lowered the concentration of total cholesterol and HDL-cholesterol to normal when compared with normal control rats and diabetic rats treated with gilbenclamide (Figures 4.4A

and 4.4C). Triglycerides was significantly ($p<0.05$) reduced only in the diabetic rats treated with 400mg/kgBW of AD extract as illustrated in Figure 4.4D. Similarly, only diabetic rats treated with gilbenclamide (5mg/kgBW) showed a significantly ($p<0.05$) decrease in serum levels of LDL-cholesterol (Figure 4.4B).

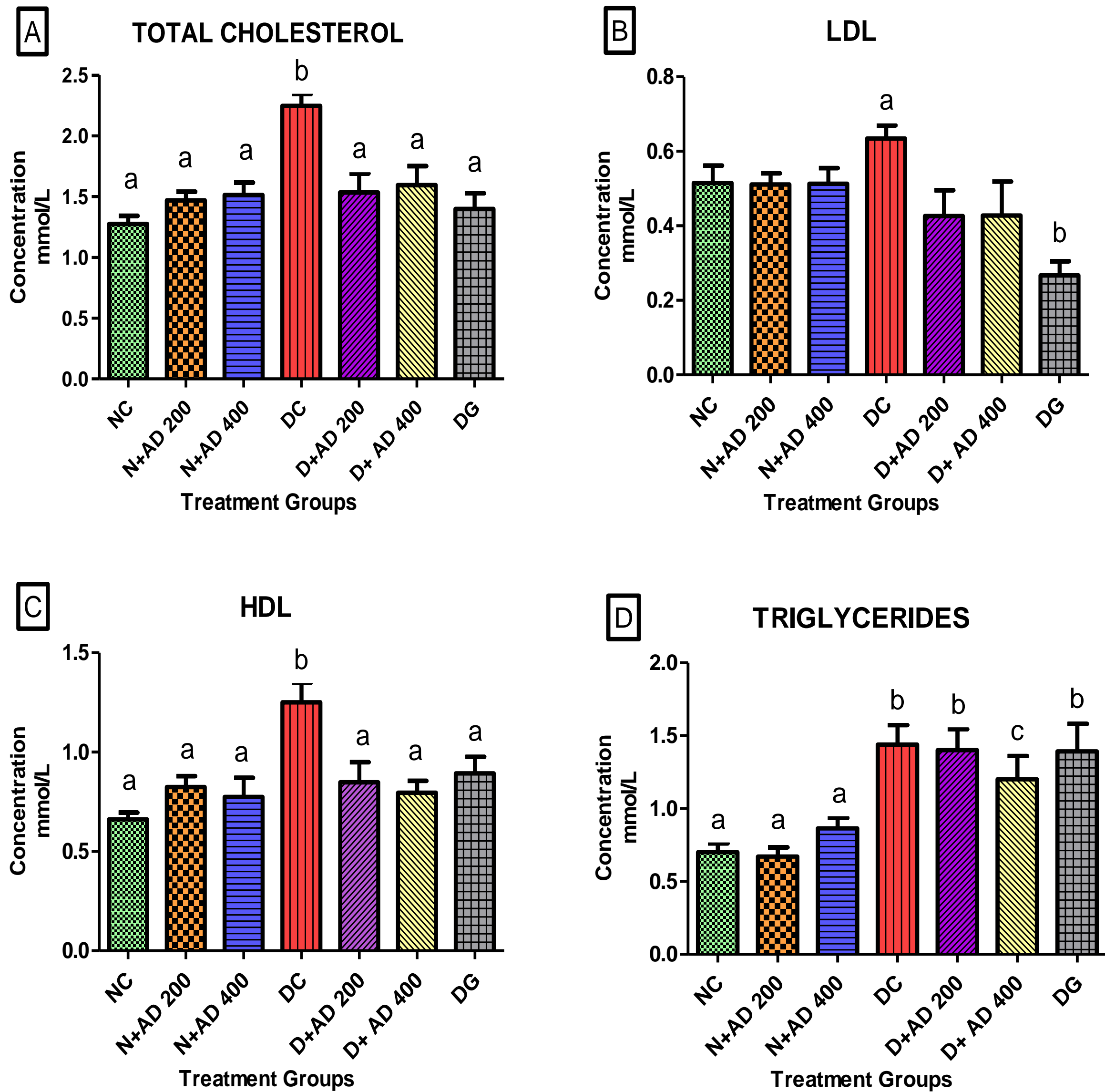


Figure 0.4: Effect of treatment with AD on the lipid profile; **(A)** Total Cholesterol **(B)** LDL-cholesterol **(C)** HDL-cholesterol and **(D)** Triglycerides in the serum of normal and diabetic rats. Bars with different letters are significantly ($p<0.05$) different from each other.

4.3.4 Impact of AD treatment on protein synthesis in STZ-induced type II diabetes

Figures 4.5A to D illustrate the concentrations of total proteins, albumin, and globulin in the serum. The figures indicate that total protein, albumin and LDH concentrations were affected by induction of diabetes as their concentrations were significantly (p<0.05) lowered. Inversely, albumin levels as illustrated in Figure 4.5B were restored to normalcy in the diabetic rats placed on 400mg/kgBW of AD extracts and gilbenclamide (5mg/kgBW). Total protein level was increased to normal in diabetic rats treated with gilbenclamide only as presented in Figure 4.5A. Treatment with 200mg/kgBW of AD significantly (p<0.05) reduced globulin levels in the serum of diabetic rats when compared with non-treated diabetic rats as exhibited in Figure 4.5C.

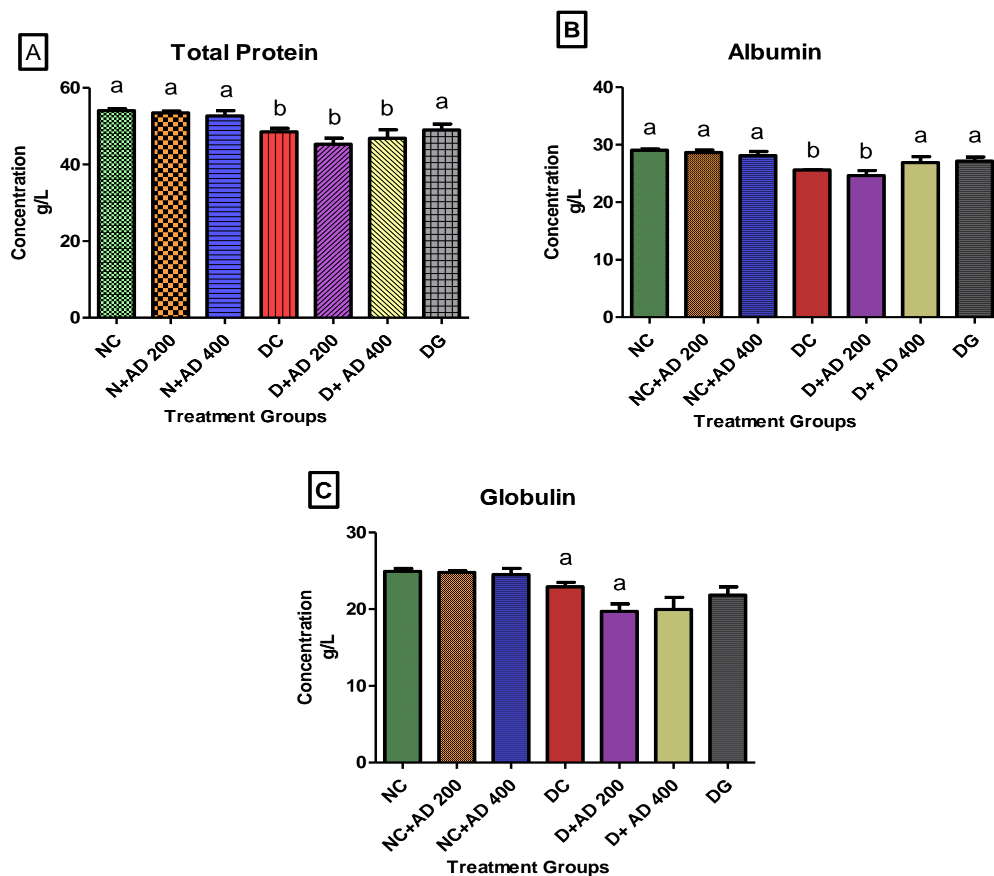


Figure 0.5: Effect of treatment with AD on **(A)** Total protein **(B)** Albumin and **(C)** Globulin in the serum of normal and diabetic rats. Bars with different letters are significantly (p<0.05) different from each other.

4.3.5 AD Intervention ameliorated lipid peroxidation, oxidative stress in type II diabetes

Constant hyperglycaemia significantly ($p<0.05$) contributes to the increased levels of TBARS in the serum of diabetic rats (Figure 4.6A). This significantly ($p<0.05$) declined in diabetic rats that were administered 200mg/kgBW of AD. Lipid peroxidation decreased (though not significant at $p<0.05$) in rats treated with 400mg/kgBW of AD when compared to diabetic untreated. Gilbenclamide administration did not reduce lipid peroxidation in diabetic rats. Furthermore, antioxidant capacity measured using FRAP diminished in diabetic rats as illustrated in Figure 4.6B. Administration of AD extracts and gilbenclamide had no significant ($p<0.05$) influence on the ferric reducing ability in the serum. Diabetes led to a significant reduction in the oxygen radical absorbance capacity of the serum of rats as exhibited in Figure 4.6C. Furthermore, groups treated with different concentrations of AD was able to restore this capacity compared to normal rats. However, gilbenclamide did not have any effect on the ORAC in the serum of diabetic rats.

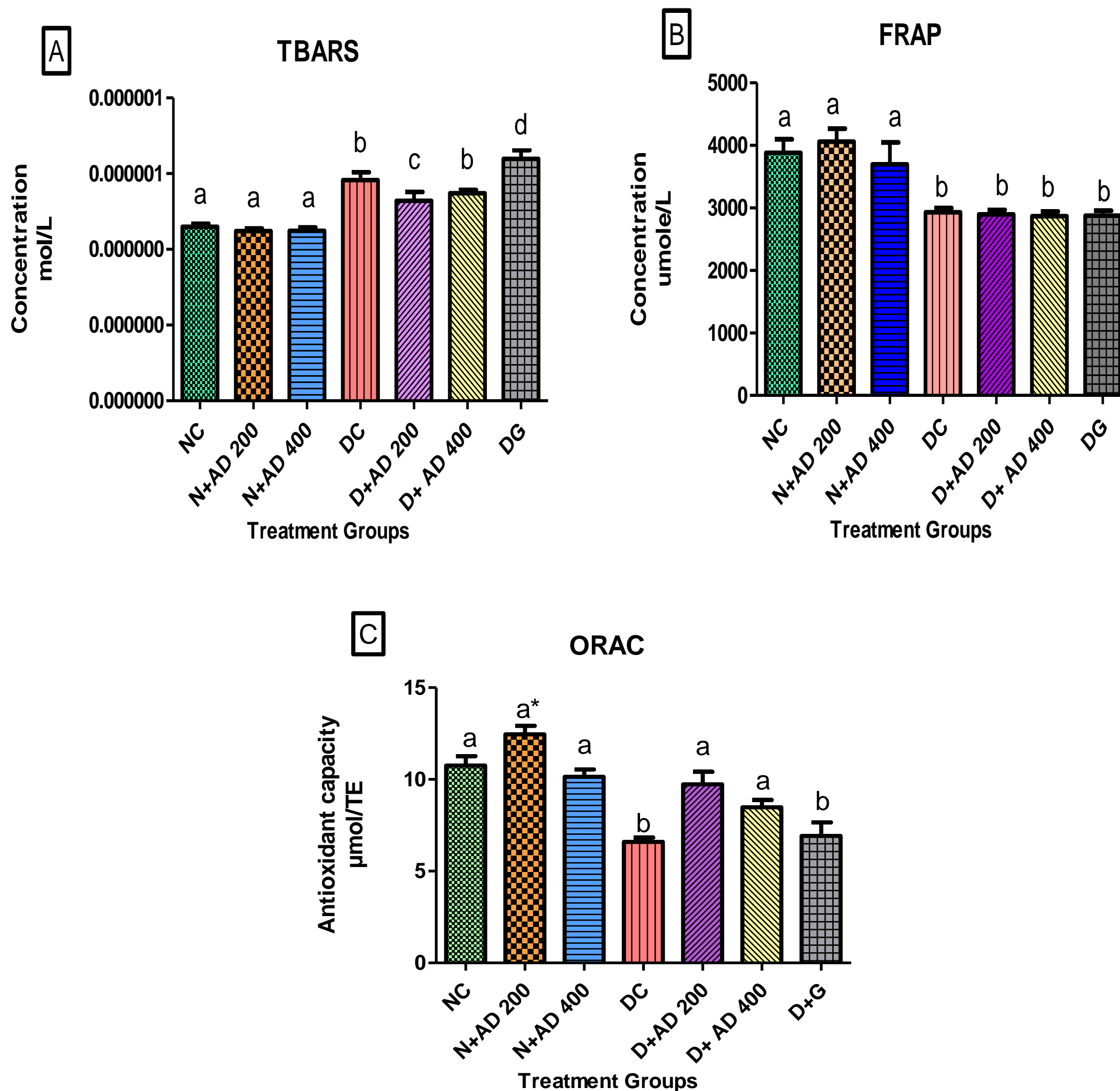
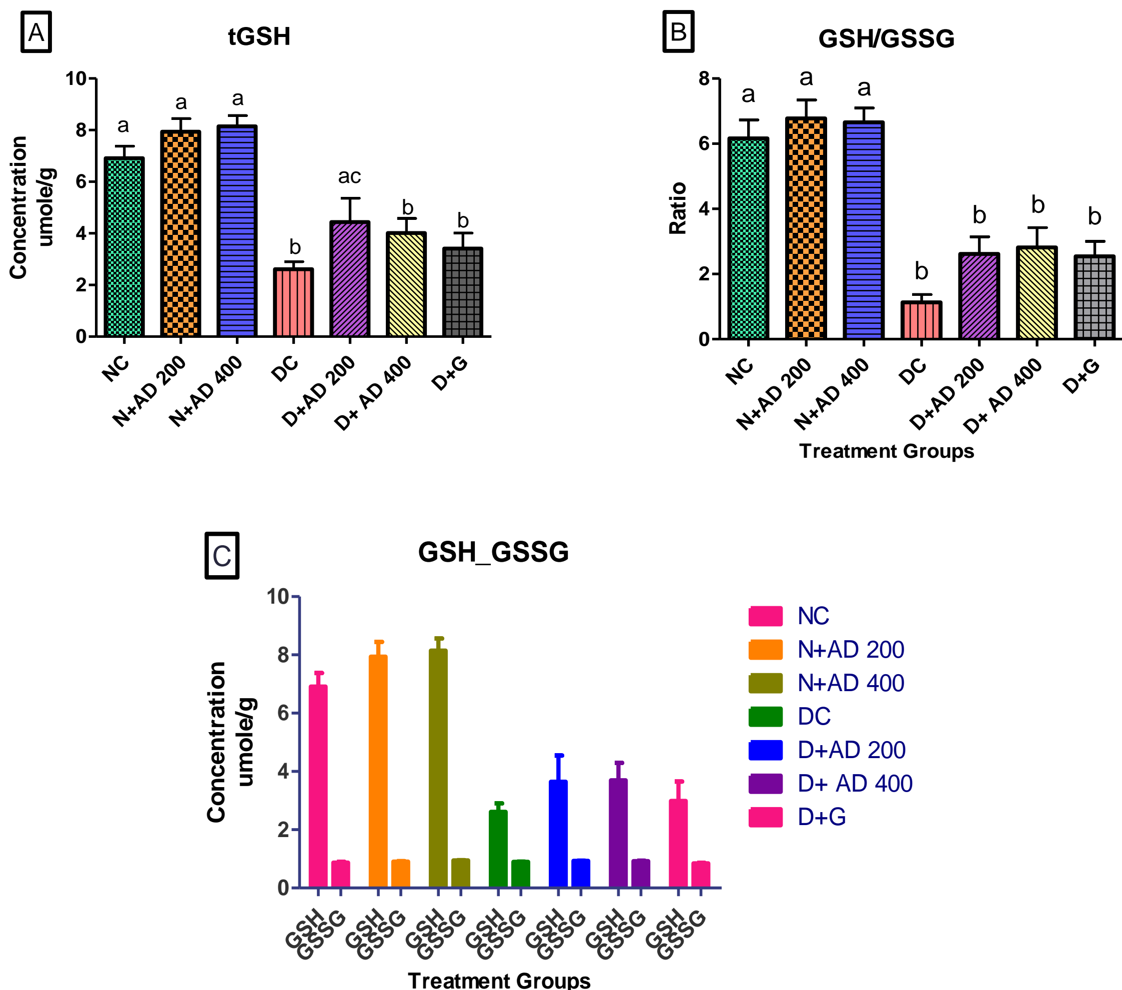


Figure 0.6: Effect of AD administration on biomarkers of lipid peroxidation and antioxidant activity; **(A)** TBARS, **(B)** FRAP and **(C)** ORAC in the serum of normal and diabetic rats. Bars with different letters are significantly (p<0.05) different from each other.

4.3.6 AD administration enhanced antioxidant status in the liver of diabetic rats

Hyperglycaemia resulted in a significant (*p*<0.05) reduction in the total glutathione levels (tGSH), glutathione to oxidized glutathione (GSH/GSSG) ratio and ORAC concentration in the liver of diabetic rats (Figure 4.7A to 4.7E). Diabetic rats who received 200mg/kgBW showed a significant (*p*<0.05) increase in the tGSH. There was an observed increase in tGSH levels and GSH/GSSG in diabetic rats that were administered 400mg/kgBW and the groups given gilbenclamide (5mg/kgBW), but not significant (*p*<0.05), this is presented in Figure

7.7A. ORAC concentrations were significantly (p<0.05) elevated in treated diabetic rats in response to AD administration as illustrated in Figure 4.7D. However, treatment with gilbenclamide did not have any positive effect on ORAC concentration in diabetic rat liver. There were no significant (p<0.05) changes in the FRAP concentration in normal rats, diabetic rats, and treated rats as exhibited in Figure 4.7E. Diabetes did not have a significant (p<0.05) effect on SOD levels in the liver as shown in Table 4.2. In addition, SOD activity was not influenced by the administration of AD and gilbenclamide. Conversely, the activity of CAT significantly (p<0.05) increased in diabetic rats when compared with the normal rats as presented in Table 4.2. Treatment with AD significantly (p<0.05) induced the activity of CAT in normal and diabetic rats in a dose dependent manner, gilbenclamide had the same effect in diabetic rats.



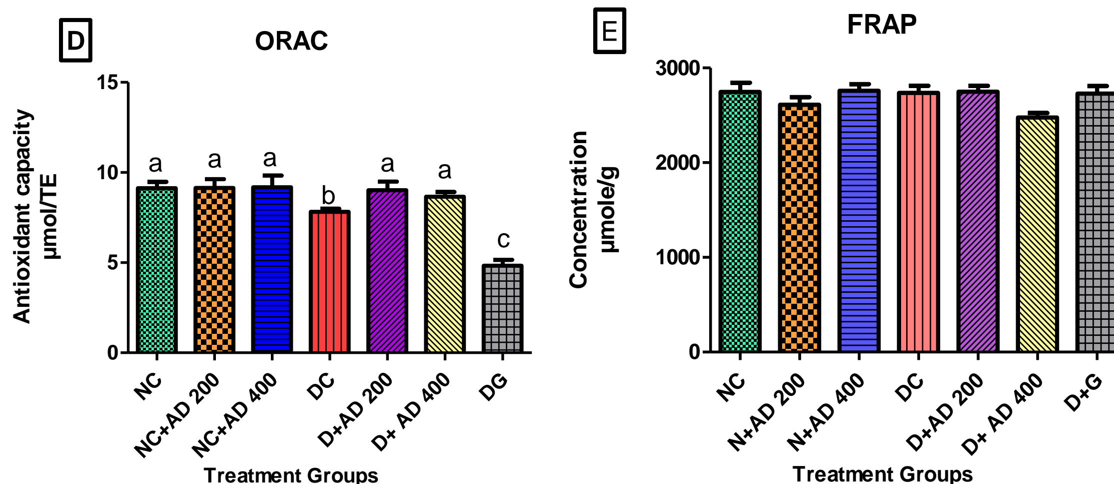


Figure 0.7: Effect of treatment on non-enzymic antioxidant indices; **(A)** total GSH, **(B)** GSH-GSSG ratio, **(C)** total GSH and GSSG, **(D)** ORAC, and **(E)** FRAP in the liver of diabetic and normal rats. Bars with different letters are significantly (p<0.05) different from each other. Letters with asterisk are significantly different from the normal control at p<0.001.

Table 0.2: Effect of treatment on antioxidant enzymes in the liver of diabetic and normal rats

Treatment Groups	SOD (U/mg)	CAT (U/mg)
NC	53.31 ± 5.81	2683 ± 206.2[a]
N+AD 200	50.98 ± 5.61	3147 ± 142.0[a]
N+AD 400	40.85 ± 1.73	6201 ± 401.2[b]
DC	36.53 ± 2.91	6010 ± 694.7[b]
D+AD 200	40.41 ± 1.80	5937 ± 734.7[b]
D+AD 400	36.34 ± 4.33	6202 ± 621.7[b]
D+G	38.46 ± 4.41	6511 ± 772.2[b]

*Values with different letters are significantly (p<0.05) different from each other

4.3.7 Treatment with AD reversed pathologies and improved the histological structure of the liver in T2D.

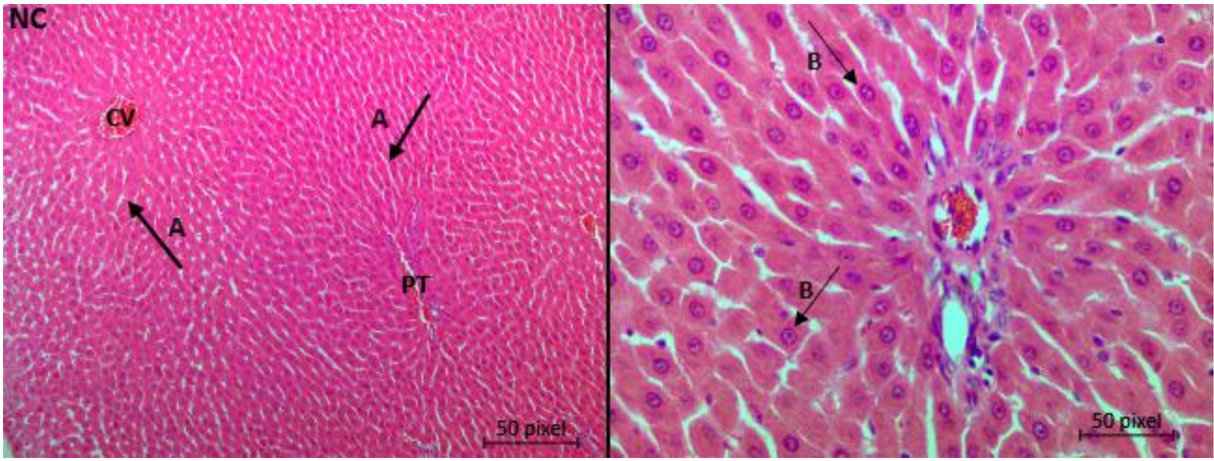
Findings from the histological examination of the liver from various treatment groups are shown in Figure 4.8. The normal control, non-diabetic rats showed normal histology, sinusoids are well divergent, the nucleus and nucleolus are clearly seen. Diabetic control

revealed enlarged sinusoids due to oedema, distorted sinusoidal arrangement, an area of focal necrosis exemplified by nuclear degradation (arrow ‘B’) and palour in the cytoplasm. Macro-vesicular steatosis can also be seen in the diabetic control rats, this was completely reversed in the diabetic rats treated with AD 200mg and 400mg/kgBW compared to normal rats. Mild centilobular necrosis and widened sinusoids were observed in the liver of diabetic rats treated with 200mg/kgBW of AD. Rats treated with 400mg/kgBW of AD showed normal histology in the liver. Steatosis and necrosis can be observed in the rats treated with gilbenclamide. The different pathologies that were observed in the hepatic tissues of different treatment groups were scored and graded as displayed in Table 4.3.

Table 0.3: Hepatic injury score in the various treatment groups

Histologic feature	**NC**	**N+AD 200**	**N+AD 400**	**DC**	**D+AD 200**	**D+AD 400**	**D+ G**
Portal inflammation	0	0	0	1	1	0	1
Steatosis	0	0	0	2	0	0	2
Sinusoidal dilatation	0	0	0	2	1	0	1
Sinusoidal Distortion	0	0	0	2	1	0	1
Centrilobular necrosis	0	0	0	3	1	0	2

The hepatic injury score was graded from 0 to 3, where 0 represents <5% damage and categorised as ‘none’, 1 represents 5%-33% damage and categorised as ‘mild’, 2 represents damage between 34%-66% and categorised as ‘moderate’ and 3 represents damage >66% of the tissue and categorised as ‘severe’.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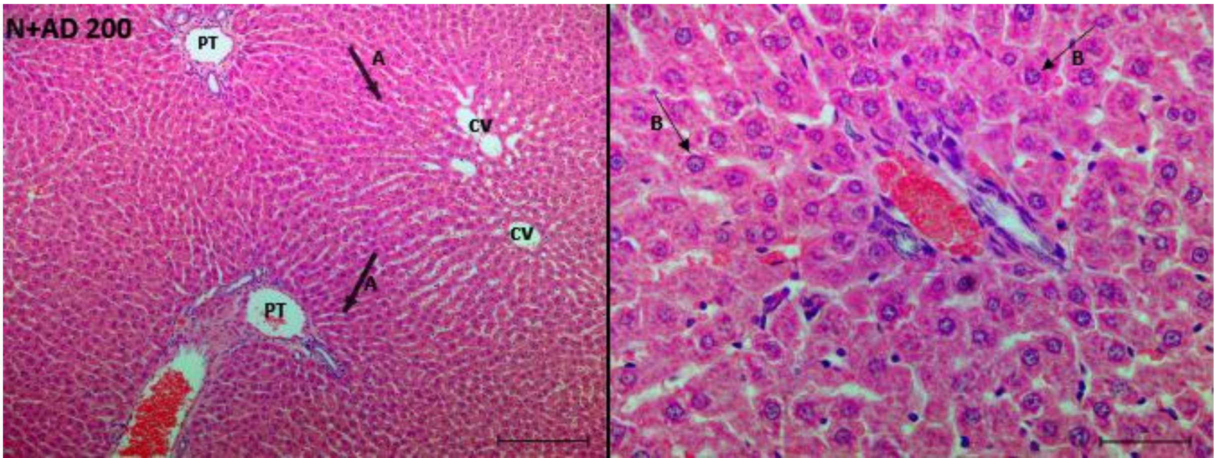
N+AD 200
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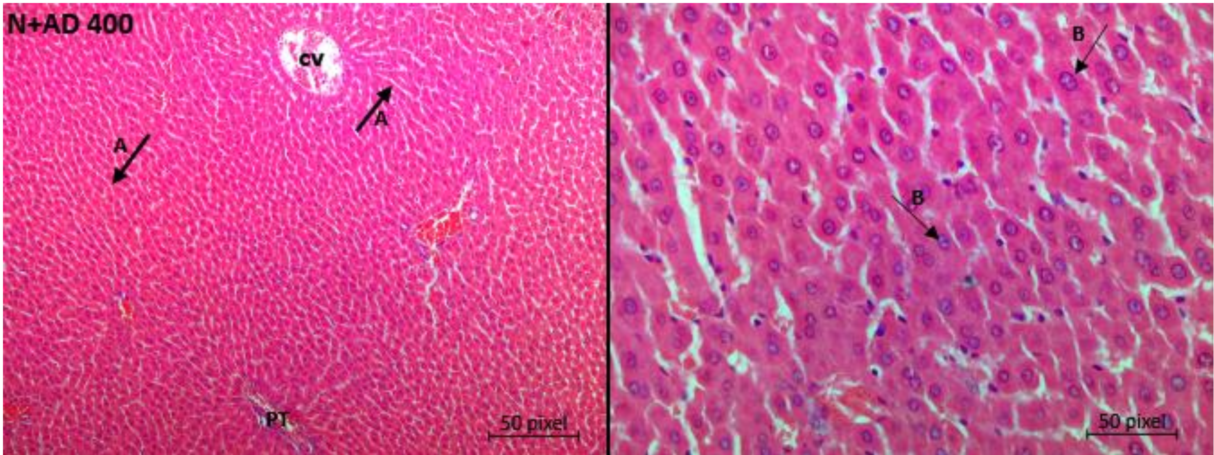
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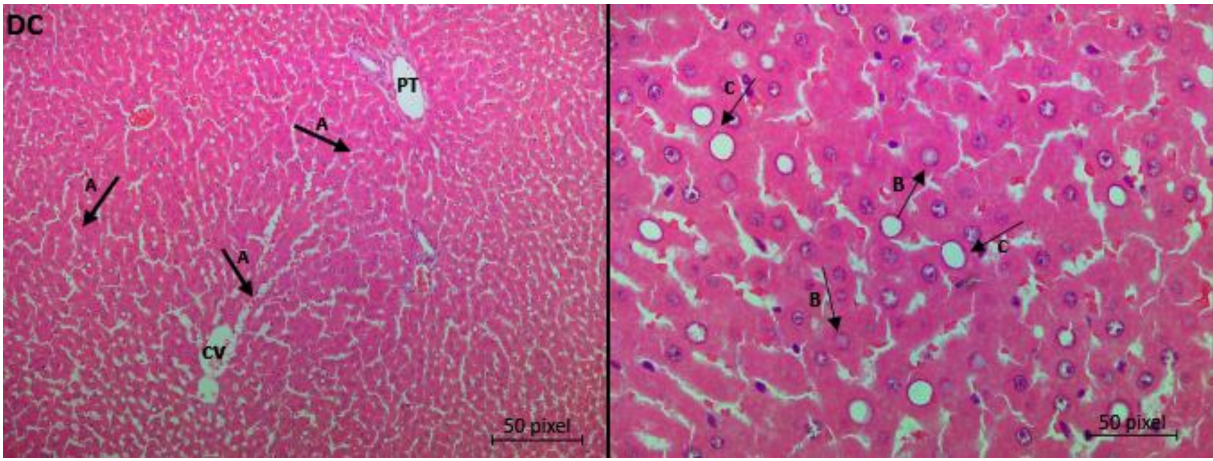
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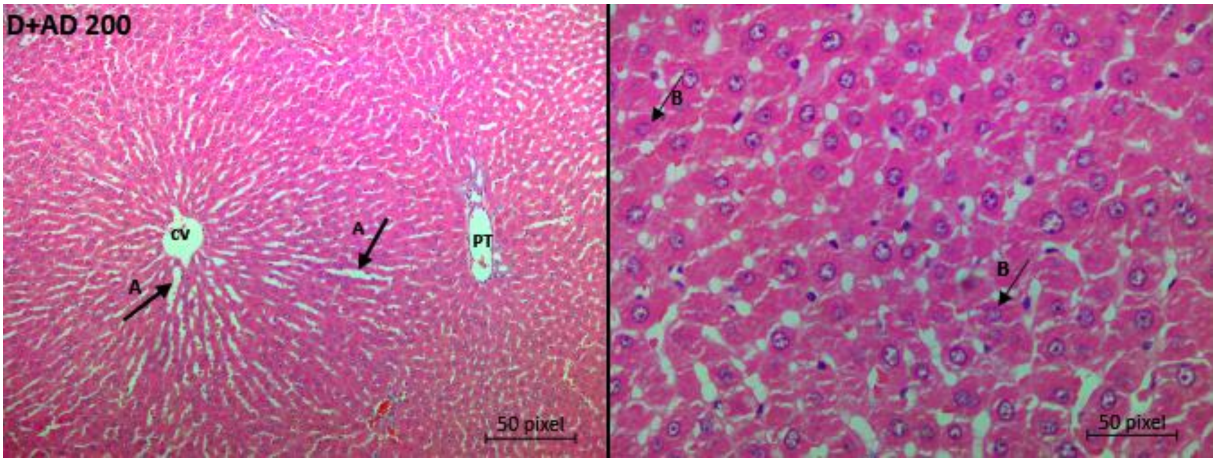
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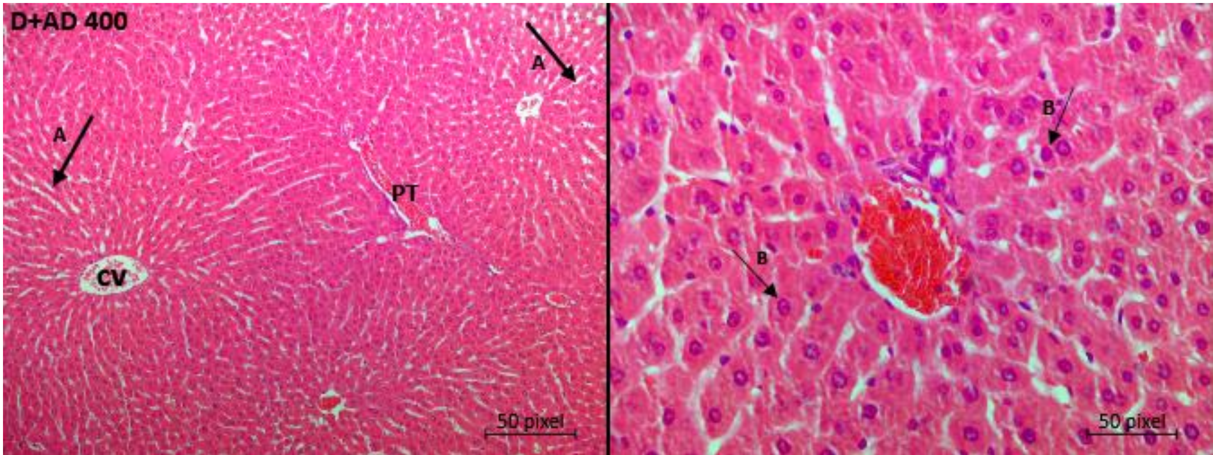
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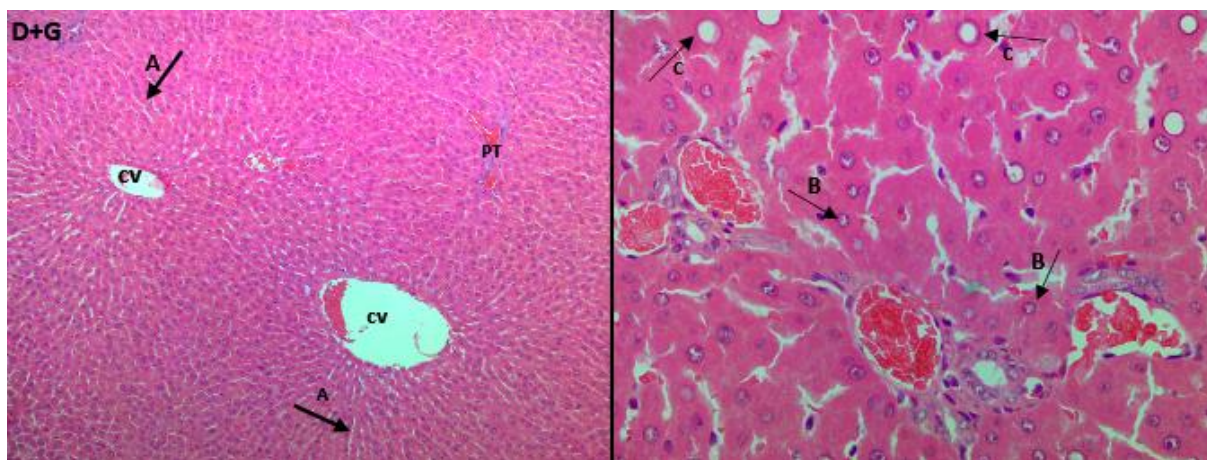

Figure 0.8: Light micrographs from liver sections of normal and diabetic rats stained with hematoxylin–eosin (x400). The black thick arrows lettered 'A' points at the sinusoids, arrows lettered 'B' points at the nucleus of the hepatocytes, and arrows lettered 'C' shows fatty droplets in the hepatocytes. Magnification 10x (left) and 40x (right). CV- central vein, PT-portal triad.

4.4 Discussion

The present study demonstrates the hypoglycaemic effect of AD aqueous leaves extract in T2D. AD showed a higher potential in lowering blood glucose in diabetic rats than the standard drug; gilbenclamide. Ethanolic extracts obtained from the roots of AD has been reported to possess hypoglycaemic effect (Adeyemi et al., 2015). Constant hyperglycaemia is associated with distortion in lipid metabolism, this results in hyperlipidemia and subsequent hepatic steatosis (Adeyemi et al., 2015). The data showed that treatment with AD and gilbenclamide significantly reduced total cholesterol to normal levels. Significant increase in HDL-cholesterol of diabetic untreated rats may be in response to the increased LDL-cholesterol in the blood, as HDL-cholesterol does reversed-cholesterol transport to get rid of excess cholesterol from the blood (Khera et al., 2011). LDL-cholesterol and triglycerides were reduced in diabetic rats with the administration of AD. This result is in agreement with a previous study which reported that root extract of AD was able to reduce plasma cholesterol in alloxan-induce diabetes (Adeyemi et al., 2015).

Abnormalities in glucose metabolism leads to an overproduction of free radicals. The presence of excess free radicals results in the oxidation of biomolecules such as lipids,

leading to breakdown of lipids especially in the cell membrane; which is majorly made up of lipids (Farombi et al., 2015). Lipid peroxidation plays an important role in the progression of diabetes and diabetic complications such as atherosclerosis which increases the risk of coronary heart disease (Ayepola et al., 2014; Ekstedt et al., 2006). Lipid peroxidation significantly increased in diabetic rats which is suggestive of cell membrane damage due to an increased production of free radicals. Treatment with AD significantly lowered lipid peroxidation in the serum of diabetic rats. Previous studies carried out in our research group shows that AD contains bioactive compounds that have antioxidant properties (Alabi et al., 2019).

Loss of cell integrity due to membrane damage results in the leakage of hepatic enzymes in diabetic rats. Hepatic enzymes have been shown to be elevated in diabetic conditions and are associated with liver pathologies (Bora et al., 2016; Chen et al., 2017). Our findings are consistent with these studies as demonstrated by the significant increase in hepatic enzymes in the serum of diabetic rats. This was however abated by the intervention with AD. Hepatic enzymes have also been used as indices of accumulation of liver fat, which was observed in the diabetic controls (Figures 4.4 and 4.8). Reports shows that increased hepatic enzymes with liver fat accumulation leads to progression of fibrosis which constitutes a clinically significant risk factor in developing end-stage liver disease (Ekstedt et al., 2006). The potency of AD in restoring elevated hepatic enzymes and total cholesterol to normalcy suggests its strong capacity to ameliorate or prevent diabetic complications associated with obesity and insulin resistance.

Proteins play a key role in cell functioning and structure. The amount of protein present in circulation is dependent on the balance between the rate of synthesis and the rate of catabolism or loss (Hamad et al., 2009). Therefore, the depletion of serum proteins may be due to increased catabolism or decreased synthesis. Abnormalities in serum proteins are observed during pathological conditions, especially diabetes mellitus (Hasan and

Abdulsattar, 2015; Suzuki, 2006). STZ-induced diabetes demonstrated a decreased concentration of total proteins and albumin in the serum. The liver is a major site of protein synthesis, therefore pathological conditions that adversely affect the liver can lead to a reduced production of proteins, hence the low concentration of total proteins observed in the diabetic groups. The observed low protein in the diabetic groups may also be as a result of an increased supply of amino acids for gluconeogenesis (Adeyemi et al., 2015). The ability of AD to restore protein levels to normalcy in diabetic rats placed on 400mg/kgBW, is suggestive of its involvement in enhancing protein synthesis. Albumin is one of the most abundant proteins in the serum (Wang et al., 2015) with numerous functions such as maintaining the colloidal osmotic pressure, acts as a source of amino acids, and in binding and transporting of substances. Low concentration of serum albumin may cause a decline in the antioxidant status of diabetic rats and is a risk factor for the development of diabetic complications (Obia et al., 2017; Park et al., 2014). The current study indicated a significant elevation in the albumin concentrations of diabetic rats treated with AD, this may have promoted the antioxidant capacity and delayed the progression of diabetic complications in the treated rats.

The oxygen radical absorbing capacity and ferric reducing antioxidant power of the diabetic rats were significantly ($p<0.05$) reduced. This is associated with the increased production of ROS as depicted by the significantly increased lipid peroxidation. However, AD significantly ($p<0.05$) elevated ORAC levels back to normal, whereas there was no effect on the FRAP levels. Administration of gilbenclamide was not able to improve the antioxidant status in the serum of rats Furthermore, there was an observable increase in the antioxidant indices in normal rats placed on AD in a dose-dependent manner, though not significant. AD increased the ORAC and FRAP levels in the serum and GSH in the liver of normal rats. This indicates the antioxidant ability of AD over gilbenclamide.

AD caused a dose-dependent induction of catalase in normal rats. The increased activity of catalase in the un-supplemented diabetic rats were likely due to the increased hydrogen peroxide and a compensatory response to the oxidative damage. Certain studies have reported a reduction in catalase activity in diabetic conditions (Erejuwa et al., 2010; Ghanbari et al., 2016). Contrarily, our findings corroborate with those of El Barky and colleagues (2016) who observed a significant increase in catalase activity in the liver of STZ-induced diabetic rats. Furthermore, this trend was also reported by Maritim and co-workers (Maritim et al., 2003). The activity of SOD in the liver of diabetic rats was not affected and administration of AD did not significantly influence SOD levels in normal and diabetic rats. Very similar observations were reported by Turk et al., (2002). Glutathione is an important inhibitor of free radical-mediated lipid peroxidation. Diminished tGSH levels and increased concentrations of TBARS are consistently observed in diabetes (Maritim et al., 2003) and our findings corroborate with these. AD was able to significantly increase tGSH levels in treated rats when compared to normal rats. The decreased tGSH levels in the liver of diabetic rats may be due to an increased utilisation in trapping the oxyradicals produced. AD administration restored antioxidant capacity in the liver of diabetic rats as expressed by the significantly increased ORAC levels in diabetic treated rats when compared to normal levels. This signifies the ability of AD to ameliorate oxidative stress-induced hepatic injuries in T2D.

T2D affected the normal histoarchitecture of the liver as shown by necrosis, macro-vesicular steatosis, dilatated sinusoids due to oedema. Treatment with AD markedly improved the histology of the liver as some of the pathologies such as steatosis were completely reversed. The observations were dose-dependent, as 400mg of AD restored the overall histoarchitecture of the liver in diabetic rats back to normal. Steatosis reflects an impairment of the normal processes of synthesis and elimination of triglycerides. Insulin resistance is a major factor that contributes to hepatic steatosis by inhibiting lipase thereby gaining control over free fatty acid release into the hepatocytes from the adipocytes (Hickman et al., 2002; Samuel and Shulman, 2016). This stimulation may be responsible for the observed significant

increase in triglyceride levels in the serum of diabetic rats, which was significantly reduced in response to treatment with AD. Also, LDL-cholesterol delivers cholesterol to the cells and this was significantly elevated in the serum of diabetic rats as was the case with total cholesterol levels. These were reversed with administration of AD.

A summary of the likely pathways involved in the antioxidant and hepatoprotective activity of AD is illustrated in Figure 4.9. The administration of fructose and STZ led to the damage of beta cells in the pancreas, this resulted into insulin resistance and hyperglycaemia. Insulin resistance brought about disturbed lipid and protein homeostasis which translated to hyperlipidaemia and decreased protein synthesis. Lipids were transported from the blood system to the hepatocytes causing steatosis. Persistent hyperglycaemia generated more ROS and caused increased lipid peroxidation which in turn resulted into oxidative damage of the cell membrane and leakage of hepatic enzymes. AD was able to attenuate these resultants effects of STZ by preventing or reducing oxidative stress, hyperglycaemia, insulin resistance, blocking fat mobilisation, and lipid peroxidation.

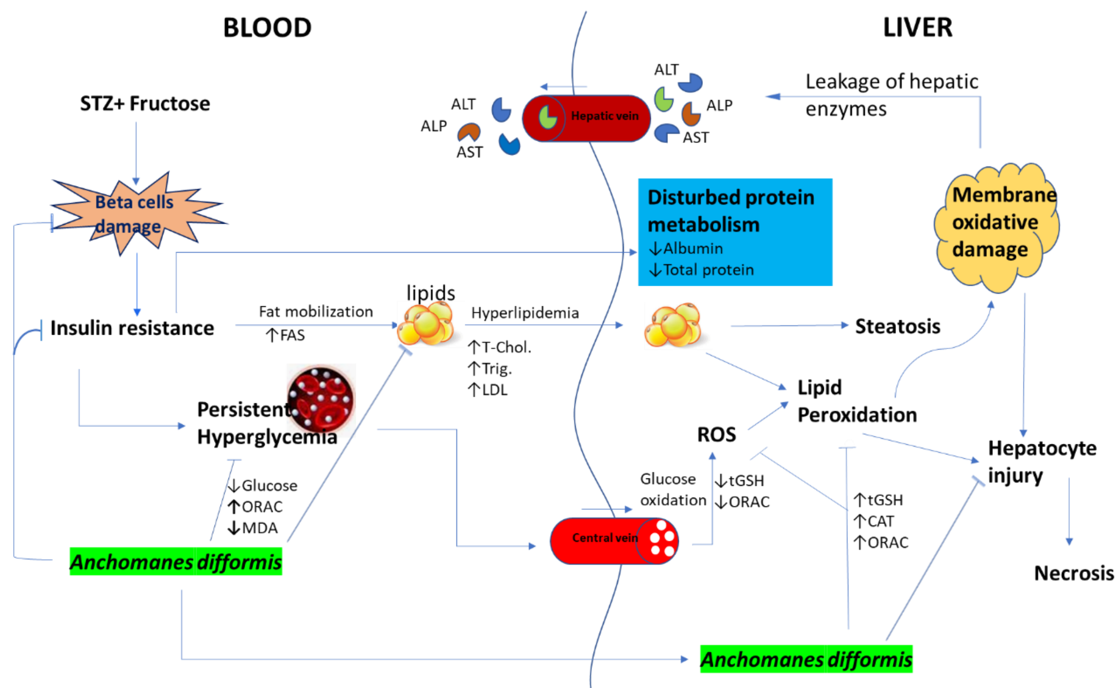

Figure 0.9: Proposed pathways involved in the antioxidant and hepatoprotective effect of Anchomanes difformis.

4.5 Conclusion

A. difformis ameliorated hepatic injury in fructose and STZ-induced diabetes in male Wistar rats. *A. difformis* exerts its hepatoprotective effects by demonstrating good glycaemic control, regulating lipid homeostasis, improving the antioxidant status, and restoring hepatic enzymes to normal levels. Treatment with *A. difformis* displayed a more antidiabetic ability than gilbenclamide. Administration of *A. difformis* in diabetes may be a potential therapeutic agent in the management of diabetes and resulted hepatic injuries.

Future Prospects

This study establishes the ability of AD aqueous extract to ameliorate hyperglycemia, hyperlipidemia, oxidative stress, liver steatosis and other pathologies in the liver. The findings will be helpful in the management of T2D using AD. However further molecular studies on the mechanisms by which AD exerts its hypolipidemic, hypoglycemic and antioxidant potentials in T2D can be explored, this will be a great tool in exploiting the full potentials of AD. Studies investigating the effect of AD on the expression of glucose transporters and other proteins involved in glucose transport and uptake, and insulin signalling is also essential. It is hoped that the current study will encourage further research on the effect of AD in other diabetic complications such as cardiomyopathy, nephropathy and reproductive dysfunctions.

Authors contributions

TD Alabi designed and preformed the experiments, analysed the experiment data and wrote the manuscript. OO Oguntibeju and NL Brooks supervised the project, helped in editing and improving the manuscript. OO Oguntibeju also coordinated the project funding. All authors read and approved the final manuscript. The authors declare no conflict of interest.

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CHAPTER FIVE

Anchomanes difformis; a potential solution to increased inflammation, apoptosis and organ toxicity in STZ-induced diabetic cardiomyopathy

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ABSTRACT

Background

Persistent hyperglycemia has been known to cause enhanced generation of reactive oxygen species in diabetes. Several inflammatory cytokines are induced by oxidative stress, and their release also leads to increased oxidative stress, this makes oxidative stress one of the important factors in the development of chronic inflammation and other immune responses. These have been implicated in the development of diabetic complications such as nephropathy and cardiomyopathy. *Anchomanes difformis* has been shown to possess antioxidant and anti-inflammatory potentials. The present study investigated the immunomodulatory potential and antiapoptotic ability of *Anchomanes difformis* to ameliorate heart toxicity and injury in type 2 diabetes.

Methods

Two weeks of fructose (10%) administration, followed by single intraperitoneal injection of streptozotocin (40mg/kg) were used to induce type II diabetes in male Wistar rats. Leaf extract (aqueous) of *Anchomanes difformis* (200 and 400mg/kg) was administered orally for six weeks. Blood glucose concentrations and body weights before and after interventions were determined. Interleukin (IL)-1β, IL-6, IL-10, IL-18, MCP-1 and TNFα were measured in the heart homogenates. CAT, SOD, total protein, ORAC, FRAP, TBARS and H-FABP levels were determined. Expressions of transcription factors (Nrf 2 and NFkB/p65) and apoptotic markers were also investigated in the heart.

Results

Anchomanes difformis administration reduced pro-inflammatory cytokines, increased anti-inflammatory markers and enhanced antioxidant defense in the heart of diabetic treated animals.

Conclusion

Anchomanes difformis is a new, promising therapeutic agent that can be explored for the treatment of pathological conditions associated with immune responses and will be a useful tool in the management of associated diabetic complications.

Keywords: *Anchomanes difformis,* apoptosis, cardiomyopathy, diabetes, glibenclamide, inflammation, oxidative stress.

ABBREVIATIONS

AD	*Anchomanes difformis*
ARE	Antioxidant response elements
Bcl2	B-cell lymphoma 2
CAT	Catalase
FRAP	Ferric reducing antioxidant power
H-FABP	Heart fatty acid binding protein
IL	Interleukin
KEAP-1	Kelch-like ECH-associated protein 1
MDA	Malondialdehyde
MCP-1	Monocyte chemoattractant protein 1
Nrf2	Nuclear factor-erythroid 2-related factor 2
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffer saline
ROS	Reactive oxygen species
SOD	Superoxide dismutase
STZ	Streptozotocin
TBARS	Thiobarbituric acid reactive substances
TNF-α	Tumor necrosis factor alpha

5.1 Introduction

Constant hyperglycemia: a major characteristic of diabetes mellitus (DM) has been linked to overproduction of ROS. Excessive generation of ROS activates cytokines production, apoptotic proteins and transcription factors, resulting in chronic inflammation and increased apoptosis; an underlying factor in the development of diabetic complications (Miranda-Díaz *et al.,* 2016). Evidence suggest that oxidative damage, pro-inflammatory responses and apoptosis are key players in pathological conditions relating to diabetic cardiomyopathy [1,2]. Diabetic cardiomyopathy (DCM) is a pathological condition of the heart observed in subjects with diabetes, and it is independent of other cardiovascular pathologies such as hypertension, ischemic heart injury, coronary artery disease, and congenital heart diseases. DCM is a major cause of heart failure [3,4]. The progression of diabetes mellitus to diabetic cardiomyopathy involves the interplay of several mechanisms; oxidative damage, inflammation, apoptosis, mitochondrial dysfunction and overactivation of Raas (Renin-angiotensin aldosterone system) as illustrated in Figure 5.1 [5,6].

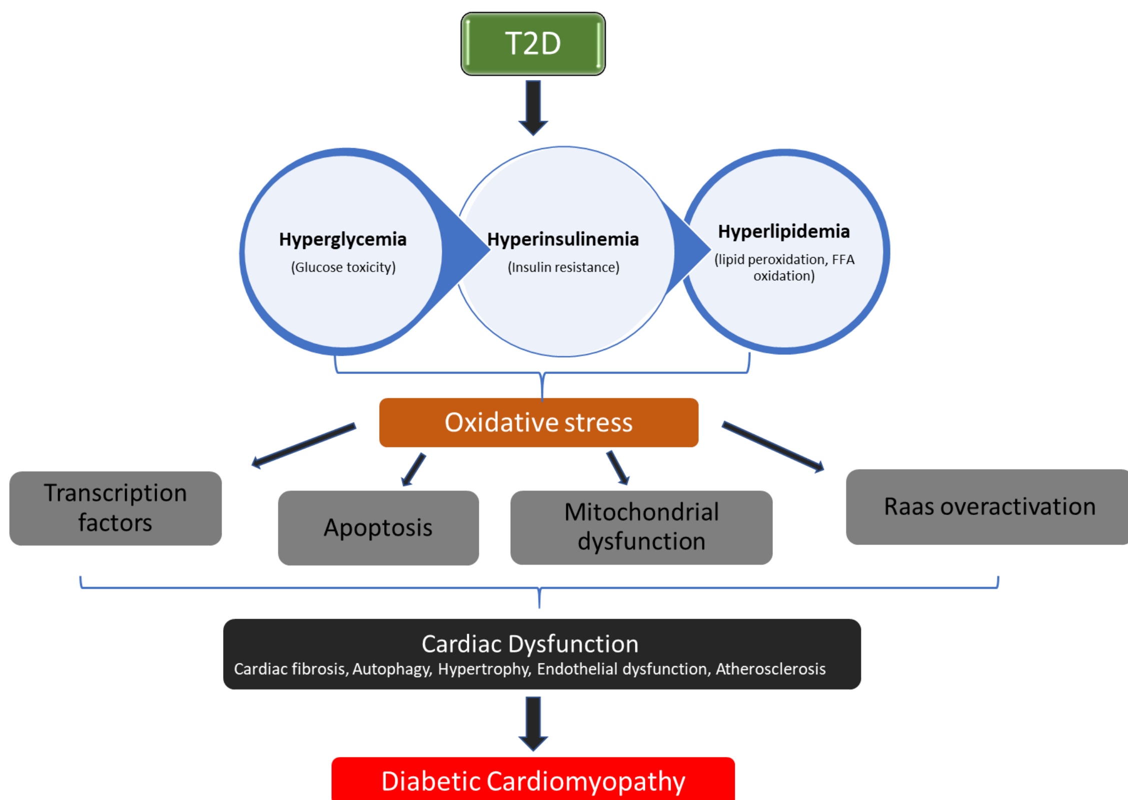


Figure 0.1: The pathophysiological pathway in the progression of diabetic cardiomyopathy.

Inflammation is a form of defense and protection from infections and tissue damage, however the uncontrolled regulation of immune responses can lead to excessive tissue damage [7,8]. Pro-inflammatory cytokines with other indices of inflammation have been shown to be elevated in the hearts of T2D subjects [9,10]. Diabetes triggers lower levels of systemic inflammation in the cardiomyocytes as an early response to myocardial injury due to the overproduction of mitochondrial ROS. This systemic inflammation triggers the recruitment of leukocytes and cause the secretion of pro-inflammatory cytokines and chemokines such as interleukin (IL)-1β, IL-6 and TNF-α [5,11]. Further exposure to high concentrations of glucose results in increased production of advanced glycation end products (AGEs). AGEs are regulators of endothelial cell permeability, migration of monocytes, and ultimately activates nuclear factor kappa-light-chain enhancers of activated B cells (NFkB) [12]. NFkB primarily stimulates the expression of more pro-inflammatory cytokines (TNF-α, IL-6, IL-18) in the heart which are associated with hypertrophy, fibrosis and left ventricular dysfunction. This cascade of reactions is repeated severally, leading to a sustained immune response thereby causing further injury and hence, cardiomyopathy [6,13].

The significance of maintaining homeostasis in multicellular organisms is pivotal and having a balance between cell death and proliferation plays a key role in homeostasis. Cell death could occur in the form of necrosis, apoptosis, autophagy. Apoptosis is a programmed cell death that serves as natural barrier against uncontrolled proliferation of cells and prevent injury that may be caused by damaged or stressed cells [14]. Excess cellular level of ROS cause damage to biomolecules, membranes and organelles which activates cell death processes such as apoptosis [15]. However, increased apoptosis has been implicated in the development of diabetic complications (DCM). Recently, Nunes and co-workers reported the influence of MCP-1 on apoptosis in cardiomyocytes. This substantiates the link between inflammation, apoptosis and DCM. [16].

During increased oxidative stress, the body system augments its antioxidant capacities to combat elevated oxidative stress. The nuclear factor E2-related factor 2 (Nrf2) is a transcription factor that is associated with mitigating oxidative stress [17]. Nrf2 is activated in the cytosol to counteract increased oxidative stress and maintain homeostasis. This is done by enhancing the expression of AREs such as CAT, SOD, GSH and GPx that will mop up the reactive oxygen and nitrogen species. Keap1 which is bound to actin in the cytosol, interacts with Nrf2, promotes ubiquitination, and eventually degrades Nrf2. This process is rapid, making the half-life of Nrf2 around 13-21 minutes [18,19]. This, however, keeps Nrf2 at relatively low basal levels. Conversely, during increased intracellular ROS which leads to cellular stress, Keap1 loses its effectiveness in degrading Nrf2. This extends the half-life of Nrf2 to about 100-120 minutes, thereby stabilizing Nrf2 levels [18,19]. Nrf2 then enters the nucleus and triggers the transcription of AREs and other cytoprotective genes [20]. Investigation on the protective role of Nrf2 showcased its significance in the regulation of inflammation and apoptosis. Nrf2 is involved in enhancing the expression of survival inflammatory markers which in turn suppresses pro-apoptotic proteins. Hematopoietic stem cells with their Nrf2 gene knocked off, showed increased oxidative stress, apoptosis and reduced expression of pro-survival genes [21]. It has recently been established that Nrf2 directly inhibits the transcription of pro-inflammatory genes coding for pro-inflammatory proteins (IL-1α, IL-1β IL-6) therefore ameliorating increased inflammation and apoptosis [22].

Streptozotocin (STZ) is a glucose analogue that is cytotoxic to the beta cells of the pancreas. It is a diabetogenic compound that is produced naturally in *Streptomyces achromogenes;* a soil bacterium. The effect of STZ administration is usually seen within 3 days depending on the dosage. STZ exhibits its selective toxicity on beta cells in rats by DNA fragmentation of the beta cells and causes death, leading to diabetic conditions which further progresses to diabetic complications if uncontrolled [23]. High dosage of STZ has been reported to result in complete destruction of the beta cells; a model of type I diabetes. Recent studies report the effective use of low dose of STZ to induce insulin resistance; a model of type II diabetes (T2D) and

subsequently, diabetic complications [24–26]. Administration of 10% fructose for two weeks, followed by 40 mg/kgBW of STZ was demonstrated by Wilson and Islam to cause partial destruction of the beta cells and insulin resistance in rats; which are typical of T2D [27].

Anchomanes difformis (AD) is a plant with numerous ethno-botanical uses in Africa for conditions such as inflammation, diabetes, asthma, microbial infections, pain, ulcerations and gastrointestinal disturbances. Some of these folkloric uses have been scientifically established while others are still indigenous claims [28]. The anti-inflammatory ability of the leaf and rhizome extracts of AD was revealed by its inhibitory activity on histamine and serotonin which are mediators in the initial phase of acute inflammation. AD showed more anti-inflammatory potential than the standard drug used; aspirin [29]. Similarly, Adebayo and colleagues also demonstrated the anti-inflammatory property of AD. The plant inhibited oedema (paw volume) in raw-egg albumin induced inflammation in chicks [30]. Studies showed that AD is effective against hyperglycemia in alloxan-induced diabetes [31,32], however the potential of AD against inflammation and apoptosis in diabetic mellitus has not been explored. This study therefore investigated the anti-inflammatory and anti-apoptotic ability of AD leaves extract on increased inflammatory response and cell death in STZ-induced diabetic cardiomyopathy in male Wistar rats. We carried out phytochemical characterization and profiling of six different extracts of AD, and 32 compounds were identified. Furthermore antioxidant capacities of the extracts were measured using ORAC, FRAP and TEAC assays, and the aqueous extract exhibited the highest antioxidant capacity [33], hence its choice for this study.

5.2 Materials and methods

5.2.1 Chemicals and reagents

STZ was purchased from Biocom Africa, South Africa. H-FABP (Cat. No: FB 4025) was obtained from Randox Laboratories (South Africa). The interleukins and TNF-α were supplied by Biorad, while MCP-1 by Merck (South Africa). Anti-Nrf2, Anti-NFkB/p65, Anti-Bcl2, Anti-Caspase-3, Anti-mouse IgG H&L and Anti-rabbit IgG H&L were bought from BiocomAfrica

(South Africa). Bicinchoninic Acid Protein Assay kit was purchased from Thermo Fisher Scientific, South Africa.

5.2.2 Plant Preparation

The harvested leaves of *A. difformis* was authenticated (LUH6623) and a specimen deposited at the herbarium, University of Lagos, Nigeria. The leaves were dried under shade, blended, de-fatted with n-hexane. Aqueous extract was from the dried leaves of AD were extracted via cold extraction (2-8°C). The extract was pulverised and stored at -20°C for further analysis.

5.2.3 Ethical Approval

This study was approved by the Faculty of Health & Wellness Sciences Research Ethics Committee (REC) of the Cape Peninsula University of Technology, Bellville, South Africa (CPUT/HW-REC 2016/A4). It was also approved (REF.04/17) by the Ethics Committee for Research on Animals at the South African Medical Research Council (SAMRC), South Africa, where the animal experiment was performed.

5.2.4 Animals

Wistar rats (male) weighing approximately 180 ± 10 g (male) were obtained from Stellenbosch University (animal facility), South Africa. The animals were acclimatized for 3-4 weeks and housed at the Primate Unit & Delft Animal Centre (PUDAC), SAMRC, Cape Town. Housing conditions were controlled: humidity- 45% to 55%, temperature between 22°C to 26°C. Standard rat chow (SRC) and water was fed to all the rats *ad libitum* and they were exposed to normal photo period (12hour dark/12hour light). Animal handling, care and other procedures were done in accordance with the standard operating procedure of SAMRC PUDAC (SOP No: 2016-R01) which conforms to the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

5.2.5 Experimental Design

Sixty-four (64) male, Wistar rats with weights ranging from 270-300 g were used for this study. The rats were randomly assigned into seven (7) groups with a minimum of eight rats in each group (8 rats in normal groups and 10 in diabetic groups) as summarised in Figure 3.2. Water served as the vehicle for fructose and AD administration, while citrate buffer was the vehicle for streptozotocin. Animals in group 1 served as the normal control (NC) and received the vehicle only. Animals in group 2 and 3 are normal rats who received 200 and 400 mg/kgBW of AD aqueous extract only (N+AD 200 and N+AD 400), these served as the treated control. Groups 4-7 consisted of animals that were placed on 10% fructose for 2 weeks followed by streptozotocin (STZ). Group 4 rats received vehicle only (DC), rats in group 5 and 6 were given 200 and 400 mg/kgBW of AD aqueous extract (D+AD 200 and D+AD 400) respectively while group 7 rats received 5 mg/kgBW of glibenclamide; an antidiabetic drug (D+G).

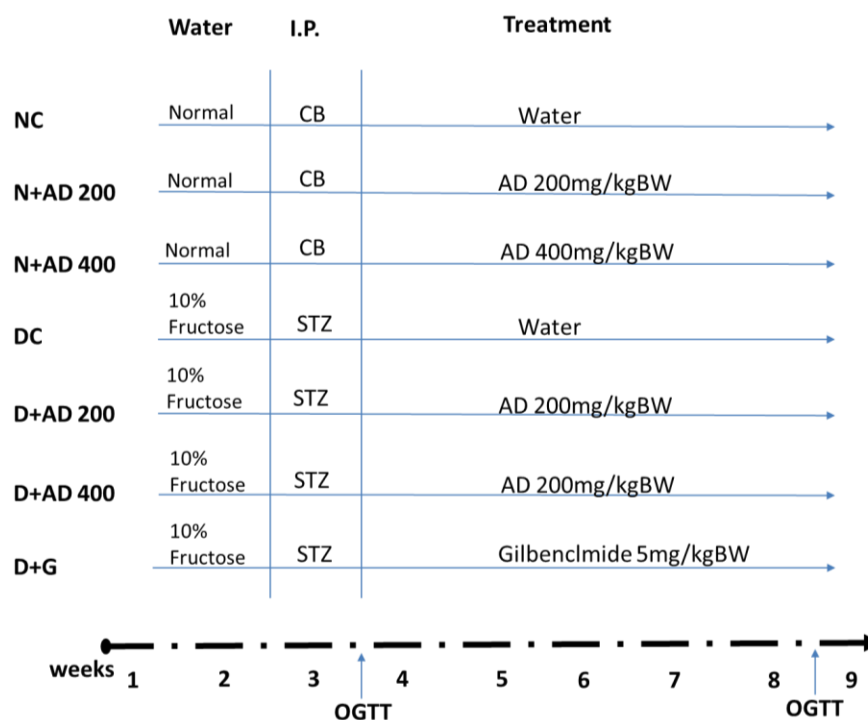


Figure 0.2: Experimental design. Animals were randomly assigned into 7 groups (n≥8). 14 days' administration of 10% fructose preceded a single-dose injection of STZ (40mg/kg). After 5 days, animals with fasting blood glucose of 15mmol/L or greater were considered diabetic. OGTT was conducted to confirm insulin resistance. Normal rats were administered the vehicle; water and citrate buffer (CB) accordingly. Treatment commenced immediately for 42 days via oral gavage. Animals were euthanized on the 43rd day (red bar).

5.2.6 Blood and Tissue Collection

The rats were anaesthetized before euthanasia, with 2% isoflurane per oxygen (1L/min flow rate) via inhalation. Blood was collected into Z-serum clot activator tubes from the abdominal vein. Blood samples were centrifuged at 4,000 g for 10 min at 4ºC. Aliquots of the supernatant were stored at -80 ºC for biochemical analysis. Under euthanasia, the heart was extracted and immediately washed in ice-cold phosphate buffered saline (PBS), dabbed and weighed. This was then frozen using liquid nitrogen and later stored at -80 ºC for further analysis.

5.2.7 Tissue Preparation

The heart samples for histological examination were fixed in 10% buffered formalin solution immediately. Samples that will be used for immunofluorescence assays were fixed with a freezing media, froze in the liquid nitrogen and further stored at -80 ºC. Homogenization of the heart tissues was done subsequently for assays requiring tissue lysate. A 200mg sample of the heart was homogenized on ice in 2mls of 50mM phosphate buffer with 0.5% triton and centrifuged at 10,000x gravitational force for 15 minutes at 4 ºC. The supernatants were aliquoted and stored at -80 ºC.

5.2.8 Determination of organ function and toxicity markers

The body weights of all the animals were measured weekly throughout the animal study. The relative heart weight was derived from the weight of the heart and the body weight of the same rat.

$$Relative\ heart\ weight\ = \frac{Heart\ weight\ (g)}{Total\ body\ weight\ (g)} \times 100\ \%$$

Heart-type fatty acid-binding protein (H-FABP) was quantified in the serum and the determination was carried out according to the standard operating procedure provided by the manufacturer. The principle of the assay was based on immunoturbidimetry.

5.2.9 Analysis of antioxidant status and lipid peroxidation indices

Activities of catalase, superoxide dismutase and levels of thiobarbituric acid reactive substances (TBARS) were assayed in the heart of normal and diabetic rats. In addition, ORAC and FRAP were also measured to evaluate antioxidant capacity in the heart. The method of Ellerby and Bredesen [33] was used to determine catalase activities in the hearts, where the rate of conversion of hydrogen peroxide to water and oxygen by catalase was measured at 240nm. SOD levels were also measured by the method of Ellerby and Bredesen [33], which measures the amount of the enzyme needed to exhibit dismutation of the superoxide radicals produced from the auto-oxidation of 6-hydroxydopamine (6-HD). TBARS levels were evaluated using the modified methods of Matsunami *et al.* and Wasowicz *et al.* [34,35]. ORAC levels were assessed according to the method of Prior *et al.*, [36] while FRAP was measured using the method described by Benzie and Strain [37]. Total protein present in the heart was quantified using Bicinchoninic Acid Protein Assay kit, and manufacturer's procedures were duly followed.

5.2.10 Estimation of pro- and anti-inflammatory biomarkers

Interleukins (IL)-1β, IL-6, and IL-10 were measured, and other chemokines including MCP-1 (monocyte chemotactic protein-1) and TNF-α (tumor necrosis factor-α) were also measured in the heart lysates of normal and diabetic rats. These inflammatory markers were measured using Bioplex Promagnetic bead-based assays (Bio-Rad Laboratories, Hercules, USA) on the Bio-plex platform (Bio-Rad). Assays were performed according to the manufacturer's instructions (BioRad and Merck Millipore). Bead acquisition and analysis of median fluorescent intensities was done using Bio-Plex Manager software, (version 6.0).

5.2.11 Evaluation of apoptotic and transcriptional proteins expression

To explore the effect of AD on Nrf2, NFkB/p65, Bcl2 and caspase-3 expression in increased oxidative stress and inflammatory process induced by hyperglycemia, we carried out an

immunohistochemical fluorescence staining on the heart tissue. The process was carried out according to the manufacturer's procedures.

5.2.11.1 Antibodies

Optimization of primary antibodies were carried out on the heart tissues, to determine the optimal staining concentrations and conditions for each antibody. Single and double stains were done for each antibody at varying dilutions and diluents. The combination that showed the highest specificity and sensitivity on positive and negative control samples was considered as the optimal staining concentration. This was done with the help of an experienced pathologist. The details of each antibody used, and their optimal concentration is presented in Table 5.1.

Table 0.1: The descriptions of the antibodies used for detection of protein expression levels, stating the host, supplier and the optimization factor

No	Marker	Antibody	Host	Source	Dilution
1.	Nrf2	Anti-Nrf2	Mouse	ABCAM, UK	1:200
2.	NFkB/p65	Anti-NFkB/p65	Rabbit	ABCAM, UK	1:250
3.	Bcl2	Anti-Bcl2	Mouse	BioLegend, SA	1:125
4.	Caspase-3	Anti-Caspase-3	Rabbit	ABCAM, UK	1:150
5.	Alexa Fluor 488	Anti-mouse IgG H&L	Goat	ABCAM, UK	1:400
6.	Alexa Fluor 594	Anti-rabbit IgG H&L	Goat	ABCAM, UK	1:400

5.2.11.2 Tissue Preparation and Staining

Heart tissues were fixed with freezing media (Leica, South Africa) and stored at -80°C. 10μm of the tissues were sectioned using a Leica CM 1860 UV Cryostat and permeabilized with PBS plus 0.025% Triton X-100 (PBS-T). Tissues were blocked with 10% normal goat serum in PBS containing 5% bovine serum albumin (BSA) for 2 hours. Incubation with respective primary antibodies (see details in Table 5.1) were done overnight at 4°C, followed by 2 washes in PBS-T. Tissues were incubated with secondary antibodies for 1 hour at room temperature, in the dark and washed thrice with PBS. Mounting was done using Dako mounting medium (Agilent

Technology Inc, South Africa). The argon multiline laser at 488nm and DPSS 561-10 laser at 561nm was used to excite the Alexa Fluor 488 (green) and Alexa Fluor 594 (red) respectively.

5.2.11.3 Imaging

Images were acquired on a Zeiss LSM780 ELYRA PS1 super-resolution, confocal microscope with a 10x/0.3 M27 objective (EC "Plan-Neofluar"). Zen 2.6 imaging software (blue and black edition, Zeiss Germany) were used for image analysis and to obtain mean fluorescent intensities (MFI) on 4 images acquired in each experimental condition. This was repeated thrice.

5.2.12 Data Analysis

Values were expressed as mean ± standard error of mean (SEM). Statistical analysis of results was performed using one-way or two-way analysis of variance (ANOVA) to find differences between groups. Bonferroni test was used for all pair-wise comparisons. Differences (*F* values) were considered statistically significant at *p* values less than 0.05. All statistical calculations were done using GraphPad Prism Version 5.00 for Windows, GraphPad Inc., San Diego, California USA.

5.3 Results

5.3.1 AD reduced weight loss and organ toxicity in STZ-induced diabetes

Induction of diabetes led to significant reduction in the mean body weight of the diabetic rats (Figure 5.3A). There was 24.1% weight loss in the untreated diabetic rats, while diabetic rats treated with glibenclamide experienced 11.2% weight loss. Body weight results of the rats treated with 200mg and 400mg of AD revealed 8.6% and 6.4% loss in body weight respectively. Interestingly, both concentrations of AD; 400mg and 200mg significantly prevented weight loss by 17.7% and 15.5% respectively, while glibenclamide reduced weight loss by 12.9%. Both concentrations of AD were able to significantly impede weight loss in STZ-induced diabetes better than the reference drug; glibenclamide (Figure 5.3A). In addition

to the increased weight loss, the heart to body weight ratio of untreated diabetic rats significantly increased by 40% when compared to the normal rats (Figure 5.3B). Administration of 400 and 200 mg/kgBW AD significantly ameliorated possible hypertrophy by reducing the heart-body weight ratio of diabetic rats by 15% and 11.95% respectively. Treatment with 5 mg/kgBW of glibenclamide did not reduce relative heart weight in diabetic hearts when compared to the untreated diabetic rats (Figure 5.3B). This demonstrated the ability of AD to protect against STZ induced organ toxicity.

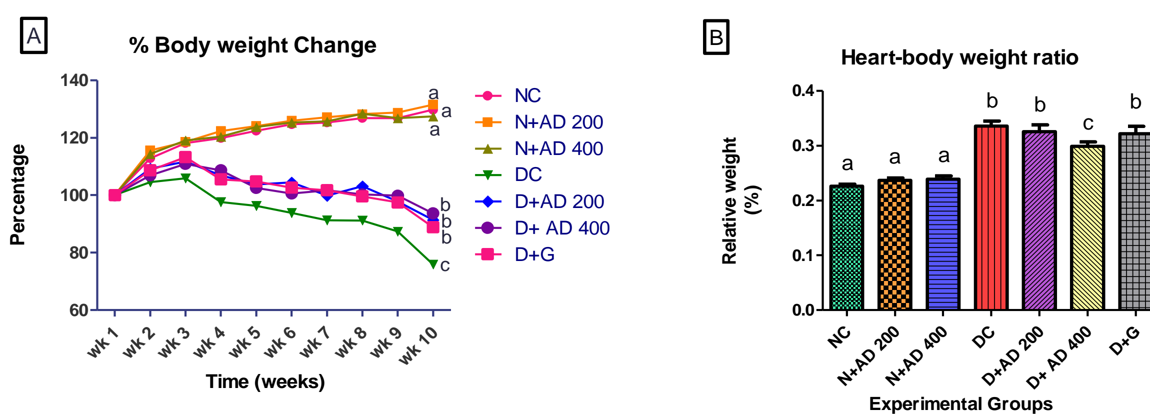


Figure 0.3: Effect of AD administration on **(A)** Bodyweight change and **(B)** Heart-body weight ratio. Points and bars are indicative of mean values ± SEM of bodyweight change and heart-body weight ratio. Bars with different letters are significantly ($p<0.05$) different from each other.

5.3.2 Effect of AD on antioxidant enzymes and protein synthesis in STZ induced diabetes

Figures 5.4A & 5.4B illustrate the effect of diabetes and administration of AD on catalase and SOD activities in the heart of normal and diabetic rats. Administration of AD led to an observable increase in the activities of catalase and SOD in the normal rats. Also, induction of diabetes by administration of STZ significantly increased the activity of catalase in the hearts of untreated diabetic rats (Figure 5.4A). However, treatment with AD attempted to revert the abnormally increased levels of catalase, however not significantly. Glibenclamide, reduced the catalase levels to near normal. There was no significant difference in the SOD levels in normal and diabetic groups (Figure 5.4B). A slight decrease in protein levels was observed in the

diabetic rats, the ingestion of AD led to increased protein levels in the treated diabetic rats as presented in Figure 5.4C. 400 mg/kgBW of AD was able to enhance protein levels in diabetic rats (*p*>0.05).

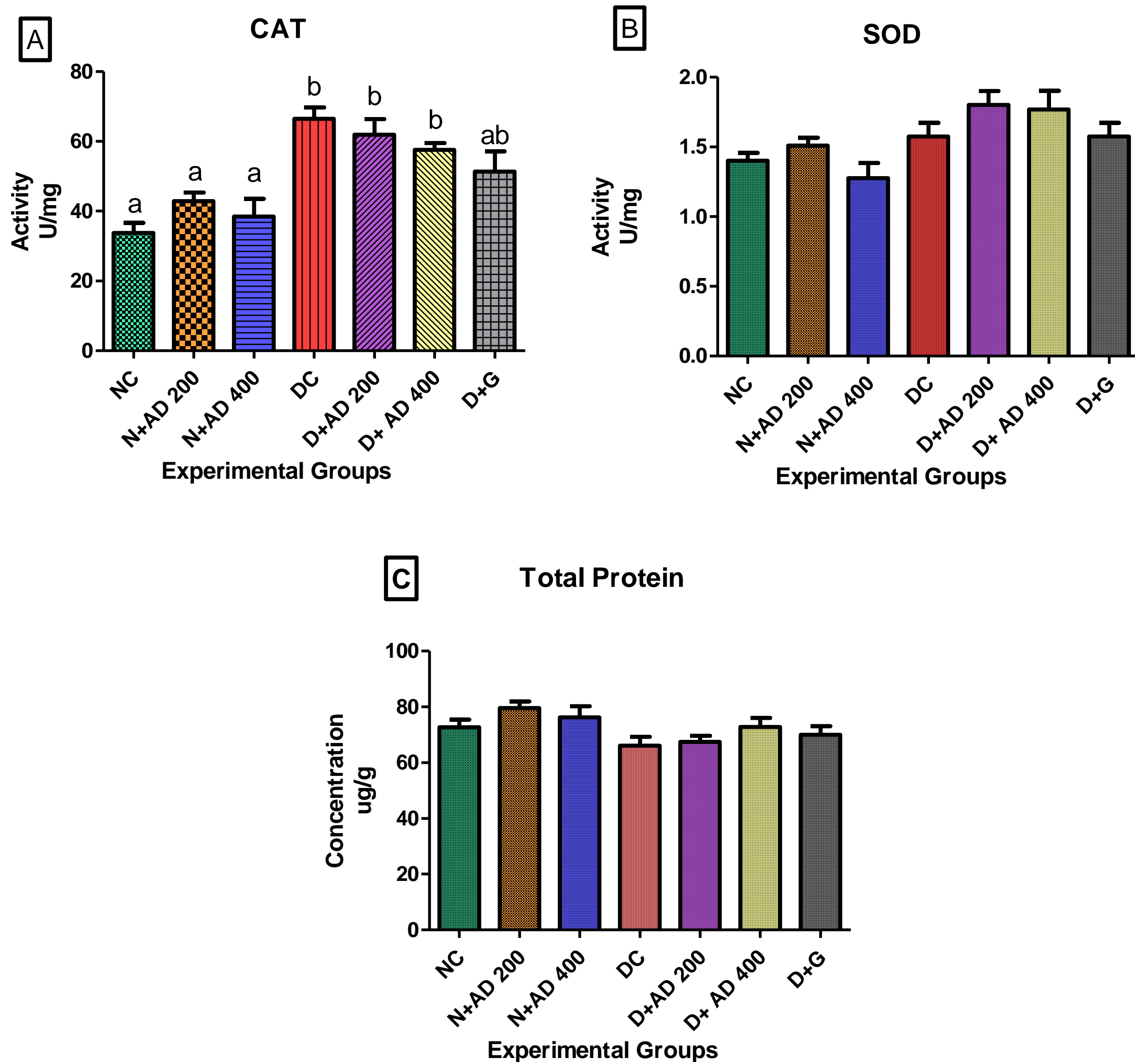


Figure 0.4: Effect of treatment with AD on the activity of **(A)** Catalase, **(B)** SOD and **(C)** Total protein in the heart of normal and diabetic rats. Bars are indicative of mean values ± SEM of group values. Bars with different letters are significantly (p<0.05) different from each other.

5.3.3 Effect of treatment with AD on antioxidant indices in the heart of normal and diabetic rats

The oxygen radical absorbing ability was significantly reduced in the hearts of untreated diabetic rats when compared to normal rats (Figure 5.5A). ORAC levels in the diabetic rats

administered 200mg/kgBW and 5mg/kgBW of AD and glibenclamide respectively were comparable to normal. Ferric reducing antioxidant power in the hearts of normal and diabetic rats were not significantly different (Figure 5.5B).

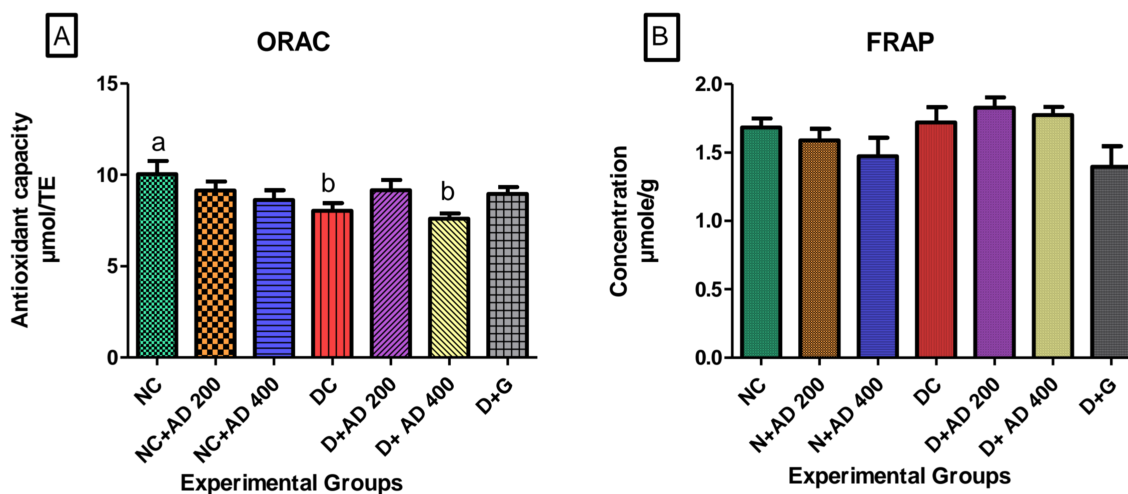


Figure 0.5: Effect of intervention with AD on the antioxidant capacities; **(A)** ORAC and **(B)** FRAP in the heart of normal and diabetic rats. Bars are indicative of mean values ± SEM of group values. Bars with different letters are significantly ($p<0.05$) different from each other.

5.3.4 AD modulated hyperglycaemia-induced immune response in the heart in T2D model

STZ injection significantly increased IL-18 in the heart of untreated diabetic rats. However, treatment with AD (400mg/kgBW) and glibenclamide (5mg/kgBW) restored it to normal levels (Figure 5.6D). Administration of 200mg/kgBW of AD caused a 16.2% reduction in IL-18 levels in diabetic hearts but not significantly at $p<0.05$. The administration of AD increased the concentration of IL-10 in the heart of normal rats, while it significantly decreased in the heart of untreated diabetic rats when compared to normal rats placed on AD (Figure 5.6C). There was no significant difference in the levels of IL-10 in normal controls and diabetic rats treated with 400mg/kgBW of AD. IL-6 and IL-1β levels were significantly reduced in the heart of diabetic rats (untreated and 200mg/kgBW) when compared with normal rats (Figure 5.6A & 5.6B). This was restored back to normal levels in diabetic rats treated with 400mg/kgBW of AD

and glibenclamide. An increase was observed in the MCP-1 levels in the hearts of untreated rats when compared with normal and treated rats, but this was not significant at 5% level of significance (Table 5.2). Injection of STZ did not have any significant difference in the TNFα concentrations in the diabetic rats compared to normal rats (Table 5.2).

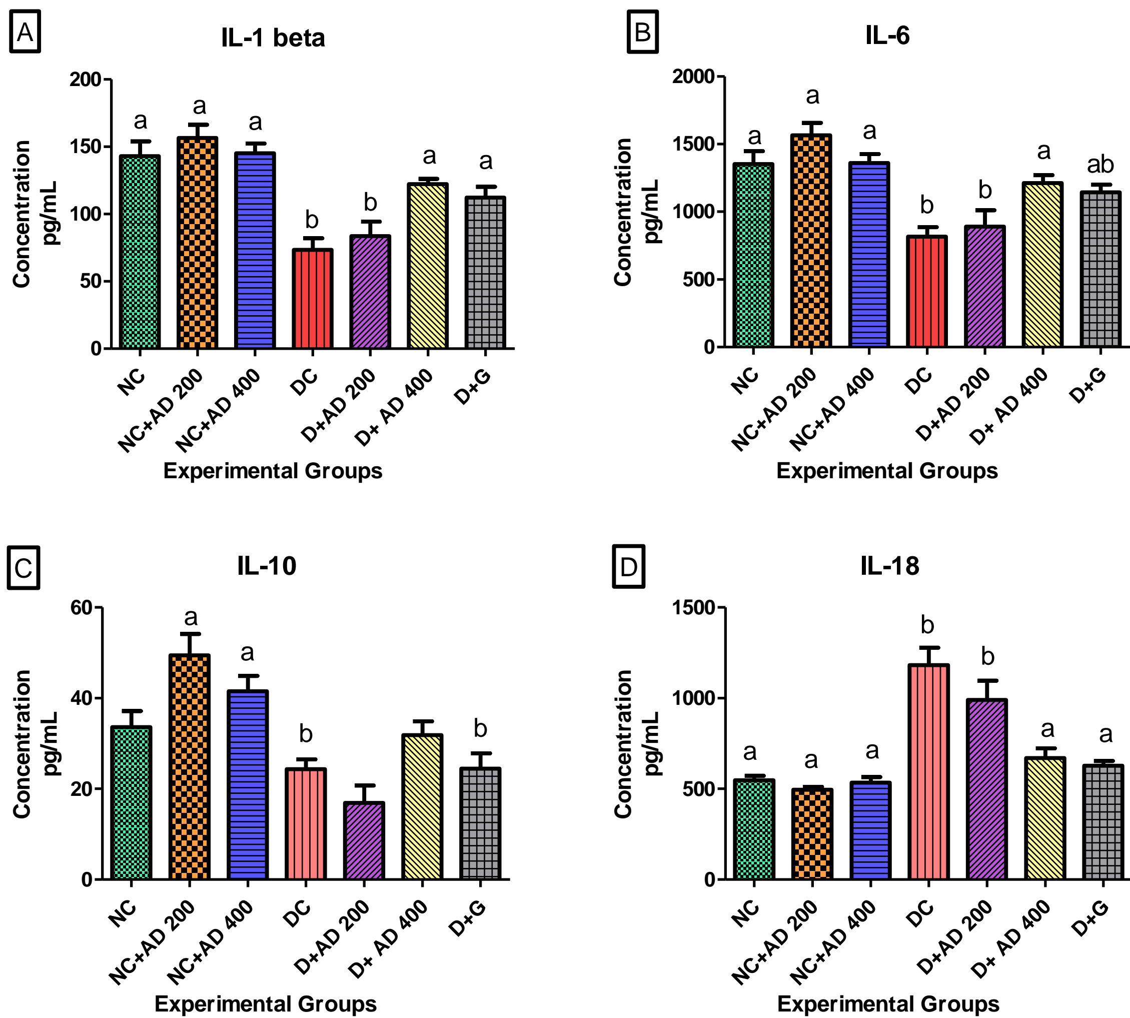


Figure 0.6: : Effect of AD administration on interleukins (IL) **(A)** IL-1β, **(B)** IL-6, **(C)** IL-10 and **(D)** IL-18 in the heart of normal and diabetic rats. Bars are indicative of mean values ± SEM of group values. Bars with different letters are significantly ($p<0.05$) different from each other.

Table 0.2: The response of TNFα and MCP-1 to treatment with AD in normal and diabetic hearts.

Experimental Groups	TNFα (pg/mL)	MCP-1 (pg/mL)
NC	56.87 ± 3.68	288.8 ± 19.34
N+AD 200	61.39 ± 2.42	272.1 ± 23.34
N+AD 400	60.05 ± 3.38	287.4 ± 15.24
DC	50.40 ± 3.06	306.3 ± 15.03
D+AD 200	48.50 ± 3.68	280.7 ± 12.25
D+AD 400	49.72 ± 2.28	301.1 ± 23.00
D+G	51.52 ± 3.06	313.1 ± 27.05

5.3.5 The effect of AD on lipid peroxidation and heart function markers

Presence of abnormal concentrations of heart fatty acid binding protein (H-FABP) in the serum is indicative of cardiomyopathy. H-FABP was measured in the serum of normal and diabetic rats. Hyperglycaemia caused a significant increase in the levels of H-FABP in the serum of diabetic rats when compared with normal rats (Table 5.3). Treatment with AD did not have any effect on the increased H-FABP in the serum of treated, diabetic rats. There was no significant difference in the TBARS concentration in normal and diabetic hearts (Table 5.3).

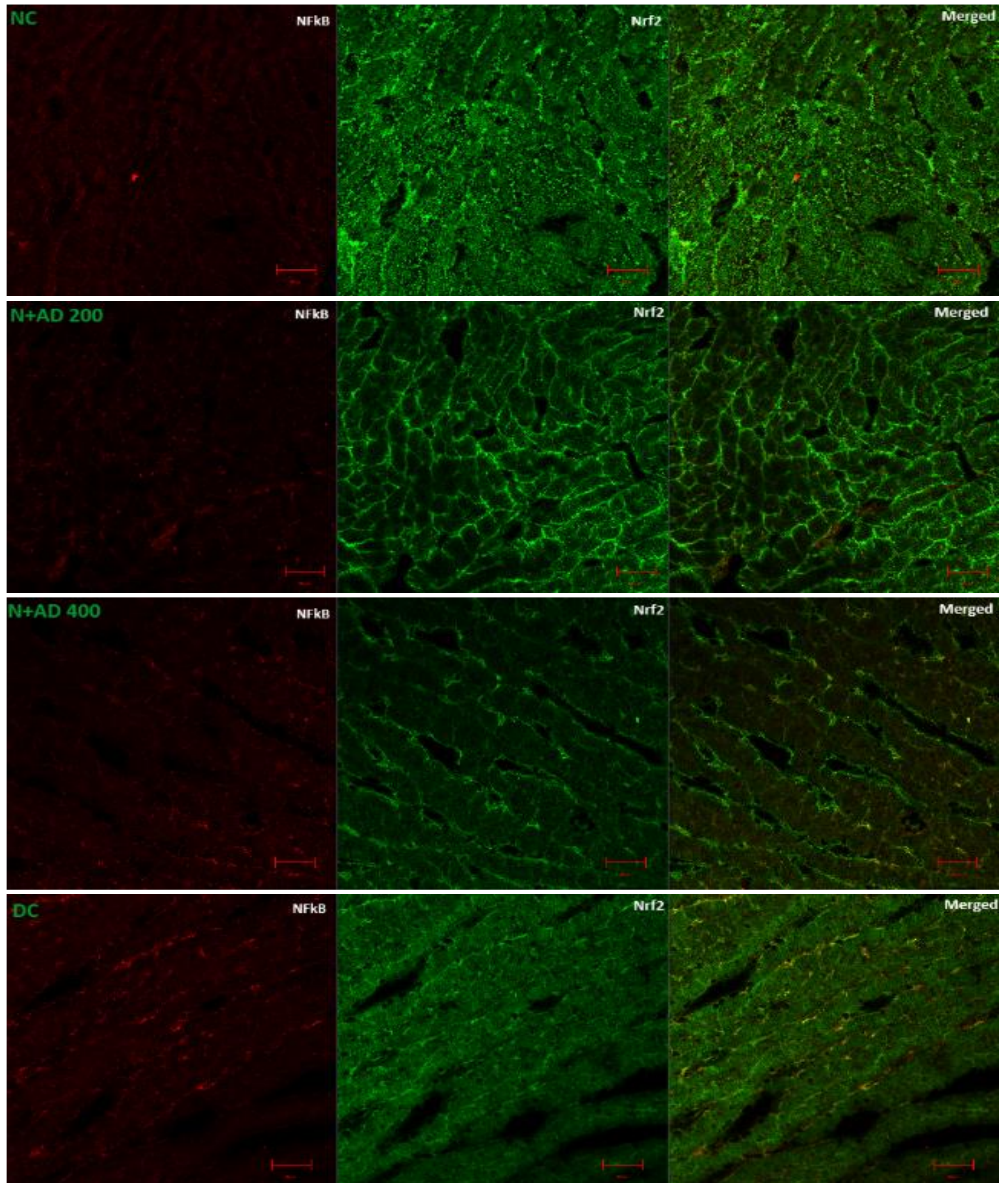
Table 0.3: Shows the effect of AD on (A) H-FABP and (B) TBARS levels in the heart of normal and diabetic hearts.

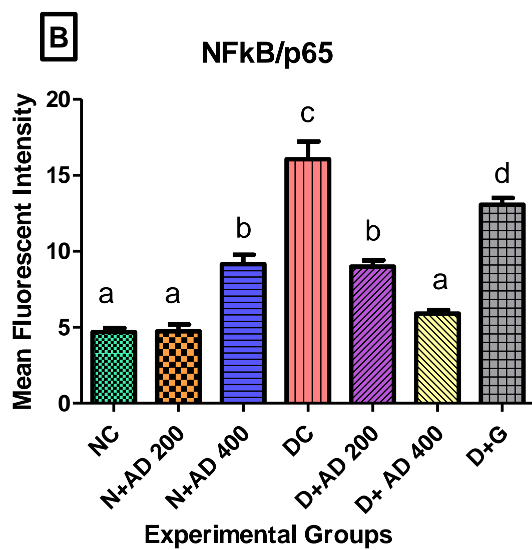
Experimental Groups	H-FABP (ng/mL)	TBARS (pmole/g)
NC	0.216 ± 0.011[a]	2.226 ± 0.15
N+AD 200	0.2784 ± 0.004	2.080 ± 0.09
N+AD 400	0.2896 ± 0.003	2.032 ± 0.16
DC	0.2955 ± 0.002[b]	1.809 ± 0.10
D+AD 200	0.2926 ± 0.006[b]	2.068 ± 0.11
D+AD 400	0.2918 ± 0.005[b]	2.371 ± 0.10
D+G	0.3121 ± 0.003[b]	1.867 ± 0.12

Values with different letters are significantly ($p<0.05$) different from each other.

5.3.6 The regulation of transcription factors by AD in T2D model

Figure 5.7A (fluorescence micrographs) demonstrates the effect of AD intervention on NFkB/p65 and Nrf2 in the hearts of normal and diabetic rats. The expression of Nrf2 and NFkB/p65 was significantly enhanced in the hearts of diabetic rats (Figure 5.7B and 5.7C). This was significantly reduced in the rats treated with 200 and 400 mg/kgBW of AD in a dose dependent manner and comparable to normal rats. 5mg/kgBW of glibenclamide had no effect on the Nrf2 expression in the diabetic hearts, but it significantly reduced the NFkB levels in treated diabetic hearts when compared to diabetic control hearts. However, the reduction of NFkB expressions by glibenclamide was not comparable to normal levels (Figure 5.7). This increase in Nrf2 and NFkB/p65 expression is suggestive of increased oxidative stress and inflammation in the diabetic rats.


NC
NFkB
Nrf2
Merged
N+AD 200
NFkB
Nrf2
Merged
N+AD 400
NFkB
Nrf2
Merged
DC
NFkB
Nrf2
Merged



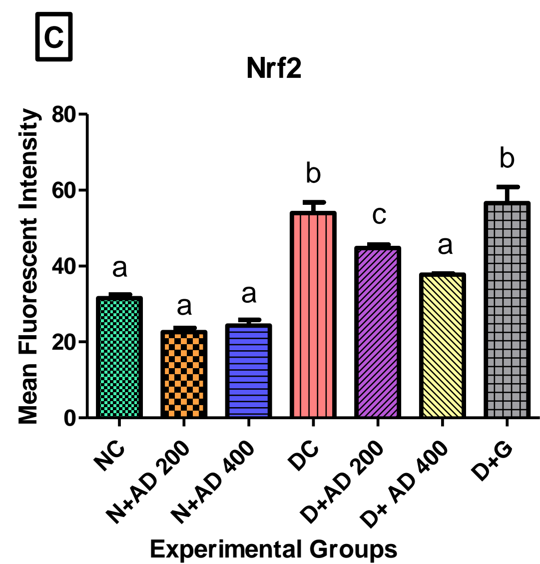
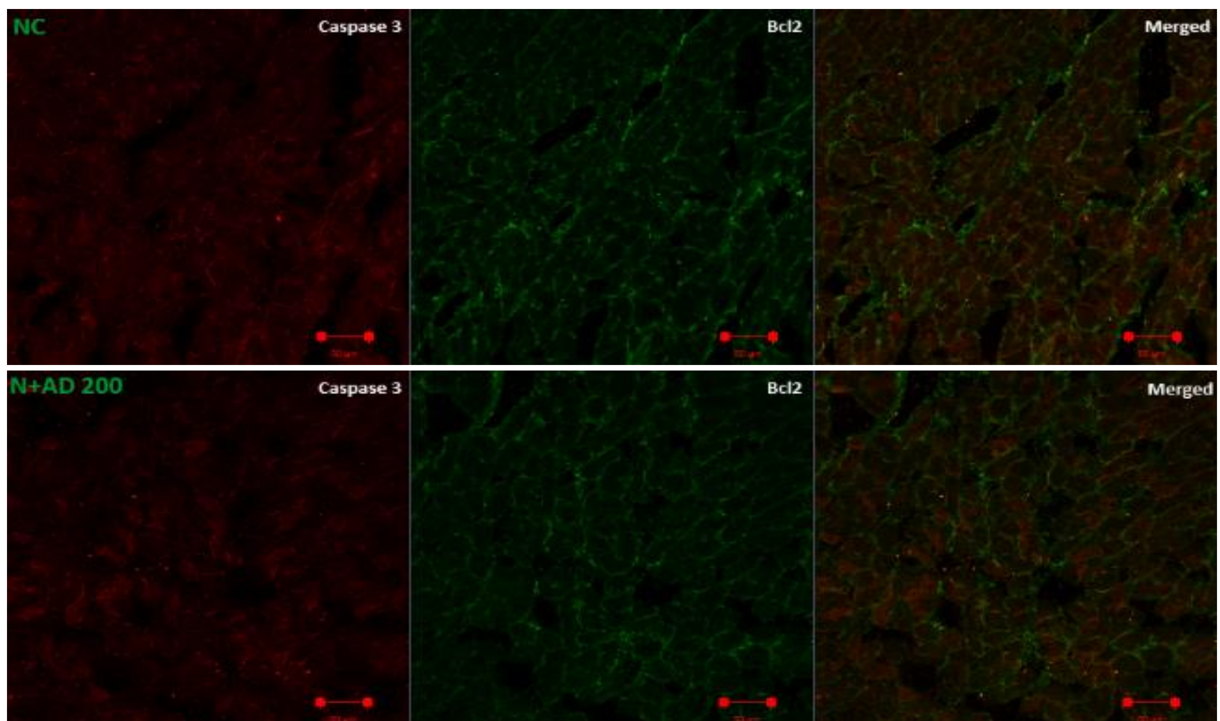


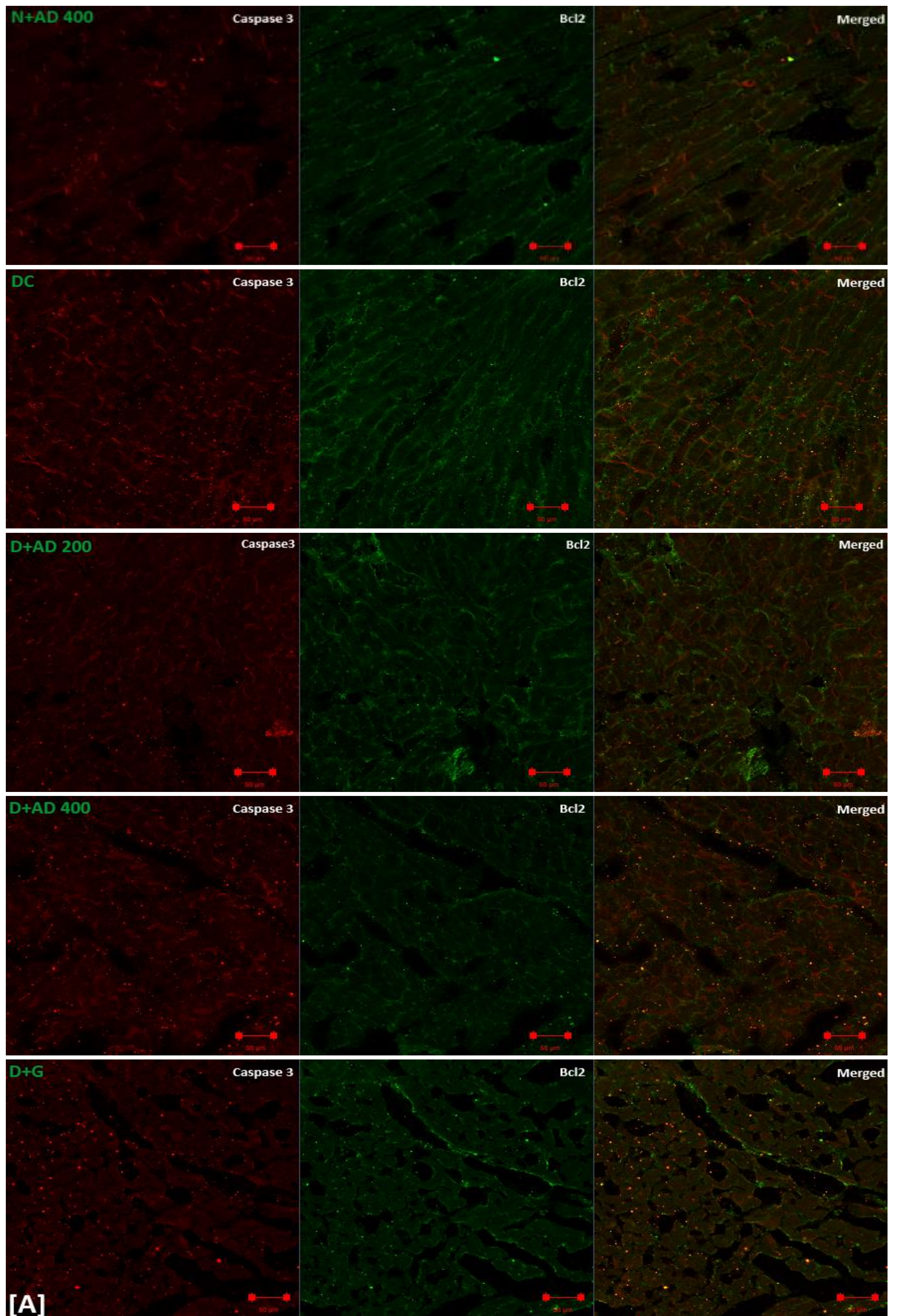
Figure 0.7: **(A)** Representatives of Confocal microscopy image showing the effect of AD on the expression of NFkB/p65 (red) and Nrf2 (green) in the heart tissues. Quantitative analysis of **(B)**

NFkB/p65 and **(C)** Nrf2 expression in the heart tissues. Bars are indicative of mean values ± SEM of group values. Bars with different letters are significantly (p<0.05) different from each other.

5.3.7 Anti-apoptotic effect of AD on T2D model

Caspase 3 is a pro-apoptotic marker, while Bcl2 promotes cell survival and inhibits the action of pro-apoptotic markers. The effect of AD administration on caspase 3 and Bcl2 in normal and diabetic rats is displayed in Figure 5.8. An increase (85.6%) was observed in the expression of caspase 3 in the diabetic hearts which was not significant at $p<0.05$ (Figure 5.8B). Conversely, there was an observable decrease (22.8%) in the levels of Bcl2 in the diabetic hearts when compared with normal rats (Figure 5.8C). Intervention with 200 and 400 mg/KgBW of AD was able to reduce the expression of Caspase 3 by 7.9%, and 9.4%, Bcl2 expression was also upregulated in the diabetic treated rats.




N+AD 400
Caspase 3
Bcl2
Merged
DC
Caspase 3
Bcl2
Merged
D+AD 200
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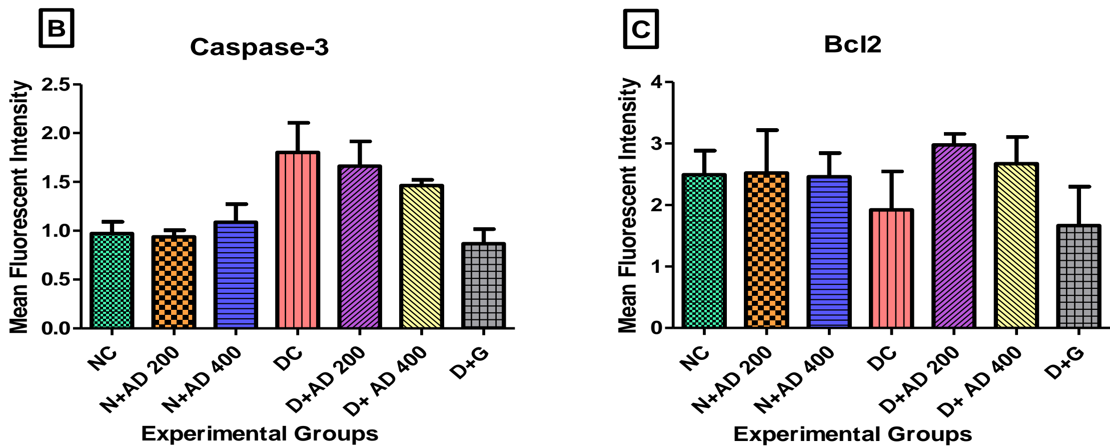

Figure 0.8: **(A)** Fluorescence micrographs showing the effect of AD intervention on apoptotic markers in the hearts of normal and diabetic rats. **(B)** Quantification of the level of expression of caspase 3 and **(C)** Bcl2 in the heart tissues.

5.4 Discussion

Organ and body weights are essential indices for evaluating the toxicity of a plant or other substances. [38]. The administration of STZ and fructose caused a significant body weight loss (24.1%), while intervention with AD significantly prevented weight loss. Rats treated with AD showed between 6.4%-8.6% weight loss. Although, 5%-10% body weight loss has been recommended as an effective strategy for glycemic control in ameliorating diabetes [39], 24.1% weight loss in diabetic control is extreme and implicative of decreased cellular metabolism and growth [40,41]. Also, the loss of insulin sensitivity prevents the utilization of glucose for energy in the cells, body burns fat and muscle as an alternative energy source [42]. Other studies have reported severe weight loss in diabetes mellitus [43,44]. An increase in relative organ weight is significant of hypertrophy [45]. Hypertrophy combined with other factors; fibrosis, oxidative stress and apoptosis in the heart are characteristics of myocardial pathology in diabetic conditions [46]. Treatment with AD was able to significantly ameliorate hypertrophy in diabetic hearts when compared with the diabetic control which alludes to the cardio-protective effect of AD against the organ impairment and related conditions caused by hyperglycemia. However, the administration of glibenclamide failed to reduce the hypertrophy in the diabetic hearts.

Activation of Nrf2 is one of the pathways initiated during oxidative stress and inflammation to mitigate the overproduction of free radicals. Increased oxidative stress due to hyperglycemia in the diabetic rats resulted in the increased activation and expression of Nrf2 in the diabetic hearts. Interestingly, certain studies reported decreased Nrf2 levels in diabetic cardiomyocytes and other associated vascular conditions [47,48]. However, the mechanism of operation by which Nrf2 is activated showed that it is constitutively expressed in higher amounts in response to oxidative stress which corroborates with our findings [18,49,50]. The expression of Nrf2 which was enhanced in the diabetic hearts was significantly reduced to near normal levels with AD therapy when compared with the untreated diabetic rats. The ability of AD to normalize Nrf2 levels can be attributed to its inherent antioxidant compounds, which scavenges the free radicals, combatting oxidative stress, hence the system has lower need to induce the expression of Nrf2. Reports showed that Nrf2 expression enhances the transcription of ARE genes thereby elevating CAT, SOD and other antioxidant capacities in the body [51]. The observed increase in the activities of CAT and SOD in this study can be related to the increased expression of Nrf2.

The insignificant decrease in lipid peroxidation in the untreated diabetic rats compared with the treated diabetic and normal rats is indicative of the increased Nrf2 expression. Studies have shown that Nrf2 inhibits lipid peroxidation and upregulates the transcription of anti-ferroptotic genes, which combats lipid peroxides and prevent ferroptotic cell death [52]. The reduced synthesis of protein in diabetic control is typical of diabetic condition and can be as a result of an increased supply of amino acids for gluconeogenesis [32,53]. Introduction of STZ altered the protein metabolism and led to decreased protein in the diabetic rats. There was no significant difference in the ORAC and FRAP levels in the heart of diabetic control, treated and normal rats. This implies that the diabetic condition and the treatment do not have significant effect on the heart ORAC and FRAP levels.

NFkB which is activated by increased oxidative stress and hyperglycemia, promotes the transcription and expression of pro-inflammatory genes and protein in the heart. These

cytokines have been linked to pathological conditions [6,13]. NFkB/p65 protein was significantly upregulated in the hearts of diabetic controls, usage of 200 and 400mg/kgBW of AD significantly diminished NFkB/p65 expression to normal levels in the diabetic hearts. The repressing effect of glibenclamide on NFkB/p65 expression in the diabetic heart is not comparable to AD. This supports ethnopharmacological claims and previous scientific findings of AD as an anti-inflammatory agent.

A relationship has been shown to exist between catalase activities and pro-inflammatory markers such as IL-1β and IL-6. The activity of catalase is inversely proportional to the concentrations of IL-1β and IL-6 [54,55]. Therefore, the significant rise in catalase activities in the untreated diabetic heart led to significantly diminished levels of IL-1β and IL-6. IL-6 can also mediate as an anti-inflammatory agent in local and systemic inflammation [56,57]. Hence, the increase in IL-6 levels in normal and diabetic rats placed on AD can suggestively be attributed to the anti-inflammatory modulation by AD. However, IL-10 concentration significantly declined in the diabetic hearts. Management with AD augments IL-10 in the diabetic heart. In addition, treatment with AD significantly abated the production of IL-18 in diabetic hearts in reference to the diabetic control. Increase of pro-inflammatory markers in the heart has been connected to cardio-dysfunction such as impaired left ventricular function [42]. The ability of AD to downregulate IL-18 and upregulate IL-10 signifies its importance in mediating cardiac pathologies associated with diabetic complications.

Mitochondrial dysfunction is one of the underlying mechanisms in the progression of DCM, and it is triggered chiefly by oxidative stress leading to distortion in the proteins, lipids and DNA in the mitochondria [58]. Mitochondrial dysfunction further upregulates pro-apoptotic proteins (cytochrome c, caspase 3, -9, -8) and cause increased apoptosis [59]. Increased ROS-mitochondrial mediated apoptosis in the cardiomyocytes, is an important factor in the development of various pathologies in diabetic hearts [60]. Bcl2 impedes mitochondrial-mediated apoptosis by blocking the activation and release of cytochrome C which in turn

initiates caspase cascades of activation that leads to cell death. Diabetic induction enhanced caspase 3 expression and led to a decline in Bcl2 expression. Supplementation with AD caused an increase in Bcl2 expression and lowered caspase 3 expression in diabetic hearts. This suggests that the ability of AD to moderate apoptosis may be one of the contributing factors in ameliorating DCM. A proposed mechanism of action for AD in ameliorating DCM by modulating apoptosis, inflammation and oxidative stress is summarized in Figure 5.9. AD reduced hyperglycemia, hence, inhibited the development of oxidative stress and was able to retard inflammation and apoptosis in diabetes by blocking the production of IL-18 and caspase 3 via the deregulation of NFkB expressions in the treated diabetic rats.

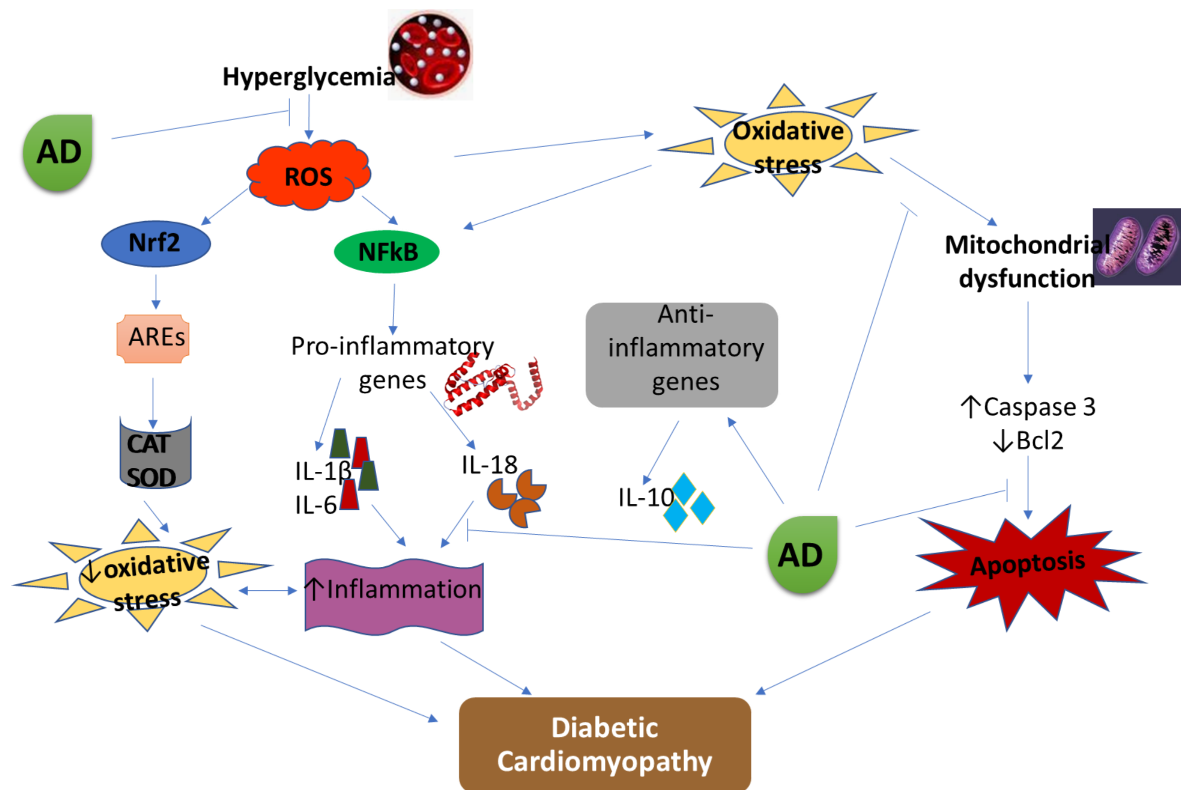

Figure 0.9: Proposed mechanism of action of AD in the management of DCM. AD-Anchomanes difformis, CAT-catalase, SOD-superoxide dismutase, ROS-reactive oxygen species, AREs-antioxidant response elements, Nrf2- nuclear factor E2-related factor 2, NFkB- nuclear factor kappa-light-chain enhancers of activated B cells

5.5 Conclusion

The ability of AD to protect against toxicity, reduce inflammation and mediate apoptosis in the heart present its potentials to mitigate diabetic cardiomyopathy. Hence, the need to consider the use of AD as an alternative therapy in the prevention and management of diabetic cardiomyopathy.

5.6 Recommendation

The ameliorating effect of AD in other diabetic complications such as nephropathy and sexual dysfunction can be explored. In addition, this study can be taken further into clinical trials to assess and validate the therapeutic effect of AD in type 2 diabetes in humans.

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Declaration OF CONFLICT

The authors do not have any conflict of interest.

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CHAPTER SIX

Treatment with *Anchomanes difformis* ameliorated kidney and pancreatic damage in type 2 diabetes

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ABSTRACT

Background

Kidney disease in diabetes is one of the common microvascular complications of diabetes mellitus implicated in end-stage renal failure. Constant hyperglycemia, oxidative stress, damaged β-cells of the pancreas, increased inflammation and apoptosis occur in diabetic nephropathy. This study explored the ability of *Anchomanes difformis* to ameliorate kidney and pancreatic damage in type 2 diabetes mellitus.

Methods

Two weeks of fructose (10%) administration, followed by single intraperitoneal injection of streptozotocin (40mg/kg) were used to induce type 2 diabetes in male Wistar rats. Leaf extract (aqueous) of *Anchomanes difformis* (200mg and 400mg/kgBW) was administered orally for six

weeks. Body weights before and after interventions were monitored. Urea and creatinine were measured as markers of kidney function. Interleukins (IL)-1β, IL-6, IL-10, IL-18, and TNFα were measured in the kidney lysate as indices of inflammation. CAT, SOD, ORAC, FRAP, and MDA levels were also evaluated in the kidney. Expressions of transcription factors (Nrf2 and NFkB/p65) and apoptotic markers (Bcl2 and caspase 3) were investigated in the kidney tissues. Histological sections of the pancreas and kidney tissues were examined for any visible pathology.

Results

Supplementation with *Anchomanes difformis* enhanced antioxidant status, modulated inflammatory response, and reduced apoptosis in the kidney. It also restored the kidney and pancreatic histoarchitecture of the treated diabetic rats.

Conclusion

The pathophysiology associated with diabetic nephropathy and pancreatic damage showcase the importance of exploring the use of antidiabetic, nephroprotective agents such as *Anchomanes difformis* to prevent or delay the progression of kidney damage in the management of type 2 diabetes.

Keywords: *Anchomanes difformis,* apoptosis, diabetes, glibenclamide, inflammation, nephropathy, oxidative stress

6.1 Introduction

Kidney disease in diabetes (refers to as diabetic nephropathy-DN) is a common complication of type 2 diabetes mellitus (T2DM) and has become one of the major causes of kidney failure [1,2]. It is widely characterised by abnormalities in the architecture of the renal tissues such as expansion of the mesangial matrix, glomerular hypertrophy, thickening of the glomerular and tubular basement membrane [3]. Abnormal values of urea, creatinine, albumin and other proteins in the serum and urine are typical observations in diabetic subjects [2]. Administration of fructose and low dose of STZ causes partial disruption of the beta cells leading to less production of insulin and hence, hyperglycemia with insulin resistance [4,5]. Hyperglycemia leads to oxidative stress by influencing the generation and overproduction of ROS through the increased activation of the polyol pathway, protein kinase C (PKC) and formation of advanced glycation end-products (AGEs) [1,6]. An imbalance in the production of ROS and the antioxidant (such as catalase and superoxide dismutase) results in oxidative stress [7,8]. Oxidative stress induced by hyperglycemia can further inhibit the production of insulin by the beta cells of the pancreas. This occurs through hyperglycemia-mediated activation of uncoupling protein-2 leading to leakage of protons and reduced ATP/ADP ratio in the beta cells. ROS produced as a result of constant hyperglycemia can also occasion lipid peroxidation in the cell membrane resulting in beta cell damage [1,9]. The excessive production of ROS such as superoxide anion in pancreatic beta cells can activate stress-signalling pathways, which further induces inflammatory mediators, cytokines and apoptotic transcription factors such as NFkB, this leads to beta cell death and reduced insulin production [9].

NFkB is upregulated during oxidative stress in most cells of the kidney including tubular cells, mesangial and endothelial cells and the podocytes [10]. The activated NFkB causes the transcription of proinflammatory genes coding for cytokines (TNFα, IL-1β, IL-2, IL-6, IL-12, and IL-18) and chemokines (MCP-1). NFkB also enhances the transcription of profibrotic genes responsible for growth factors (TGF-β) and leukocyte adhesion molecules (E-selectin, VCAM1,

and ICAM-1). The expression of these proinflammatory and profibrotic proteins triggers inflammation, atherosclerosis and vascular dysfunction [11].

Cell death is a key factor in the progression of DN [12]. Chronic exposure of tissues to oxidative stress sufficiently induces a wide range of pathophysiological events that leads to eventual cell death [3]. In normal tissues, unwanted cells are removed by apoptosis thereby maintaining tissue homestasis, however, in damaged cells, apoptosis is triggered thereby activating cell-death receptors such as TNFRs [12–14]. As a result of the inappropriate activation of these intracellular signalling pathways, an imbalance occurs between cell death and cell proliferation in the damaged tissues in diabetes [15,16]. The expression of Bcl2; an anti-apoptotic protein, is majorly detected in the proximal and distal tubules and in the capsular parietal cells in normal rats and humans [17]. Interestingly, a loss of balance has been recorded in DN between pro- and anti-apoptotic markers, such as the decreased expression of anti-apoptotic Bcl2 protein [18]. Furthermore, increased expression of Growth factors such as TGF-B are induced by hyperglycemia and oxidative stress in the glomerulus and other tissues. The upregulation of TGF-B promotes further, the generation of ROS by activating NADPH oxidase and mitochondrial respiratory process resulting to aggravated TGF-B-induced apoptosis and detachment of the podocyte [19,20].

ROS-mediated renal injury finally progresses into chronic kidney disease by evoking hemodynamic dysregulation and abnormalities in the structure and function of the nephron [21]. The damaged glomerulus and its filtration barrier increase permeability of plasma proteins such as albumin (albuminuria); a crucial process involved in the etiology of DN [3]. Elevated proteins (in the tubular ultrafiltrate) in the presence of increased ROS elicit various aberrant signaling pathways [3]. The increased activation of these impaired signalling mediators which includes transcription factors, inflammatory agents, growth factors, and vasoactive molecules causes several pathological events in the glomerulus and the tubules promoting renal injury

from a progressive state to an end-stage renal failure [3]. Figure 6.1 summarizes the key pathways and mediators involved in the progression and pathogenesis of DN.

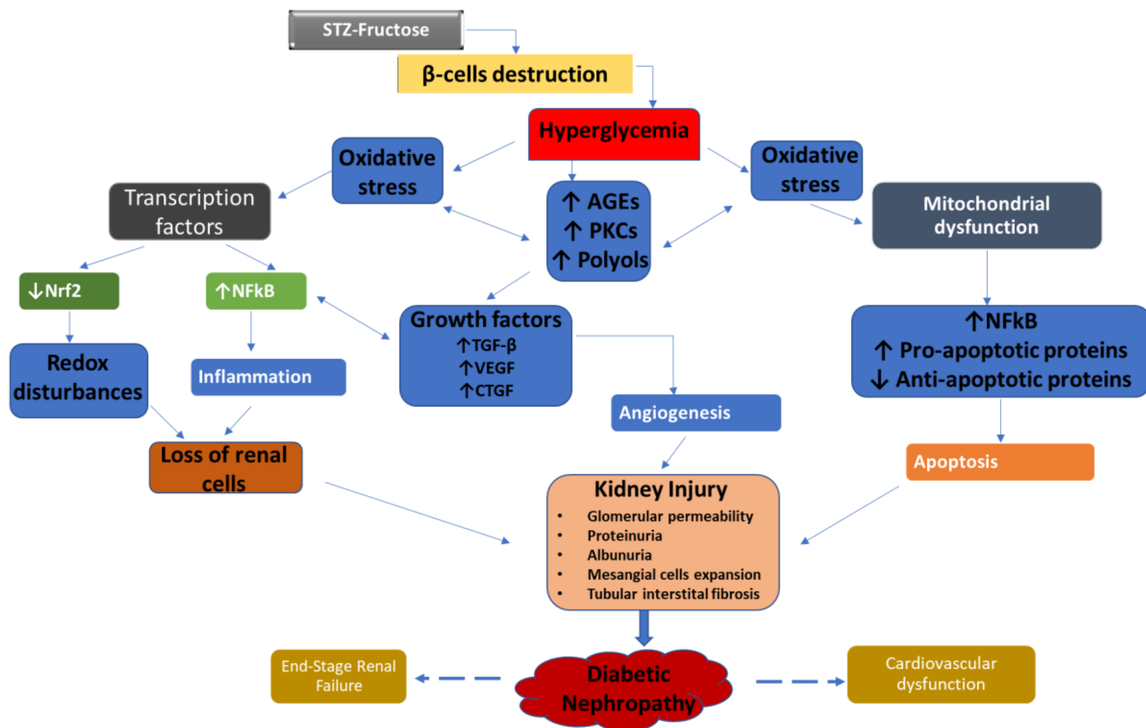


Figure 0.1: Pathogenesis of diabetic nephropathy.

There is a paradigm shift towards the use of medicinal plants in the management of pathological conditions and diseases, due to its cost effectiveness, with little or no adverse effects when appropriately used. It is also more available when compared with conventional drugs [22,23]. *Anchomanes difformis* (AD) has been used traditionally as a treatment against varying pathological conditions including diabetes, kidney pains, asthma, pain and wounds, microbial infections, and gastrointestinal related problems. Most of these folkloric uses and claims have been proven with scientific studies, while others are still indigenous claims [24]. We carried out a study on the identification and characterization of the bioactive compounds present in six different extracts of AD using HPLC and UPLC-MS, antioxidant capacities of these extracts were investigated. Aqueous extract exhibited the highest antioxidants ability [25]. AD extracts especially aqueous, contains phytochemicals such as quercetin, phloridzin, kaempferol, rutin, chlorogenic acids amongst others which are active against hyperglycaemia,

oxidative stress, inflammation and apoptosis [25,26]. The wide range of biological properties of compounds present in AD has necessitated the need to further investigates its potentials against diabetic complications. Antidiabetic ability of AD has been established and reported [27,28], however, no study has been conducted to investigate the potency of AD leaves on the kidney and pancreas pathology in diabetes mellitus. This study therefore investigates the ameliorative potential of AD in hyperglycemia-induced kidney and pancreatic damage using in diabetic male Wistar rats.

6.2 Methodology

6.2.1 Plant preparation

The leaves of AD were harvested and authenticated at the Herbarium; University of Lagos, Nigeria and a specimen was deposited (LUH6623) in the Herbarium. The harvested leaves were dried under shade, milled and de-oiled using n-hexane (10% $^{W}/_{V}$) for 2 days. The aqueous extraction was carried out using cold-stirred extraction method, where the defatted leaves were soaked in water (5% $^{W}/_{V}$) for 2 days at 2-8°C. The extract was freeze-dried and stored at -20°C for further analysis.

6.2.2 Ethical consideration

Ethical approval for this study was granted by the Research Ethics Committee (REC) of the Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville, South Africa (CPUT/HW-REC 2016/A4) and the Ethics Committee for Research on Animals at the South African Medical Research Council (SAMRC), South Africa (REF.04/17), where the animal experiment was conducted.

6.2.3 Animals

64 male Wistar rats weighing approximately 180 ± 10g were procured for this study from the Animal facility in Stellenbosch University, South Africa. The animals were accommodated at the Primate Unit & Delft Animal Centre (PUDAC), SAMRC and made to adapt to the

environment for 3-4 weeks. Housing conditions were controlled: humidity- 45% to 55%, temperature- 22°C to 26°C. They were exposed to normal photo period (12hour dark/12hour light) and fed with standard rat chow (SRC). Animal handling, care and other procedures were done in accordance with the standard operating procedure of SAMRC PUDAC (SOP No: 2016-R01) which conforms to the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

6.2.4 Modelling and grouping

After acclimatization, the rats (with weights between 270g and 300g) were indiscriminately grouped into 7 with a minimum of eight rats in each group (Figure 6.2). The diabetic models received 10% fructose for 2 weeks followed by intraperitoneal injection of 40mg/KgBW streptozotocin (STZ). Water and citrate buffer served as vehicles for fructose and STZ in normal rats. Group 1 served as the negative controls (non-diabetics) and received vehicle only (NC). Animals in group 2 and 3 served as treatment controls (non-diabetic treated rats) and were placed on 200mg and 400mg/KgBW of AD (N+AD 200 and N+AD 400 respectively). Group 4 was the positive control (untreated diabetic rats) and were given vehicle only (DC). Group 5, 6 and 7 were treated diabetic groups placed on 200mg, 400mg of AD and 5mg glibenclamide respectively (D+AD 200, D+AD 400 and D+G).

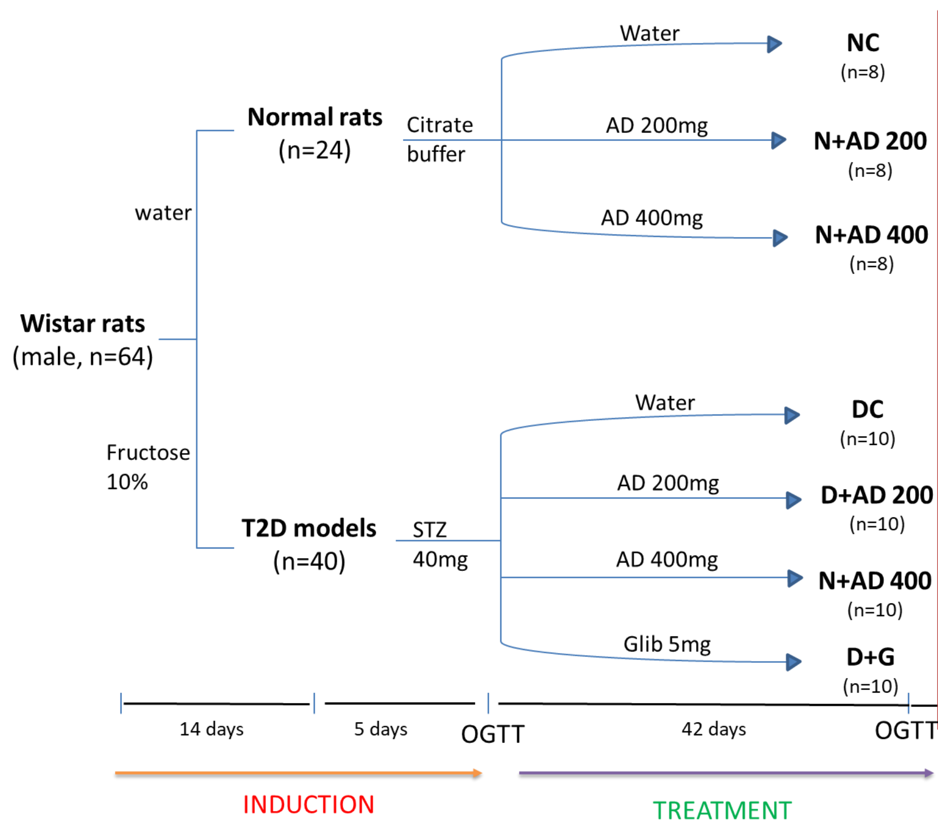


Figure 0.2: Experimental design. Animals were randomly assigned into 7 groups (n≥8). 14 days' administration of 10% fructose preceded a single-dose injection of STZ (40mg/kg). After 5 days, animals with fasting blood glucose of 15mmol/L or greater were considered diabetic. OGTT was conducted to confirm insulin resistance. Normal rats were administered the vehicle; water and citrate buffer (CB) correspondingly. Treatment commenced immediately for 42 days via oral gavage. Animals were euthanized on the 43rd day (red bar).

6.2.5 Sample collection

The rats were euthanised after the treatment period. The rats were anaesthetized prior to euthanasia with 2% isoflurane per oxygen (1L/min flow rate) via inhalation. Blood was collected from the abdominal vein into the Z-serum clot activator tubes. The kidneys and pancreas were immediately harvested, washed in ice-cold phosphate buffer and weighed. Blood samples were centrifuged at 4,000 g for 10 min at 4°C for serum yield and aliquoted. The serum and tissues were frozen in liquid nitrogen and later stored at -80^{0}C for further analysis.

6.2.6 Tissue Preparation

The kidney and pancreas samples for histological examination were immediately fixed in 10% buffered formalin solution. Kidney samples that will be used for immunofluorescence assays were fixed with a freezing media, froze in the liquid nitrogen and stored at -80 ˚C. Homogenization of kidney tissues (200mg) for other assays was carried out on ice in 2mls of 50mM phosphate buffer with 0.5% triton and centrifuged at 10,000 g for 15 minutes at 4^0C. The supernatants were aliquoted and stored at -80 ˚C.

6.2.7 Estimation of organ function and toxicity markers

Serum levels of urea and creatinine were measured in the normal and diabetic rats as indices of organ function. The estimation of urea and creatinine in the serum was done on an ABX Pentra 400 Chemistry Analyzer (Horiba) using Horiba kits (Montpellier, France) and performed following the manufacturer's guidelines. The body weight measurements of all the animals were recorded every week till euthanasia. Relative kidney or pancreas weight for each rat was calculated using the kidney weight or pancreas weight and the weight of the same animal.

$$Relative\ kidney/pancreas\ weight = \frac{Kidney/pancreas\ weight\ (g)}{Total\ body\ weight\ (g)} \times 100\ \%$$

6.2.8 Evaluation of antioxidant status and oxidative stress markers

The activities of antioxidant enzymes; CAT and SOD was done in the kidney homogenates according to the method of Ellerby and Bredesen [29]. Other non-enzymic antioxidant indices such as ORAC and FRAP were used to also evaluate the antioxidant status in the kidney. ORAC assay was carried out following the method of Prior and colleagues [30], while FRAP was determined using the method described by Benzie and Strain [31]. Lipid peroxidation was measured to assess oxidative stress status in the kidney by determining TBARS levels in the kidney homogenates using the combined methods of Matsunami *et al.* and Wasowicz *et al.* [32,33].

6.2.9 Measurement of inflammatory markers

The determination of the levels of interleukins (IL)-1β, IL-6, IL-10, IL-18 and TNFα were determined in the kidney lysate of normal and diabetic rats. Bioplex Promagnetic bead-based assays (Bio-Rad Laboratories, Hercules, USA) were used for the measurement of these inflammatory markers. Assays were performed according to the manufacturer's instructions (BioRad and Merck Millipore). Bead acquisition and analysis of median fluorescent intensities was done using Bio-Plex Manager software, (version 6.0).

6.2.10 Quantification of the expression of transcription and apoptotic proteins

Immunofluorescence staining and imaging was done in the kidney tissues to estimate the expression of transcription factors (Nrf2 and NFkB) and apoptotic proteins (Caspase-3 and Bcl2). Frozen tissues were sectioned (10μm) on a cryostat (Leica CM 1860 UV Cryostat) and permeabilized with 1x PBS containing 0.025% Triton X-100 (PBS-T). Blocking of tissues was done using 10% normal goat serum in PBS and 5% bovine serum albumin for 2 hours. Tissues were incubated with primary antibodies overnight at 4°C and secondary antibodies for 1 hour in the dark. Washes were done after each incubation with PBS-T and PBS respectively. The slides were mounted (Dako mounting medium, Agilent Technology Inc, South Africa) and imaged with a Zeiss LSM780 ELYRA PS1 super-resolution, confocal microscope with a 10x/0.3 M27 objective (EC "Plan-Neofluar"). The argon multiline laser at 488nm and DPSS 561-10 laser at 561nm was used to excite the Alexa Fluor 488 (green) and Alexa Fluor 594 (red) respectively. Zen 2.6 imaging software (blue and black edition, Zeiss Germany) were used for image analysis and to obtain mean fluorescent intensities (MFI) on four images acquired in each experimental condition of three biological repeats.

6.2.11 Histological examination of the kidney and pancreas

The fixed tissues were dehydrated using graded series of alcohol, embedded in paraffin wax, sectioned (5μm), deparaffinized, rehydrated and stained with haematoxylin and eosin (H&E)

dyes. Slides were mounted and examined under a light microscope. Photomicrographs were taken using digital camera (Motic software).

6.2.12 Statistical analysis

Values are expressed as mean ± standard error of mean (SEM). Statistical analysis of the results was performed using one-way analysis of variance (ANOVA) to find differences between groups. Bonferroni test was used for all pair-wise comparisons. Differences (F values) were considered statistically significant at p values less than 0.05. All statistical calculations were done using GraphPad Prism Version 5.00 for Windows, GraphPad Inc., San Diego, California USA.

6.3 Results

6.3.1 Effect of treatment with AD on the relative weight of the kidney and pancreas

Figure 6.3 shows the relative weight of the kidney and pancreas in normal and diabetic rats. Induction of diabetes using fructose and STZ led to a significant increase in the relative weights of the kidney and pancreas in the untreated diabetic rats (positive control). However, intervention with 200mg and 400mg/KgBW AD lowered relative kidney weight by 9.1% and 10.7% respectively (Figure 6.3A). Glibenclamide (5mg/KgBW) also reduced relative kidney weight by 6.4% in the treated diabetic rats. Similarly, relative pancreatic weight was significantly increased in the positive control when compared with the normal rats (Figure 6.3B). The relative pancreatic weight was minimized in the treated rats by 15.9% and 14.6% following the administration of 200mg and 400mgKgBW AD. This is comparable to the activity of glibenclamide which had a 20% reduction on the relative pancreatic weight (Figure 6.3B).

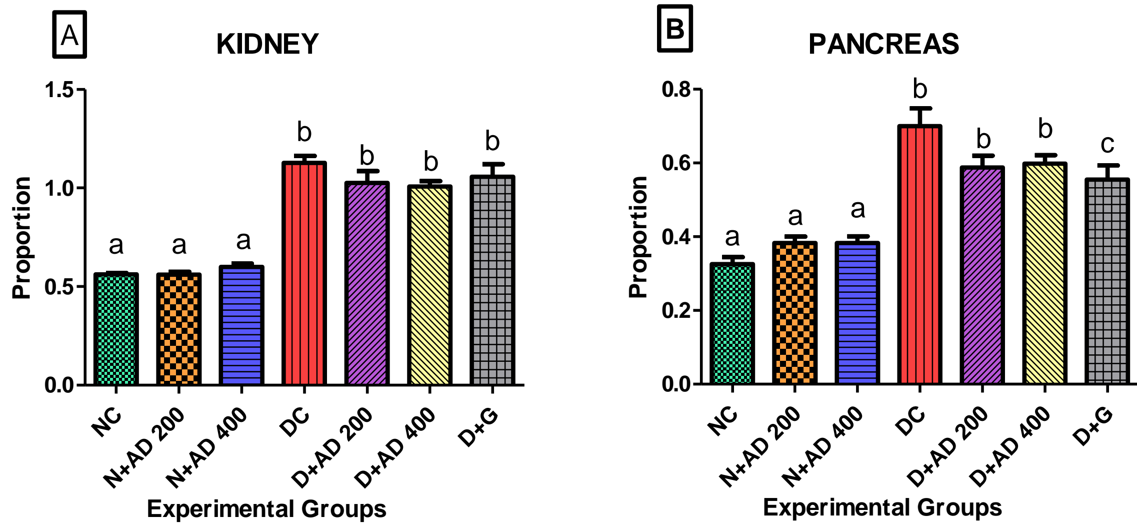


Figure 0.3: Effect of AD administration on the **(A)** relative kidney weight and **(B)** relative pancreas weight of normal and diabetic rats. Bars are indicative of mean values ± SEM of group values. Bars with different letters are significantly ($p<0.05$) different from each other.

6.3.2 Effect of AD administration on kidney function markers

Hyperglycemia caused by STZ and fructose administration resulted in significant increase (by 3 folds) in the serum levels of urea in the diabetic rats. Treatment with 400mg/kgBW and 200mg AD abated the urea concentration by 22.5% and 4.5% in treated diabetic rats when compared with untreated diabetic rats (Figure 6.4A). Administration of 5mg/kgBW glibenclamide did not reduce urea concentration in diabetic kidneys when compared to the untreated diabetic kidneys. Creatinine levels in the serum was not significantly affected by either hyperglycemia or treatment with AD (Figure 6.4B).

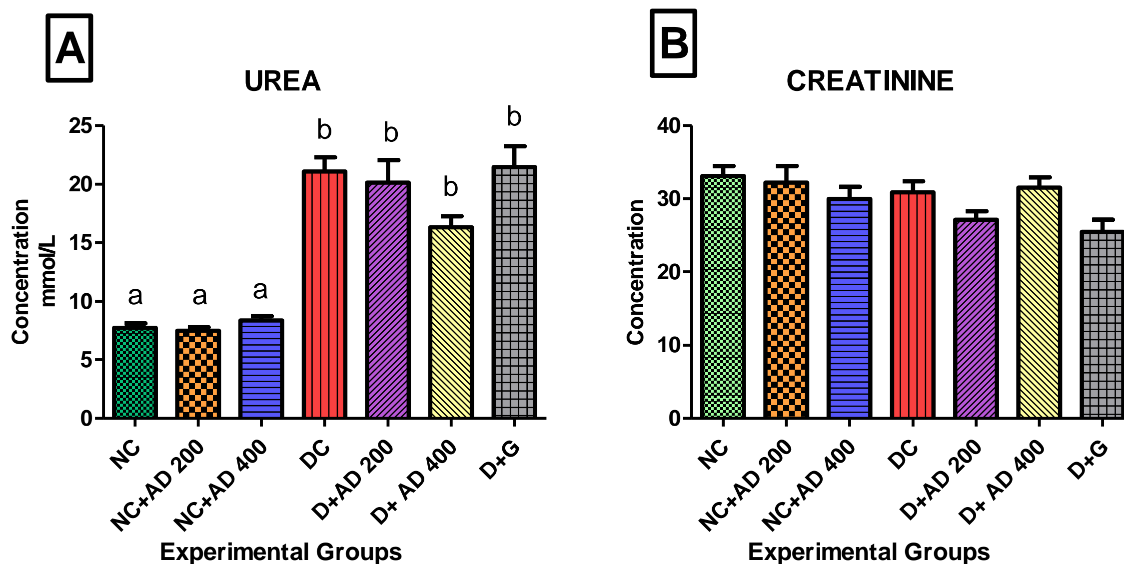


Figure 0.4: Effect of AD administration on the **(A)** urea and **(B)** creatinine concentration in the serum of normal and diabetic rats. Bars are indicative of mean values ± SEM group values. Bars with different letters are significantly ($p<0.05$) different from each other.

6.3.3 AD enhanced the antioxidant status in the kidney

The effect of AD and glibenclamide on the antioxidant indices (CAT, SOD, ORAC and FRAP) and lipid peroxidation marker (TBARS) is presented in Figure 6.5(A-E). The administration of AD significantly increased CAT levels in the normal rats treated with 200 and 400mg of AD (Figure 6.5A). Conversely, a significant decrease (20.2%) in the activity of CAT was observed in the diabetic control when compared with the normal rats. However, supplementation with 200mg and 400mg/KgBW of AD led to about 13% increase in the activity of CAT in the treated groups when compared with untreated diabetic rats (Figure 6.5A). In addition, the activity of SOD was increased by 27% and 25% in the normal rats treated with 200mg and 400mg/KgBW respectively when compared with the normal control. There was no significant difference in the ORAC, FRAP and TBARS levels in the kidney of diabetic control, treated and normal rats.

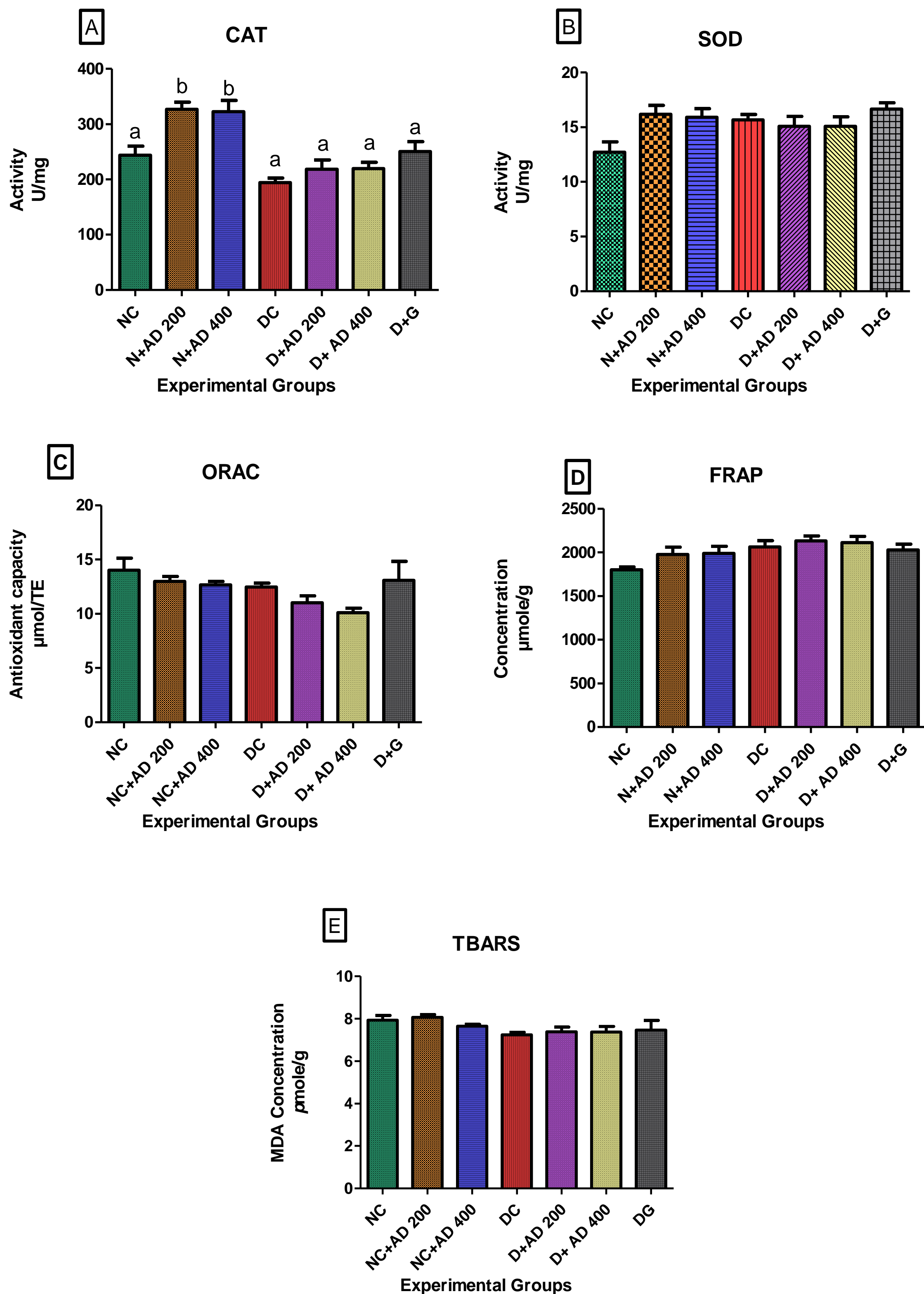


Figure 0.5: Effect of intervention with AD on the antioxidant capacities; **(A)** CAT, **(B)** SOD, **(C)** ORAC **(D)** FRAP and **(E)** Lipid peroxidation in the kidney of normal and diabetic rats (TBARS). Bars are indicative of mean values ± SEM of group values. Bars with different letters are significantly ($p<0.05$) different from each other.

6.3.4 AD modulated hyperglycaemia-induced immune response in the kidney

Induction of diabetic condition triggered increased inflammatory response in the diabetic rats as IL-1β and IL-6 were significantly elevated in untreated diabetic rats (Figure 6.6A & 6.6B). Intervention with 400mg AD and 5mg glibenclamide showed a significant reduction in the IL-1β levels in the treated diabetic kidney. An appreciable decrease (11%) was observed in the kidney levels of IL-6 of rats treated with both concentrations of AD and glibenclamide, when compared with untreated diabetic rats. There was an 8.6% increase in the kidney levels of IL-18 in the untreated diabetic rats (Figure 6.6D). However, 200mg and 400mg/KgBW of AD significantly abated IL-18 in the kidney of treated diabetic rats by 14.7% and 6.3% respectively when compared with the positive control. It is noteworthy that supplementation with 200mg and 400mg/KgBW AD decreased IL-18 levels by 12.2% and 10.9% in the normal treated kidneys when compared with the normal control rats (Figure 6.6D). This same trend was observed in the TNFα levels in the treated normal kidneys (Figure 6.6E). 200mg and 400mg/KgBW AD curtailed the expression TNFα by 10% and 5% respectively in the non-diabetic treated rats. Diabetes triggered TNFα response (15%) in untreated diabetic kidney. 200mg and 400mg/KgBW AD supplementation reduced TNFα by 14.4% and 6.3% respectively in treated diabetic rats when compared with diabetic control (Figure 6.6E). IL-10 levels were significantly increased in the kidney of diabetic control rats, this was modulated in diabetic rats placed on AD extract, when compared to the diabetic control (Figure 6.6C). Similar trend was observed in the diabetic rats treated with glibenclamide.

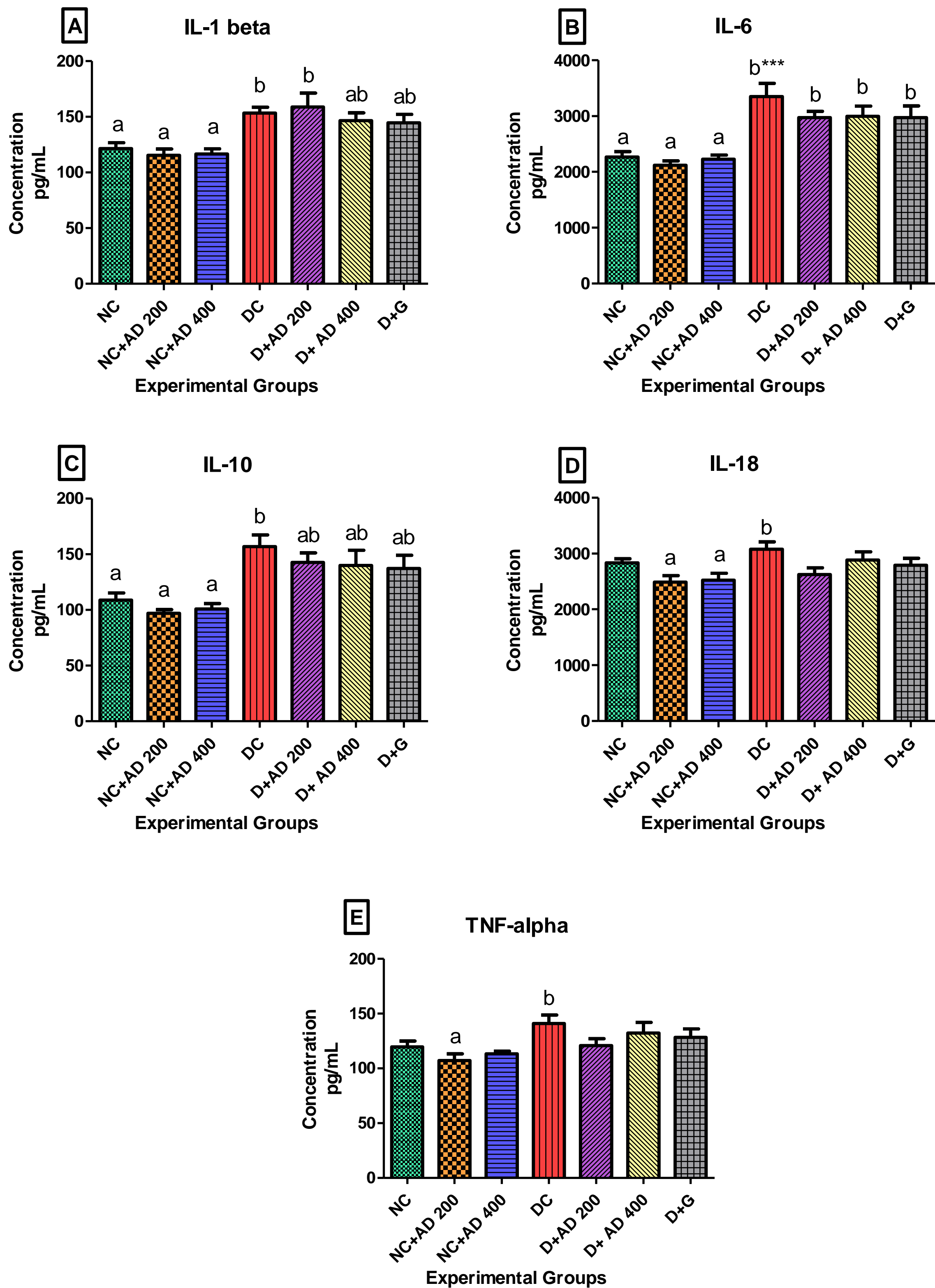
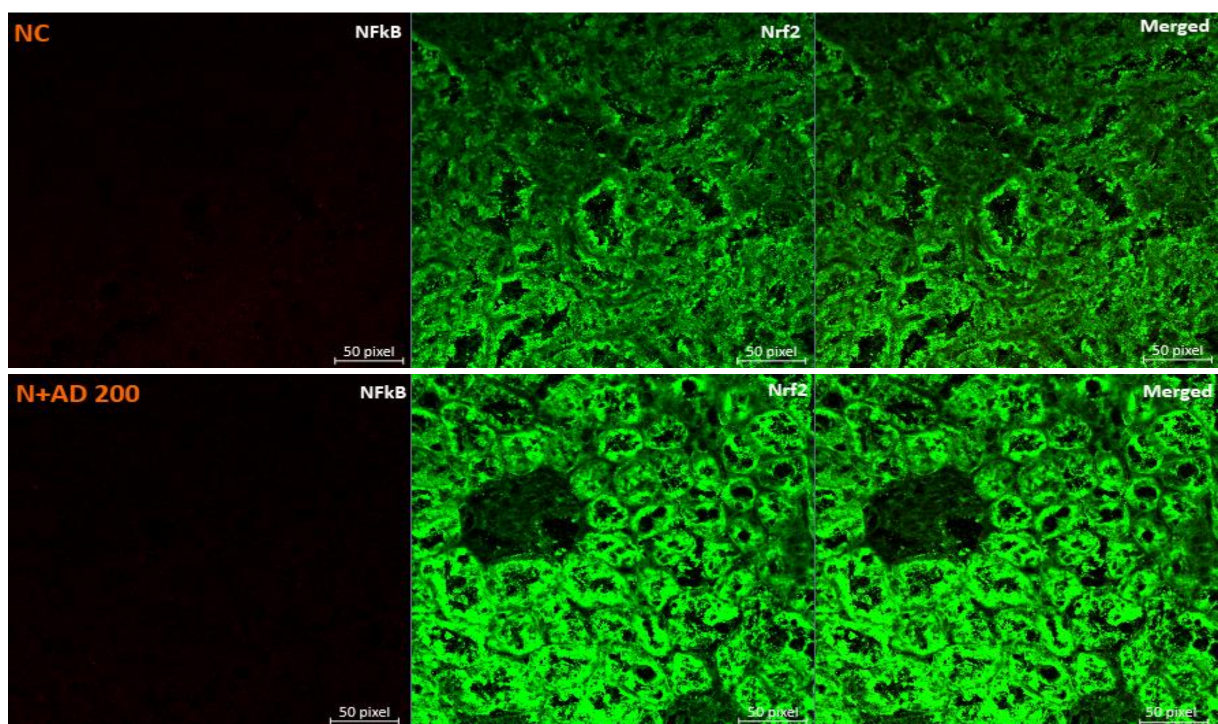
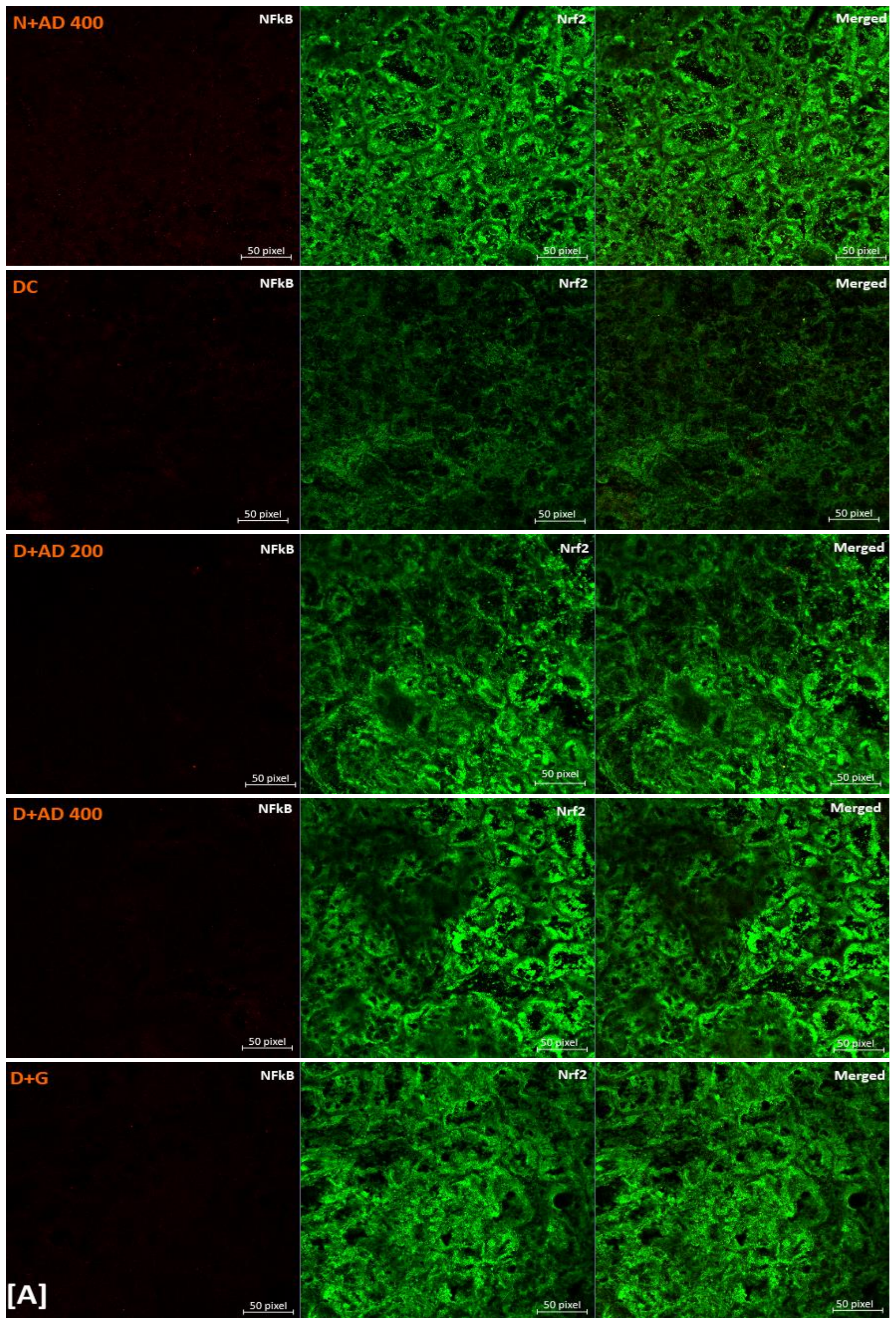


Figure 0.6: Effect of AD administration on interleukins (IL) **(A)** IL-1β, **(B)** IL-6, **(C)** IL-10 **(D)** IL-18 and **(E)** TNF-alpha in the kidney of normal and diabetic rats. Bars are indicative of mean values ± SEM of group values. Bars with different letters are significantly ($p<0.05$) different from each other, while bars with *** is significantly different at $p<0.0001$.

6.3.5 Effect of AD supplementation on transcription factors in normal and diabetic rats

NFkB expressions in the kidney of untreated diabetic rats were upregulated as depicted by the 35% increase in the MFI when compared with the normal control rats (Figure 6.7B). Management with 200mg and 400mg/KgBW of AD brought about 50% and 25% reduction in the NFkB/p65 levels in the treated diabetic kidney. Treatment with glibenclamide also led to 43% reduction in the expressions of NFkB/p65. This was comparable to the levels observed in the normal rats (Figure 6.7B). The expressions of Nrf2 were significantly enhanced in the treated normal rats placed on 200mg and 400mgKgBW of AD (Figure 6.7A). This was significantly dwindled (40%) in the untreated diabetic rats. Intervention with 200mg and 400mg/KgBW AD elevated Nrf2 expressions (40% and 27.3% respectively) in the treated diabetic kidney (Figure 6.7C). A similar upregulation (33%) was observed in the groups placed on 5mg/KgBW glibenclamide.

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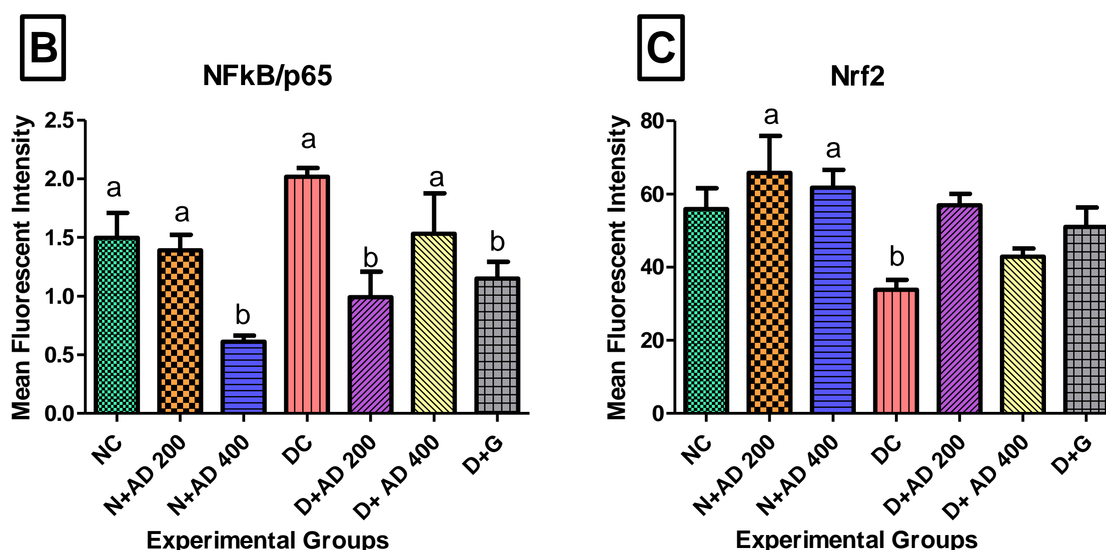
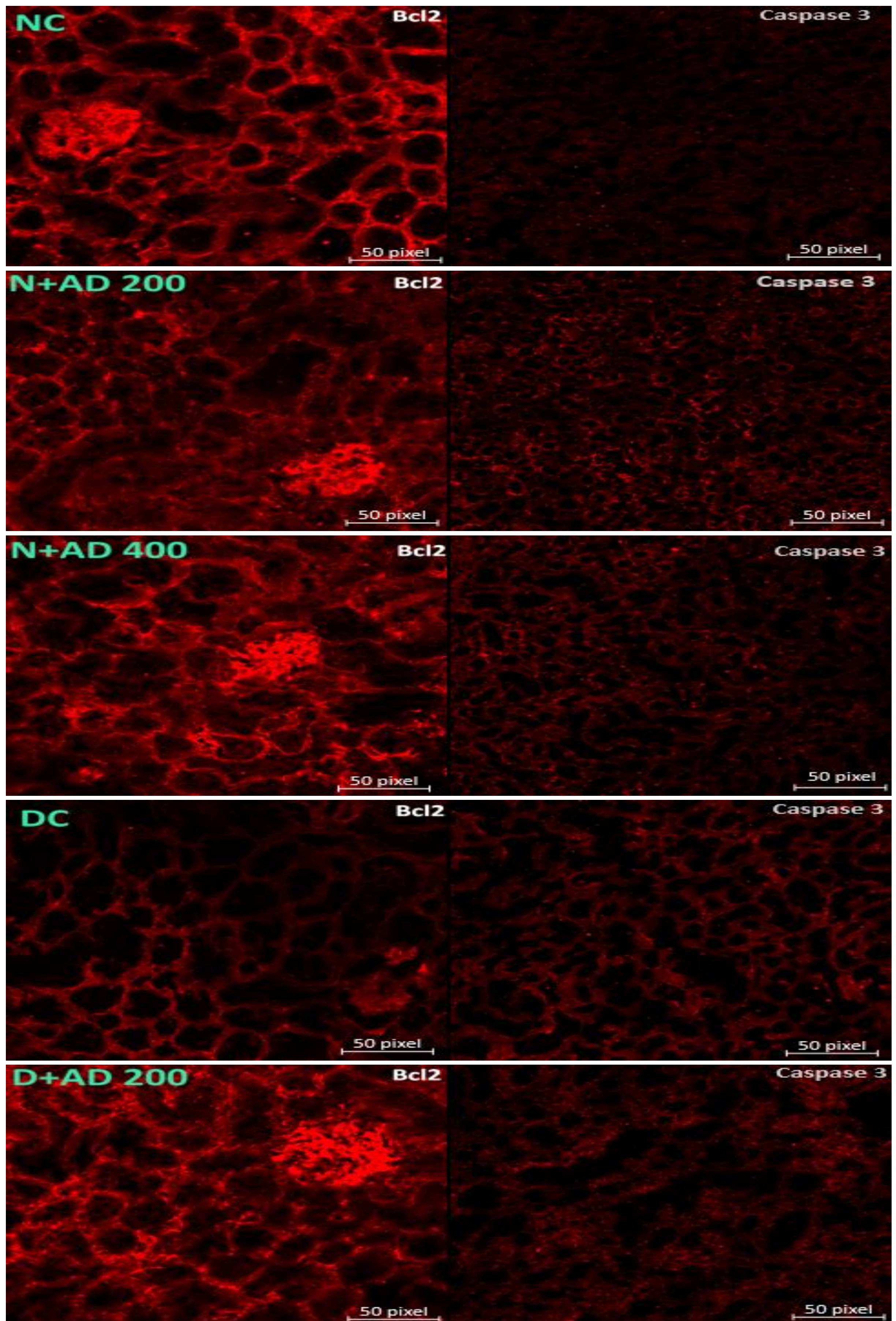


Figure 0.7: (A) Confocal microscopy image showing the effect of AD on the expression of NFkB/p65 (red) and Nrf2 (green) in the kidney tissues. Quantitative analysis of **(B)** NFkB/p65 and **(C)** Nrf2 expression in the Kidney tissues. Bars are indicative of mean values ± SEM of group values. Bars with different letters are significantly ($p<0.05$) different from each other.

6.3.6 AD administration upregulated the expressions of Anti-apoptotic proteins in diabetic rats

The induction of diabetes altered Bcl2 expressions in the untreated diabetic kidneys. This led to a significant decline in the MFI of Bcl2 in the untreated diabetic rats when compared with normal control rats (Figure 6.8A). A regimen of 200mg and 400mg/KgBW of AD significantly restored the Bcl2 levels as comparable to the observed levels in the normal control rats (Figure 6.8B). STZ-induction of diabetes or treatment with AD did not have any significant effects on the expressions of caspase 3 (Figure 6.8A & 6.8C).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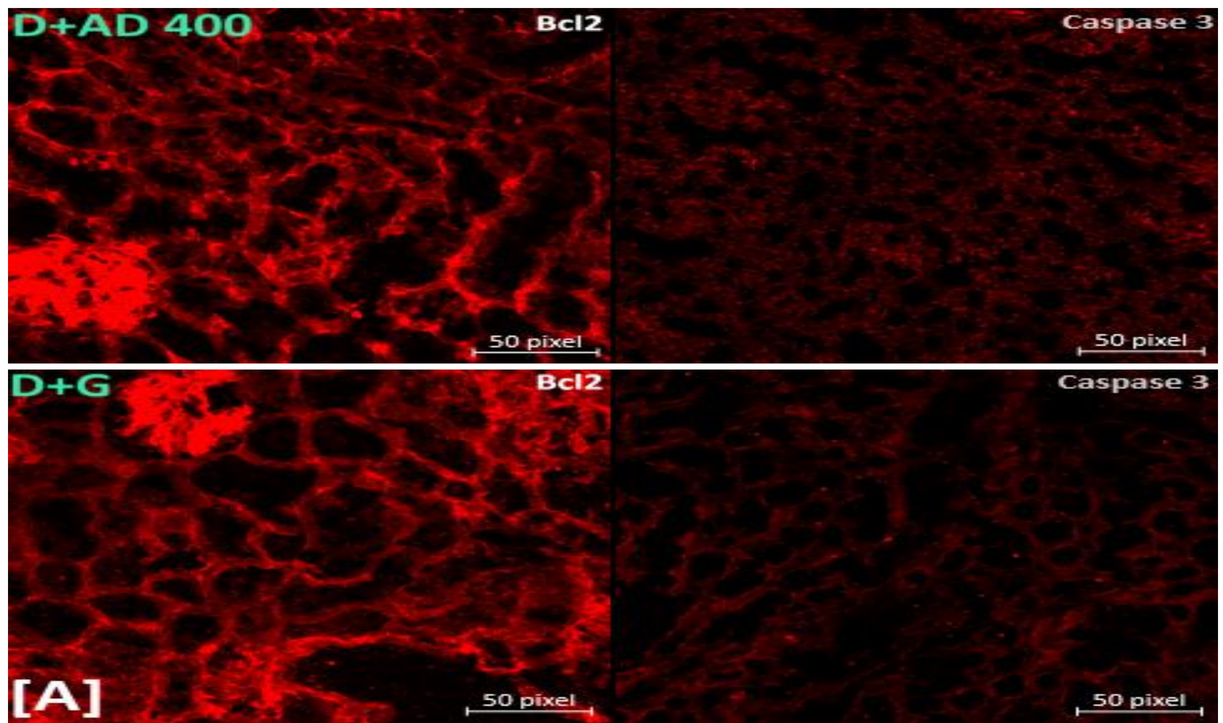
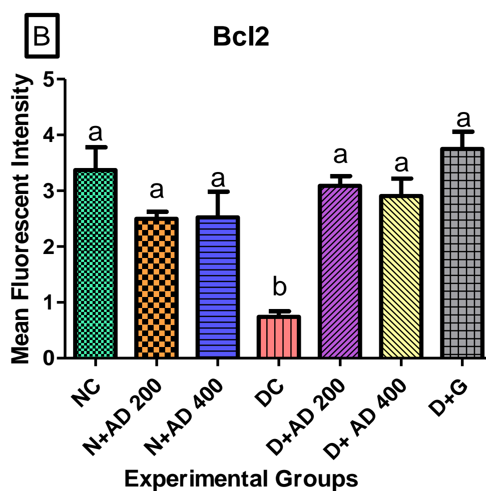




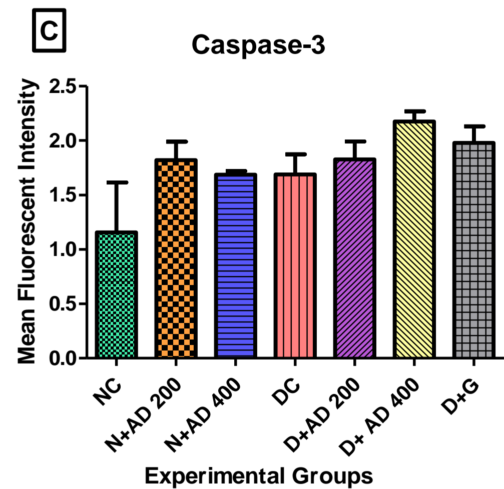
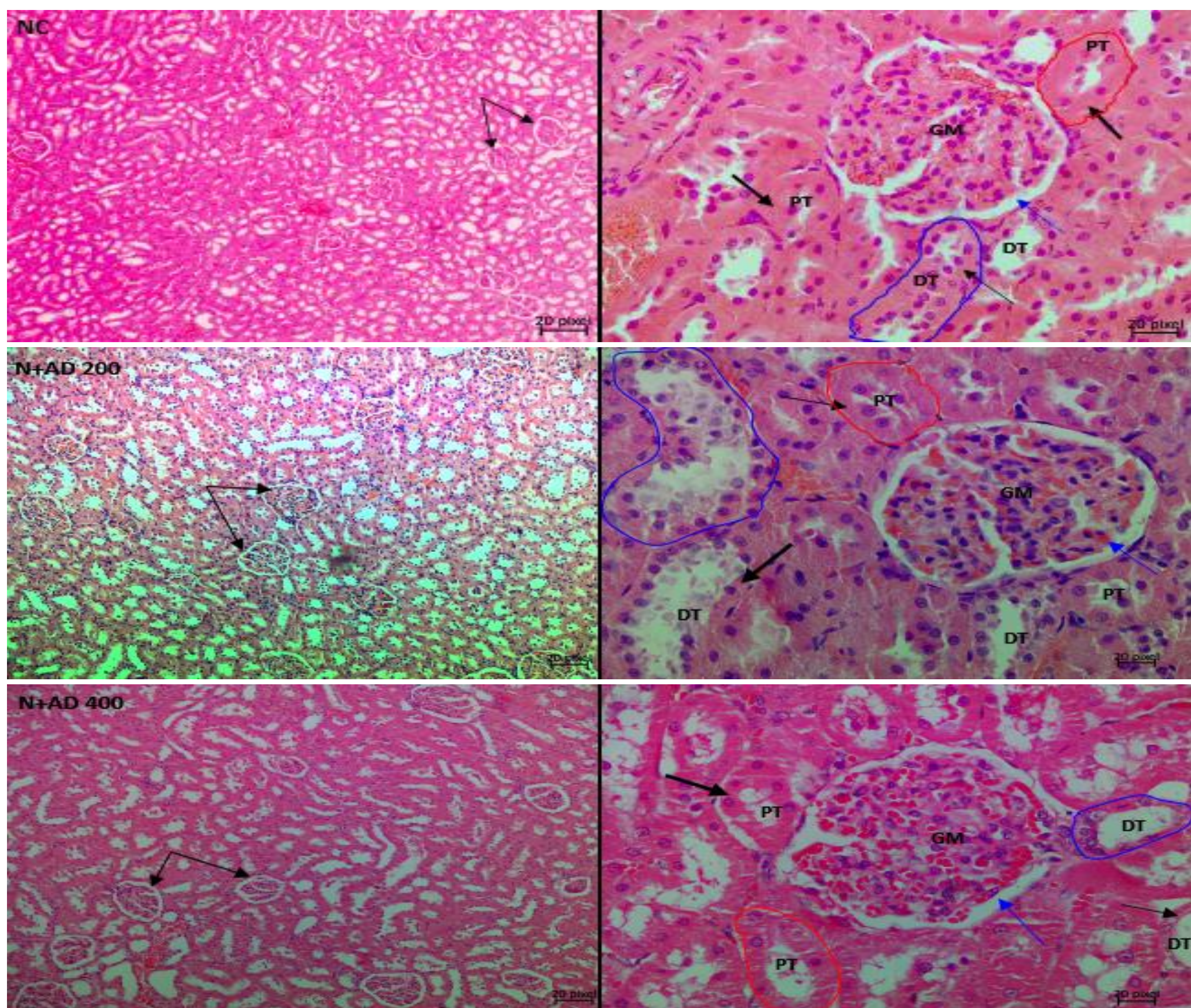


Figure 0.8: **(A)** Fluorescence micrographs showing the effect of AD intervention on apoptotic markers in the kidney tissues of normal and diabetic rats. Quantification of the level of expression of **(B)** caspase 3 and **(C)** Bcl2 in the kidney tissues.

6.3.7 Intervention with AD improved histoarchitecture of the kidney and pancreas in type2 diabetes

Figures 6.9 & 6.10 are representative micrographs of the histological examination in the kidney and pancreas tissues of the normal, treated and untreated diabetic rats. Normal control and treated normal rats showed normal kidney architecture. Pathological conditions such as disappearing of the glomerular tuft comprising of the glomerular capillaries, podocytes and

mesangial cells were observed in the kidney tissues of untreated diabetic rats. This led to widened Bowman's space in the diabetic control rats when compared with the kidney of normal rats. Furthermore, endothelial cells were markedly detached from the basement membrane of the glomerulus in the untreated diabetic kidneys. Loss of tubular cells especially of the proximal tubules (PT) and increased blood flux were revealed in the kidney sections of untreated diabetic rats. Treatment with 200mg/KgBW showed mild loss of tubular cells in the PT, while glomerular capillaries were clearly restored and less occurrence of glomerular shrinkage. 400mg/KgBW AD ameliorated glomerular and tubular damage in the kidney of treated diabetic rats. 400mg/KgBW prevented loss of tubular cells of the proximal tubules and minimized loss of glomerular tuft in the treated diabetic rats as comparable to normal. Administration of glibenclamide showed widened bowman's space due to shrinkage of the glomerular tuft and moderate loss of tubular cells of the PT.



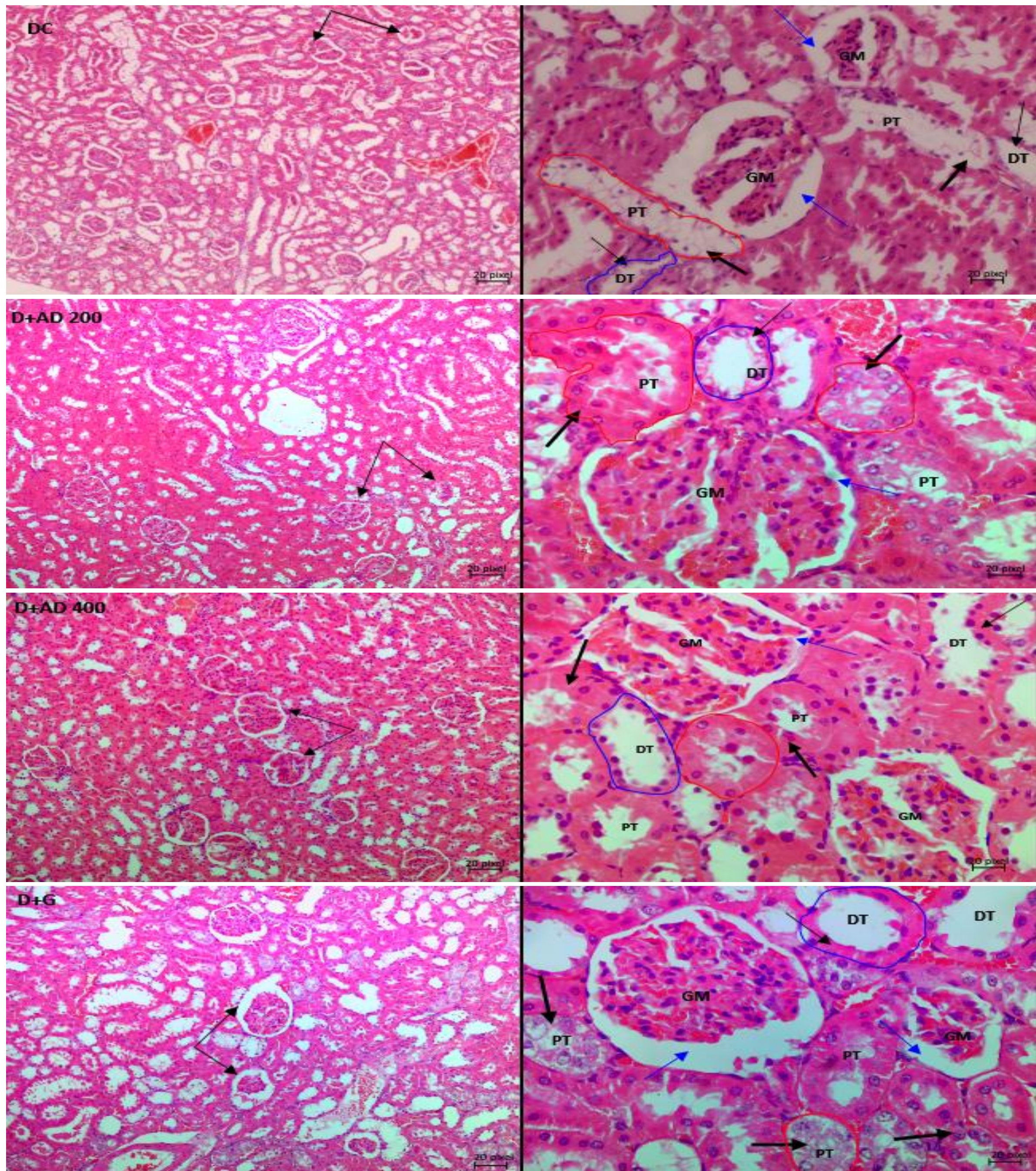
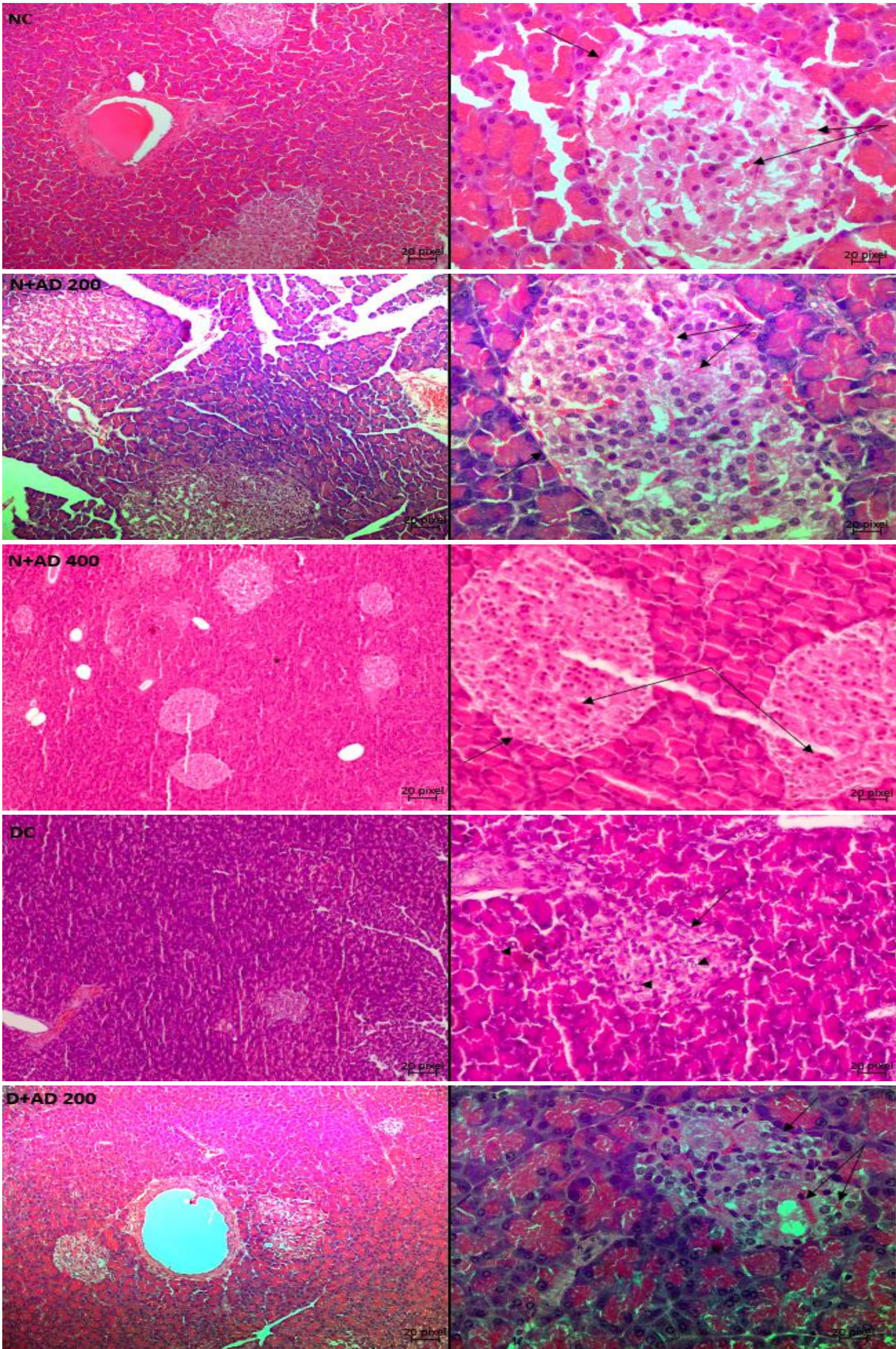


Figure 0.9: Light photomicrographs of haematoxylin and eosin-stained kidney cortex of normal and diabetic rats. Double arrows are pointing to the bowman's capsule and glomerulus (GM), while single thin arrows are showing the cells of the distal convoluted tubule (DT). The thick single arrows reveal the cells of the proximal convoluted tubule (PT). the blue arrows points at the bowman's space. Magnification 10x (left) and 40x (right).

The histological examination of the pancreatic tissue in the non-diabetic rats showed a normal histology of the pancreas (Figure 6.10). The exocrine region clearly distinct from the endocrine region as the basement membrane of the islets of Langerhans is visible and the islet appeared lightly stained. The shape and structure of the islets is not distorted, and the pancreatic lobules

are intact, separated by interlobular and intralobular spaces. The islet was well vascularized (double arrows). STZ induction of diabetes revealed pathological changes in both the endocrine and exocrine regions of the pancreas in the positive control rats. The structure and shape of the islet of Langerhans were distorted as the basement membrane is not visible (single arrow), severe destruction of the islet cells especially the beta cells are also observed. Vacuolation of the nucleus is seen in almost all the acinar cells and the islet cells. Distortion of the berry-like shaped acini was displayed, the pyramidical shape of the acinar cells were also distorted. Treated diabetic rats placed on 200mg/KgBW of AD showed a better structured acinus, acinar cells and the islet with restoration of beta cells when compared to the untreated diabetic rats (Figure 6.10). However, pathological alterations such as moderate vacuolation of the acinar and islet cells were seen. Supplementation with 400mg/KgBW of AD restored the architecture of the pancreas to normal when compared with the untreated diabetic rats. The islets were well vascularized, the structure and shape of the islet were normal, and the basement membrane is clearly visible. There was no observable damage such as vacuolation in the acinar and islet cells. Also, the structure and shape of the acinar cells were restored. Treatment with glibenclamide improved the histology of the pancreas but not comparable to normal. The shape of the acini and its cells were moderately distorted, and the basement membrane of the islet not distinct enough.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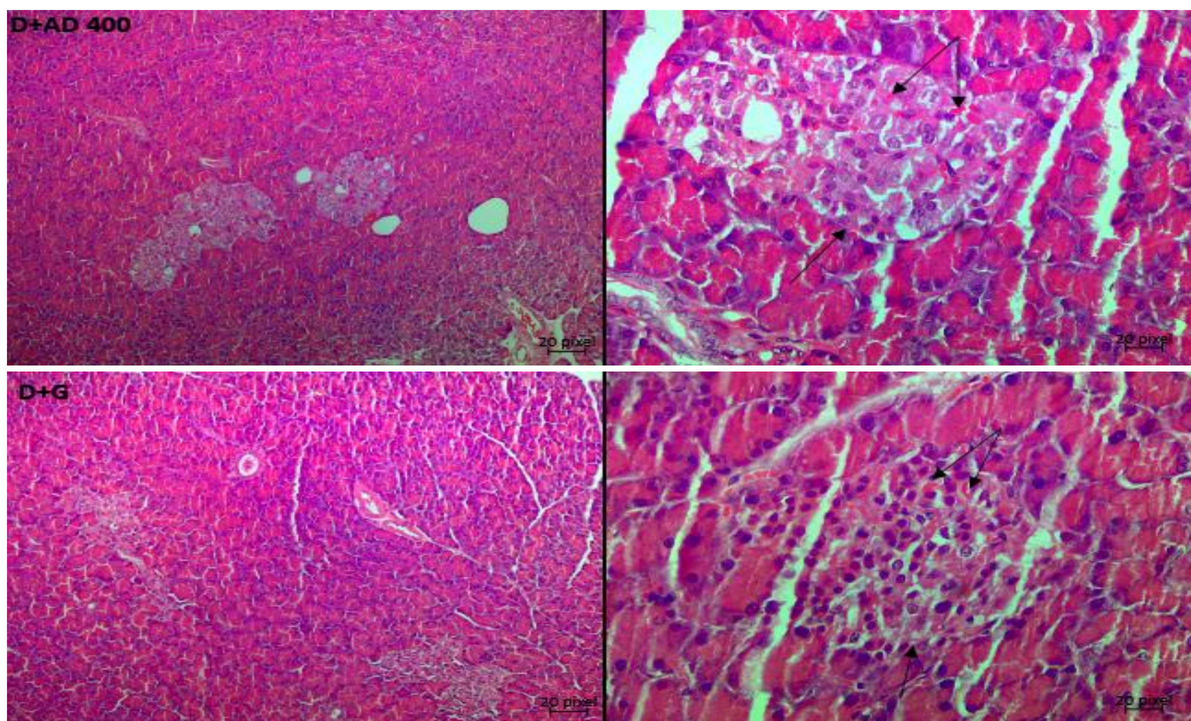

Figure 0.10: Light photomicrographs of haematoxylin and eosin-stained pancreatic tissue of normal and diabetic rats. Single arrows points to the basement membrane of the islets, while double thin arrows are showing the islet capillaries. Arrow heads show vacuolated nuclei in the acinar and islet cells. Magnification 10x (left) and 40x (right).

6.4 Discussion

The current study exhibits the protective effect of AD against organ toxicity, immunotoxicity and oxidative stress exerted by STZ on the kidney and pancreas in T2D. Organ weights have been used as important markers in investigating the toxicity of xenobiotics [34] and are indicative of hypertrophy if significantly increased [35]. Relative weight changes of internal organs are indices of pathology in the organs [36]. Introduction of fructose and STZ brought about significant increase in the relative weight of the kidney and pancreas of diabetic rats. Renal toxicity, tubular hypertrophy is known to be reflective of changes in the kidney weight [37]. Also, constant hyperglycemia results in the formation of AGEs and hyperfiltration leading to glomerular hypertrophy [38,39]. Administration of AD apparently reduced kidney toxicity as revealed by its ability to decrease relative kidney weight by 10%; an improvement over treatment with glibenclamide, which had no effect. Similar trends were observed in the relative weight of the pancreas in the diabetic control which increased significantly when compared to normal rats. However, AD abated abnormally elevated pancreatic weights in treated diabetic rats

comparable to glibenclamide. This depicts that AD may be useful in minimising or preventing kidney and pancreas toxicity.

Urea and creatinine are one of the major metabolic waste excreted in the urine [3]. Abnormal levels of urea in the serum is indicative of kidney dysfunction (Mishra *et al.*, 2015). Serum urea levels was significantly increased in the diabetic controls when compared to normal rats. There were severe pathological changes seen in the glomerulus of the diabetic control such as disappearing glomerulus tuft and capillaries, widened bowman's space which are essential in glomerular filtration. Abnormal glomerular filtration may lead to increased urea and creatinine in the serum. Also, tubular damage especially in the proximal tubules was observed in the kidney tissues of untreated diabetic rats. Proximal tubules are very crucial in the reabsorption process as most essential substances in the filtrate including urea are reabsorbed in the proximal tubule [3,40]. Therefore, the elevated levels of serum urea were likely due to the glomerular and tubular damage. Similar results were revealed by other studies [41,42] where serum urea was significantly raised in diabetic subjects. Conversely, the administration of AD, diminished serum concentration of urea in treated diabetic rats, especially the 400mg/KgBW treatment which reduced serum urea by 22.5%. This is expected as 400mg/KgBW of AD restored the architecture of the kidney as comparable to normal rats. It is noteworthy that AD was more effective in restoring organ function in the diabetic rats than glibenclamide, as glibenclamide did not have any reducing effect on the serum urea. Also, corresponding pathologies were seen in the tubules and glomeruli of rats placed on glibenclamide.

Oxidative stress triggered by persistent hyperglycemia, play an important role in the progression of diabetic nephropathy [39,43]. Oxidative stress occurs as a result of imbalance in the production of ROS and antioxidants. AD was shown to induce CAT and SOD activities in normal rats, thereby improving the antioxidant status in the rats. The significant decline in the CAT activity in the untreated diabetic kidneys is indicative of oxidative stress. Contrarily, AD elevated CAT levels in the kidneys of treated diabetic rats. Increased activities of CAT and

SOD by AD, suggestively reduced oxidative stress in the diabetic kidneys thereby ameliorating injury to cells and resultant cell death that may have occurred as revealed in Figure 6.9. Previous studies on the phytochemical content of AD confirmed the presence of antioxidant compounds [25]. A slight impaired induction of SOD levels was observed in the diabetic control which is likely as a response to the hyperglycaemic-induced oxidative stress. Lim and colleagues [44] reported a similar finding of impaired induction of SOD and a corresponding reduction in SOD activity in diabetic nephropathy.

Increased Nrf2 levels are known to be enhanced by oxidative stress, however, Nrf2 levels can be activated by in the presence of phytochemicals with antioxidant ability [45]. Supplementation with AD stimulated increased production of Nrf2 in normal and diabetic rats (Figure 6.7C). One of the possible mechanisms by which AD exerts its antioxidant ability may be that the antioxidant compounds present in AD trigger the dissociation of Nrf2/Keap1, activating Nrf2. Activated Nrf2 binds to the promoter region of the AREs gene, enhancing the transcription of AREs genes that code for antioxidant enzymes such as CAT and SOD. Figure 6.5A showed that there was a corresponding increase in the CAT and SOD activities of rats treated with AD, which follows the pattern of Nrf2 induction in the same rats. The significant decrease of Nrf2 observed in untreated diabetic rats concurs with the reduced CAT activities in the same rats. Benipal and co-researchers [46] hypothesized that repetitive ROS production downregulates the expression of Nrf2.

NFkB activated by oxidative stress; plays a central role in inflammatory mediation and its deregulation is strongly associated with inflammatory diseases, hence its role in the progression of renal damage [47,48]. NFkB expression was upregulated in the kidney of diabetic control rats, which was downregulated in the diabetic rats placed on AD. The activation of NFkB usually promotes the expression of pro-inflammatory markers and apoptosis. The increased production of the inflammatory markers such as TNFα further activates NFkB leading to prolonged and persistent inflammatory response [49,50].

Pro-inflammatory indices; IL-1β and IL-6 were significantly increased in the kidneys of diabetic control rats when compared to normal rats. Proinflammatory cytokines; IL-1β, IL-6, IL-18 and TNFα which play major roles in the development and progression of DN are triggered by hyperglycemia and oxidative stress [51]. Increased expression of IL-1β in the glomeruli of STZ-induced diabetes and significant increase of IL-6 in diabetic subjects especially in the mesangium, interstitium and the tubules have been documented [52]. The study on kidney biopsies of DN subjects carried out by Navarro-Gonzalez and Mora-Fernandez [52], showed a positive correlation of IL-6 expression in the mesangium and podocytes with glomerular injuries. IL-18; a strong proinflammatory agent that influences the production of other proinflammatory cytokines (IL-1β and TNFα) is also elevated in DN, which in turn increases urinary albumin excretion thereby increasing kidney susceptibility to renal damage [53–56]. High levels of TNFα in DN patients is implicated in microalbuminuria [53], it promotes the generation of ROS locally in cells such as mesangial cells and activates transcription factors, growth factors, receptors and other cytokines [3,52] in the renal cells. TNFα can also alter the glomerular filtration rate, cause a dysfunction in endothelial permeability and eventual induction of apoptosis [3]. However, the administration of AD led to decrease in the levels of these markers, as comparable to the glibenclamide in the diabetic treated rats. Similar trends were observed in the levels of IL-18 and TNFα. Supplementation with AD is a potential therapy against inflammation-induced renal injury in T2D as also supported by its suppression of NFkB and reduction of pro-inflammatory cytokines in the normal rats (Figures 6.7(A-B) and 6.6(A-E)). The abnormal increase in the levels of IL-10 in the diabetic rats may be due to compensatory response, this was significantly repressed in the rats administered AD and glibenclamide.

Bcl2 acts as an anti-apoptotic protein, regulates apoptosis and promotes cell survival by inhibiting pro-apoptotic proteins [57]. Bcl2 also acts as an anti-inflammatory protein by inhibiting NFkB and its controlled genes. The expression of Bcl2 was significantly reduced in the untreated diabetic rats. Studies have reported decreased Bcl2 expression in diabetic subjects [17,46]. This is expected as poor glycemic control represses the expression of Bcl2 in diabetes [57].

Reduced expression of Bcl2 leads to increased inflammation and apoptosis which contributes to the progression of renal pathology in diabetic subjects [57]. AD regimen significantly restored the expressions of Bcl2 to normal in treated diabetic rats. Lau and co-workers [58] investigated the underlying mechanisms through which apoptosis occur in DN. They found out that Bcl2-modifying factor (Bmf); a pro-apoptotic protein is induced by hyperglycemia via ROS and growth factor (TGFβ1) and upregulated in diabetic kidneys of rats and humans. Activated Bmf translocates from the cytoskeleton to the mitochondria where it binds and inhibits Bcl2 and other apoptotic proteins. This ultimately generates mitochondria transmembrane potential, which activates the intrinsic signalling pathway of apoptosis. It was also observed that Bmf can activate caspase 3 leading to apoptosis [57].

The major pathological alterations observed in the kidney tissues of untreated diabetic rats were disappearing glomerular tuft due to loss of podocytes, mesangial cells and glomerular capillaries. Also, loss of tubular cells especially the proximal tubules was observed in diabetic controls. This was ameliorated in the diabetic rats treated with AD. Apoptotic cell death plays a very crucial role in the loss of mesangial cells and podocytes [59,60]. Studies have reported the apoptotic death of renal tubular cells across various diabetic models such as mice, rats and humans [58]. One of the main causes of renal and glomerular apoptosis is increased inflammation. NFkB; a pivotal inflammatory factor is activated in the podocytes, mesangial cells and tubular cells [10]. Increased expression and activation of NFkB in these cells results in the production of pro-inflammatory factors and eventually apoptosis [49]. Decreased apoptosis was reported in NFκB tubular epithelial-specific knockout mice due to less activation of NFκB and reduced chemokine expression [50]. This supports the central role played by NFkB in the apoptosis of the tubular and glomerular cells. A strong correlation has been established between NFkB activation and the severity of renal disease [10]. In addition, the expression of Bcl2 is majorly detected in the proximal and distal tubules and in the capsular parietal cells in normal rats and humans [17]. In this study Bcl2 expression was significantly reduced in the kidney parenchyma of diabetic control rats, thereby aiding apoptosis. Figure 6.11 illustrates

the suggested mechanisms and pathways that may be involved in the ameliorative effect of AD against kidney damage.

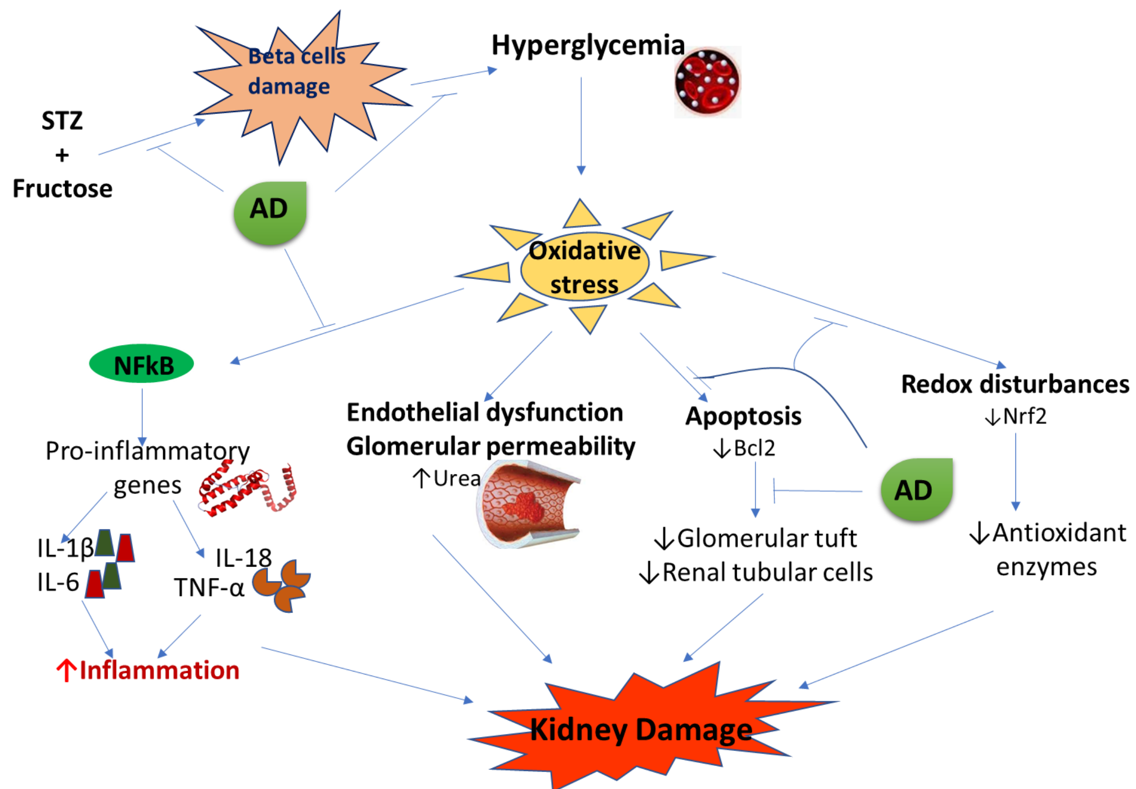

Figure 0.11: Proposed mechanisms by which AD ameliorates diabetic nephropathy and pancreatic damage. AD-Anchomanes difformis.

6.5 Conclusion

AD enhanced antioxidant status, reduced apoptosis and modulated inflammatory response in diabetic nephropathy. These biological activities of AD in the kidney might be a useful tool in the prevention and management of diabetic nephropathy and other associated diabetic complications.

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Declaration of conflict

The authors do not have any conflict of interest.

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CHAPTER SEVEN

The beneficial role of *Anchomanes difformis* in STZ-induced reproductive dysfunction in male Wistar rats

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ABSTRACT

Background

Progression of diabetes mellitus has increasingly led to several diabetic complications in the body system. Diabetes is one of the major factors implicated in reproductive system damage. Recent approaches such as the use of medicinal plants have been explored in the management of diabetes and its associated complications. *Anchomanes difformis* has been shown to possess anti-diabetic ability. Therefore, this study seeks to investigate the potency of *Achomanes difformis* to ameliorate diabetes-induced reproductive dysfunction.

Method

Type 2 diabetes was induced with 10% fructose administration for 2 weeks and intraperitoneal injection of 40mg/kgBW of STZ. The 200mg and 400mg/kgBW aqueous extract of *Anchomanes difformis* (leaves) were administered daily for 6 weeks. The impact of diabetes and treatment was investigated by estimating sperm concentration, motility indices, viability and morphological parameters in the normal, treated and diabetic rats. Histological examination of the testes and epididymis was performed.

Results

The administration of *Anchomanes difformis* significantly (p<0.005) increased sperm concentration and sperm viability while it also significantly improved the percentage of morphologically normal in diabetic rats. *Anchomanes difformis* also ameliorated impaired testicular damage in the diabetic testes when compared to the diabetic controls.

Conclusion

The potency of *Anchomanes difformis* displayed against diabetic-induced damage in the reproductive system might be a new and promising tool in the management of male reproductive dysfunctions and associated complications that arise in diabetes mellitus.

Keywords: *Anchomanes difformis,* concentration, morphology, motility, reproductive, streptozotocin, viability

7.1 Introduction

Diabetes mellitus is one of the many diseases that contribute to male infertility in the present age, as an estimated 51% of diabetic subjects are suffering from different sexual dysfunctions (Omolaoye & du Plessis, 2018; Han et al., 2019). Oxidative stress; especially hyperglycemia-induced, has been implicated in the pathogenesis of reproductive complications arising from diabetes (Shoorei et al., 2019). Some of the ways by which oxidative stress results in reproductive dysfunction include increased lipid peroxidation of PUFA, damaging the sperm membrane which are prone to reactive oxygen species (ROS) attack (Aitken, 2017). Increased lipid peroxidation further degenerate to DNA damage in the spermatozoa (Makker et al., 2009). Furthermore, oxidative stress leads to damage in the reproductive system through other mechanisms such as mitochondrial DNA damage and DNA fragmentation (Omolaoye & du Plessis, 2018). Other pathways implicated in the pathogenesis of male infertility in diabetes mellitus include the production of advanced glycation end products, the progression of diabetic nephropathy and endocrine disorder which affects the hormones (Omolaoye & du Plessis, 2018).

Hormonal control is an important factor in male fertility as spermatogenesis; the processes involved in the production and development of spermatozoa from germ cells (Alves et al., 2013), are controlled and regulated by the hypothalamic-pituitary-gonadal hormones. The hypothalamus produces gonado-tropin-releasing hormone (GnRH) which in turn triggers the secretion of follicle stimulating hormone (FSH) and luteinising hormone (LH) from the anterior pituitary lobe (Manocha et al., 2018). The release of LH stimulates the production of testosterone from the Leydig cells, while FSH prompts the Sertoli cells to supply nutrients to the germ cells and activate spermatogenesis (Johnson et al., 2019). Any imbalance or impairment in this array of hormones can cause dysfunction in the reproductive system. Decreased LH and FSH levels have been reported in diabetic conditions (Ballester et al., 2004; Arokoyo et al., 2017).

The use of streptozotocin (STZ) to induce hyperglycemia-mediated reproductive toxicity has been documented (Navarro-Casado et al., 2010; Omolaoye & du Plessis, 2018); Omolaoye and du Plessis, 2019), and has been known to affect the antioxidant status, the spermatogenic processes, sperm functions and the reproductive organs (Arokoyo et al., 2018; Johnson et al., 2019). The contributory role of medicinal plants in combating diabetic-induced reproductive impairment have been emphasized in recent times. *Anchomanes difformis* (AD) has a strong ethnopharmacological relevance and it is known for its numerous biological activities such as its potency against diabetes, nephropathy, inflammation, microbial activities, and gastrointestinal pathologies. These folkloric claims have been scientifically established and more investigations to explore its other potentials are ongoing (Ahmed, 2018; Udje et al., 2018). Agyare and colleagues investigated the potentials of leaves and rhizome extracts of AD on histamine and serotonin; mediators of acute inflammation (Agyare et al., 2015). AD demonstrated more anti-inflammatory ability when compared with aspirin as reference drug (Agyare et al., 2015). Adebayo also reported the anti-inflammatory potentials of AD leaves against raw-egg albumin-induced inflammation in chicks (Adebayo et al., 2014). An ethanolic extract of AD rhizome displayed hypoglycemic effects in alloxan-induced diabetes using Wistar rats (Adeyemi et al., 2015). Egwurugwu and co-workers proposed the effect of AD rhizome against uterine fibroid showing its ability to modulate female sex hormones implicated in uterine fibroid (Egwurugwu et al., 2016). We carried out previous studies on the antioxidant capacities and phytochemical analysis of six different extracts of AD, which revealed that AD is a natural source of antioxidant with aqueous leaves extract exhibiting the highest antioxidant ability and contained the highest concentration of polyphenols (Alabi et al., 2019). Phytochemical characterization of the leaves aqueous extract was also performed using ultra performance liquid chromatography mass spectrometry (UPLC-MS) and high performance liquid chromatography (HPLC). The results showed the presence of bioactive compounds including phloridzin, quercetin, rutin, and kaempferol which are antioxidants and have therapeutic effect against diabetes, inflammation and apoptosis (Alabi et al., 2019). The antioxidant, hypoglycaemic, anti-inflammatory activities of AD and its impact in modulating female sex

hormone indicates its potentials in attenuating reproductive dysfunctions associated with diabetes. However, no study has investigated the possible effects of AD leaves extract on sperm functional parameters as well as, testicular and epididymal damage in diabetes. This paucity in the literature was the major drive that led to this study which focusses on assessing the ameliorative effect of *Anchomanes difformis* on reproductive dysfunction mediated by diabetes mellitus in male Wistar rats.

7.2 Methodology

7.2.1 Plant collection and extraction

The fresh leaves of AD were from the south-western part of Nigeria and authenticated at the University of Lagos Herbarium, Nigeria. A specimen was deposited in the herbarium; LUH6623. The harvested leaves were dried under shade, ground into fine particles and defatted by soaking in n-hexane (10%$^{W}/_{V}$) for 48 hours. Following defatting, the leaves were subjected to a cold-stirred extraction in water (5% $^{W}/_{V}$) for 48 hours at 2-8°C. The extract was pulverised and stored at -20°C.

7.2.2 Ethical consideration

Ethical approval for this study was granted by the Research Ethics Committee (REC) of the Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, Bellville, South Africa (CPUT/HW-REC 2016/A4) and the Ethics Committee for Research on Animals at the South African Medical Research Council (SAMRC), South Africa (REF.04/17), where the animal experiment was conducted.

7.2.3 Animal care

Male Wistar rats (n=64) with approximate weights of 180 ± 10g were secured from the Animal facility at Stellenbosch University, South Africa. The animals were accommodated at the Primate Unit & Delft Animal Centre (PUDAC), SAMRC and acclimatize for 3-4 weeksbefore commencement of the study. Housing conditions were controlled: humidity- 45% to 55%,

temperature- 22°C to 26°C. They were exposed to normal photo period (12hour dark/12hour light) and fed with standard rat chow (SRC). Animal handling, care and other procedures were done in accordance with the standard operating procedure of SAMRC PUDAC (SOP No: 2016-R01) which conforms to the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

7.2.4 Induction of diabetes

Type 2 diabetes was induced with 10% fructose and streptozotocin (STZ) according to the method of Wilson and Islam (Wilson & Islam, 2012). The diabetic model rats were placed on 10% fructose water *ad libitum* for two weeks. A single dose of 40mg/kgBW STZ was administered intraperitoneally. After 5 days, animals with fasting blood glucose level above 15mmol/l were considered diabetic. Oral glucose tolerance test (OGTT) was performed to confirm insulin resistance.

7.2.5 Experimental design

Wistar rats with weights ranging from 270-300g was used for this study. The rats were randomly grouped into seven with a minimum of eight rats in each (8 rats in normal groups and 10 in diabetic groups) as summarised in Figure 7.1. Water served as the vehicle for fructose and AD administration, while citrate buffer was the vehicle for streptozotocin. Animals in group 1 served as the normal control (NC) and received vehicle only. Animals in group 2 and 3 are normal rats who received 200 and 400mg/kgBW of AD aqueous extract only (N+AD 200 and N+AD 400) and served as the treated control. Group 4-7 consist of animals that were placed on 10% fructose for 2 weeks followed by streptozotocin (STZ). Group 4 received vehicle only (DC), group 5 and 6 were given 200 and 400mg/kgBW of AD aqueous extract (D+AD 200 and D+AD 400) respectively while group 7 received 5mg/kgBW of gilbenclamide; an antidiabetic drug (D+G).

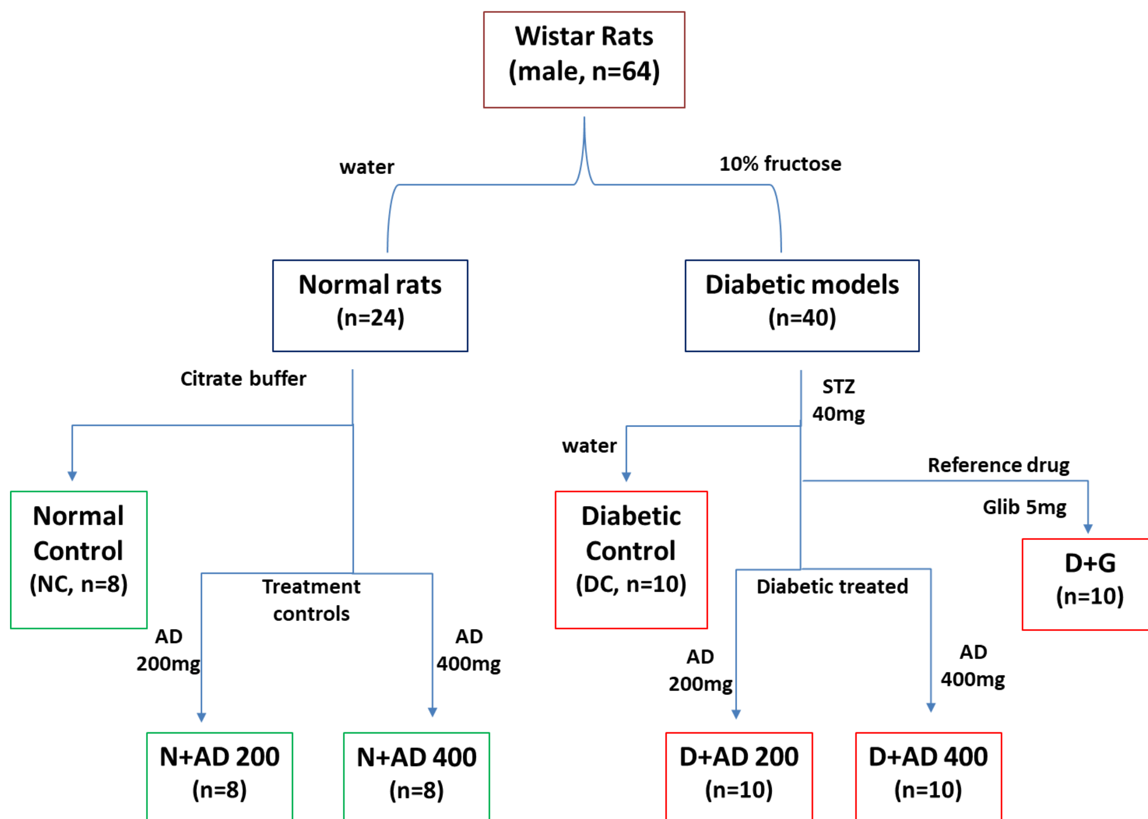


Figure 0.1: Experimental design. Water and citrate buffer served as vehicles of administration, AD-*Anchomanes difformis* leaf extract, Glib-glibenclamide (standard antidiabetic drug), STZ-streptozotocin

7.2.6 Sample collection

Rats were anaesthetized by means of 2% isoflurane (Biofarm, Cape Town, South Africa) per oxygen (1L/min) inhalation and thereafter terminated via exsanguination. Both testes, epididymis and vas deferens were excised. The dissected male reproductive tract was cleaned by carefully trimming off all unnecessary blood vessels and fat tissue until all parts of the reproductive tract could be clearly seen and differentiated. The cauda epididymis, comprising the larger convoluted tubes, was removed and placed in a pre-warmed petri dish containing 200µL Ham's F10 (Invitrogen, Cape Town, South Africa). The testes and epididymis were washed and weighed accordingly. The relative weights of the testes and epididymis were calculated using the body weight of the same rats against the weight of the testes or epididymis.

$$Relative\ testis/epididymis\ weight\ = \frac{Testes/epididymis\ weight\ (g)}{Total\ body\ weight\ (g)} \times 100\ \%$$

7.2.7 Sperm isolation (Swim out method)

The swim-out technique was used to select the motile spermatozoa. Small incisions were made at the distal end of the cauda epididymis and the motile spermatozoa could swim out of the ducts into the surrounding medium (Ham's F10 supplemented with 3% bovine serum albumin). forming a cloud of spermatozoa.

7.2.8 Measurement of sperm concentration and motility

Semen samples were taken from the edge of the spermatozoan cloud to measure sperm concentration, motility and other related parameters. The semen (5 µL) was pipetted into a 20µm deep chamber Leja slide; SC 20-01-04B (Leja Products B.V., Nieuw-Vennep, The Netherlands). The slide was pre-heated on the microscope's warming stage at 37°C. For assessment of sperm concentration, the semen sample was diluted with Ham's F10 in the ratio 1:20. To measure sperm motility, 2-10 fields were captured until a total number of 200 spermatozoa were analyzed. Other motility parameters evaluated include curved-linear velocity (VCL), straight-line velocity (VSL), average-path velocity (VAP), beat-cross frequency (BCF), linearity (LIN), straightness (STR), oscillation index; also known as wobble (WOB), and amplitude of lateral head displacement (ALH). These measurements are presented as an average percentage of all the captured fields. The sperm concentration and motility were determined with the Rodent SCA® CASA (sperm class analyser, computed assisted sperm analysis) system version 6.2.0.15 (Microptic S.L, Barcelona, Spain). Photomicrographs were captured with a Basler A602fc digital camera (Microptic S.L, Barcelona, Spain) that was mounted (C-mount) onto a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa), equipped with phase contrast optics and a heated stage.

7.2.9 Evaluation of sperm morphology and viability

7.2.9.1 Preparation of sperm smears

Sperm smears for morphology and vitality were prepared from the swim-out sperm preparation after 10 minutes of incubation to confirm the motility of the selected spermatozoa to be smeared. 10µL of swim-up sperm was pipetted to make duplicate smears, making it about about 2-10 spermatozoa per field when viewed at a 10^3x magnification. Smears were allowed to dry.

7.2.9.2 Morphology

Dried smears were stained with Rapid SpermBlue (a combination of fixative and stain) following the method described by van der Horst and Maree (Van Der Horst & Maree, 2010). Smears were vertically placed into the staining tray (coplin jar) containing SpermBlue for 40-50 seconds at room temperature. Excess stain/fixative were drained off by holding the slides at an angle between 60 to 80 degrees. Slides were washed by slowly dipping in another coplin jar containing distilled water for six seconds, excess fluid was allowed to run off on tissues paper. Stained smears were dried at room temperature for 24 hours and mounted with DPX. Sperm morphology was assessed using SCA® on a Nikon E200 microscope, with a blue filter and phase 'A' setting, and 60x magnification. Spermatozoa with background staining or that overlap another were excluded and a total of fifty spermatozoa were analyzed per slide. Other indices associated with sperm morphology were estimated. These include; area, width, angle of the head and midpiece, while perimeter, chord and roughness were assessed in the head only.

7.2.9.3 Viability

BrightVit; a nigrosin-eosin based solution (Sigma Aldrich, Cape Town, South Africa) was used to stain the sperm smears for the analysis of sperm viability. Briefly, 40µL of BrightVit was pre-heated to 37°C and mixed with 10µL of semen for 5 minutes at 37°C. The stained smears were left to dry in the dark room at room temperature and mounted with DPX. Spermatozoa were

viewed at 20x magnification under a light microscope. Percentage viability was calculated as the percentage of live spermatozoa from a total of 100 spermatozoa analyzed per slide. Dead spermatozoa appeared pink/red due to the eosin stain while live spermatozoa appeared white (unstained) due to the ability of the intact membranes to exclude dyes.

7.2.9.4 Sperm deformity index (SDI)

Sperm deformity index was estimated following the method described by Ahmad and Tariq (2011) (Ahmad & Tariq, 2011). SDI was calculated using the sum of sperm morphological deformities observed and the total numbers of sperm randomly selected and counted in a sperm population.

$$Sperm\ deformity\ index\ = \frac{Total\ number\ of\ sperm\ defects}{Total\ number\ of\ sperm\ counted}$$

7.2.10 Histological analysis of the gonadal tissues

The testes and epididymis were excised, washed, weighed and fixed in 10% buffered formalin. After fixation, the tissues were passed through ascending series of alcohol for dehydration, cleared in xylene and embedded in paraffin. Sections (5 μm) of each tissue were deparaffinized, rehydrated and stained with hematoxylin and eosin. The stained tissues were mounted and examined under a light microscope.

7.2.11 Statistical analysis

Values are expressed as mean ± standard error of mean (SEM). Data were analysed using GraphPad Prism Version 5.00 for Windows, GraphPad Inc., San Diego, California USA. Differences between means of groups were determined using one-way analysis of variance (ANOVA). The Bonferroni test was used for all pair-wise comparisons. Differences (F values) were considered statistically significant at p values less than 0.05.

7.3 Results

7.3.1 Treatment with AD alleviates organ toxicity in the testis and epididymis

Figure 7.2 presents the weights of the testis, epididymis and their relative weights. Induction of diabetes with STZ and fructose resulted in significant decrease in the weight of the epididymis in the diabetic controls (Figure 7.2C), while no significant changes were observed in the weight of the testes in the normal, diabetic and treated rats (Figure 7.2A). The relative testicular weight of the diabetic controls was significantly increased (54.1%) when compared with the weights of the testes in the normal rats. Treatment with 200 and 400mg/kgBW of AD reduced relative testis weight by 7.8% and 10.4% respectively, while 5mg/kgBW of glibenclamide reduced relative testis weight by 4.2% (Figure 7.2B) when compared with the diabetic control. Inversely, induction of diabetes led to a significant decrease in the relative weight of the epididymis when compared with the epididymis of the normal rats. However, the administration of 200 and 400mg/kgBW of AD and glibenclamide significantly increased the relative epididymal weight to be comparable to normal (Figure 7.2D).

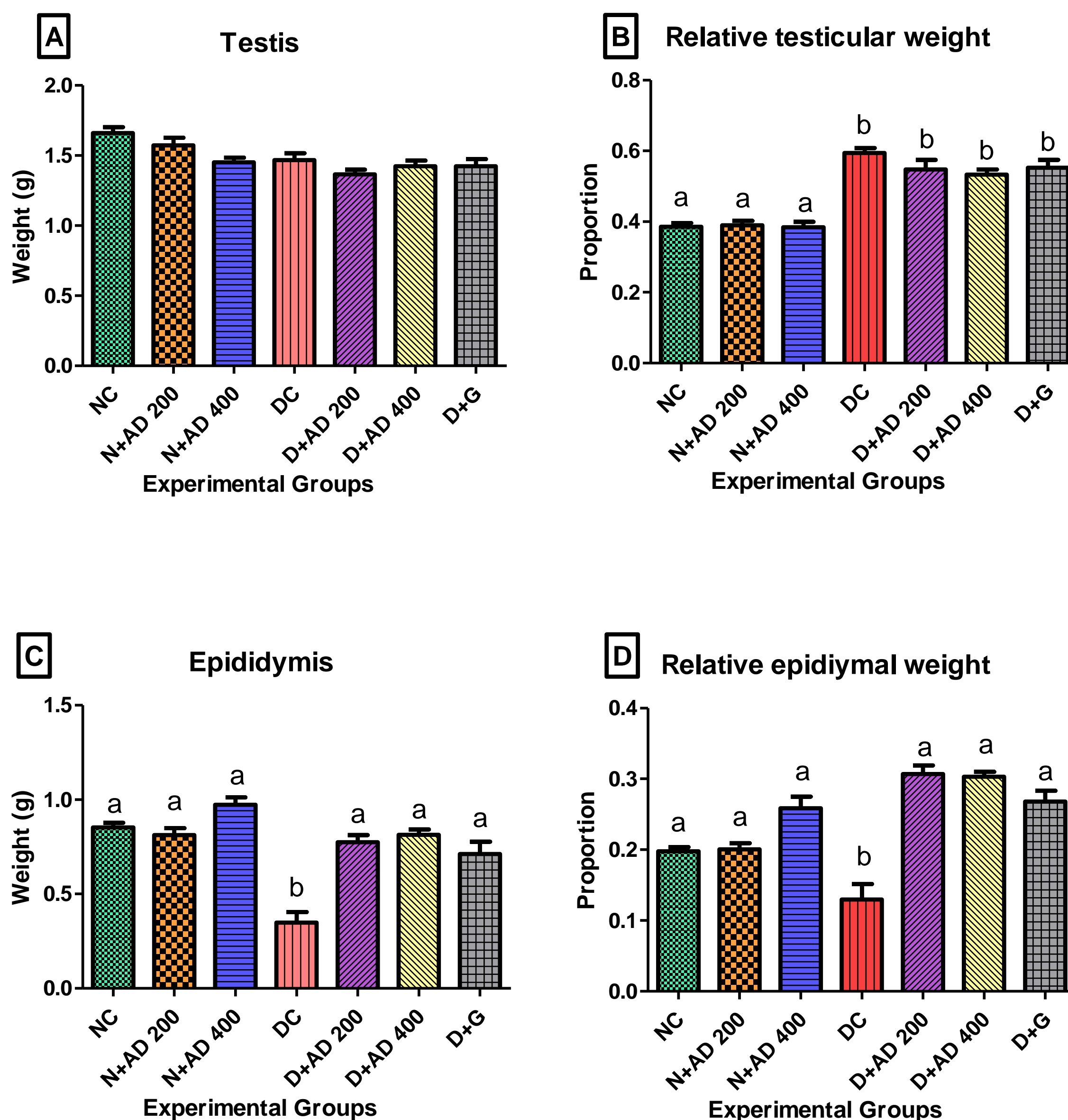


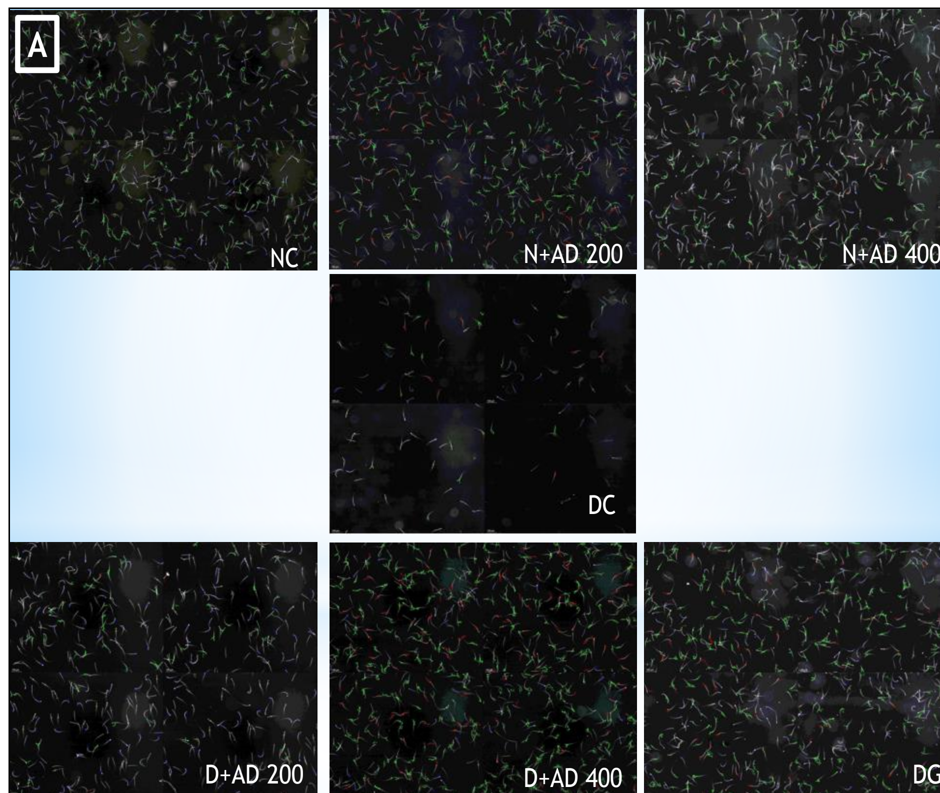
Figure 0.2: : Effect of AD administration on the **(A)** testicular weight **(B)** relative weight of the testis **(C)** weight of the epididymis and **(D)** the relative epididymal weight of normal and diabetic rats. Bars are indicative of mean values ± SEM of group values. Bars with different letters are significantly ($p<0.05$) different from each other.

7.3.2 AD improved sperm function in normal and diabetic rats

Sperm concentration was significantly reduced in diabetic control rats when compared with the normal rats. Supplementation with 200 and 400mg/kgBW of AD significantly restored sperm concentration back to normal in treated diabetic rats (Figure 7.3B). Administration of 200 and 400mg/kgBW triggered a 10% and 28.5% increase respectively in the sperm

concentration of normal rats serving as treatment controls. Figure 7.3A is a representative micrograph showing the sperm density in the normal, diabetic and treated rats. The sperm population in the normal controls (NC) and treated normal rats were densely packed, while it was sparsely populated in the untreated diabetic rats (DC). This is the same trend observed in the quantitative measurement of the sperm concentration (Figure 7.3B) where treatment with AD increased sperm concentration in the non-diabetic treated rats.

The effect of diabetes, treatment with AD and glibenclamide on indices of sperm function and quality is presented in Figure 7.4. Sperm morphology was significantly distorted in the untreated diabetic rats when compared with the normal rats (Figure 7.3A). Sperm morphology of diabetic rats that received 200 and 400mg/kgBW of AD were restored to normal. In addition, there was a significant increase in the sperm deformity index (SDI) in the diabetic controls when compared with normal rats (Figure 7.3B). SDI was significantly abated to normal with 200 and 400mg/kgBW AD treatment in treated diabetic rats as well as the standard drug. Induction of diabetes also caused a significant decrease in sperm viability of diabetic controls (Figure 7.3C). This was significantly enhanced in diabetic rats treated with both concentrations of AD, however, 400mg/kgBW increased sperm viability, as comparable to normal. There was no significant difference in the percentage progressive sperm motility in normal, diabetic and treated rats (Figure 7.3D).



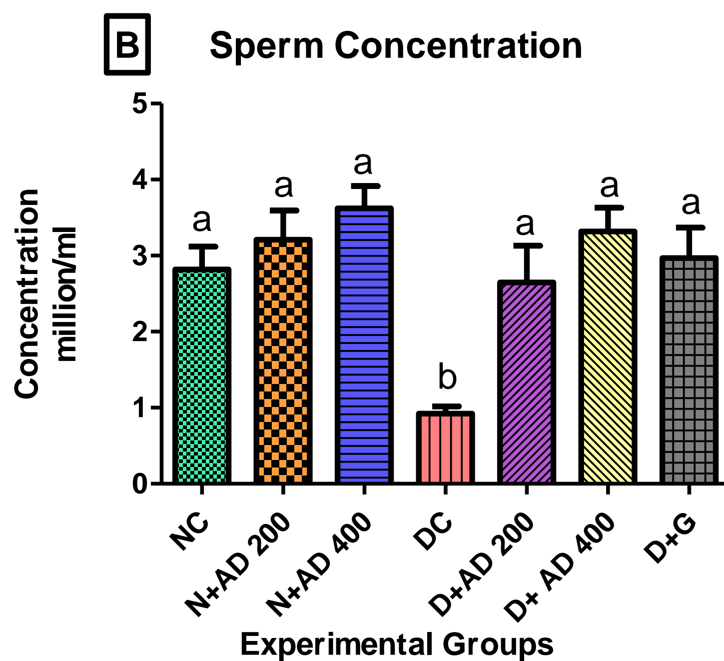


Figure 0.3: Sperm concentration of normal, diabetic and treated diabetic rats; **(A)** Representative micrographs and **(B)** quantitative graphical representation. Bars are indicative of mean values ± SEM of group values. Bars with different letters are significantly ($p<0.05$) different from each other. Each plate in Figure A comprises of four different fields of the semen sample.

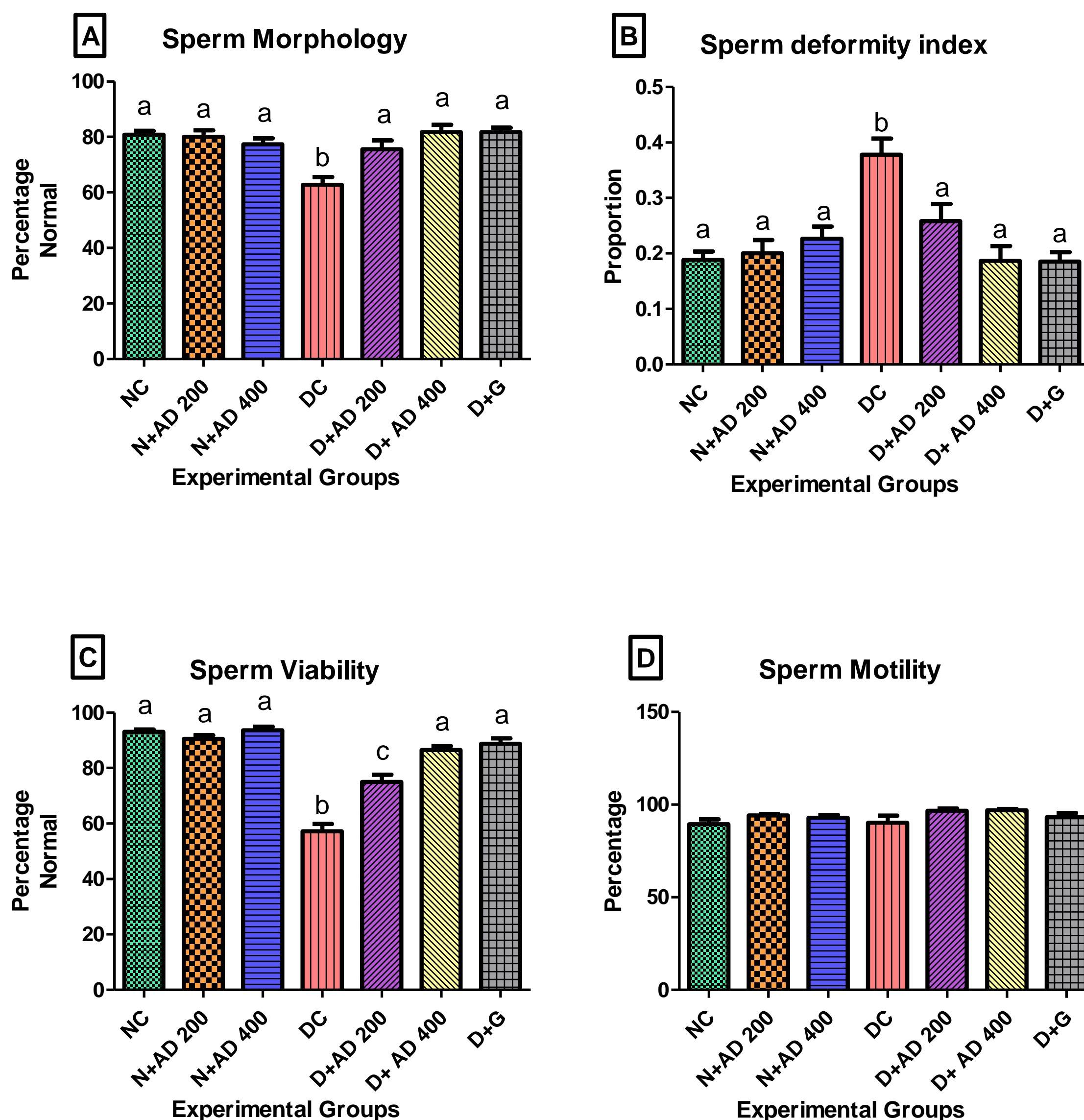


Figure 0.4: Effect of AD administration on the sperm function indices **(A)** sperm morphology **(B)** sperm deformity index **(C)** sperm viability and **(D)** sperm motility. Bars are indicative of mean values ± SEM of group values. Bars with different letters are significantly ($p<0.05$) different from each other.

7.3.3 AD enhanced sperm velocities and kinematics in STZ-induced diabetes

Other parameters associated with motility such as velocities, linearity, straightness and oscillation index were measured in the semen of normal, treated and untreated diabetic rats. The curved linear velocity (VCL) observed in the semen of diabetic rats was not significantly different from the normal controls and the treated rats (Figure 7.5A). The straight-line velocity (VSL) was significantly reduced in the diabetic controls when compared with the normal controls (Figure 7.5B), while VSL was enhanced in the rats treated with 200 and 400mg/kgBW

of AD. Spermatozoa of diabetic controls had a 28.5% reduced average path velocity (VAP) when compared with spermatozoa from normal rats. An observable increase of 15.7% and 23.7% in the VAP was noticed in the sperm of rats treated with 200 and 400mg/kgBW (Figure 7.5C). Linearity (LIN), straightness and oscillation index (WOB) of the spermatozoa were significantly affected and decreased in diabetic control. Administration of both concentrations of AD improved the LIN, WOB and straightness of the sperm cell in treated diabetic rats (Figures 7.6 (A-C)).

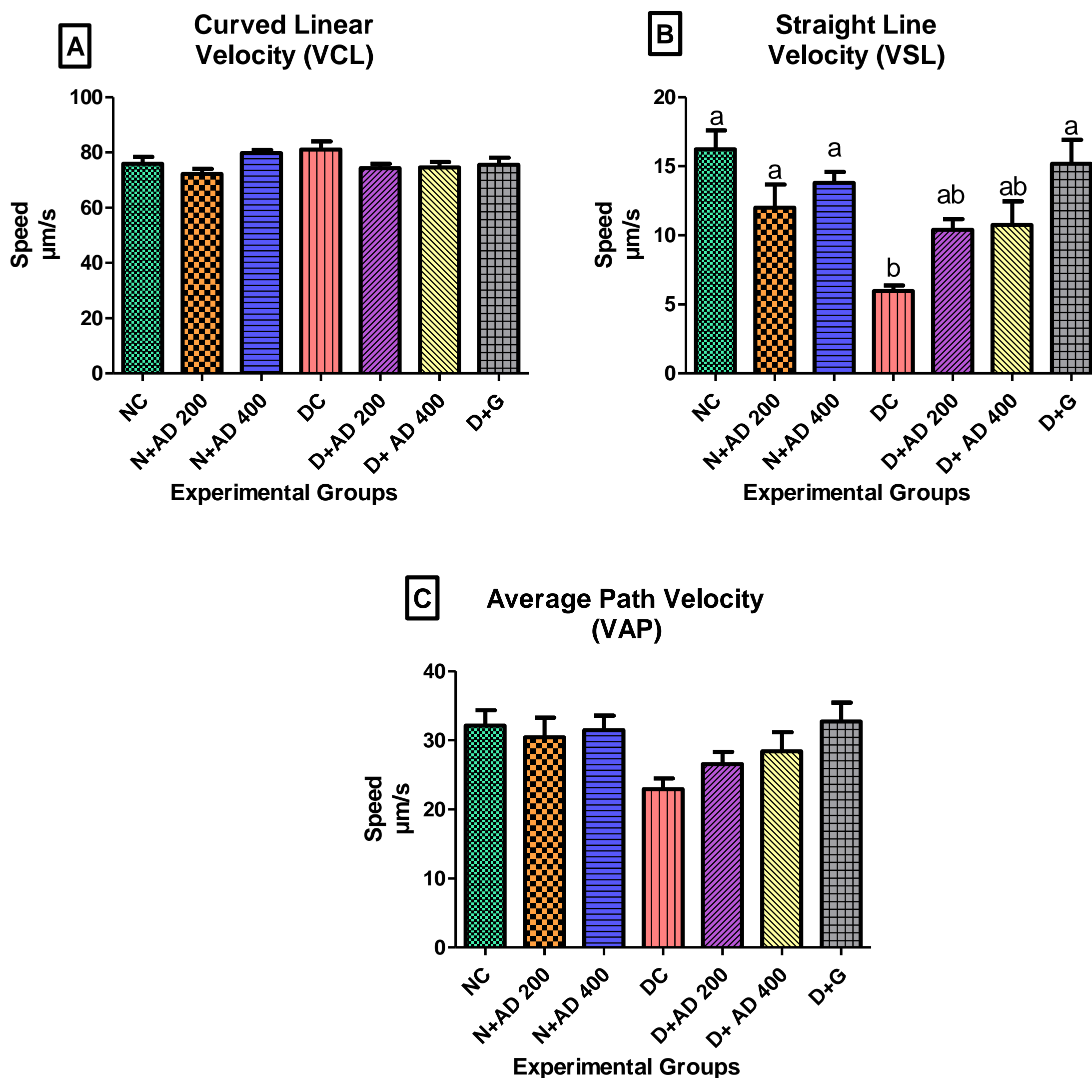


Figure 0.5: Effect of AD administration on indices of sperm velocities **(A)** VCL **(B)** VSL **(C)** VAP and **(D)** BCF of spermatozoa in normal and diabetic rats. Bars are indicative of mean values ± SEM of group values. Bars with different letters are significantly ($p<0.05$) different from each other.

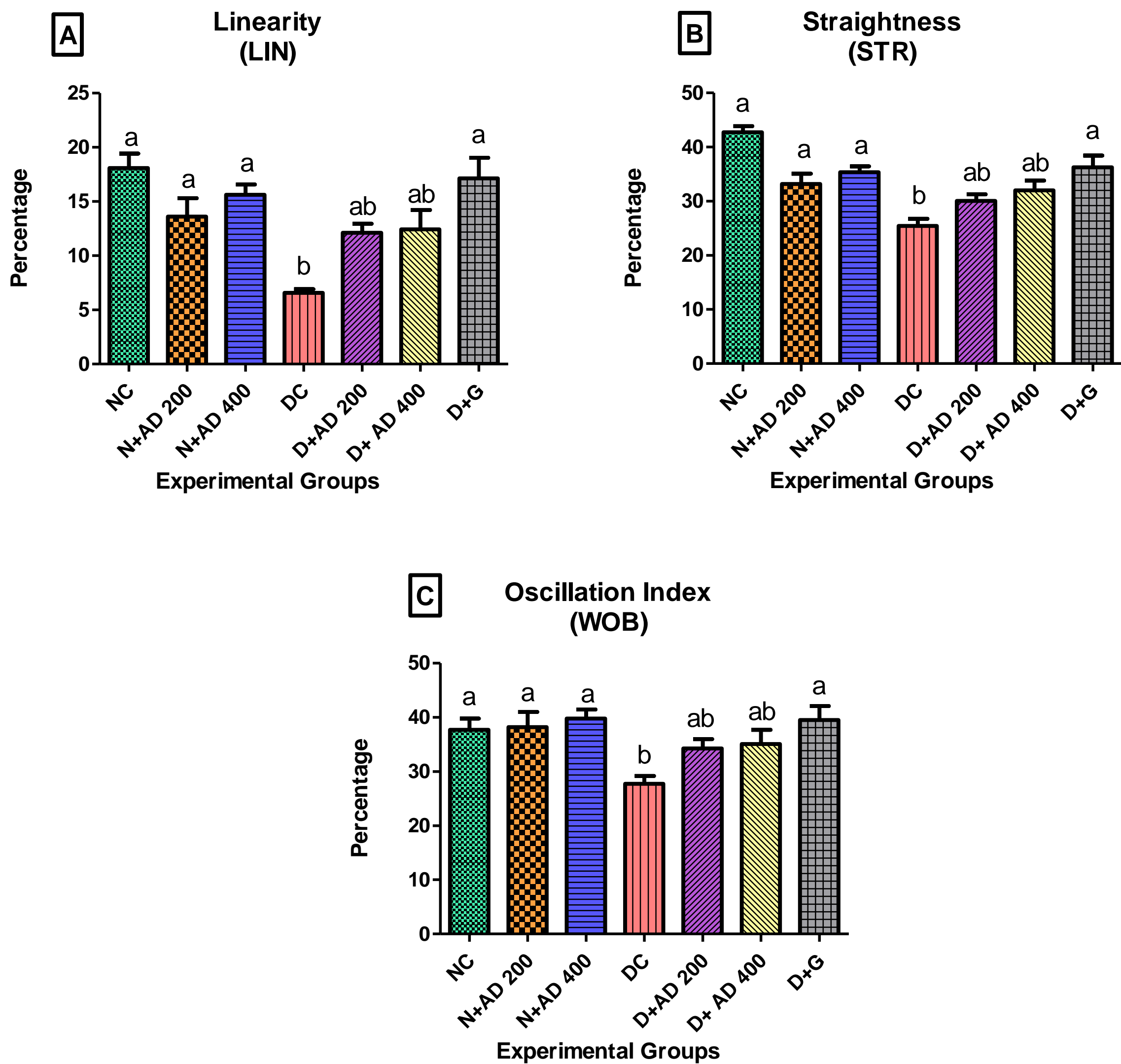


Figure 0.6: Effect of AD administration on sperm kinematics; **(A)** LIN **(B)** STR and **(C)** WOB of spermatozoa in normal and diabetic rats. Bars are indicative of mean values ± SEM of group values. Bars with different letters are significantly ($p<0.05$) different from each other.

The impact of STZ-induction of diabetes and treatment on the factors affecting WOB is presented in Figure 7.7. ALH was increased by 20% in the diabetic controls and diabetic rats treated with 200mg/kgBW when compared with normal rats. The administration of 400mg/kgBW led to a 25% reduction in the abnormal ALH values in treated diabetic rats comparable to normal and standard drug (Figure 7.7A). There were no significant changes in the sperm BCF values in the normal, diabetic and treated rats

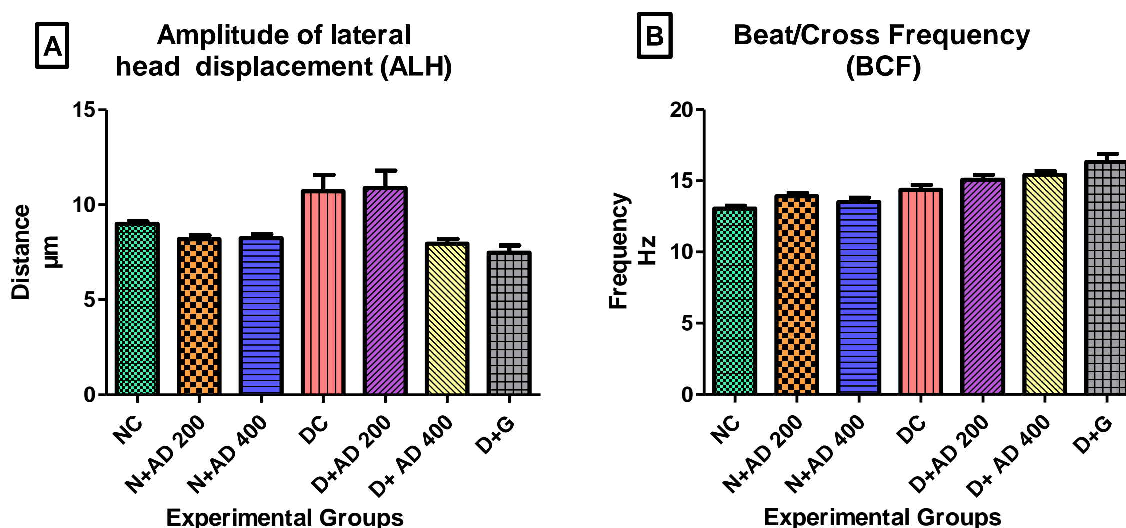


Figure 0.7: Effect of AD administration on indices of oscillation index; **(A)** ALH and **(B)** BCF of sperm cells in normal and diabetic rats. Bars are indicative of mean values ± SEM of group values.

7.3.4 The Effect of AD administration on sperm morphometric indices in normal and diabetic rats

To further investigate the effect of diabetes, and treatment with AD and glibenclamide, certain parameters relating to morphology of the sperm cell were measured in the normal, diabetic and treated rats. The arc, width, area, perimeter, angle, roughness and chord of the sperm head, together with the area, width and angle of the sperm midpiece were measured (Table 7.1). Result showed that there are no significant changes in the spermatozoa head and midpiece of normal, diabetic and treated rats.

Table 0.1: Morphology parameters of sperm cells from normal and diabetic rats

HEAD	NC	N+AD 200	N+AD 400	DC	D+AD 200	D+AD 400	D+G
Arc	21.36±0.14	21.46±0.16	21.23±0.10	20.86±0.21	20.71±0.14	21.20±0.1	20.89±0.14
Width	1.415±0.02	1.442±0.03	1.513±0.02	1.518±0.03	1.485±0.03	1.500±0.02	1.474±0.03
Area	18.01±0.26	18.81±0.22	18.82±0.19	18.52±0.19	17.94±0.31	18.55±0.25	18.44±0.38
Perimeter	45.48±0.33	45.60±0.3	45.36±0.20	44.52±0.37	44.42±0.26	45.23±0.18	44.64±0.31
Angle	66.18±1.39	63.26±1.39	62.71±1.61	61.81±1.01	62.75±0.80	63.13±0.92	62.75±0.79
Roughness	0.110±0.00	0.113±0.00	0.117±0.00	0.116±0.00	0.115±0.00	0.116±0.00	0.117±0.00
Chord	11.34±0.07	11.36±0.16	10.99±0.10	11.12±0.15	10.91±0.13	11.06±0.16	10.86±0.09

MID PIECE							
Area	2.19±0.16	2.68±0.13	2.38±0.14	2.70±0.16	2.17±0.11	2.41±0.11	2.62±0.15
Width	0.67±0.02	0.69±0.02	0.707±0.02	0.701±0.02	0.675±0.01	0.690±0.02	0.702±0.02
Angle	11.11±0.56	11.44±0.71	10.78±0.43	13.39±0.49	12.16±0.93	11.42±0.68	10.04±0.64

7.3.5 The effect of intervention with AD on gonadal tissues; testis and epididymis in STZ-induced diabetes

Figures 7.8 and 7.9 present the findings from the microscopic examination of the testes and epididymal tissues. The normal and treatment control gonadal tissues showed normal architecture, the structure of the germinal epithelium and spermatozoa were intact. Autolytic changes characterized with vacuolation within the germinal epithelium and loss of the Sertoli and germ cells were observed in the testes of diabetic control rats (Figure 7.8). These pathological changes led to a severe disruption in spermatogenesis depicted by degeneration of spermatogonia, spermatocytes and spermatids. The testes of the rats administered 200mg/kgBW of AD revealed moderate vacuolation while the testes of rats treated with 400mg/kgBW had very mild vacuolation. Treatment with AD repaired the germinal epithelium of the treated diabetic rats to near normal when compared with the diabetic control.

The structures of the epithelia lining the tubules of the epididymis were clearly seen with nicely arranged cilia and high intraluminal sperm density in the normal controls and treated controls. In diabetic controls, several sloughed germ cells were observed in the lumen of the epididymis, and a reduced sperm density. Also, the brush border; ciliated epithelium was disrupted (Figure 7.9). Treatment with 200mg and 400mg/kgBW of AD markedly impede sloughing of germ cells and restored the epithelium of the epididymis with the cilia visible and nicely arranged. Administration of AD further increased the intraluminal sperm density when compared with diabetic control.

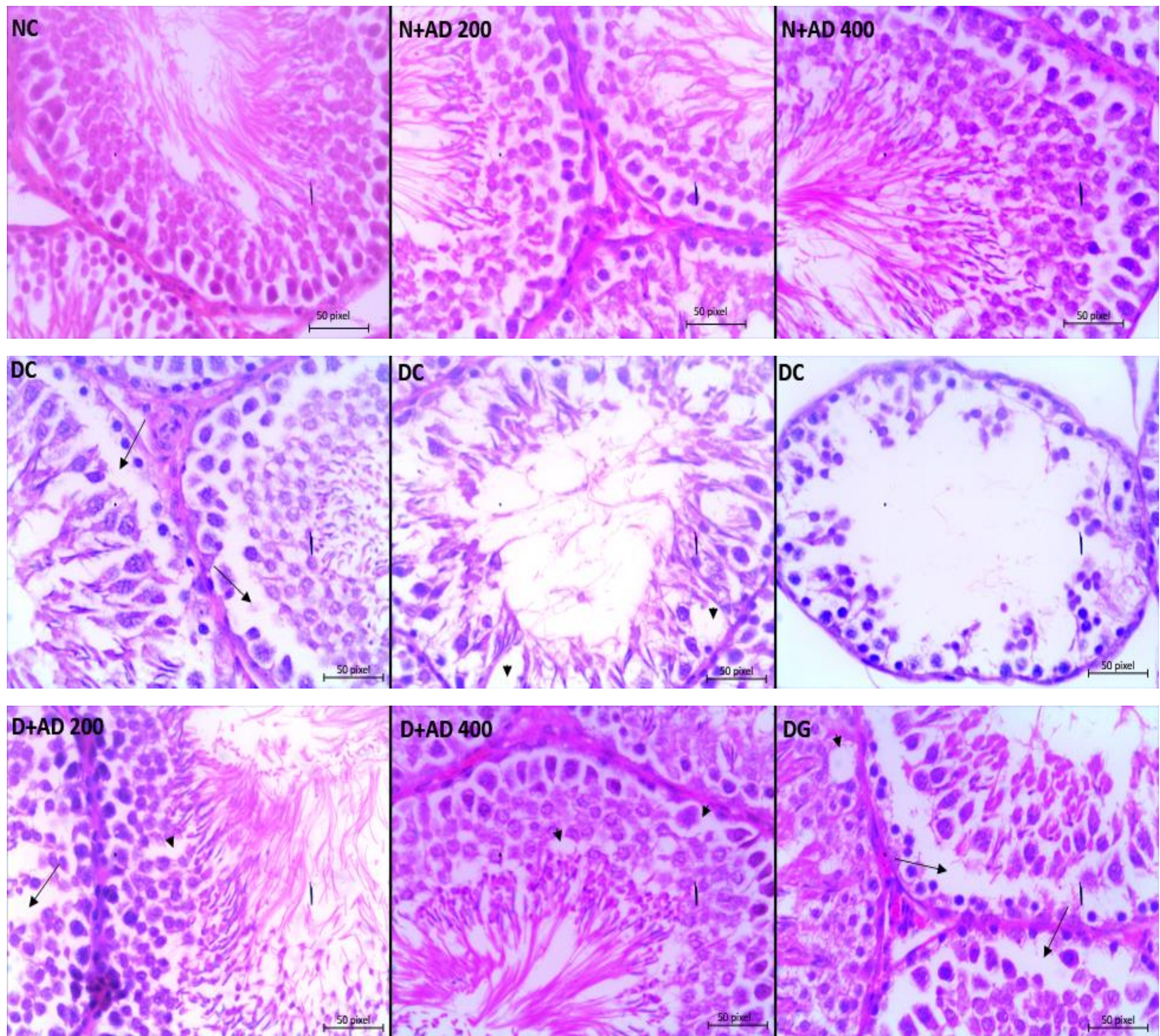
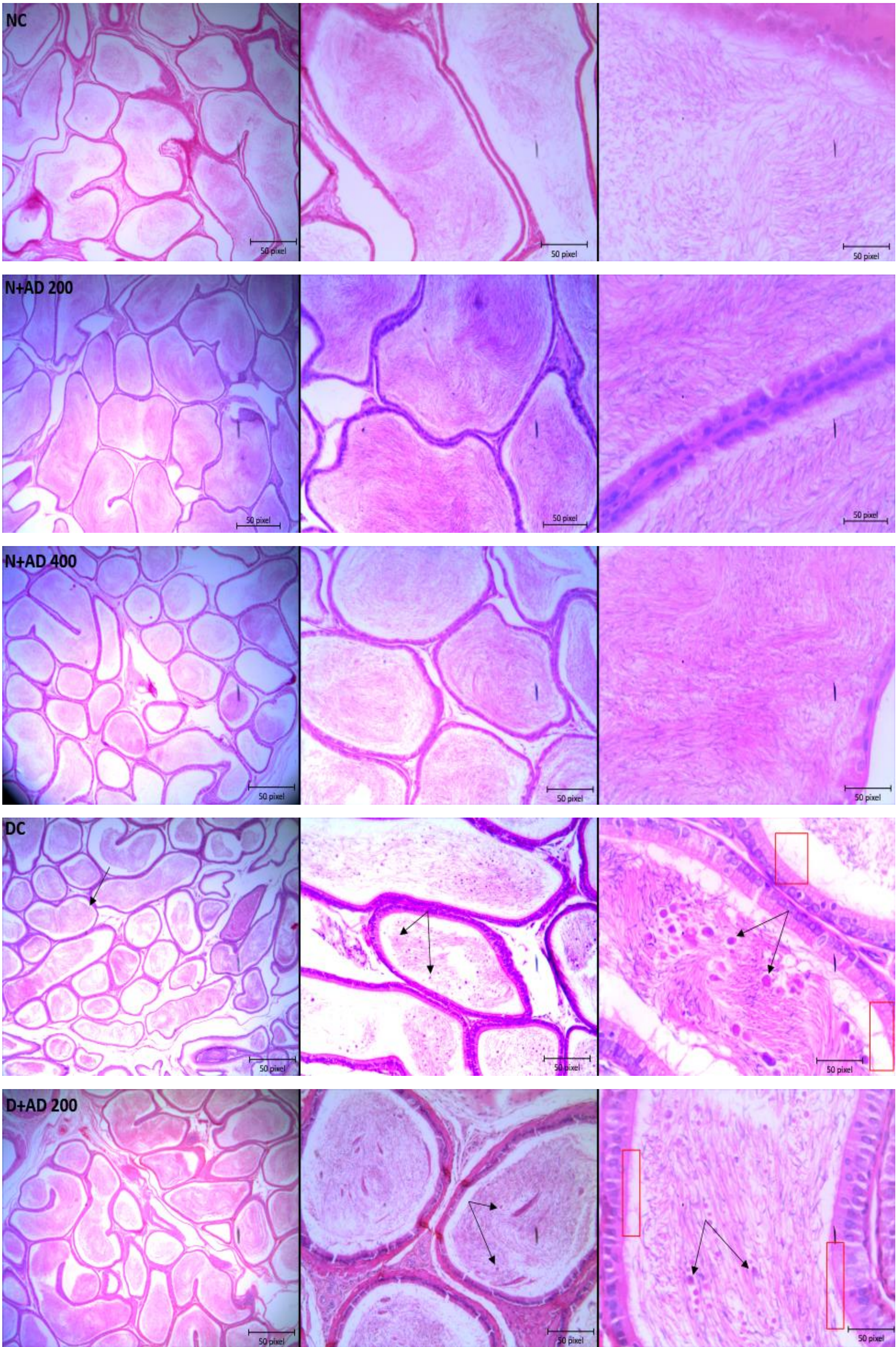


Figure 0.8: Light micrographs of the testes of normal and diabetic rats stained with hematoxylin–eosin (x400). The arrowhead reveals vacuolation within the germinal epithelium, the long arrow shows loss of germ cells and distorted spermatogenesis (40x Magnification).


NC
50 pixel
50 pixel
50 pixel
N+AD 200
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DC
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D+AD 200
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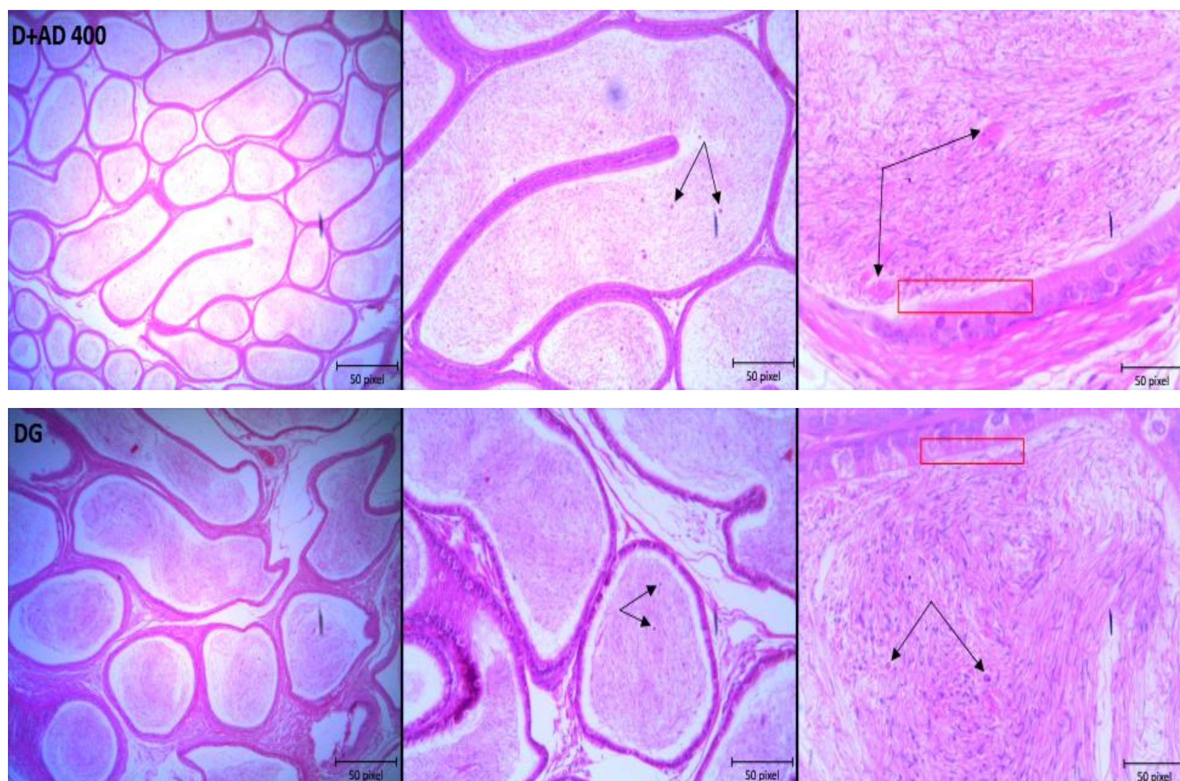

Figure 0.9: Light micrographs of the testes of normal and diabetic rats stained with hematoxylin–eosin (x400). The double arrows reveal sloughed germ cells within the epididymal lumen, while the red rectangle focuses on the cilia and brush border of the epithelium of the epididymis. (4x, 10x and 40x Magnification).

7.4 Discussion

Diabetes mellitus is known to affect sexual behaviour and reproductive tract functions which ultimately leads to a reduced fertility (Han et al., 2019). Male reproductive irregularities associated with diabetes and the ability of various medicinal plants to ameliorate diabetes and testicular dysfunction in diabetic rats have been explored (Suresh et al., 2010; Arokoyo *et al.*, 2018; Shoorei et al., 2019). The findings from this study established the potency of AD in ameliorating diabetes-induced reproductive dysfunction. Persistent hyperglycemia resulted in significant increase in the relative testicular weight and a significant decrease in the weight and relative weight of the epididymis. Similar findings have been reported in diabetic studies involving the use of STZ (Arokoyo et al., 2017; Omolaoye et al., 2018). Progression of diabetic condition is known to cause significant changes in the weight of the body and internal organs including the gonads (Cintra et al., 2017; Han et al., 2019). Disparity in relative organ weights

serve as one of the crucial factors in assessing the presence of toxicity and pathology in an organ (Akhigbe, 2014; Al-Malki & El Rabey, 2015). The ability of AD to increase the total and relative epididymal weight, and also the reduce relative testicular weight in treated diabetic rats comparable to standard drug establishes its potency against diabetes induced testicular and epididymal toxicity.

There was no significant difference in the percentage sperm motility of the normal, diabetic and treated rats. This was also reported in a study conducted by Arokoyo and colleagues (Arokoyo et al., 2017) on diabetic-induced reproductive dysfunction. In the present study, it is worthy of note that no tail defects were observed in the morphology assessment across the groups, especially seeing that the tails are important structures that drive sperm motility (Lehti & Sironen, 2017). The absence of tail defects, especially in the diabetic controls might explain the non-disparity in the percentage sperm motility in the normal, diabetic and treated rats. Interestingly, a significant decrease was observed in the sperm velocities and kinematics of untreated diabetic rats and this was significantly increased with AD supplementation in treated diabetic rats (Figure 7.5 & 7.6). A very similar result was documented by Mukhopadhyay and colleagues, (Mukhopadhyay et al., 2010), where routine semen analyses from patients exposed to heavy metals and cigarette smokes showed normal motility. However, further probing on CASA system using the same semen samples, showed that VCL, STR and ALH which are sperm velocity parameters were significantly reduced. This highlights the possibilities of impaired sperm motion which may not reflect in the overall percentage motility but observed in the significant differences in sperm velocities and kinematics as observed in the diabetic control rats. Furthermore, STZ administration can affect sperm velocities and kinematics (Omolaoye et al., 2018). However, AD seems to prevent these anomalies in a similar manner to the standard drug.

A relationship has been shown to exist between sperm velocities, kinematics and concentration (Farooq et al., 2018), sperm concentration is negatively correlated to VCL, VSL

and VAP. The reported VAP values were twice the VSL values (Figure 7.5B and 7.5C), and these are observed in cases of irregular sperm motion path which have significant effects on the WOB, STR, ALH and BCF of sperms (Lu et al., 2014). Sperm velocities directly correlates to LIN, STR, WOB, ALH and BCF (Farooq et al., 2018), which further strengthens our findings in this study.

Sperm concentration, motility, morphology and viability are important factors to consider when assessing the sperm or semen quality (de Villiers, 2018). The significant reduction in the sperm concentration, viability, morphology and motility parameters in the diabetic controls may be linked to impaired spermatogenesis observed in the seminiferous tubules as it affects the quantity and quality of the spermatozoa produced (Bo et al., 2015). Spermatogenesis is usually disrupted in diabetes through hyperglycemia-induced oxidative stress (Armstrong et al., 1999). Antioxidants have shown to increase sperm viability and reduce sperm defects by alleviating oxidative stress (Suresh et al., 2010). Phytochemical characterisation carried out on the aqueous extract of AD leaves established the presence of bioactive compounds that have high antioxidant and antidiabetic properties such as quercetin, kaempferol, rutin, and phloridzin amongst others (Alabi et al., 2019). The significant reduction in sperm concentration corresponds with the significantly reduced weight of the epididymis and the relative epididymal weight as the epididymis stores spermatozoa until maturation. The ability of AD to improve hyperglycemia-induced sperm functions is most likely due to its possession of these bioactive compounds.

Male fertility largely depends on the complete development of spermatozoa in the testis and their maturation in the epididymis (Yeung & Cooper, 2002), inferentially, sperm quality is compromised in the case of pathological alterations in the testis or epididymis. The severe pathological features observed in the testis and epididymis of diabetic controls were associated with the decreased sperm functions in the same animals. The germinal cells observed in the epididymal lumen of diabetic rats were immature germ cells that were sloughed from the

germinal epithelium of the testis and were transported with the spermatozoa into the epididymis. It is evident from the findings that the level of damage in the germinal epithelium of the testes was commensurate to the proportion of sloughed germ cells observed in the epididymal lumen of diabetic control rats. These were noticeably reduced in the diabetic rats treated with AD. Sertoli cells are potential targets for toxicants (Monsees et al., 2000), severe loss of these cells as noticed in our study is possibly due to the administration of STZ. The loss of Sertoli and germ cells in the diabetic controls were accountable for the reduced sperm production, as the number of Sertoli cells are strongly related to sperm concentration. Sertoli cells play a key role in the attachment, development and sustenance of germ cells, they supply the nutrients and hormones required by the germ cells (Monsees et al., 2000). Hence, damage or the loss of Sertoli cells results in disrupted spermatogenesis, loss of germ cells, decreased sperm production and reproductive dysfunction in the diabetic control which was repaired with treatment with AD. The potentials of AD to prevent germinal epithelial loss and improve sperm quality and functions provides a natural alternative and therapy in attenuating diabetes related sexual dysfunctions. These findings however provide the basis for further studies that will aim at investigating the underlying mechanisms by which AD exerts its pro-fertility abilities in diabetic and non-diabetic conditions.

7.5 Conclusion

The potency of *Anchomanes difformis* displayed against diabetic-induced damage in the reproductive system might be a new and promising tool in the management of sexual dysfunctions and associated complications that arise in diabetes mellitus.

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Declaration of conflict

The authors do not have any conflict of interest.

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CHAPTER EIGHT
GENERAL DISCUSSION AND CONCLUSION

8.1 General discussion

Natural therapy is a promising approach in the prevention and treatment of several ailments and diseases prevalent among humans and animals today. The use of medicinal plants in the management of diabetes is getting more attention, especially with the increasing prevalence of diabetes, its social and economic significance. The preference of alternative medicine over conventional drugs is due to its availability, cost effectiveness and little or no associated adverse effect (Wang *et al.,* 2003; Fimognari *et al.,* 2006; Hinterthuer, 2008). *Anchomanes difformis* is one of such medicinal plants with strong ethnopharmacological relevance including its use in diabetic conditions (Ahmed, 2018). However, there is paucity of scientific evidence on its antidiabetic potentials and the underlying mechanisms. Hinged on these, the hypoglycemic, hypolipidemic, anti-inflammatory, anti-apoptotic abilities of *Anchomanes difformis*, and its potentials against reproductive dysfunction was investigated in this study.

Trending reports on the biological activities of *Anchomanes difformis,* its relation to diabetes and oxidative stress was reviewed. The aetiology and pathogenesis of type 2 diabetes and diabetic complications were critiqued. The present study was partitioned into two; the *in vitro* analyses on the *Anchomanes difformis* extracts and the *in vivo* investigations on the effect of *Anchomanes difformis* in diabetic male Wistar rats.

The *in vitro* phase of this study focused on the screening and selection of the more effective plant-part of *Anchomanes difformis* and its most efficacious extract. This was carried out by evaluating antioxidant capacities; ORAC, FRAP and TEAC on three solvent extracts (aqueous, ethanol and ethyl acetate) each from the leaves and the rhizome of *Anchomanes difformis.* Quantitative phytochemical analyses of the polyphenolic content, flavonol and flavanol content were also conducted on these extracts. HPLC and UP-LCMS was used for identification and

characterization of bioactive compounds present in these various extracts. All the six extracts demonstrated antioxidant properties and contain polyphenols, flavonol and flavanols. However, aqueous extract exhibited the highest antioxidant ability in ORAC, FRAP and TEAC assays that were evaluated. This is likely attributed to its polyphenolic content as it contained the highest polyphenols. Polyphenols have been positively and strongly correlated with antioxidant power (Locatelli *et al.*, 2017) and reported for its biological activities which include anti-inflammatory, antidiabetic, antioxidant, anticancer, profertility potentials (Li *et al.*, 2014; Abbas *et al.,* 2017). Hence, aqueous extract was selected to further investigate the potentials of *Anchomanes difformis* in diabetes using male Wistar rats.

The *in vivo* experiment of this study centered on the ameliorative potentials of *Anchomanes difformis* in type 2 diabetes and associated complications in the liver, heart, kidney and reproductive system. Fructose and STZ was used to induce type 2 diabetes in male Wistar rats (Wilson and Islam, 2012). Treatment with two doses of *Anchomanes difformis*; 200 and 400 mg/kgBW was administered in comparison with a standard drug; glibenclamide (5 mg/kgBW). The ethanolic extract from the rhizome of *Anchomanes difformis* has been shown to exhibit hypoglycemic ability in alloxan-induced diabetes in wistar rats (Adeyemi *et al.,* 2015) The results from the *in vivo* diabetic experiment validated the hypoglycemic activity and unveiled the hypolipidemic properties of *Anchomanes difformis* over the reference drug; glibenclamide. Both doses of aqueous leaves extract of *Anchomanes difformis* significantly reduced the blood glucose concentration in the treated diabetic rats, and this was more effective than glibenclamide. Constant hyperglycemia led to significant weight loss in the diabetic rats. Intervention with both doses of *Anchomanes difformis* significantly prevented weight loss in the treated diabetic rats. 200 and 400 mg/kgBW of *Anchomanes difformis* demonstrated greater capacity to prevent weight loss than glibenclamide. Significant changes in the weights and relative weights of organs are reflective of organ toxicity (Tanna *et* al., 2015). Induction of diabetes caused significant increase in the weights of the heart, kidney pancreas and testes, while a significant decrease was observed in the epididymis in the diabetic controls.

Treatment with *Anchomanes difformis* reduced the relative weights of the heart, kidney pancreas and testes while it increased the relative weight of the epididymis to normal in treated diabetic rats.

Oxidative stress plays a pivotal role in the pathogenesis and progression of diabetes (Pan, 2008). For this study, the oxidative stress level and the antioxidant status in the serum and the organs were measured. Lipid peroxidation (TBARS) and GSH-GSSG ratio were measured in the serum and the liver respectively, in addition, ORAC and FRAP levels were determined in the serum and the liver of normal, diabetic and treated rats. Treatment with *Anchomanes difformis* significantly reduced lipid peroxidation in diabetic rats, while glibenclamide did not exhibit any reducing effect. Antioxidant status was also restored to normal in the serum and the liver of diabetic rats placed on *Anchomanes difformis,* as observed by ORAC levels which were restored to normal and increased GSH-GSSG ratio. Furthermore, the administration of *Anchomanes difformis* increased tGSH in a dose-dependent manner when compared to the normal control rats. *Anchomanes difformis* also increased ORAC and FRAP levels and reduced TBARS levels in non-diabetic rats thereby enhancing antioxidant status and diminishing oxidative stress. This suggests that *Anchomanes difformis* has preventive ability against increased oxidative stress. More investigations on the impact of *Anchomanes difformis* in attenuating diabetic hepatopathy, revealed the ability of *Anchomanes difformis* to significantly reduce the leakage of ALP, AST and ALT from the hepatocytes in diabetic treated rats. *Anchomanes difformis* displayed its protective role on the liver by increasing the antioxidant status and reducing oxidative stress and prevented further oxidative damage to the liver. The glycemic control displayed by *Anchomanes difformis* can be attributed to its antioxidant and lipid-lowering ability, as these are important factors in persistent hyperglycemia (Adeyemi *et al.,* 2015).

The investigations on the impact of *Anchomanes difformis* in diabetes extended to its ameliorative potentials in complications associated with diabetes such as cardiomyopathy,

nephropathy and sexual dysfunctions. Increased oxidative stress, inflammation, and apoptosis are implicated in the development of diabetic complications (Miranda-Díaz *et al.,* 2016), therefore, the effect of *Anchomanes difformis* on these factors were assessed in the heart and kidney. As a result of the increased oxidative stress, Nrf2; a transcription factor that triggers the transcription of antioxidant enzymes was significantly expressed in the heart of diabetic controls. This led to the significant, increased production of catalase and SOD in the diabetic controls. An inverse relationship has been established between antioxidant enzymes and some inflammatory markers, especially between catalase, IL-1β and IL-6 (Mathy-Hartert *et al.,* 2008; Gutierrez-Ruiz *et al*., 2001). The increasing activities of catalase led to a decrease in the production of IL-1β and IL-6, this was modulated by intervention with *Anchomanes difformis* in the diabetic treated rats. NFkB; a chief player in the inflammatory response was significantly elevated in the heart of diabetic controls which led to a corresponding increase in the production of IL-18 in the diabetic hearts. IL-10 levels were also decreased in the diabetic controls. NFkB and IL-18 expressions were significantly reduced and IL-10 increased following treatment with *Anchomanes difformis.* NFkB has also been implicated in increased apoptosis (Jin *et al*., 2018), an observable increase in caspase 3 expression and decrease in Bcl2 expression was seen in the diabetic control. The effect of the administration of *Anchomanes difformis* was also observed on apoptosis, as Bcl2 expression was increased and Caspase 3 expression was downregulated in the treated diabetic rats. *Anchomanes difformis* attenuated pathological conditions in the heart by down-regulating inflammation and reducing apoptosis in the heart tissues.

Furthermore, the effect of *Anchomanes difformis* in the diabetic kidney damage was assessed. The results obtained revealed significant increase in markers of kidney injury and toxicity such as urea and relative kidney weight in the diabetic controls. This was reduced in the diabetic rats treated with *Anchomanes difformis*, especially 400 mg/kgBW. *Anchomanes difformis* enhanced the expression of Nrf2 and the activities of catalase in the treatment controls (non-diabetic rats). The expression of Bcl2 was significantly downregulated in diabetic controls,

management with *Anchomanes difformis* markedly restored Bcl2 to normal levels in diabetic treated rats. Increased proinflammatory markers (IL-1β and IL-6), were reduced in diabetic rats following treatment with *Anchomanes difformis. Anchomanes difformis* ameliorated kidney damage through antioxidant-mediated roles such as up-regulation of transcription factor Nrf2 which articulates the expression of antioxidant enzymes. Also, *Anchomanes difformis* modulated inflammatory response and repressed apoptosis in the kidney tissues.

The prevalence of male infertility in prolonged diabetes mellitus prompted the exploration of the impact of *Anchomanes difformis* on sperm function in diabetes (Han *et al.,* 2019). The induction of diabetes mellitus led to significant reductions in sperm concentration, viability and morphology in the diabetic controls. This was completely reversed to normal level with the administration of *Anchomanes difformis* in the treated diabetic rats. There was no significant difference in the percentage motility of the sperm cells in normal, diabetic and treated rats. However, further investigations on the velocities and kinematics of the sperm cells revealed a significant decrease in the sperm velocities and kinetics in the diabetic rats. This was also observed by Mukhopadhyay and colleagues, (Mukhopadhyay et al., 2010), where semen routine analyses of sperm cells from patients exposed to heavy metals and cigarette smokes showed normal motility. However, further probing on CASA system using the same semen samples, showed that VCL, STR and ALH which are sperm velocity parameters were significantly reduced. *Anchomanes difformis* administration had a positive effect on sperm velocities and kinematics in the treated diabetic rats. It is interesting to note that *Anchomanes difformis* may possess pro-fertility abilities as depicted by its capacity to increase sperm concentration in normal rats administered 200 and 400 mg/kgBW in a dose-dependent manner.

Histological examination for any visible pathological changes was conducted in the liver, kidney, pancreas, testis and epididymis in normal, treated and diabetic rats. Histology of the liver; the main organ involved in drug metabolism and most affected by it revealed some

pathological changes in the diabetic rats such as portal inflammation, steatosis, sinusoidal dilatation and centrilobular necrosis. Treatment with 400 mg/kgBW of *Anchomanes difformis* reversed these pathological conditions, while it was significantly reduced in the liver of rats placed on 200 mg/kgBW of *Anchomanes difformis* and this was more effective than glibenclamide. Persistent hyperglycemia resulted in the shrinkage and loss of glomerulus tuft, loss of tubular cells, prominently in the proximal tubules, and increased blood flux in the renal parenchyma. Treatment with 200 mg/KgBW showed mild loss of tubular cells in the PT, while glomerular capillaries were clearly restored and less occurrence of glomerular shrinkage. 400 mg/KgBW AD ameliorated glomerular and tubular damage in the kidney of treated diabetic rats. 400 mg/KgBW prevented loss of tubular cells of the proximal tubules and minimized loss of glomerular tuft in the treated diabetic rats as comparable to normal. In the pancreas, STZ administration led to distortion in the shape and loss of pancreatic islets, severe destruction of the beta cells in the islets and vacuolation in the acinar and islet cells. Intervention with *Anchomanes difformis* improved the structure of the islets of Langerhans and the surrounding acinar cells and minimized vacuolation in the treated diabetic rats in a similar manner as glibenclamide.

Prolonged hyperglycemia affected the structure of the testes in diabetic rats as evident by loss of Sertoli cells and different spermatogenic phases. The brush border of the epididymis was also distorted in the diabetic controls. The administration of *Anchomanes difformis* enhanced spermatogenesis and impeded germinal cell loss in the testes thereby boosting sperm functions; sperm concentration, viability, and morphology and repaired the brush border in the epididymis.

Summarily, findings from this study revealed that *Anchomanes difformis* exerts its antidiabetic potentials by combating oxidative stress, hyperlipidemia, inflammation and apoptosis which are key factors in the aetiology of diabetes and its complications. It is noteworthy that *Anchomanes difformis* displayed preventive roles against oxidative stress, inflammation and apoptosis in non-diabetic rats (treated controls) and enhanced antioxidant status and sperm

functions. Interestingly, *Anchomanes difformis* exhibited more ameliorative potentials in this model than the reference antidiabetic drug; glibenclamide.

8.2 Conclusion

This study focused on the medicinal activities of *Anchomanes difformis* in type 2 diabetes and diabetic complications. This study was able to establish the antidiabetic and hypolipidemic potentials of *Anchomanes difformis.* Furthermore, this study was able to demonstrate the ability of *Anchomanes difformis* to ameliorate and delay the progression of diabetic complication with its novelty in showcasing the mode of action and mechanisms of *Anchomanes difformis* in type 2 diabetes. This discovery will be a useful tool in the management of diabetes and its associated complications.

8.3 Recommendation

The therapeutic competency of *Anchomanes difformis* has been established in this study using Wistar rats, however, there is the need to consider its efficacy in humans through clinical studies. Further research is recommended in this regard. The possible effect of *Anchomanes difformis* on other diabetic complications such as retinopathy, neuropathy and wound healing can be explored, especially those related to its folklore. Studies involving the isolation, characterization and the biological activities of the bioactive compounds present in *Anchomanes difformis* can be designed, this will help identify the compounds responsible for the medicinal properties and if they work in synergy or isolation.

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ADDENDUM

ADDENDUM 1: RESEARCH OUTPUTS

PUBLISHED ARTICLES

- **Toyin D. Alabi,** Nicole L. Brooks, Oluwafemi O. Oguntibeju (2018). Medicinal Activities of *Anchomanes difformis* and its potentials in the treatment of diabetes mellitus and other disease conditions: A Review, In the Book titled "Bioactive Compounds of Medicinal Plants: Properties and Potential for Human Health" 2018, Vol 1, pp 219-235.
- **Toyin D. Alabi,** Nicole L. Brooks, Oluwafemi O. Oguntibeju (2019). Antioxidant Capacity, Phytochemical Screening, and Identification of Active Compounds in *Anchomanes difformis. The Natural Products Journal,* 9(1).
- **Alabi TD**, Chegou NN, Brooks NL, Oguntibeju OO (2020). Effects of Anchomanes difformis on Inflammation, Apoptosis, and Organ Toxicity in STZ-Induced Diabetic Cardiomyopathy. *Biomedicines*, 8(2):1-22

ARTICLES UNDER REVIEW

- Toyin D. Alabi, Nicole L. Brooks, Oluwafemi O. Oguntibeju. Improved antioxidant status and hepato-protective role of *Anchomanes difformis* in streptozotocin-induced diabetes in male Wistar rats. *Journal of Ethnopharmacology*
- Toyin D. Alabi, Nicole L. Brooks, Oluwafemi O. Oguntibeju. *Anchomanes difformis* ameliorated kidney and pancreatic damage in type 2 diabetes? *European Journal of Integrative Medicine*
- Toyin D. Alabi, Nicole L. Brooks, Oluwafemi O. Oguntibeju. The beneficial role of *Anchomanes difformis* in STZ-induced reproductive dysfunction. *Andrologia*

CONFERENCES ATTENDED

- 6th Annual Meeting of the International Cytokine and Interferon Society (ICIS); "Cytokines 2018". 27-30 October 2018, Boston, USA. "*Anchomanes difformis*; a

potential solution to increased inflammation in type 2 diabetes and complications" **Toyin D Alabi**, Nicole L Brooks, Oluwafemi O Oguntibeju (poster presentation).

- 6th U6-International Conference; "Research, Innovation and Technology for African Development". 4th-6th September 2018, Cape Town, South Africa. "Improved antioxidant status and Protective role of *Anchomanes difformis* on the liver in type 2 diabetes" **Toyin D Alabi**, Nicole L Brooks, Oluwafemi O Oguntibeju (oral presentation).
- The South African Annual Pharmacology (SAPHARM) Conference; "Advances in Pharmacological Sciences". 1st-4th October 2017, Bloemfontein, South Africa. "Antioxidant Capacity, Phytochemical Screening and Identification of active compounds in *Anchomanes difformis*" **Toyin D Alabi**, Nicole L Brooks, Oluwafemi O Oguntibeju (oral presentation).

ADDENDUM 2: ETHICS CERTIFICATES

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa
Symphony Road Bellville 7535
Tel: +27 21 959 6917
Email: sethn@cput.ac.za

12 February 2019
REC Approval Reference No:
CPUT/HW-REC 2016/A4 (renewal)

Faculty of Health and Wellness Sciences – Biomedical Sciences Department

Dear Ms Toyin Dorcas Udje

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC on 21 June 2016 to Ms Udje for ethical clearance. This approval is for research activities related to the Department of Biomedical Sciences at this Institution.

TITLE: Effects of Anchomanes difformis extract on biochemical and histological parameters in streptozotocin-induced diabetes and diabetic complications.

Supervisor: Prof OO Oguntibeju and Dr N Brooks

Comment:
Approval will not extend beyond 13 February 2020. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Dr Navindhra Naidoo
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences

Cape Peninsula University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa
Symphony Road Bellville 7535
Tel: +27 21 959 6917
Email: sethn@cput.ac.za

15 September 2017
REC Approval Reference No:
CPUT/HW-REC 2016/A4

Faculty of Health and Wellness Sciences – Biomedical Sciences Department

Dear Ms Toyin Dorcas Udje

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC on 14 September 2017 to Ms Udje for ethical clearance. This approval is for research activities related to the Department of Biomedical Sciences at this Institution.

TITLE: Effects of Anchomanes difformis extract on biochemical and histological parameters in streptozotocin-induced diabetes and diabetic complications.

Supervisor: Prof OO Oguntibeju and Dr N Brooks

Comment:
Approval will not extend beyond 16 September 2018. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Mr. Navindhra Naidoo
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences

Cape Peninsula
University of Technology

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC)
Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa
Symphony Road Bellville 7535
Tel: +27 21 959 6917
Email: sethn@cput.ac.za

23 August 2016
REC Approval Reference No:
CPUT/HW-REC 2016/A4

Faculty of Health and Wellness Sciences – Biomedical Sciences Department

Dear Ms Toyin Dorcas Udje

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC on 21 June 2016 to Ms Udje for ethical clearance. This approval is for research activities related to the Department of Biomedical Sciences at this Institution.

TITLE: **Effects of Anchomanes difformis extract on biochemical and histological parameters in streptozotocin-induced diabetes and diabetic complications.**

Supervisor: **Prof OO Oguntibeju and Dr N Brooks**

Comment:
Approval will not extend beyond 24 August 2017. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Mr. Navindhra Naidoo
Chairperson – Research Ethics Committee
Faculty of Health and Wellness Sciences

ETHICS COMMITTEE FOR RESEARCH ON ANIMALS (ECRA)

29 March 2017

Mrs T Udje
Biomedical Sciences
CAPE PENINSULA UNIVERSITY OF TECHNOLOGY

Dear Mrs T Udje

CERTIFICATE OF FINAL APPROVAL – REF. 04/17 "Effect of *Anchomanes difformis* extract on biochemical and histological parameters in streptozotocin-induced diabetes and diabetic complications in male Wistar rats"

The ECRA Committee reviewed your corrected Application and it was final approved.

Attached herewith please find your Certificate of Approval for the period 30 March 2017 – 30 September 2017. Should this study period not be long enough, please write a letter to the ECRA Committee requested an extension on the study.

Should you encounter any difficulties duties your study or sudden death of the animals, please do not hesitate to inform the ECRA thereof as well as the reason for the death. You will need to submit every 6 months an interim report on the study to the ECRA Committee. The secretariat will inform you thereof in time.

Kind regards.

PROF D DU TOIT
Chairperson : ECRA Committee

THE SOUTH AFRICAN MEDICAL RESEARCH COUNCIL

Animal Ethics Approval Certificate

Decision of the Animal Ethics Committee for the use of living vertebrates for research, diagnostic procedures and product development

APPROVAL PERIOD: 30 March 2017 – 30 September 2017

PROJECT NUMBER:	04/17			
PROJECT TITLE:	**"Effect of *Anchomanes difformis* extract on biochemical and histological parameters in streptozotocin-induced diabetes and diabetic complications in male Wistar rats"**			
PROJECT LEADER:	Mrs Toyin D. Udje			
DIVISION:	Biomedical Sciences – Cape Peninsula University of Technology			
CATEGORY:	Diabetes			
SPECIES OF ANIMAL:	Rattus norvegicus (Wistar)	3 months old Males	250-280gr	
NUMBER OF ANIMALS:	64			
NOT APPROVED:	n/a			
APPROVED:	29 March 2017			

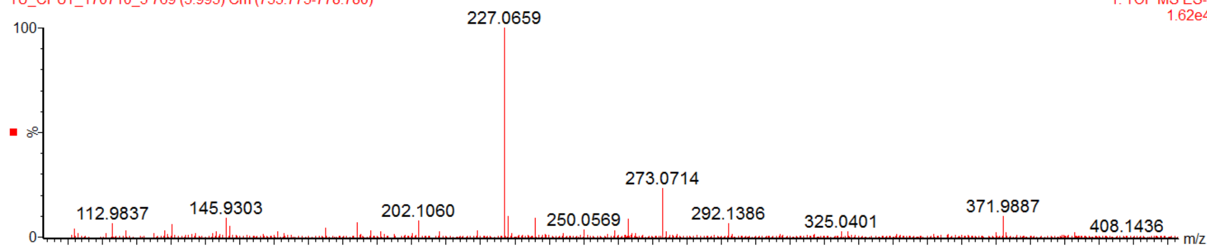
PLEASE NOTE: Should the number or species of animal(s) required, or the experimental procedure(s) change, please submit a revised animal ethics clearance form to the animal ethics committee for approval before commencing with the experiment

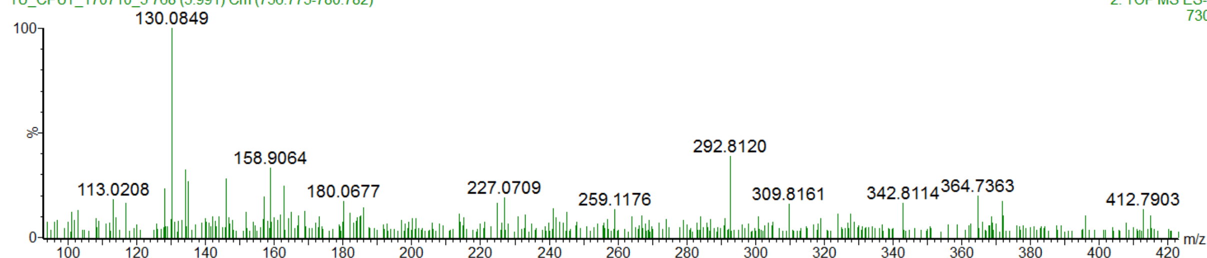
PROF D DU TOIT **DATE** **29 March 2017**

CHAIRPERSON ANIMAL ETHICS COMMITTEE

ADDENDUM 3: CHROMATOGRAMS

Chromatograms of compounds identified in AD, showing the unfragmented, the fragmented spectra, the elemental analysis and the UV spectra.





Elemental Composition

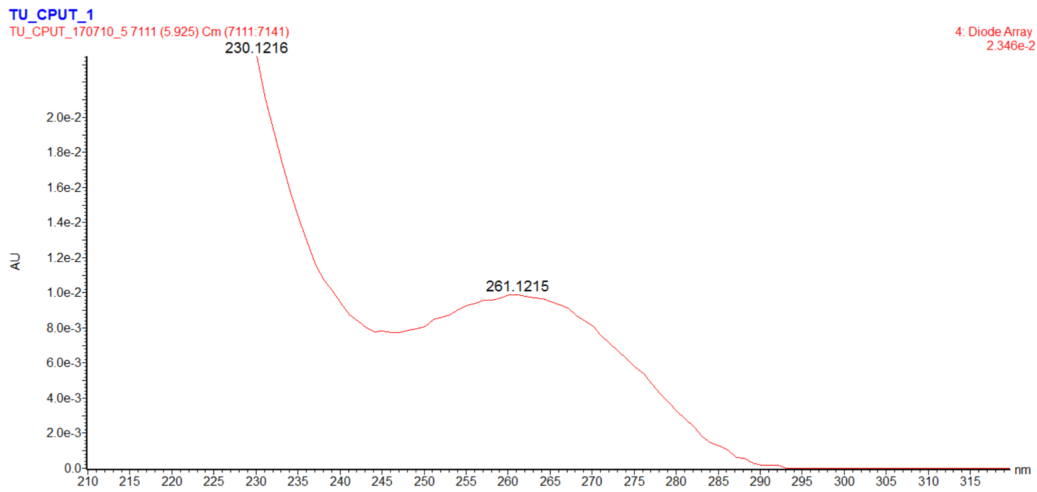
File Edit View Process Help

Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
248 formula(e) evaluated with 44 results within limits (all results (up to 1000) for each mass)
Elements Used:

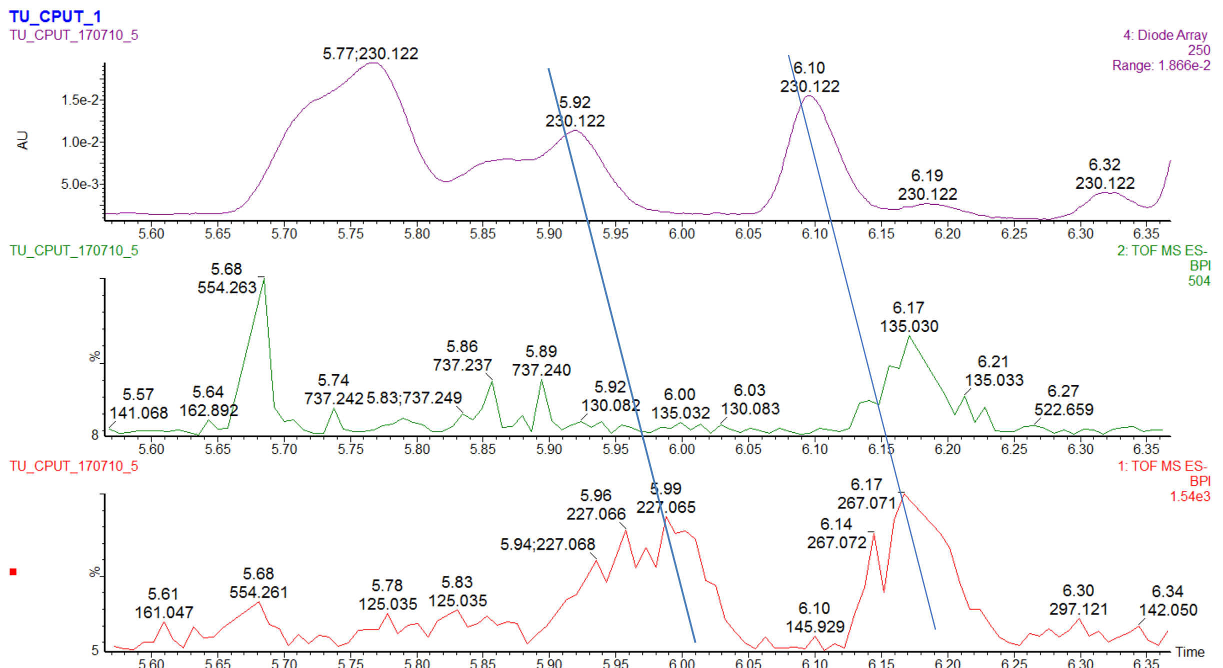
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
737.2288	737.2293	-0.5	-0.7	14.5	C34 H41 O18	8.18	34	41	18
	737.2269	1.9	2.6	45.5	C59 H29	0.04	59	29	
	737.2328	-4.0	-5.4	36.5	C52 H33 O5	0.10	52	33	5
	737.2234	5.4	7.3	23.5	C41 H37 O13	1.29	41	37	13
	737.2352	-6.4	-8.7	5.5	C27 H45 O23	7.14	27	45	23
	737.2199	8.9	12.1	1.5	C23 H45 O26	2.09	23	45	26
	737.2387	-9.9	-13.4	27.5	C45 H37 O10	0.41	45	37	10
	737.2175	11.3	15.3	32.5	C48 H33 O8	0.20	48	33	8

TU_CPUT_1
TU_CPUT_170710_5 768 (5.991) Cm (756:775-780:782)
2: TOF MS ES-
7.30e+002
130.0849
737.2288
292.8120
158.9064
162.8908
128.0336
113.0208
102.9435
227.0709
241.1064
342.8114
364.7363
412.7903
452.1780
508.0375
553.1732
603.9980
656.0779
714.3566
738.2394
790.1284
869.2743
911.4977
942.3420
m/z

For Help, press F1

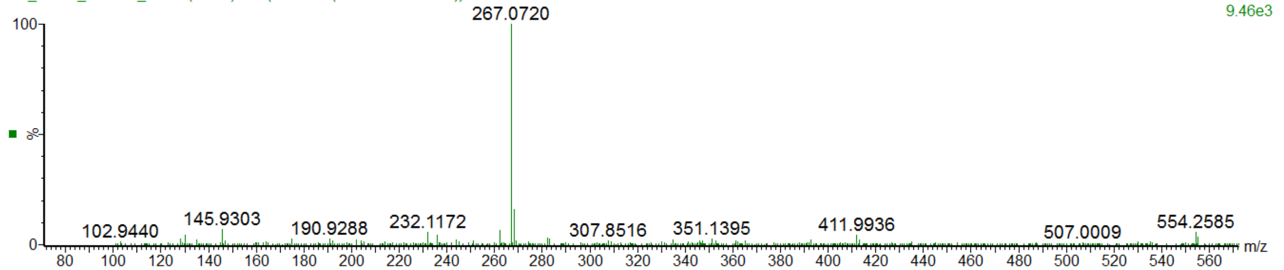


Unknown 737 m/z at 5.99 minutes

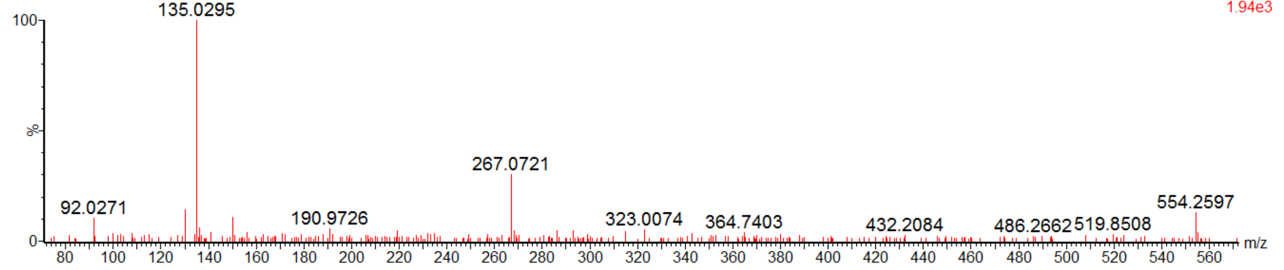


TU_CPUT_1

TU_CPUT_170710_5 793 (6.194) Cm (786:799-(781:782+804:811)) 1: TOF MS ES- 9.46e3



TU_CPUT_170710_5 793 (6.198) Cm (787:796-(779:781+802:806)) 2: TOF MS ES- 1.94e3



Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

40 formula(e) evaluated with 6 results within limits (all results (up to 1000) for each mass)

Elements Used:

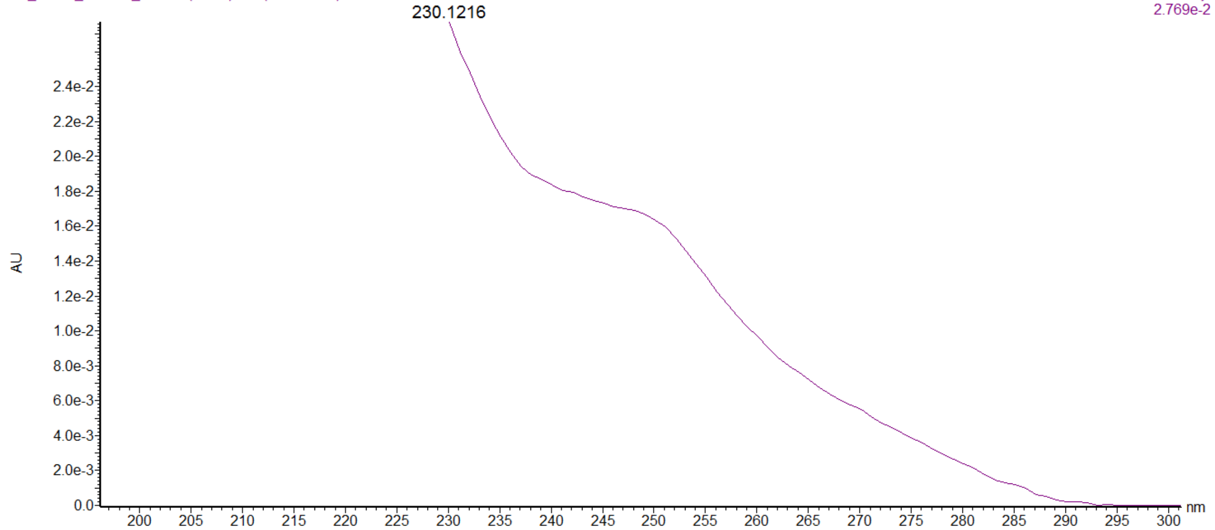
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
267.0720	267.0716	0.4	1.5	2.5	C9 H15 O9	5.20	9	15	9
	267.0657	6.3	23.6	11.5	C16 H11 O4	26.24	16	11	4
	267.0810	-9.0	-33.7	15.5	C20 H11 O	0.53	20	11	1
	267.0869	-14.9	-55.8	6.5	C13 H15 O6	58.44	13	15	6
	267.0564	15.6	58.4	-1.5	C5 H15 O12	0.28	5	15	12
	267.0505	21.5	80.5	7.5	C12 H11 O7	9.32	12	11	7

TU_CPUT_1

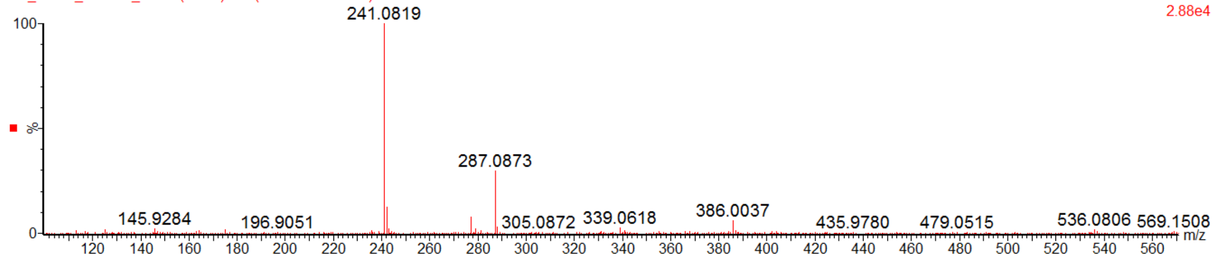
TU_CPUT_170710_5 793 (6.194) Cm (786:799-(781:782+804:811)) 1: TOF MS ES- 9.46e+003

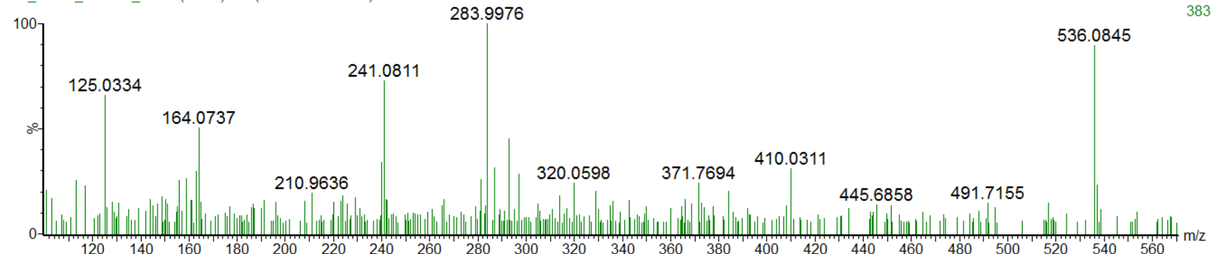
For Help, press F1

TU_CPUT_1



TU_CPUT_1





Elemental Composition

File Edit View Process Help

Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
38 formula(e) evaluated with 5 results within limits (all results (up to 1000) for each mass)
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
241.0819	241.0865	-4.6	-19.1	9.5	C15 H13 O3	0.01	15	13	3
	241.0923	-10.4	-43.1	0.5	C8 H17 O8	0.13	8	17	8
	241.0712	10.7	44.4	5.5	C11 H13 O6	99.86	11	13	6
	241.0653	16.6	68.9	14.5	C18 H9 O	0.00	18	9	1
	241.1017	-19.8	-82.1	13.5	C19 H13	0.00	19	13	

TU_CPUT_1
TU_CPUT_170710_5 973 (7.579) Cm (967:985-1017:1040)
1: TOF MS ES-
3.24e+004

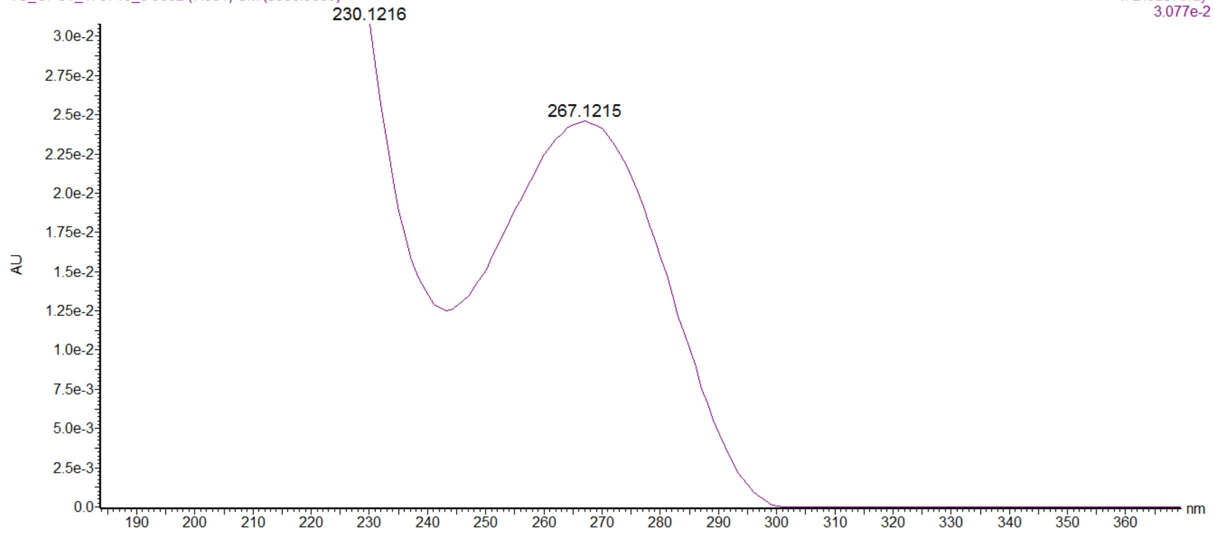
241.0819, 287.0871, 242.0853, 288.0902, 386.0047, 125.0338, 147.9410, 222.0768, 341.0082, 387.0090, 479.0522, 536.0816, 579.1471, 632.0905, 678.1354, 717.1517, 743.6697, 788.1659, 841.1191, 862.6988, 900.0271, 952.3036, 974.2837 m/z

For Help, press F1

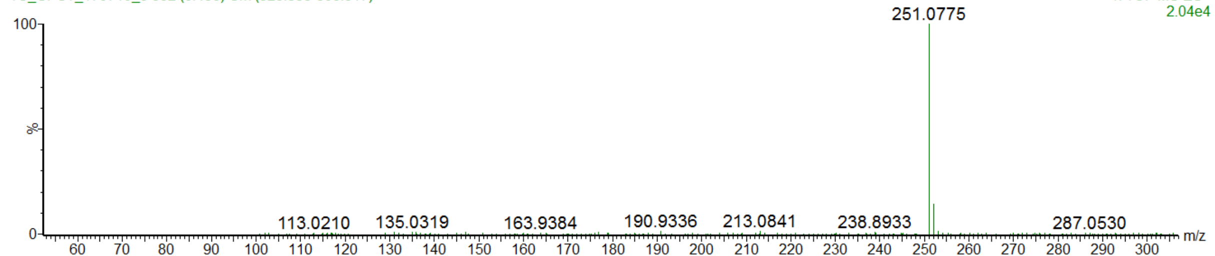
4-Vinylsyringol

251

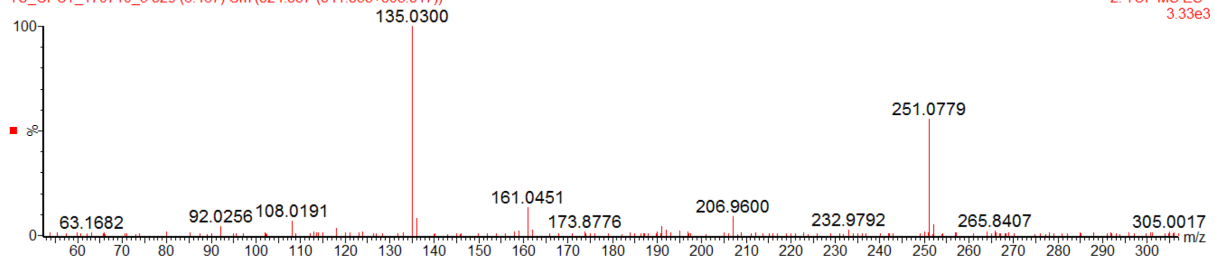
TU_CPUT_1
TU_CPUT_170710_5 9002 (7.501) Cm (8988:9030)



TU_CPUT_1
TU_CPUT_170710_5 832 (6.486) Cm (825:838-803:817)



TU_CPUT_170710_5 829 (6.467) Cm (824:837-(841:853+803:817))



Elemental Composition

File Edit View Process Help

Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
36 formula(e) evaluated with 6 results within limits (all results (up to 1000) for each mass)
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
251.0775	251.0767	0.8	3.2	2.5	C9 H15 O8	1.31	9	15	8
	251.0708	6.7	26.7	11.5	C16 H11 O3	18.90	16	11	3
	251.0861	-8.6	-34.3	15.5	C20 H11	3.53	20	11	
	251.0919	-14.4	-57.4	6.5	C13 H15 O5	58.80	13	15	5
	251.0614	16.1	64.1	-1.5	C5 H15 O11	0.05	5	15	11
	251.0556	21.9	87.2	7.5	C12 H11 O6	17.41	12	11	6

TU_CPUT_1
TU_CPUT_170710_5 832 (6.486) Cm (825:838-803:817)

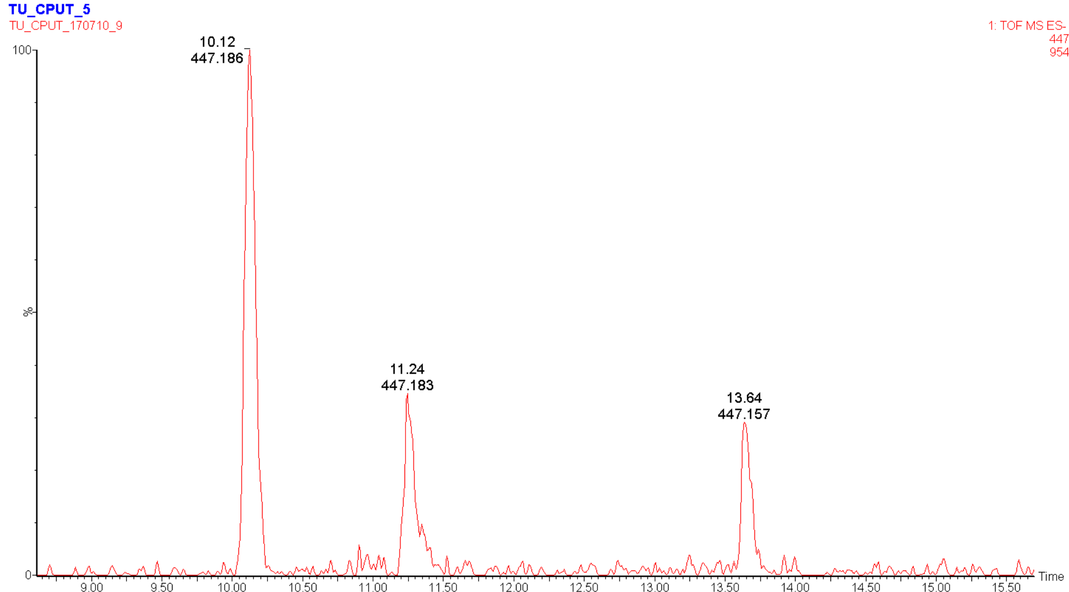
1: TOF MS ES-
2.04e+004

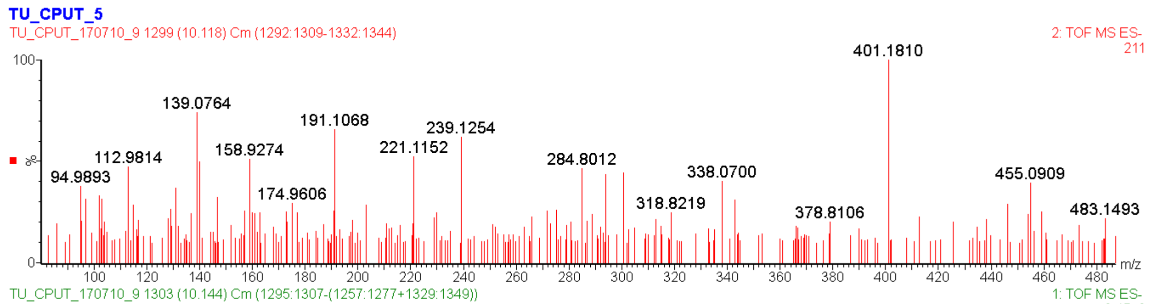
251.0775
252.0800
253.0845
113.0210 131.0359 135.0319 146.9416 150.9118 163.9384 176.9406 190.9336 207.0501 213.0841 216.9187 230.0668 238.8933 271.9343 275.8552 287.0530 298.0503 302.1370

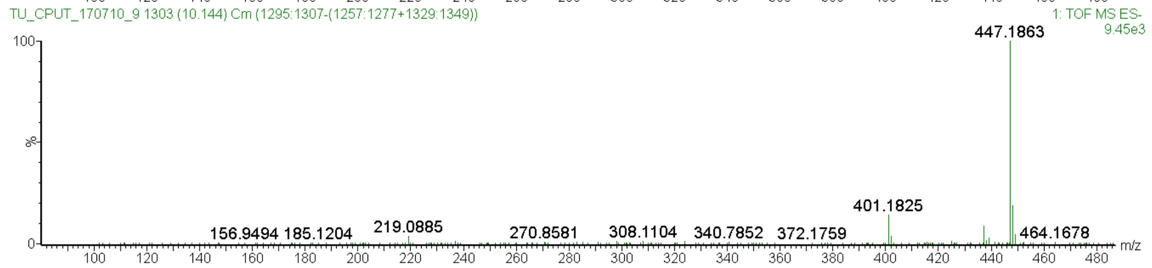
100 % 0
105 110 115 120 125 130 135 140 145 150 155 160 165 170 175 180 185 190 195 200 205 210 215 220 225 230 235 240 245 250 255 260 265 270 275 280 285 290 295 300 305 m/z

For Help, press F1

447







File Edit View Process Help

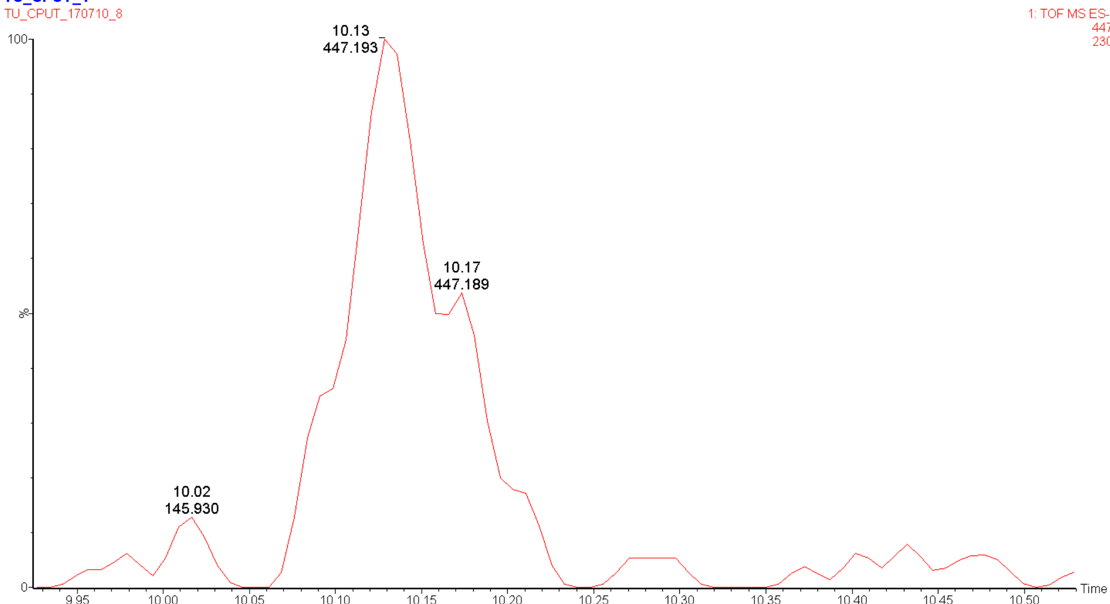
Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
99 formula(e) evaluated with 14 results within limits (all results (up to 1000) for each mass)
Elements Used:

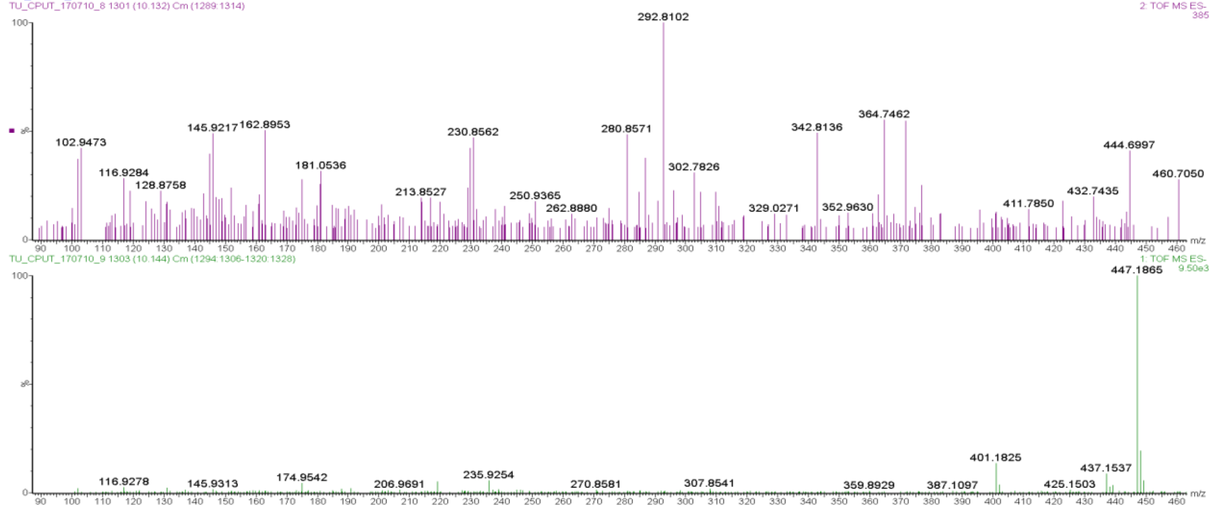
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
447.1863	447.1866	-0.3	-0.7	5.5	C20 H31 O11	1.59	20	31	11
	447.1808	5.5	12.3	14.5	C27 H27 O6	0.05	27	27	6
	447.1960	-9.7	-21.7	18.5	C31 H27 O3	0.02	31	27	3
	447.1749	11.4	25.5	23.5	C34 H23 O	0.01	34	23	1
	447.1714	14.9	33.3	1.5	C16 H31 O14	47.48	16	31	14
	447.2019	-15.6	-34.9	9.5	C24 H31 O8	0.08	24	31	8
	447.1655	20.8	46.5	10.5	C23 H27 O9	0.10	23	27	9
	447.2078	-21.5	-48.1	0.5	C17 H35 O13	49.73	17	35	13
	447.2113	-25.0	-55.9	22.5	C35 H27	0.00	35	27	

TU_CPUT_5
TU_CPUT_170710_9 1303 (10.144) Cm (1295:1307-(1257:1277+1329:1349))
1: TOF MS ES-
9.45e+003
447.1863
448.1899
401.1825
402.1893
449.1932
156.9494 219.0885 237.0375 308.1104 323.8124 515.1775 549.1199 614.0791 646.0295 675.3562 754.7655 830.5596 887.2070 945.2483 978.3166
m/z

For Help, press F1

447





Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

99 formula(e) evaluated with 14 results within limits (all results (up to 1000) for each mass)

Elements Used:

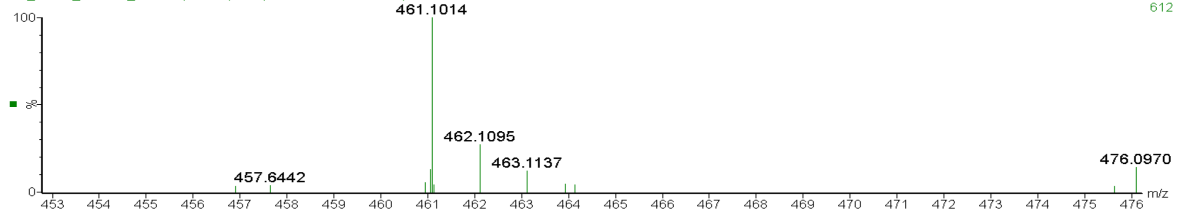
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
	447.1655	18.4	41.1	10.5	C23 H27 O9	8.87	23	27	9
	447.2078	-23.9	-53.4	0.5	C17 H35 O13	2.33	17	35	13
	447.1596	24.3	54.3	19.5	C30 H23 O4	10.49	30	23	4
	447.2113	-27.4	-61.3	22.5	C35 H27	3.49	35	27	
	447.2171	-33.2	-74.2	13.5	C28 H31 O5	8.92	28	31	5
	447.1503	33.6	75.1	6.5	C19 H27 O12	2.36	19	27	12
	447.2230	-39.1	-87.4	4.5	C21 H35 O10	2.93	21	35	10
	447.1444	39.5	88.3	15.5	C26 H23 O7	7.00	26	23	7

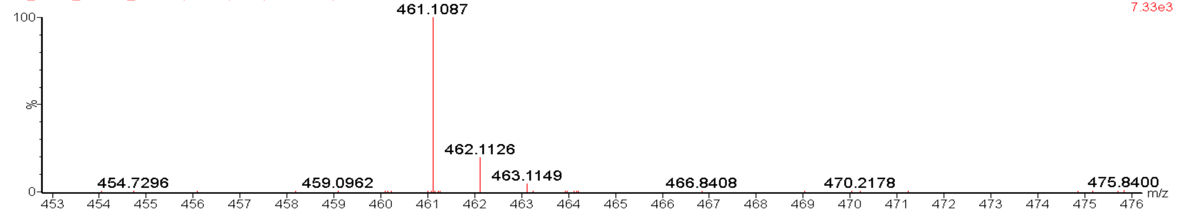
TU_CPUT_4
TU_CPUT_170710_8 1304 (10.151) Cm (1294:1304-1332:1343)
1: TOF MS ES-
1.49e+003

447.1839
448.1893
392.1584
449.1869
145.0507
174.9534
135.0481
227.1022
272.9396
335.1297
388.8003
402.1745
500.0890
569.2007
619.7083
686.1344
716.4336
792.6569
818.7360
854.8157
910.3690
960.6490
m/z

For Help, press F1

461





File Edit View Process Help

Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
109 formula(e) evaluated with 21 results within limits (all results (up to 1000) for each mass)
Elements Used:

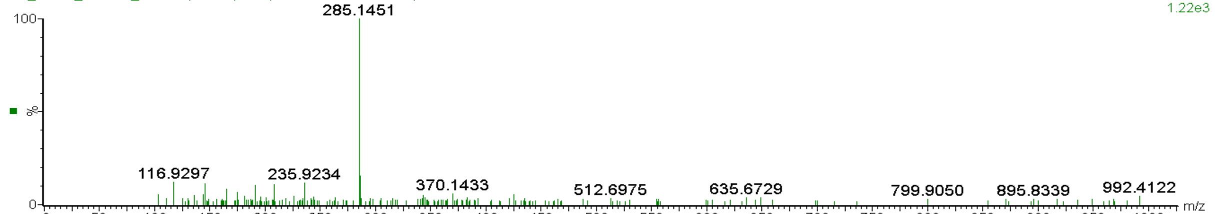
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
461.1087	461.1084	0.3	0.7	12.5	C22 H21 O11	0.65	22	21	11
	461.1143	-5.6	-12.1	3.5	C15 H25 O16	2.64	15	25	16
	461.1025	6.2	13.4	21.5	C29 H17 O6	0.02	29	17	6
	461.1178	-9.1	-19.7	25.5	C33 H17 O3	0.01	33	17	3
	461.0990	9.7	21.0	-0.5	C11 H25 O19	0.04	11	25	19
	461.0966	12.1	26.2	30.5	C36 H13 O	0.00	36	13	1
	461.1236	-14.9	-32.3	16.5	C26 H21 O8	0.04	26	21	8
	461.0931	15.6	33.8	8.5	C18 H21 O14	83.21	18	21	14
	461.1295	-20.8	-45.1	7.5	C19 H25 O13	11.55	19	25	13

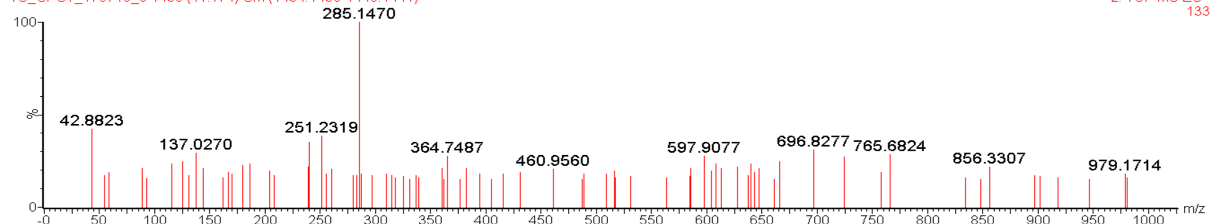
TU_CPUT_3
TU_CPUT_170710_7 2715 (21.131) Cm (2713:2728)
1: TOF MS ES-
7.33e+003
461.1087
462.1126
463.1149
For Help, press F1

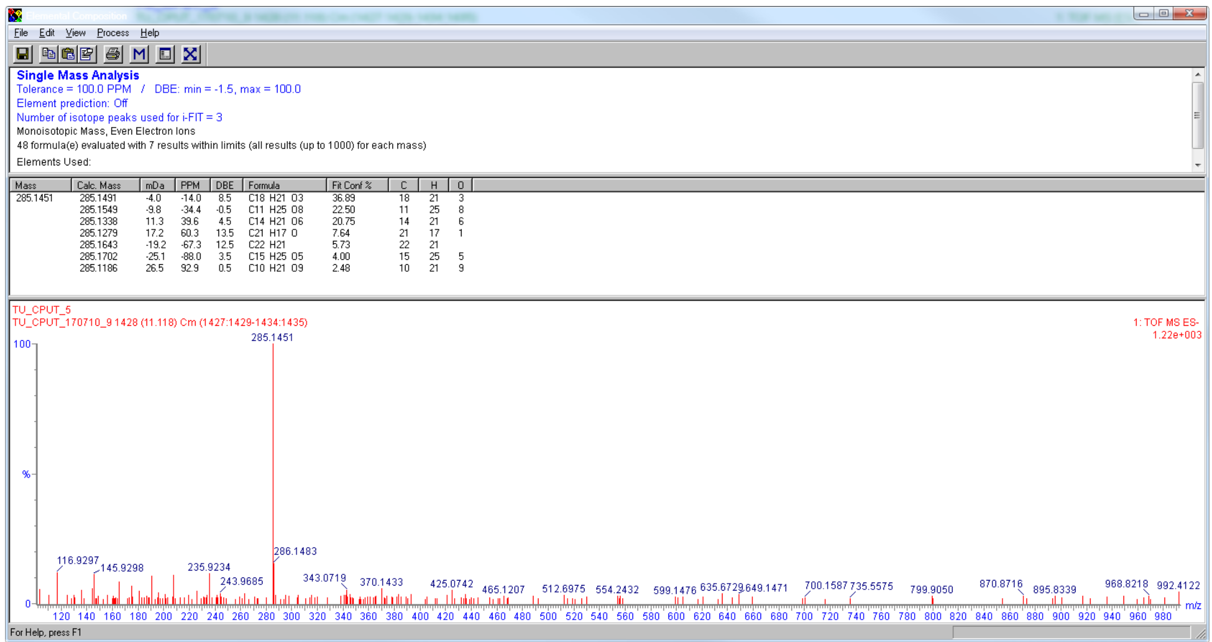
Chrysoeriol 7-O-glucoside

Isorhamnetin 3-O-rutinoside

285

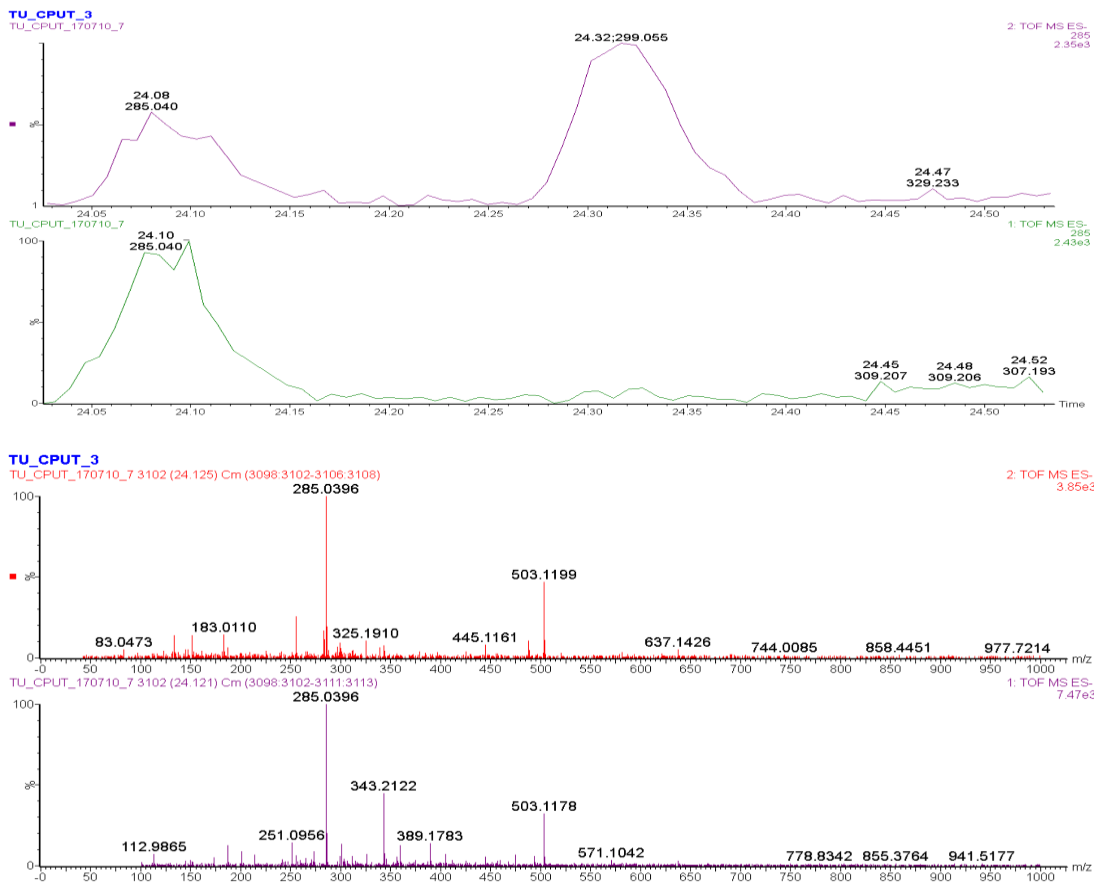


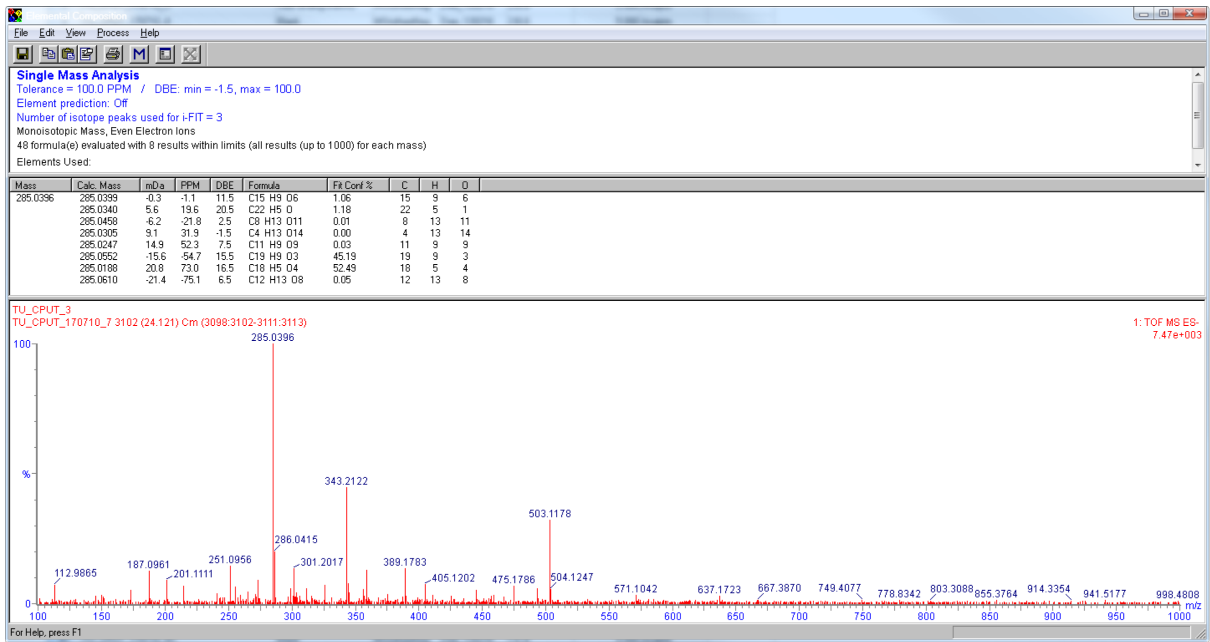




Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
285.1451	285.1491	-4.0	-14.0	8.5	C18 H21 O3	36.89	18	21	3
	285.1549	-9.8	-34.4	-0.5	C11 H25 O8	22.50	11	25	8
	285.1338	11.3	39.6	4.5	C14 H21 O6	20.75	14	21	6
	285.1279	17.2	60.3	13.5	C21 H17 O	7.64	21	17	1
	285.1643	-19.2	-67.3	12.5	C22 H21	5.73	22	21	
	285.1702	-25.1	-88.0	3.5	C15 H25 O5	4.00	15	25	5
	285.1186	26.5	92.9	0.5	C10 H21 O9	2.48	10	21	9

285





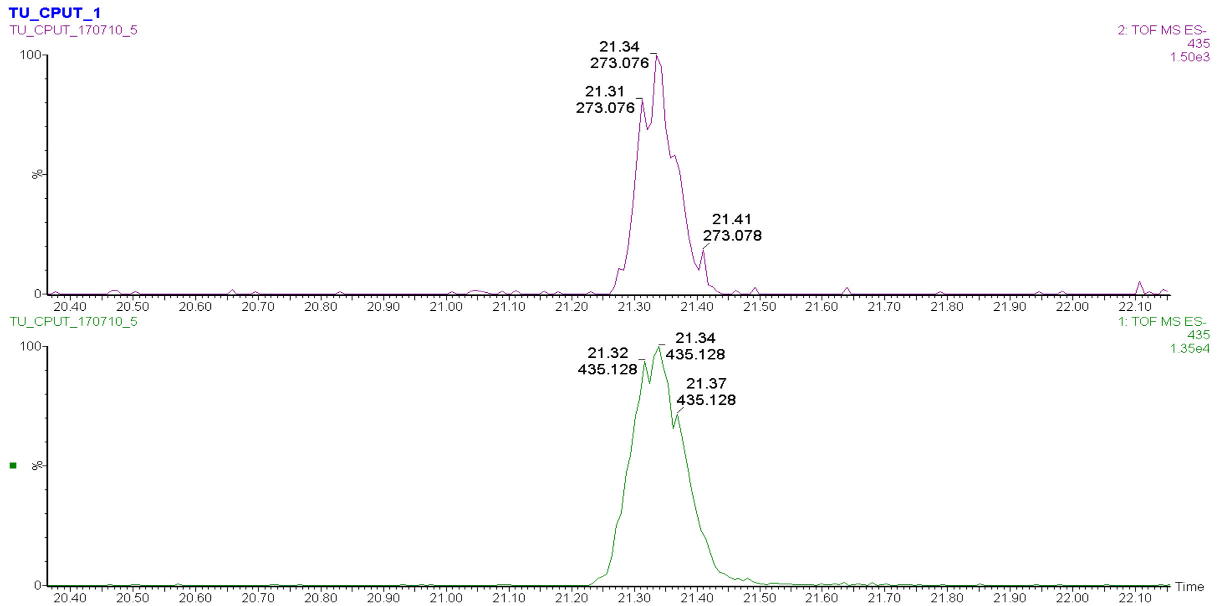
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
285.0396	285.0399	-0.3	-1.1	11.5	C15 H9 O6	1.06	15	9	6
	285.0340	5.6	19.6	20.5	C22 H5 O	1.18	22	5	1
	285.0458	-6.2	-21.8	2.5	C8 H13 O11	0.01	8	13	11
	285.0305	9.1	31.9	-1.5	C4 H13 O14	0.00	4	13	14
	285.0247	14.9	52.3	7.5	C11 H9 O9	0.03	11	9	9
	285.0552	-15.6	-54.7	15.5	C19 H9 O3	45.19	19	9	3
	285.0188	20.8	73.0	16.5	C18 H5 O4	52.49	18	5	4
	285.0610	-21.4	-75.1	6.5	C12 H13 O8	0.05	12	13	8

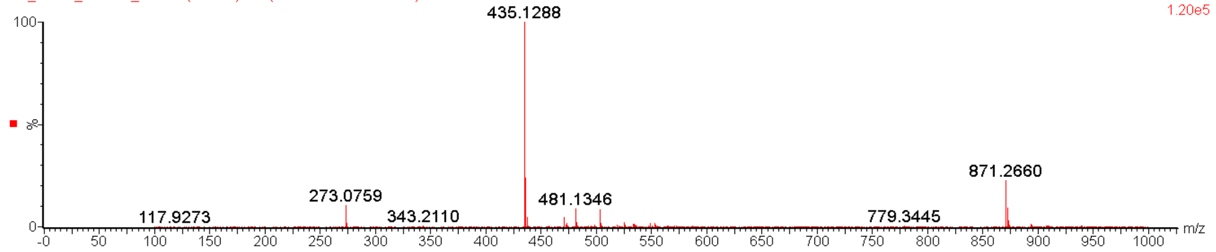
Luteolin

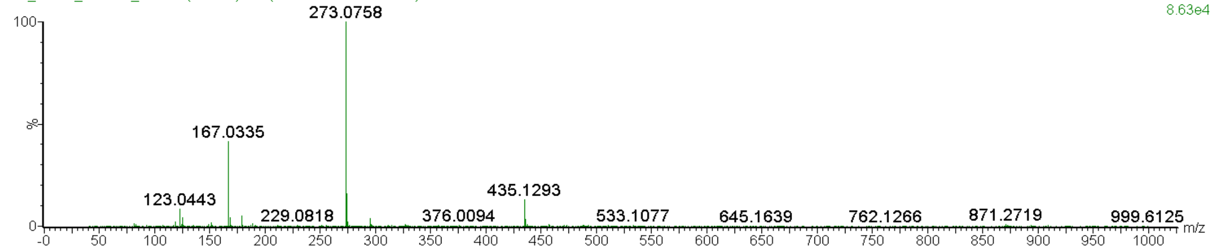
Scutellarein

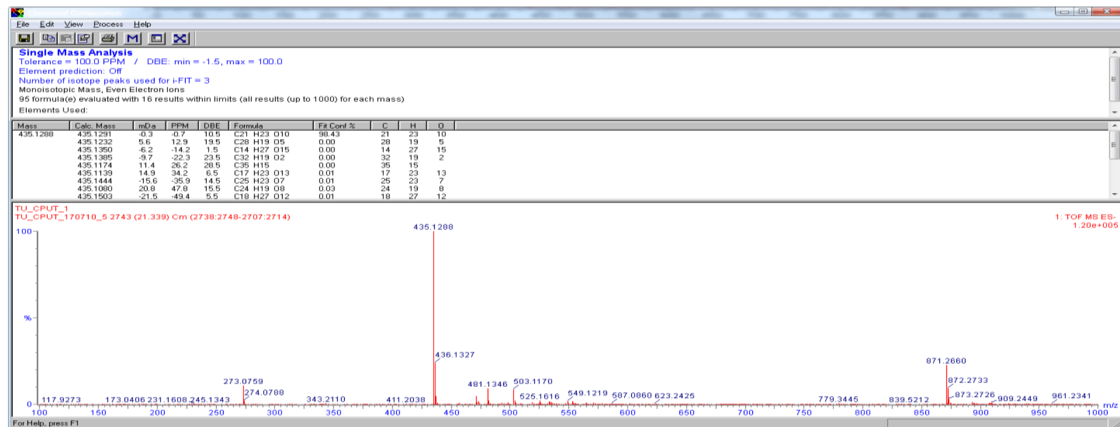
Kaempferol

435





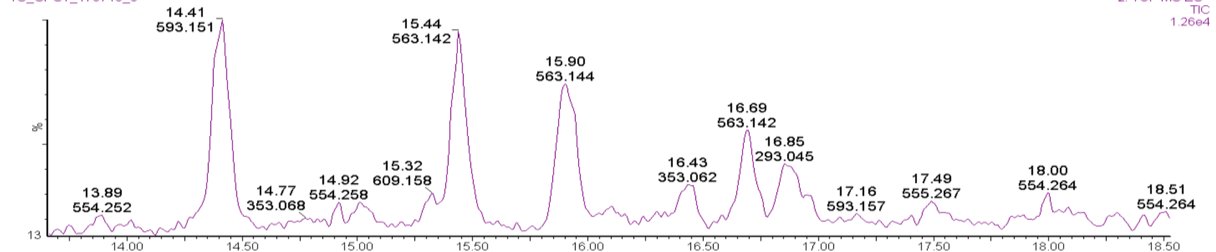


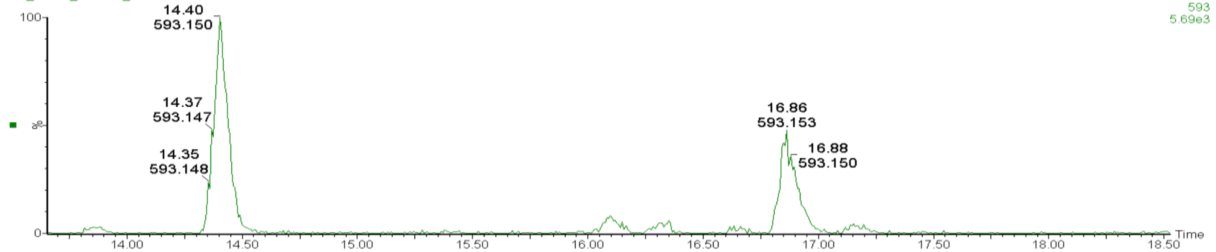


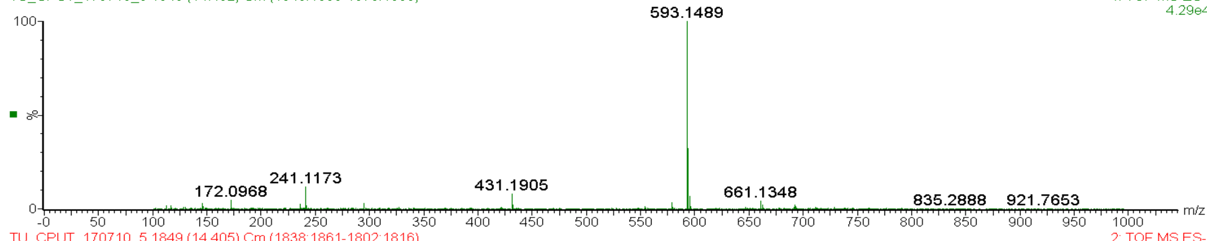
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
435.1288	435.1291	-0.3	-0.7	10.5	C21 H23 O10	98.43	21	23	10
	435.1232	5.6	12.9	19.5	C28 H19 O5	0.00	28	19	5
	435.1350	-6.2	-14.2	1.5	C14 H27 O15	0.00	14	27	15
	435.1385	-9.7	-22.3	23.5	C32 H19 O2	0.00	32	19	2
	435.1174	11.4	26.2	28.5	C35 H15	0.00	35	15	
	435.1139	14.9	34.2	6.5	C17 H23 O13	0.01	17	23	13
	435.1444	-15.6	-35.9	14.5	C25 H23 O7	0.01	25	23	7
	435.1080	20.8	47.8	15.5	C24 H19 O8	0.03	24	19	8
	435.1503	-21.5	-49.4	5.5	C18 H27 O12	0.01	18	27	12

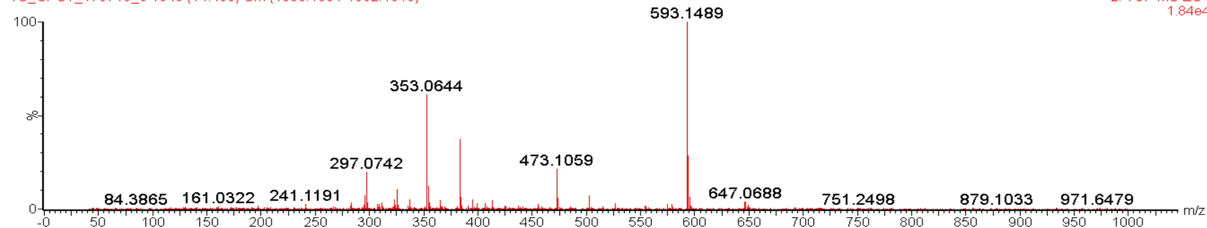
Phloridzin

593









File Edit View Process Help

Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
170 formula(e) evaluated with 31 results within limits (all results (up to 1000) for each mass)
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
593.1489	593.1506	-1.7	-2.9	13.5	C27 H29 O15	37.62	27	29	15
	593.1448	4.1	6.9	22.5	C34 H25 O10	0.57	34	25	10
	593.1542	-5.3	-8.9	35.5	C45 H21 O2	0.00	45	21	2
	593.1565	-7.6	-12.8	4.5	C20 H33 O20	0.57	20	33	20
	593.1413	7.6	12.8	0.5	C16 H33 O23	0.16	16	33	23
	593.1389	10.0	16.9	31.5	C41 H21 O5	0.02	41	21	5
	593.1600	-11.1	-18.7	26.5	C38 H25 O7	0.05	38	25	7
	593.1354	13.5	22.8	9.5	C23 H29 O18	1.47	23	29	18
	593.1330	15.9	26.8	40.5	C48 H17	0.00	48	17	

TU_CPUT_1
TU_CPUT_170710_5 1849 (14.402) Cm (1840:1863-1878:1890)
1: TOF MS ES-
4.29e+004

593.1489, 594.1524, 595.1535, 596.1503, 241.1173, 145.9292, 172.0968, 235.9231, 242.1185, 295.1375, 327.0700, 421.1602, 431.1905, 432.1896, 547.2086, 579.1326, 661.1348, 692.0696, 729.1202, 760.0499, 835.2888, 869.9338, 921.7653, 939.7047

For Help, press F1

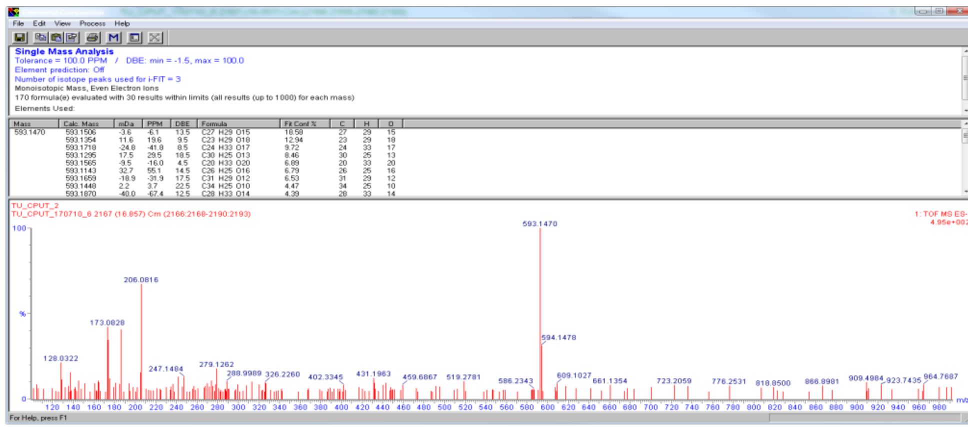
Apigenin 6,8-di-C-glucoside

Chrysoeriol 7-O-apiosyl-glucoside

Luteolin 7-O-rutinoside

Kaempferol 3-O-galactoside 7-O-rhamnoside

Kaempferol 3-O-rutinoside

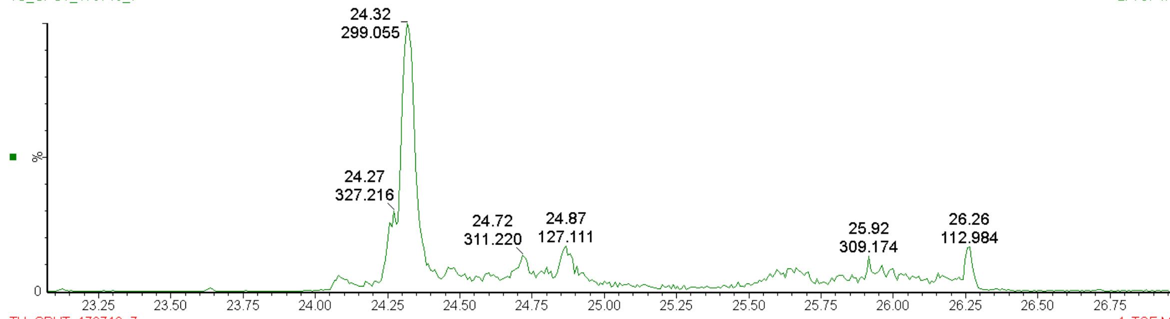


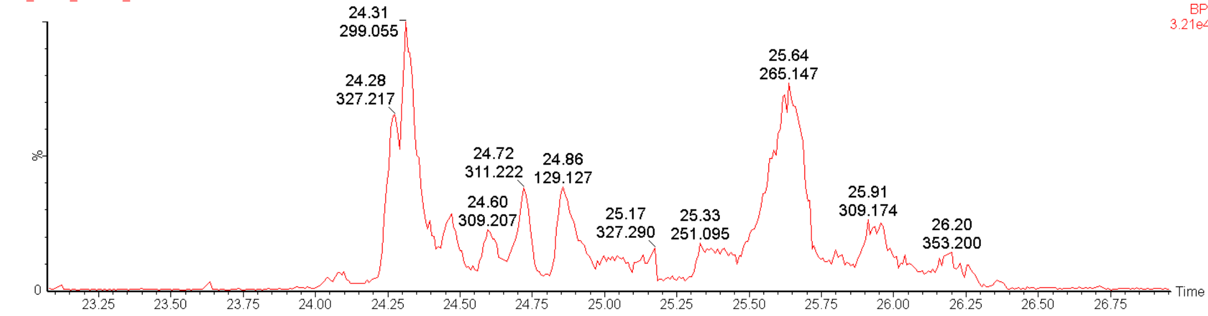
File Edit View Process Help

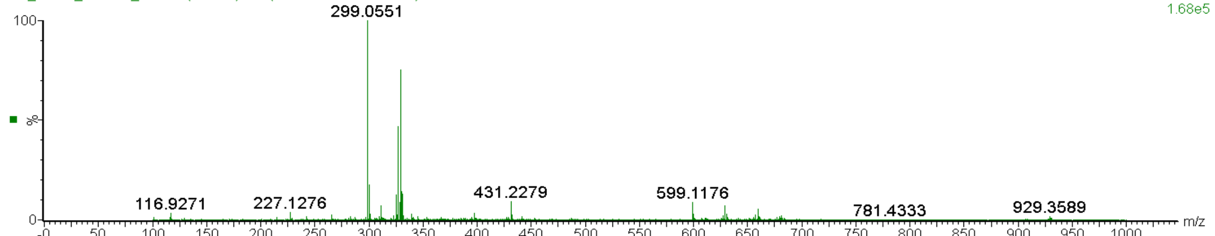
Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
170 formula(e) evaluated with 30 results within limits (all results (up to 1000) for each mass)
Elements Used:

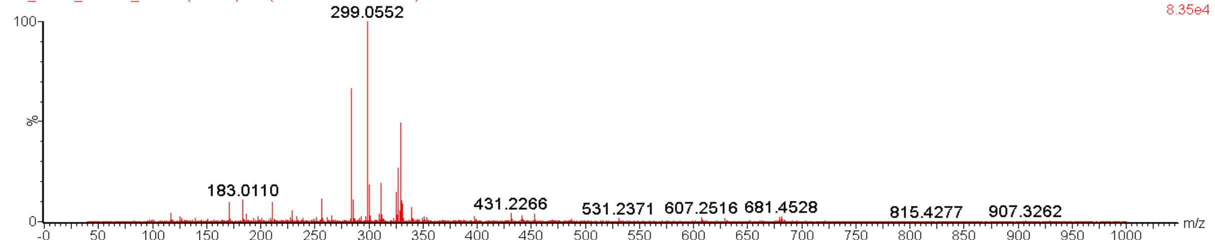
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
593.1470	593.1506	-3.6	-6.1	13.5	C27 H29 O15	18.58	27	29	15
	593.1354	11.6	19.6	9.5	C23 H29 O18	12.94	23	29	18
	593.1718	-24.8	-41.8	8.5	C24 H33 O17	9.72	24	33	17
	593.1295	17.5	29.5	18.5	C30 H25 O13	8.46	30	25	13
	593.1565	-9.5	-16.0	4.5	C20 H33 O20	6.89	20	33	20
	593.1143	32.7	55.1	14.5	C26 H25 O16	6.79	26	25	16
	593.1659	-18.9	-31.9	17.5	C31 H29 O12	6.53	31	29	12
	593.1448	2.2	3.7	22.5	C34 H25 O10	4.47	34	25	10
	593.1870	-40.0	-67.4	12.5	C28 H33 O14	4.39	28	33	14

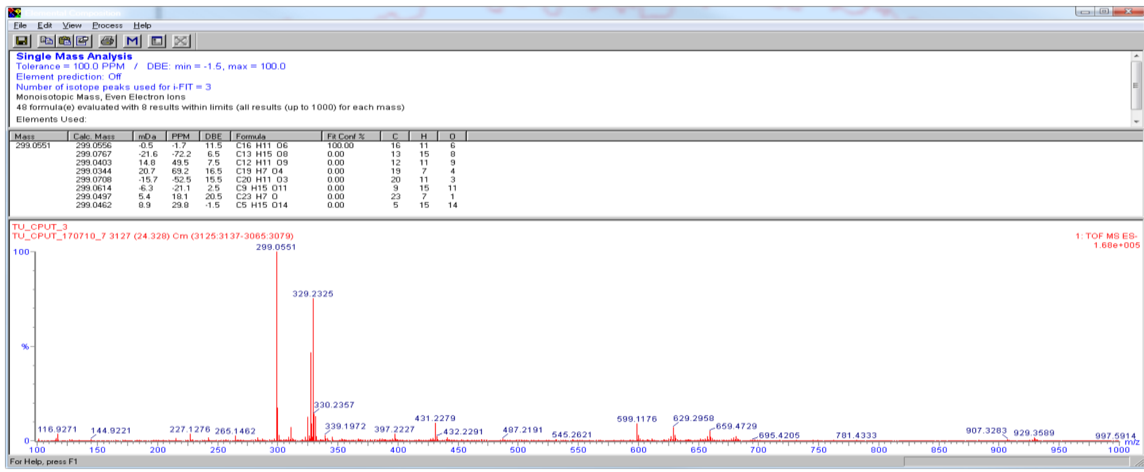
299









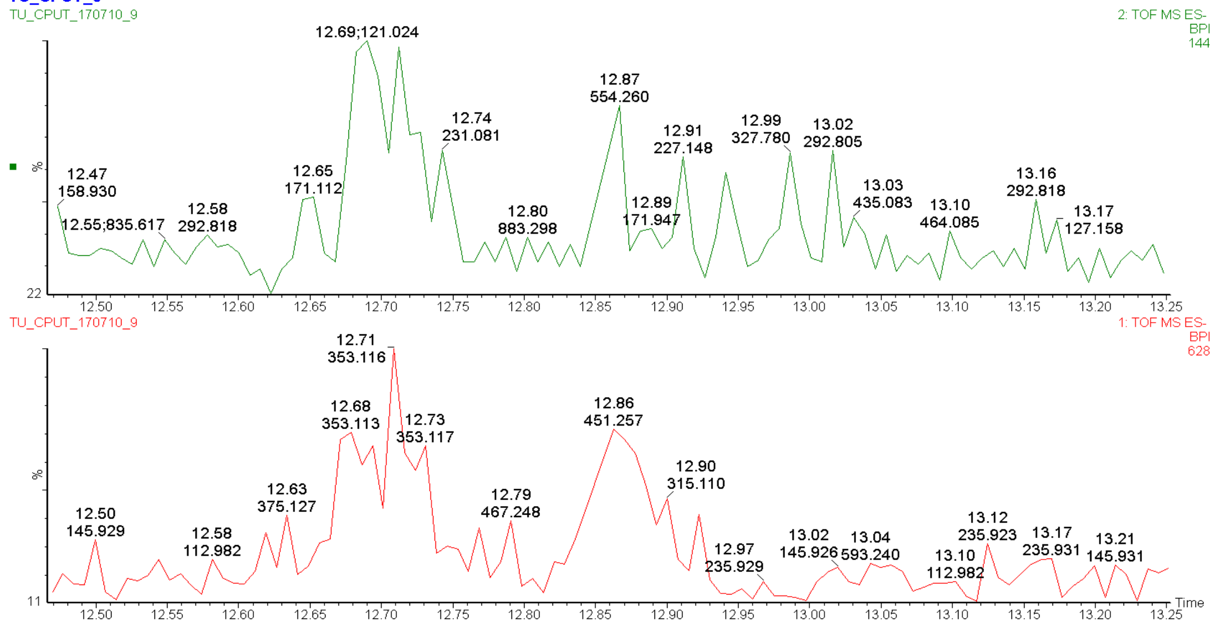

File Edit View Process Help

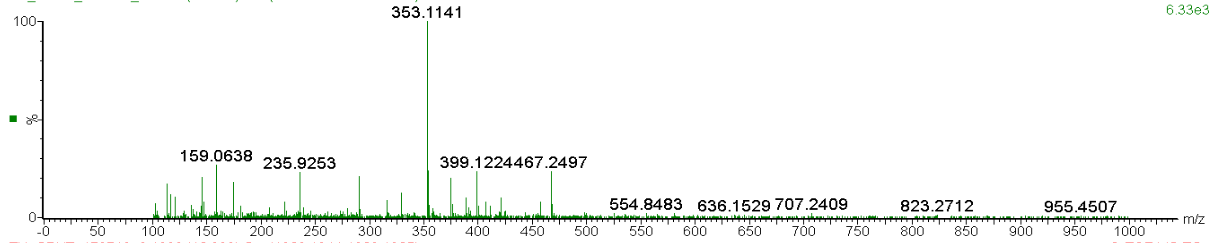
Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
48 formula(e) evaluated with 8 results within limits (all results (up to 1000) for each mass)
Elements Used:

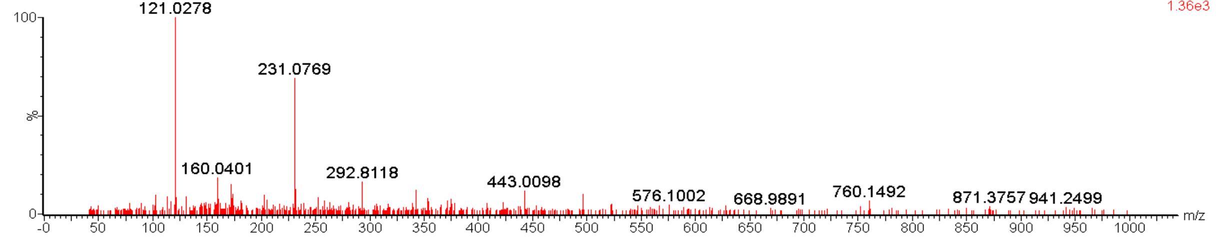
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
299.0551	299.0556	-0.5	-1.7	11.5	C16 H11 O6	100.00	16	11	6
	299.0767	-21.6	-72.2	6.5	C13 H15 O8	0.00	13	15	8
	299.0403	14.8	49.5	7.5	C12 H11 O9	0.00	12	11	9
	299.0344	20.7	69.2	16.5	C19 H7 O4	0.00	19	7	4
	299.0708	-15.7	-52.5	15.5	C20 H11 O3	0.00	20	11	3
	299.0614	-6.3	-21.1	2.5	C9 H15 O11	0.00	9	15	11
	299.0497	5.4	18.1	20.5	C23 H7 O	0.00	23	7	1
	299.0462	8.9	29.8	-1.5	C5 H15 O14	0.00	5	15	14

Hispidulin

353







Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
70 formula(e) evaluated with 12 results within limits (all results (up to 1000) for each mass)
Elements Used:

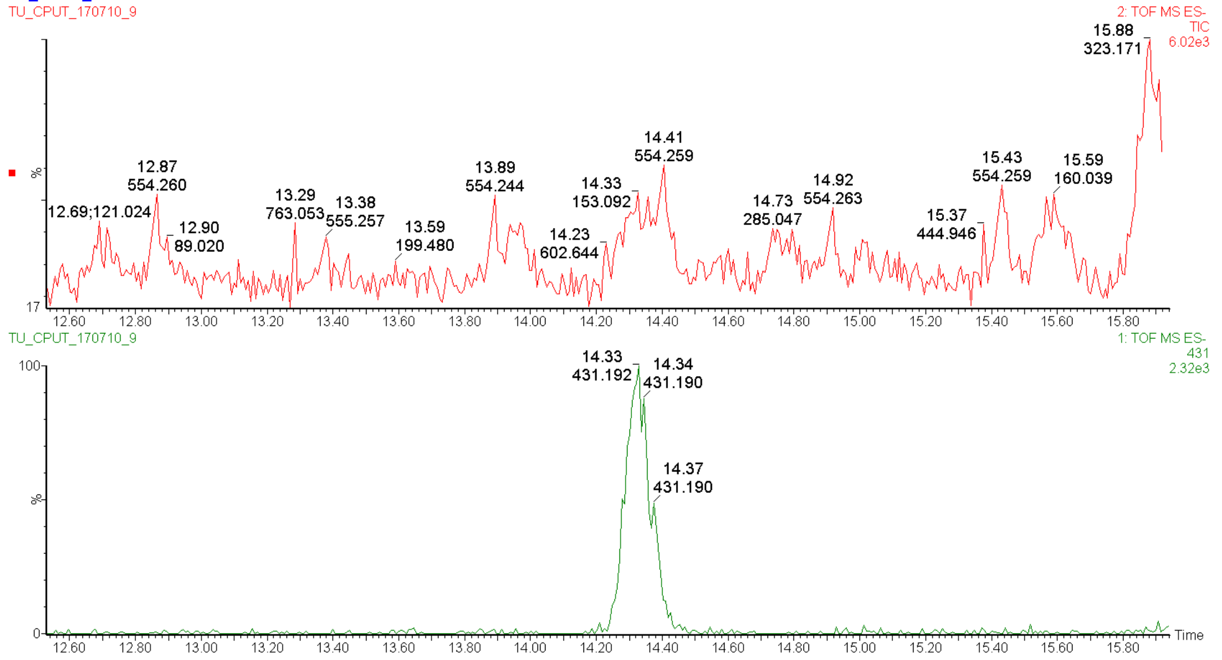
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
353.1141	353.1178	-3.7	-10.5	16.5	C24 H17 O3	17.39	24	17	3
	353.1084	5.7	16.1	3.5	C13 H21 O11	4.87	13	21	11
	353.1236	-9.5	-26.9	7.5	C17 H21 O8	13.73	17	21	8
	353.1025	11.6	32.9	12.5	C20 H17 O6	20.70	20	17	6
	353.1295	-15.4	-43.6	-1.5	C10 H25 O13	1.83	10	25	13
	353.0966	17.5	49.6	21.5	C27 H13 O	4.35	27	13	1
	353.1330	-18.9	-53.5	20.5	C28 H17	3.10	28	17	
	353.0931	21.0	59.5	-0.5	C9 H21 O14	0.87	9	21	14
	353.1389	-24.8	-70.2	11.5	C21 H21 O5	16.58	21	21	5

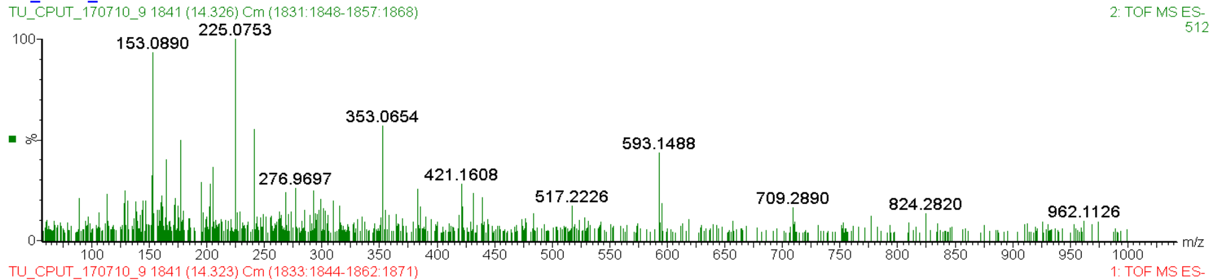
TU_CPUT_5
TU_CPUT_170710_9 1631 (12.694) Cm (1619:1644-1602:1609)
1: TOF MS ES- 6.33e+003

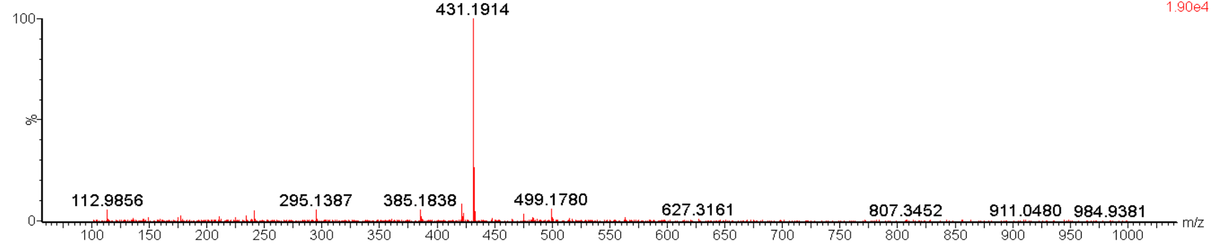
For Help, press F1

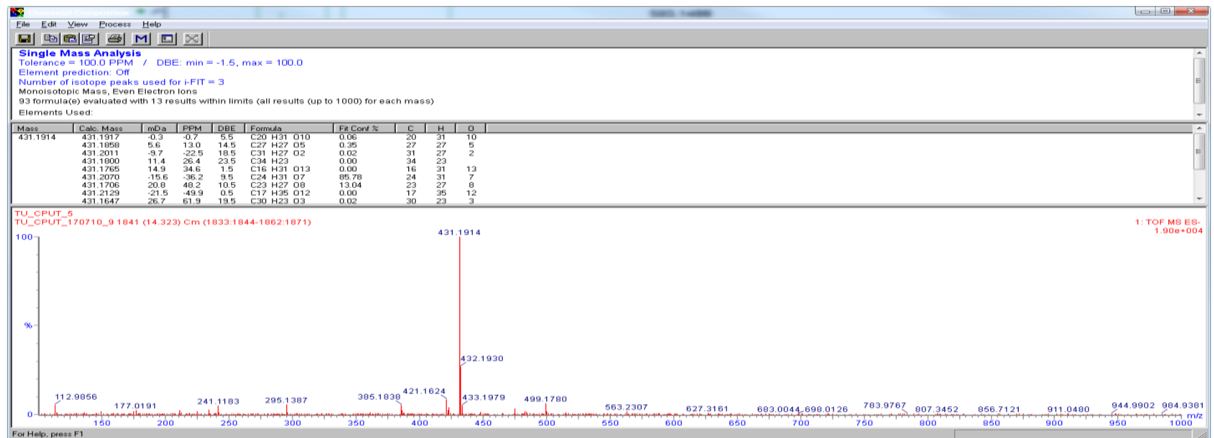
Unknown

431





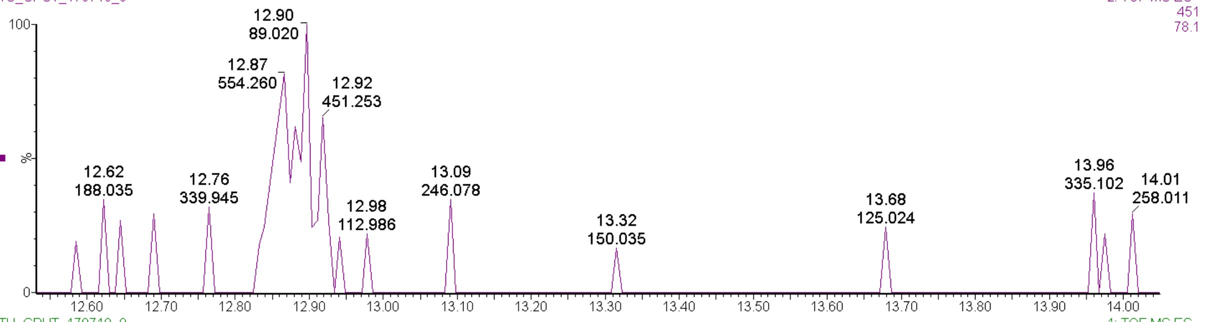


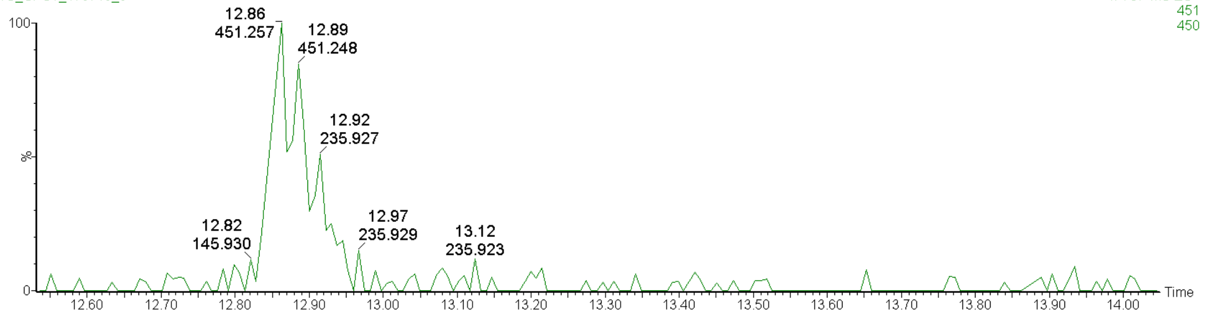


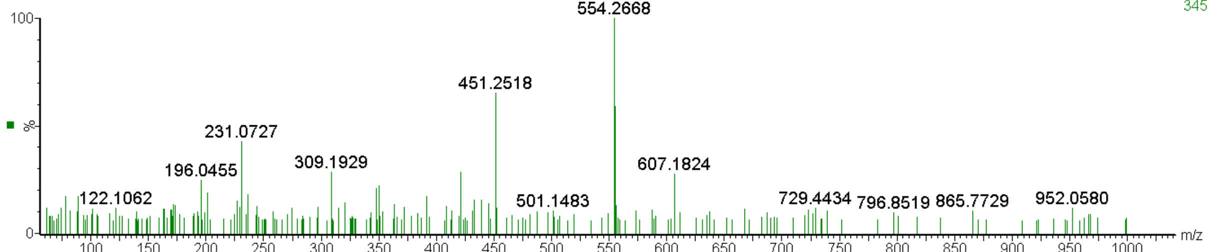
Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
93 formula(e) evaluated with 13 results within limits (all results (up to 1000) for each mass)
Elements Used:

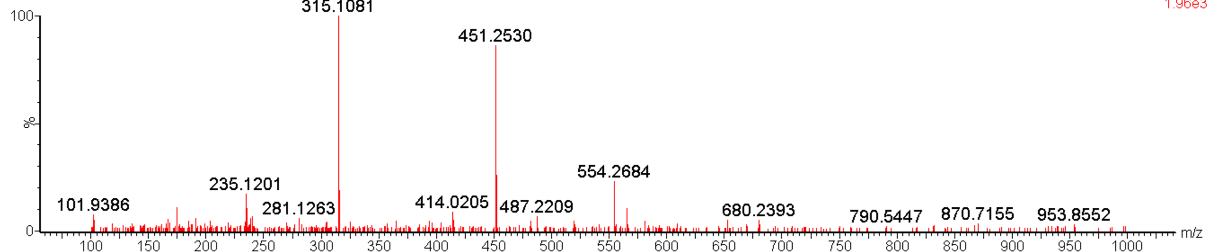
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
431.1914	431.1917	-0.3	-0.7	5.5	C20 H31 O10	0.06	20	31	10
	431.1858	5.6	13.0	14.5	C27 H27 O5	0.35	27	27	5
	431.2011	-9.7	-22.5	18.5	C31 H27 O2	0.02	31	27	2
	431.1800	11.4	26.4	23.5	C34 H23	0.00	34	23	
	431.1765	14.9	34.6	1.5	C16 H31 O13	0.00	16	31	13
	431.2070	-15.6	-36.2	9.5	C24 H31 O7	85.78	24	31	7
	431.1706	20.8	48.2	10.5	C23 H27 O8	13.04	23	27	8
	431.2129	-21.5	-49.9	0.5	C17 H35 O12	0.00	17	35	12
	431.1647	26.7	61.9	19.5	C30 H23 O3	0.02	30	23	3

451









File Edit View Process Help

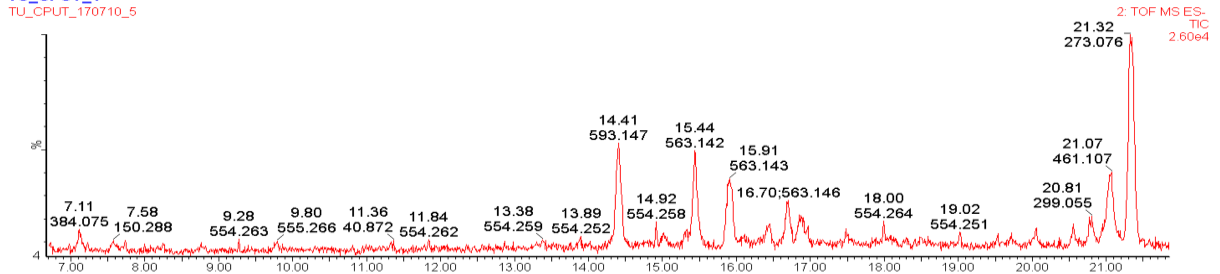
Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
101 formula(e) evaluated with 12 results within limits (all results (up to 1000) for each mass)
Elements Used:

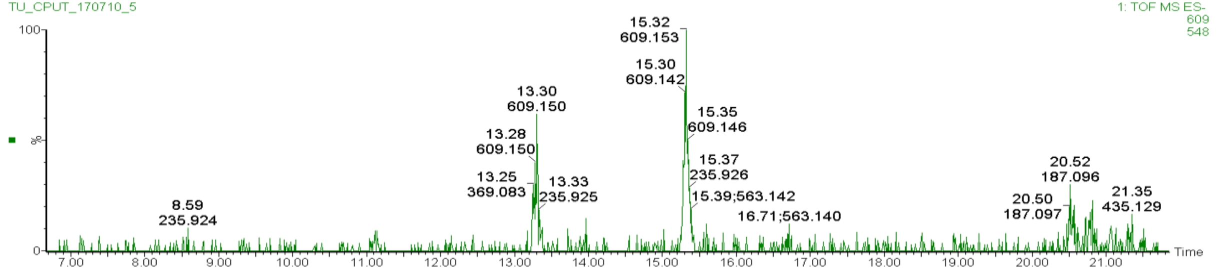
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
451.2530	451.2543	-1.3	-2.9	2.5	C21 H39 O10	11.96	21	39	10
	451.2484	4.6	10.2	11.5	C28 H35 O5	9.10	28	35	5
	451.2426	10.4	23.0	20.5	C35 H31	3.43	35	31	
	451.2637	-10.7	-23.7	15.5	C32 H35 O2	5.29	32	35	2
	451.2391	13.9	30.8	-1.5	C17 H39 O13	7.77	17	39	13
	451.2696	-16.6	-36.8	6.5	C25 H39 O7	11.30	25	39	7
	451.2332	19.8	43.9	7.5	C24 H35 O8	11.41	24	35	8
	451.2273	25.7	57.0	16.5	C31 H31 O3	5.54	31	31	3
	451.2848	-31.8	-70.5	10.5	C29 H39 O4	7.03	29	39	4

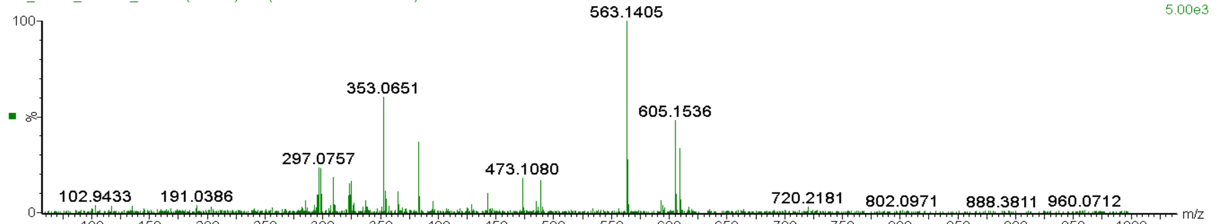
TU_CPUT_5
TU_CPUT_170710_9 1651 (12.863) Cm (1651:1656-1630:1634)
1: TOF MS ES-
1.96e+003
315.1081
451.2530
452.2608
554.2684
316.1114
565.2257
235.1201
101.9386
174.9546
235.9229
190.9305
365.1794
414.0205
487.2209
519.2488
652.6722
680.2393
709.1874
790.5447
832.1874
870.7155
938.8615
953.8552
998.3884
m/z

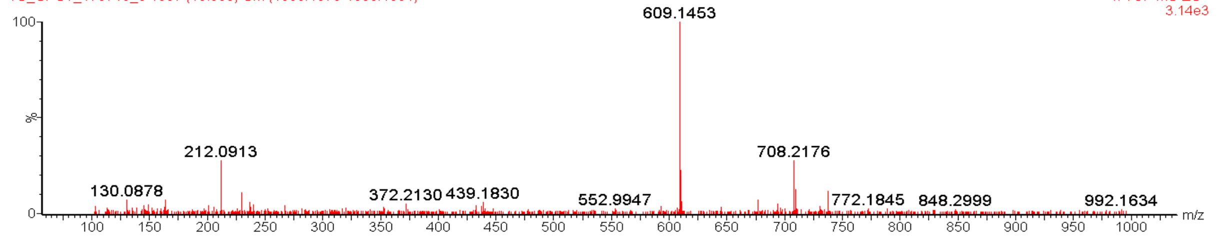
For Help, press F1

609









File Edit View Process Help

Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
178 formula(e) evaluated with 32 results within limits (all results (up to 1000) for each mass)
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
609.1453	609.1456	-0.3	-0.5	13.5	C27 H29 O16	0.27	27	29	16
	609.1491	-3.8	-6.2	35.5	C45 H21 O3	0.01	45	21	3
	609.1397	5.6	9.2	22.5	C34 H25 O11	0.03	34	25	11
	609.1514	-6.1	-10.0	4.5	C20 H33 O21	42.40	20	33	21
	609.1362	9.1	14.9	0.5	C16 H33 O24	1.43	16	33	24
	609.1549	-9.6	-15.8	26.5	C38 H25 O8	0.02	38	25	8
	609.1338	11.5	18.9	31.5	C41 H21 O6	0.01	41	21	6
	609.1303	15.0	24.6	9.5	C23 H29 O19	3.41	23	29	19
	609.1608	-15.5	-25.4	17.5	C31 H29 O13	0.06	31	29	13

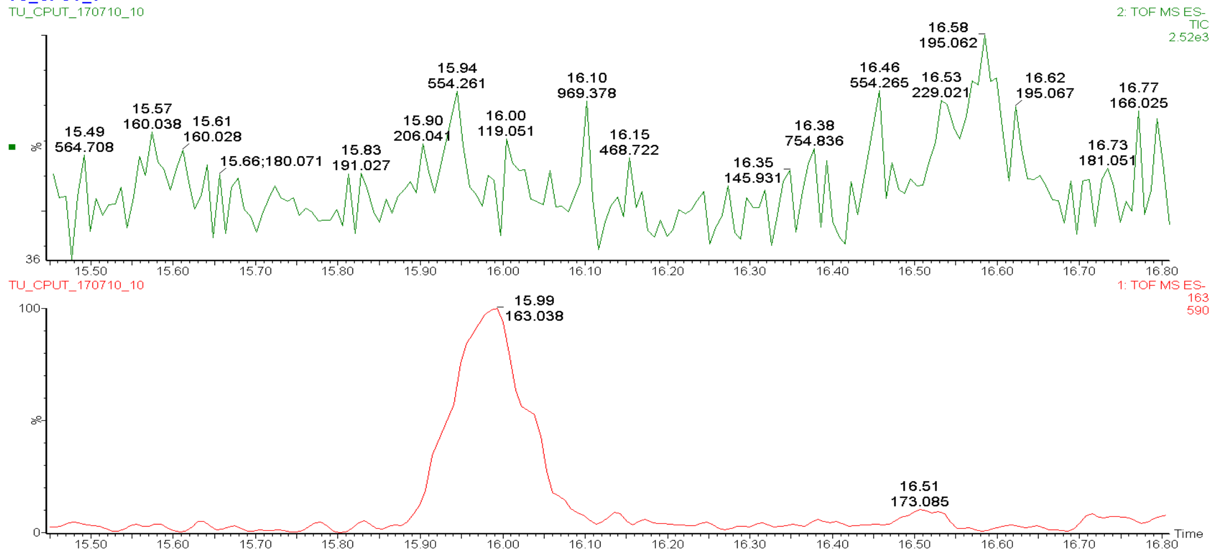
TU_CPUT_1
TU_CPUT_170710_5 1967 (15.303) Cm (1960:1973-1938:1951)
1: TOF MS ES-
3.14e+003

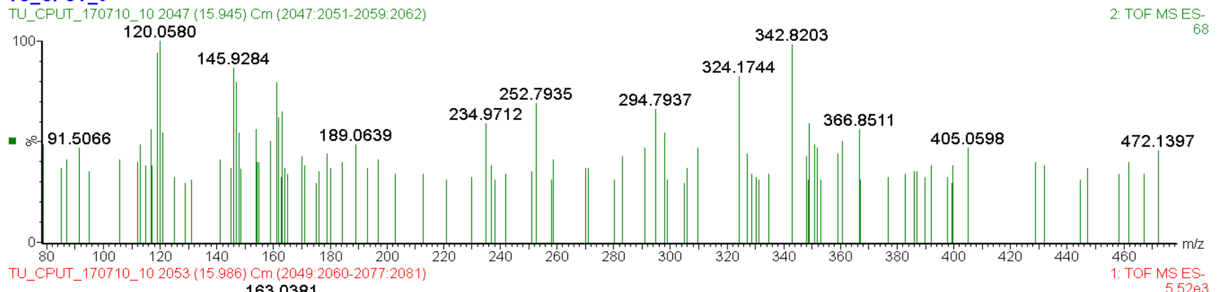
609.1453 · 212.0913 · 610.1483 · 708.2176 · 709.2329 · 130.0878 · 163.9370 · 230.1004 · 239.9034 · 320.0678 · 352.8516 · 372.2130 · 439.1830 · 478.2360 · 552.9947 · 593.1588 · 611.1565 · 677.1564 · 772.1845 · 789.2114 · 848.2999 · 930.3680 · 992.1634

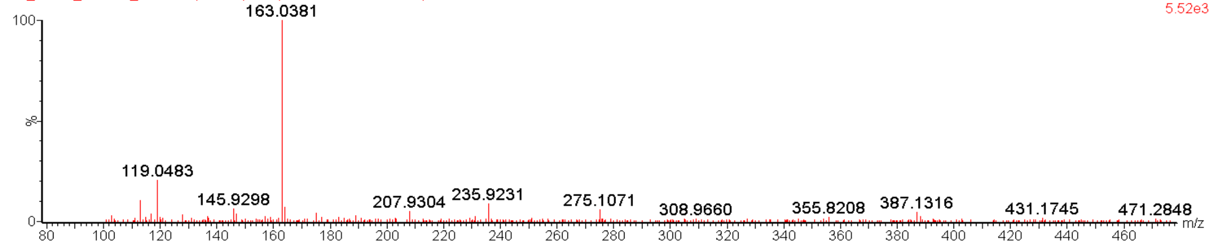
For Help, press F1

Kaempferol 3,7-O-diglucoside
Kaempferol 3-O-sophoroside
Quercetin 3-O-galactoside 7-O-rhamnoside
Quercetin 3-O-rhamnosyl-galactoside
Quercetin 3-O-rutinoside

163





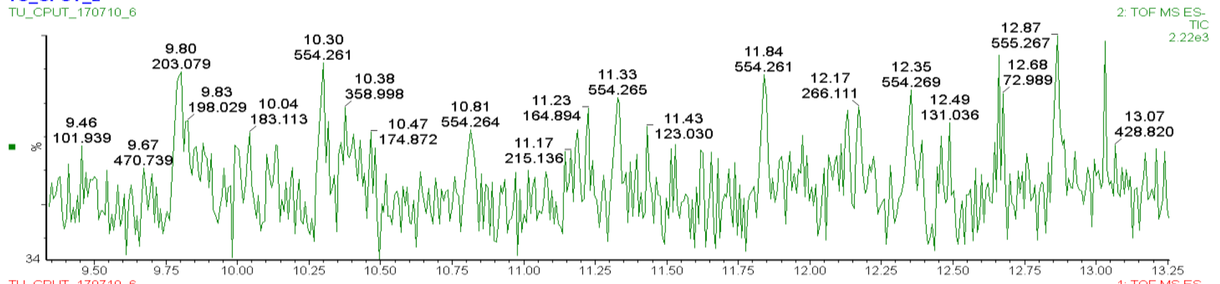


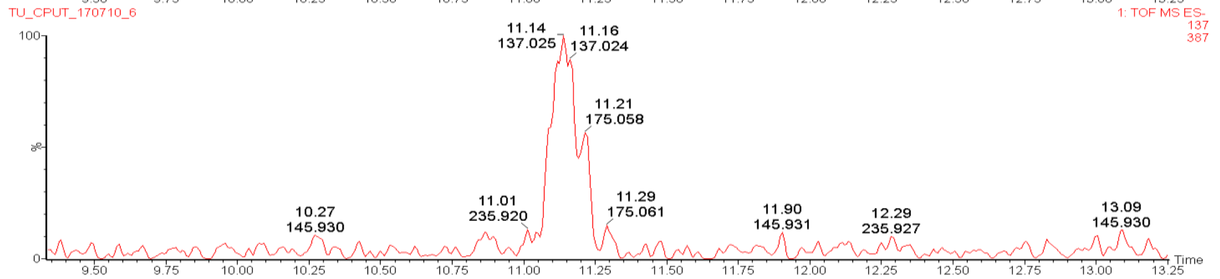
m-Coumaric acid

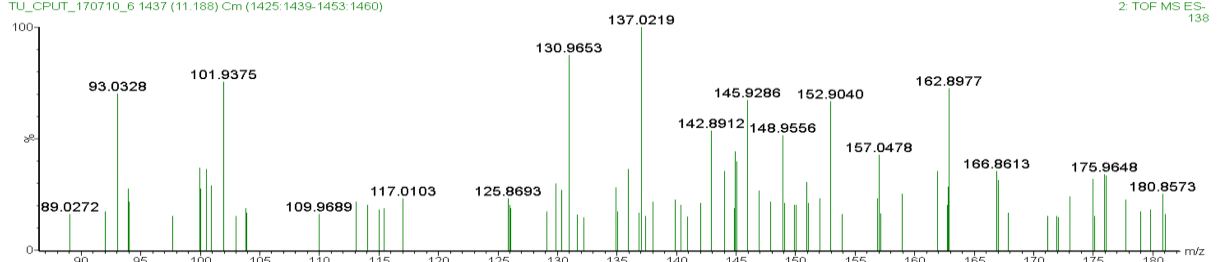
o-Coumaric acid

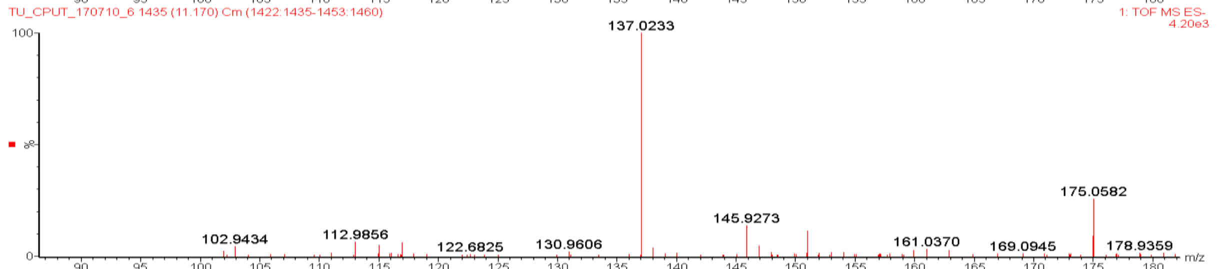
p-Coumaric acid

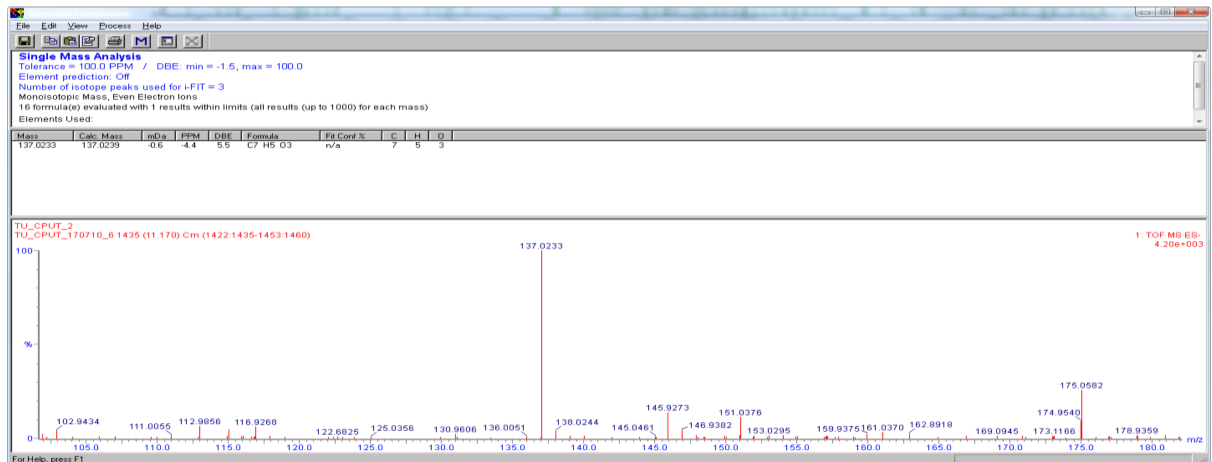
137









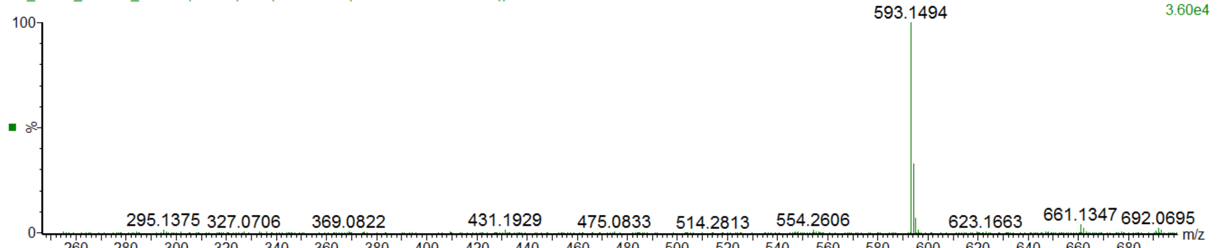


Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	O
137.0233	137.0239	-0.6	-4.4	5.5	C7 H5 O3	n/a	7	5	3

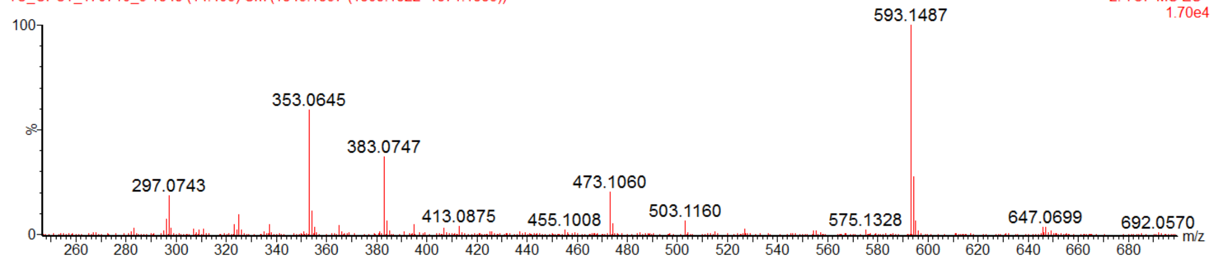
Sesamol
Protocatechuic aldehyde
2-Hydroxybenzoic acid
3-Hydroxybenzoic acid
4-Hydroxybenzoic acid

TU_CPUT_1

TU_CPUT_170710_5 1849 (14.402) Cm (1848:1861-(1869:1895+1810:1821)) 1: TOF MS ES- 3.60e4



TU_CPUT_170710_5 1849 (14.405) Cm (1845:1857-(1809:1822+1871:1885)) 2: TOF MS ES- 1.70e4



593 m/z at 14.40 minutes: $C_{27}H_{30}O_{15}$

Elemental Composition

File Edit View Process Help

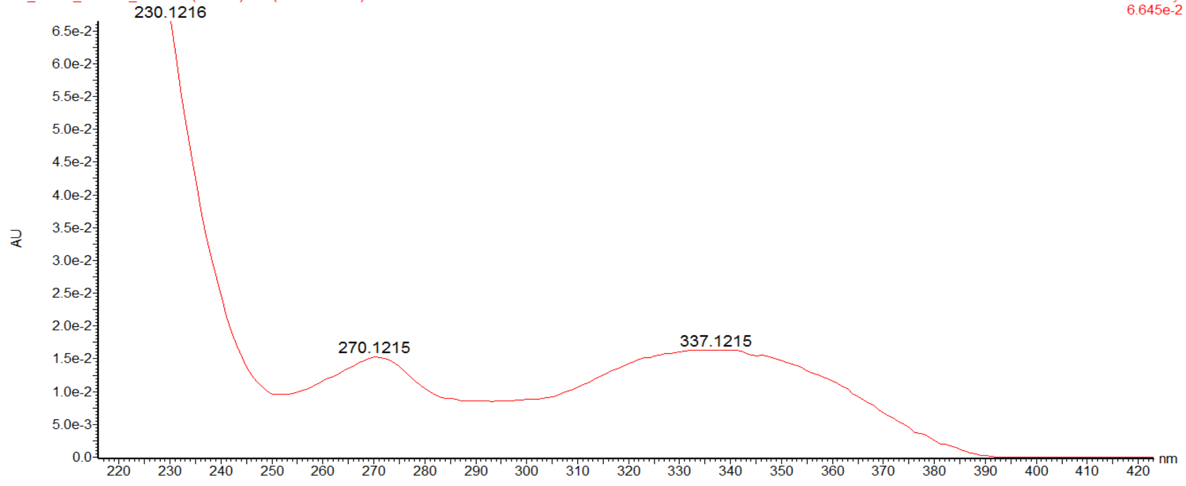
Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
330 formula(e) evaluated with 31 results within limits (all results (up to 1000) for each mass)
Elements Used:

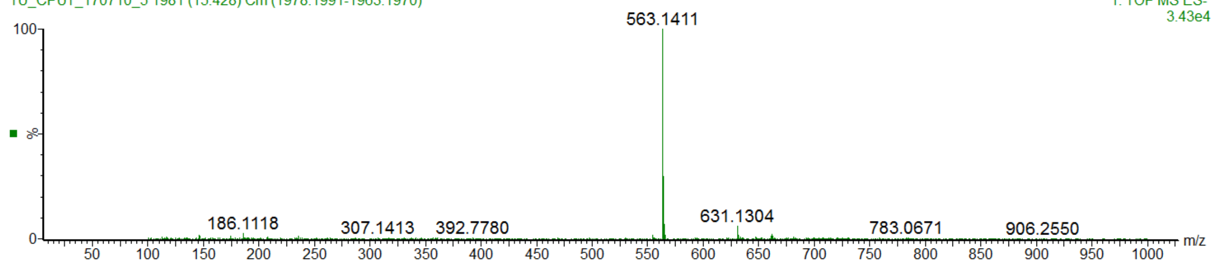
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	N	O
593.1494	593.1506	-1.2	-2.0	13.5	C27 H29 O15	28.89	27	29		15
	593.1448	4.6	7.8	22.5	C34 H25 O10	0.62	34	25		10
	593.1542	-4.8	-8.1	35.5	C45 H21 O2	0.00	45	21		2
	593.1565	-7.1	-12.0	4.5	C20 H33 O20	0.48	20	33		20
	593.1413	8.1	13.7	0.5	C16 H33 O23	0.15	16	33		23
	593.1389	10.5	17.7	31.5	C41 H21 O5	0.02	41	21		5
	593.1600	-10.6	-17.9	26.5	C38 H25 O7	0.05	38	25		7
	593.1354	14.0	23.6	9.5	C23 H29 O18	1.14	23	29		18

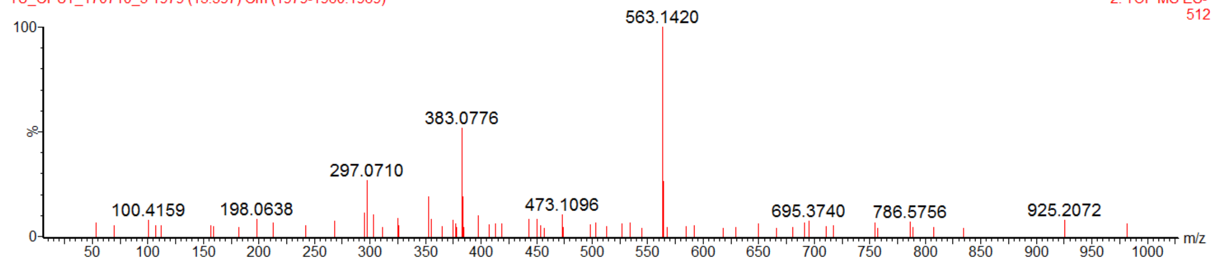
TU_CPUT_1
TU_CPUT_170710_5 1849 (14.402) Cm (1848:1861-(1869:1895+1810:1821)) 1: TOF MS ES- 3.60e+004

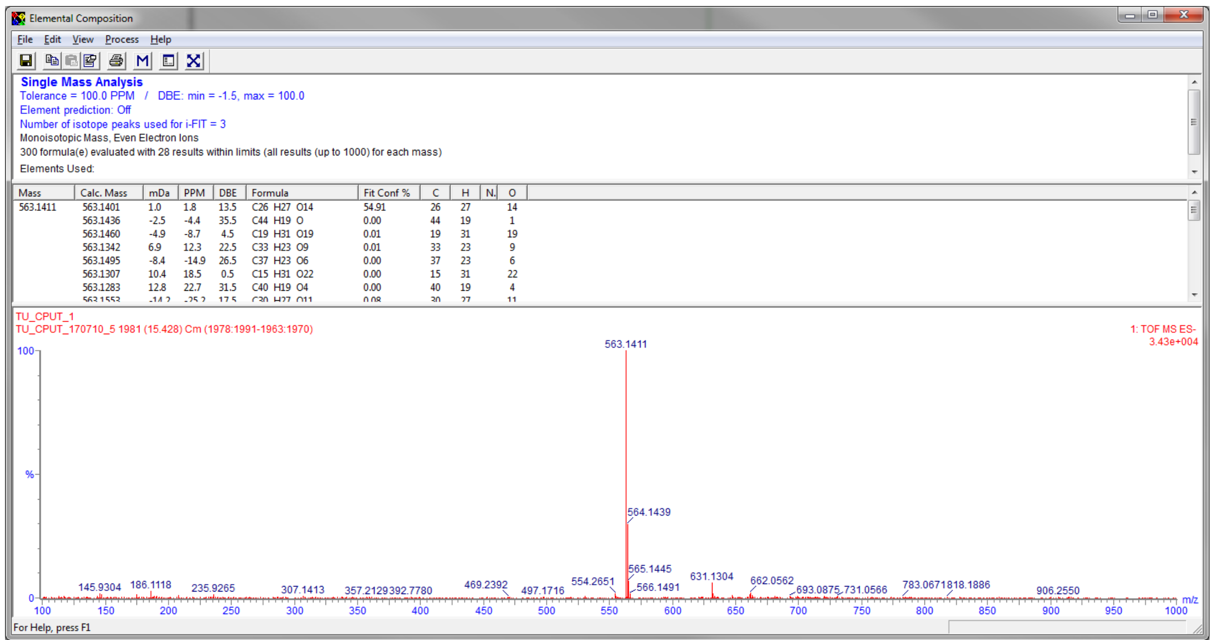
593.1494, 594.1526, 595.1536, 596.1500, 254.9614, 295.1375, 327.0706, 336.1040, 369.0822, 409.1723, 431.1929, 458.7940, 475.0833, 503.5781, 514.2813, 547.2083, 554.2606, 576.2264, 647.0891, 661.1347, 692.0695

For Help, press F1

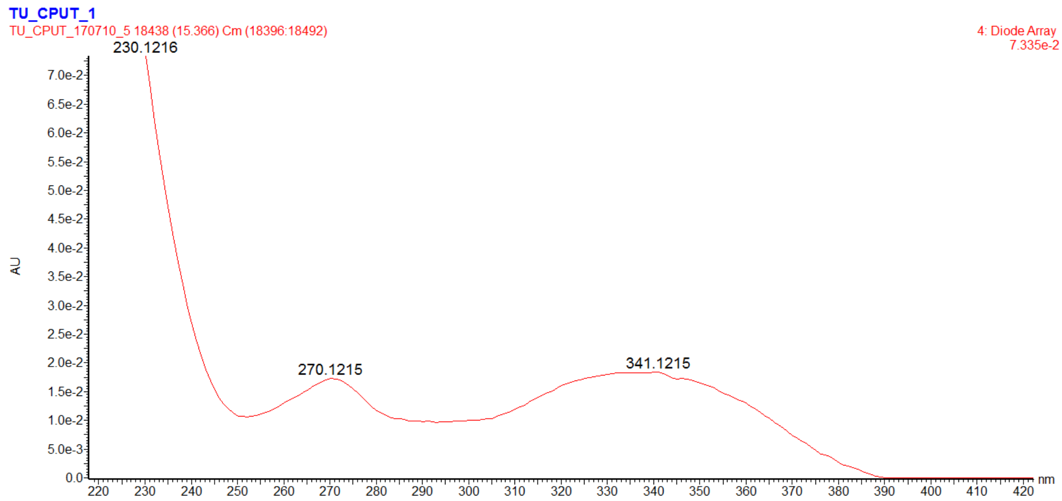

TU_CPUT_1
TU_CPUT_170710_5 17209 (14.342) Cm (17209:17266)
4: Diode Array
6.645e-2
230.1216
270.1215
337.1215
AU
nm

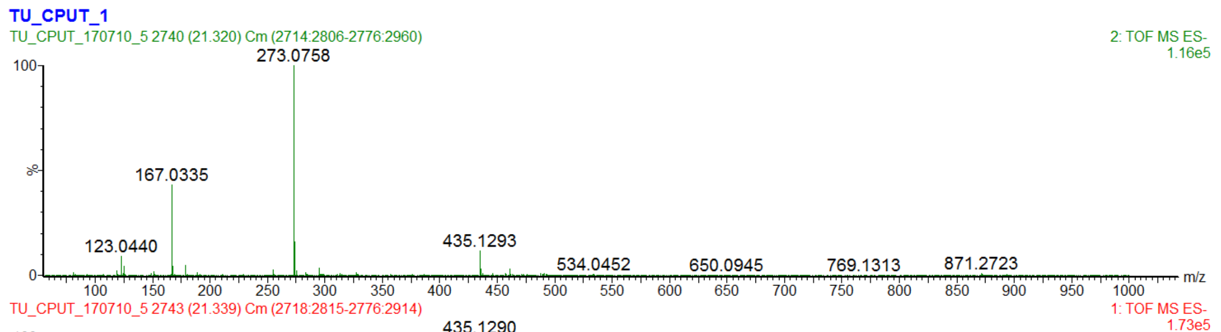

TU_CPUT_1
TU_CPUT_170710_5 1981 (15.428) Cm (1978:1991-1963:1970)
1: TOF MS ES-
3.43e4
563.1411
186.1118
307.1413
392.7780
631.1304
783.0671
906.2550
m/z

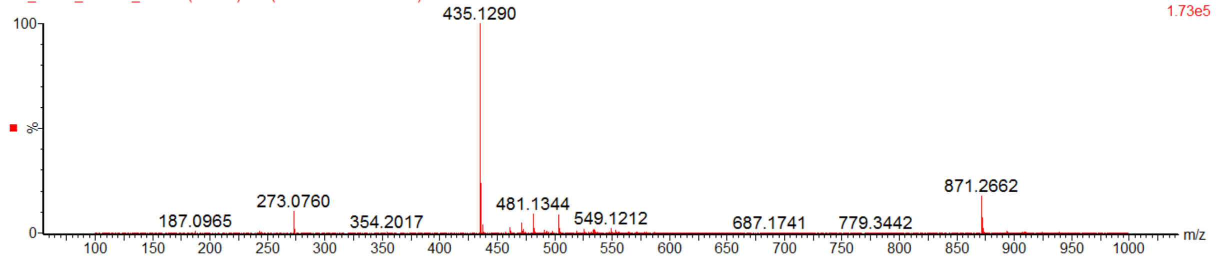

TU_CPUT_170710_5 1979 (15.397) Cm (1979-1960:1969)
2: TOF MS ES-
512
563.1420
383.0776
297.0710
100.4159
198.0638
473.1096
695.3740
786.5756
925.2072
m/z

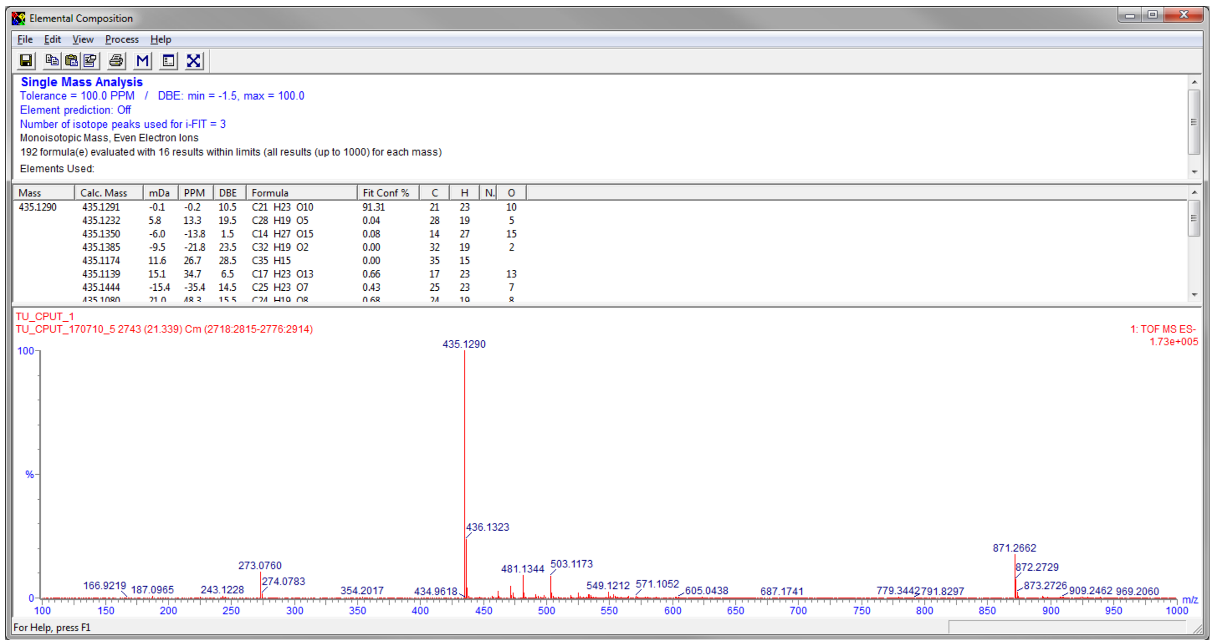


Apigenin



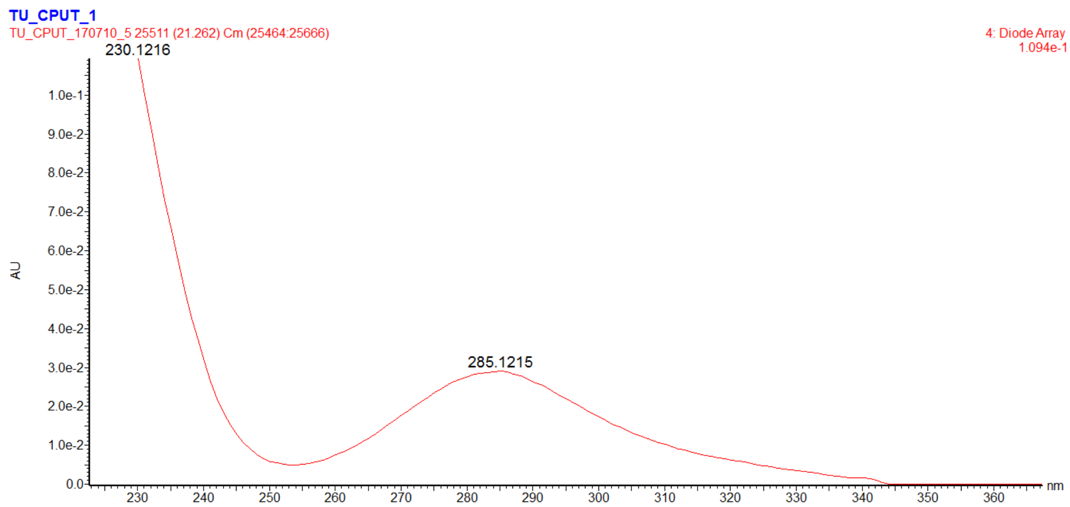






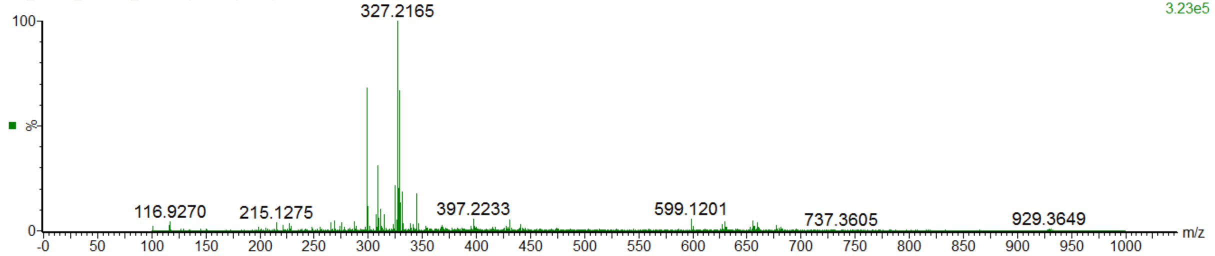
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	N	O
435.1290	435.1291	-0.1	-0.2	10.5	C21 H23 O10	91.31	21	23		10
	435.1232	5.8	13.3	19.5	C28 H19 O5	0.04	28	19		5
	435.1350	-6.0	-13.8	1.5	C14 H27 O15	0.08	14	27		15
	435.1385	-9.5	-21.8	23.5	C32 H19 O2	0.00	32	19		2
	435.1174	11.6	26.7	28.5	C35 H15	0.00	35	15		
	435.1139	15.1	34.7	6.5	C17 H23 O13	0.66	17	23		13
	435.1444	-15.4	-35.4	14.5	C25 H23 O7	0.43	25	23		7

Phloridzin

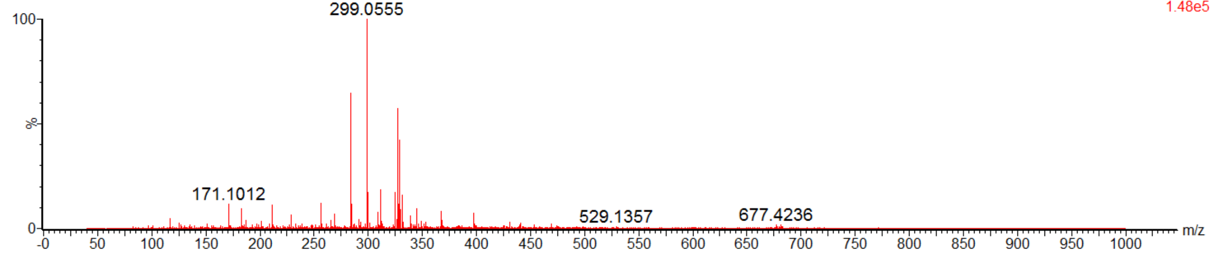


TU_CPUT_2

TU_CPUT_170710_6 3125 (24.312) Cm (3109:3154-2967:3062) 1: TOF MS ES- 3.23e5



TU_CPUT_170710_6 3126 (24.324) Cm (3109:3145-2977:3083) 2: TOF MS ES- 1.48e5



Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

114 formula(e) evaluated with 6 results within limits (all results (up to 1000) for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	N.	O
327.2165	327.2171	-0.6	-1.8	3.5	C18 H31 O5	15.56	18	31		5
	327.2113	5.2	15.9	12.5	C25 H27	22.28	25	27		
	327.2019	14.6	44.6	-0.5	C14 H31 O8	12.01	14	31		8
	327.2324	-15.9	-48.6	7.5	C22 H31 O2	19.08	22	31		2
	327.1960	20.5	62.6	8.5	C21 H27 O3	18.95	21	27		3
	327.2383	-21.8	-66.6	-1.5	C15 H35 O7	12.12	15	35		7

TU_CPUT_2

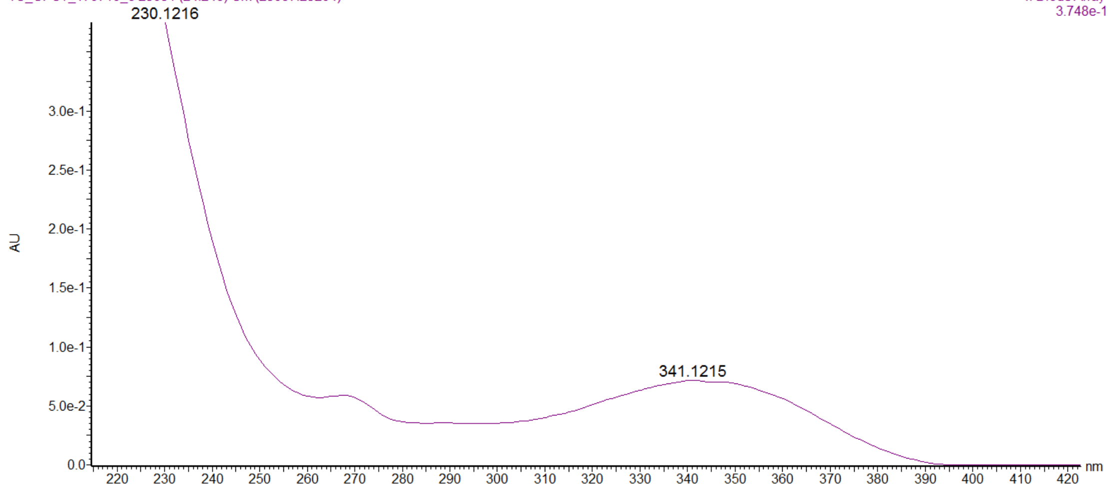
TU_CPUT_170710_6 3125 (24.312) Cm (3109:3154-2967:3062) 1: TOF MS ES- 3.23e+005

327.2165, 299.0555, 329.2319, 331.2473, 116.9270, 199.1323, 215.1275, 269.0449, 397.2233, 431.2281, 474.2848, 545.2692, 599.1201, 655.4389, 677.4246, 737.3605, 781.4933, 807.4908, 929.3649, 981.6141

For Help, press F1

TU_CPUT_2

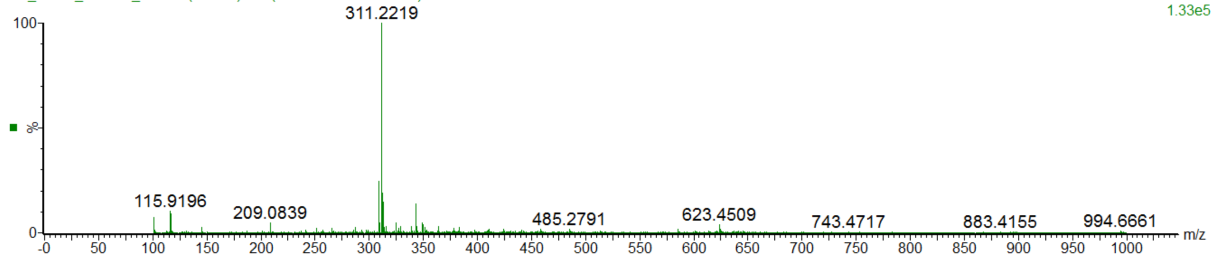
TU_CPUT_170710_6 29091 (24.246) Cm (29057:29204) 4: Diode Array 3.748e-1



TU_CPUT_2

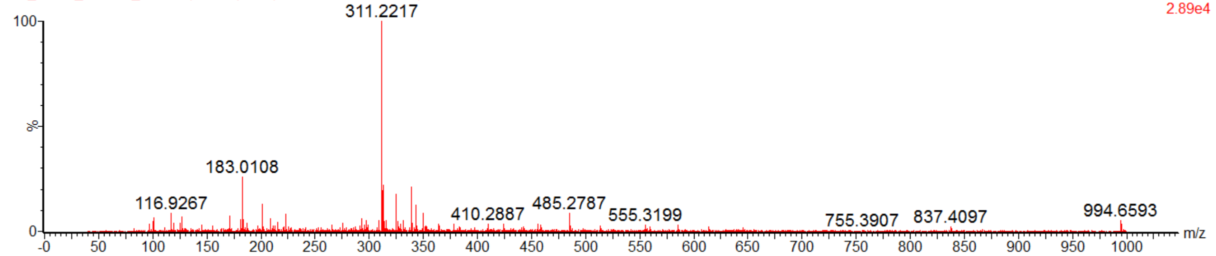
TU_CPUT_170710_6 3176 (24.713) Cm (3170:3183-3185:3189)

1: TOF MS ES-
1.33e5



TU_CPUT_170710_6 3177 (24.724) Cm (3169:3181-3163:3165)

2: TOF MS ES-
2.89e4



Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

104 formula(e) evaluated with 5 results within limits (all results (up to 1000) for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	N	O
311.2219	311.2222	-0.3	-1.0	3.5	C18 H31 O4	21.35	18	31		4
	311.2070	14.9	47.9	-0.5	C14 H31 O7	14.78	14	31		7
	311.2375	-15.6	-50.1	7.5	C22 H31 O	23.47	22	31		1
	311.2011	20.8	66.8	8.5	C21 H27 O2	24.89	21	27		2
	311.2434	-21.5	-69.1	-1.5	C15 H35 O6	15.51	15	35		6

TU_CPUT_2
TU_CPUT_170710_6 3176 (24.713) Cm (3170:3183-3185:3189)

1: TOF MS ES-
1.33e+005

311.2219, 309.2061, 312.2255, 313.2366, 115.9196, 144.9218, 209.0839, 265.1469, 287.2230, 349.2136, 383.1898, 458.2923, 485.2791, 513.3117, 585.3264, 623.4509, 645.4385, 677.4257, 743.4717, 867.5269, 883.4155, 994.6661

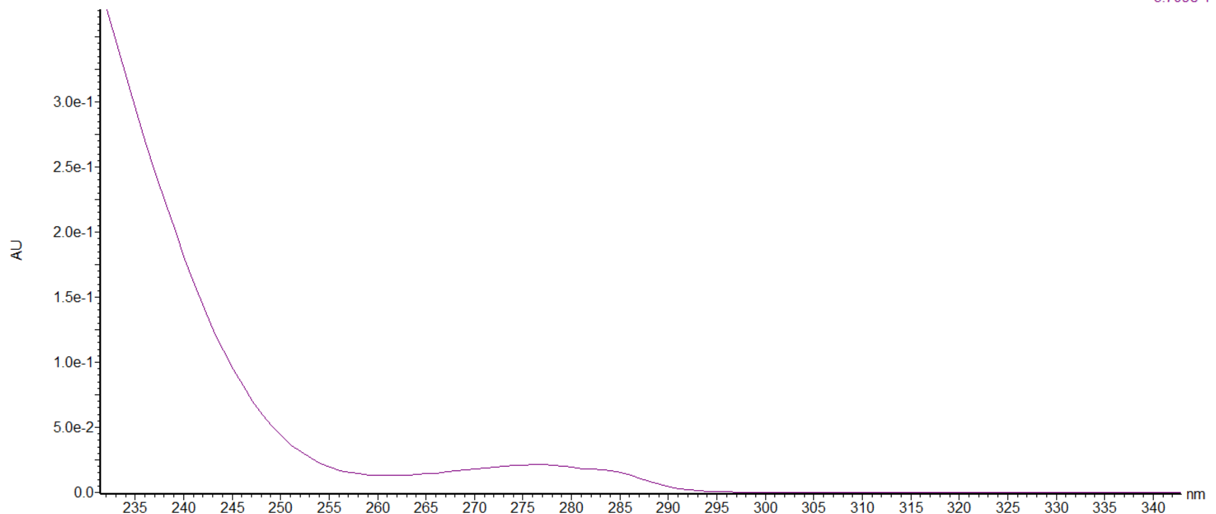
m/z: 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000

For Help, press F1

TU_CPUT_2

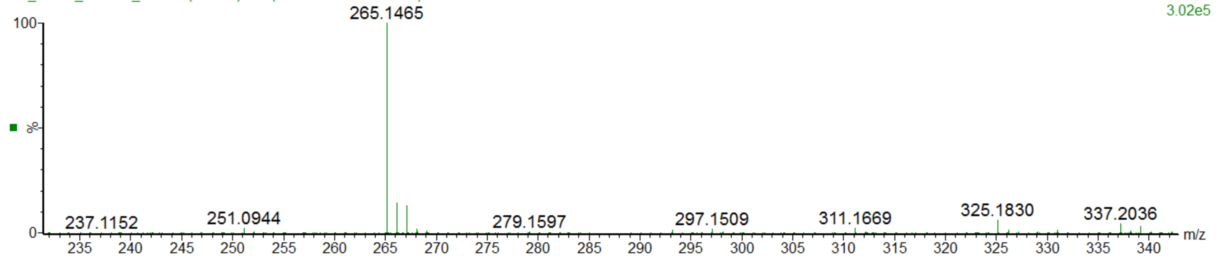
TU_CPUT_170710_6 29596 (24.667) Cm (29576:29617)

4: Diode Array
3.709e-1

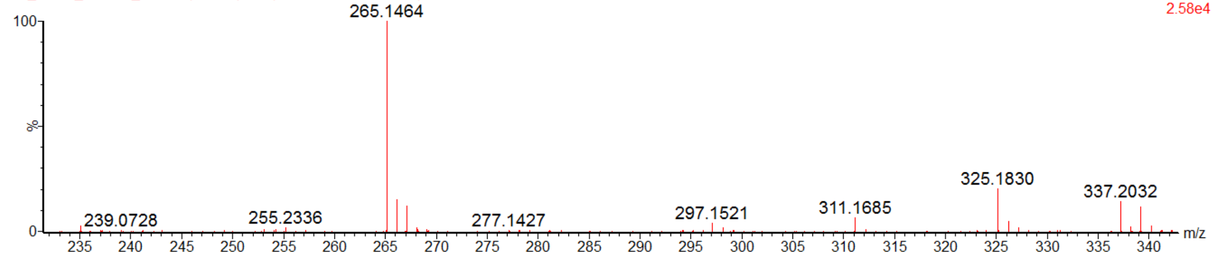


TU_CPUT_2

TU_CPUT_170710_6 3301 (25.687) Cm (3293:3316-3246:3261) 1: TOF MS ES- 3.02e5



TU_CPUT_170710_6 3301 (25.690) Cm (3292:3316-3244:3262) 2: TOF MS ES- 2.58e4



Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

81 formula(e) evaluated with 5 results within limits (all results (up to 1000) for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	N.	O
265.1465	265.1440	2.5	9.4	5.5	C15 H21 O4	19.27	15	21		4
	265.1592	-12.7	-47.9	9.5	C19 H21 O	5.26	19	21		1
	265.1287	17.8	67.1	1.5	C11 H21 O7	33.61	11	21		7
	265.1651	-18.6	-70.1	0.5	C12 H25 O6	34.77	12	25		6
	265.1229	23.6	89.0	10.5	C18 H17 O2	7.08	18	17		2

TU_CPUT_2

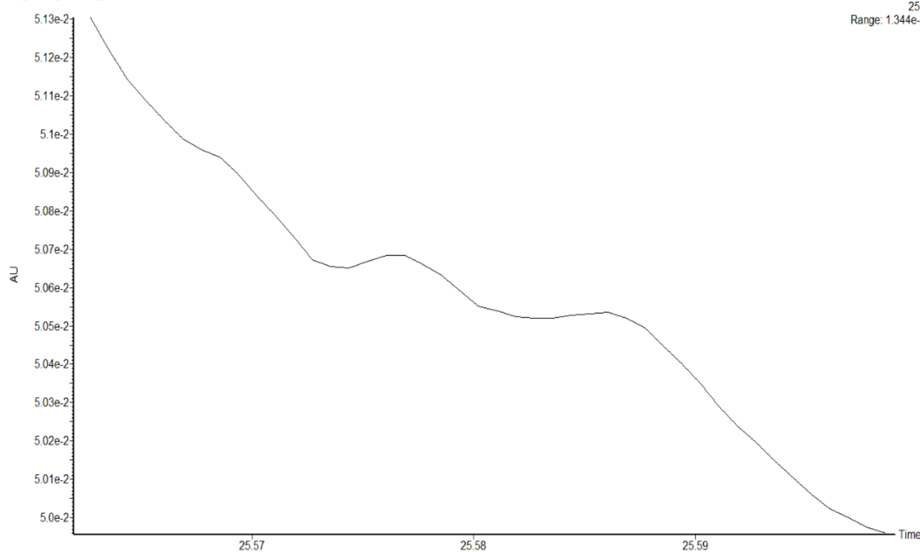
TU_CPUT_170710_6 3301 (25.687) Cm (3293:3316-3246:3261) 1: TOF MS ES- 3.02e+005

265.1465, 266.1496, 267.1447, 268.0911, 237.1152, 248.9751, 251.0944, 257.2083, 265.0905, 279.1597, 283.0660, 293.1781, 297.1509, 299.2596, 309.1707, 311.1669, 318.9778, 325.1830, 330.9773, 337.2036, 339.1989

For Help, press F1

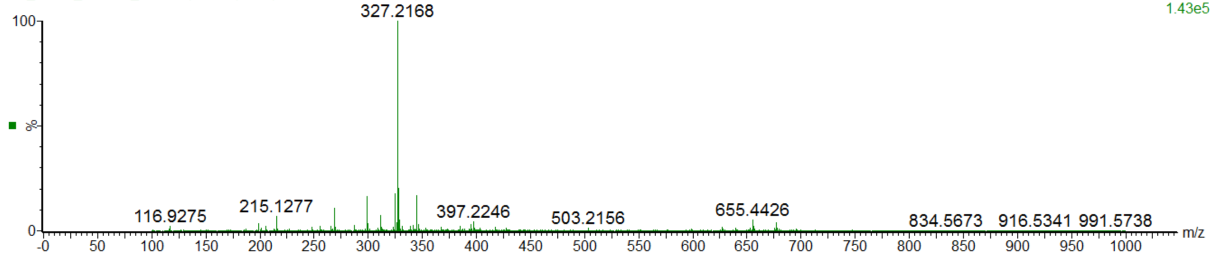
TU_CPUT_2

TU_CPUT_170710_6

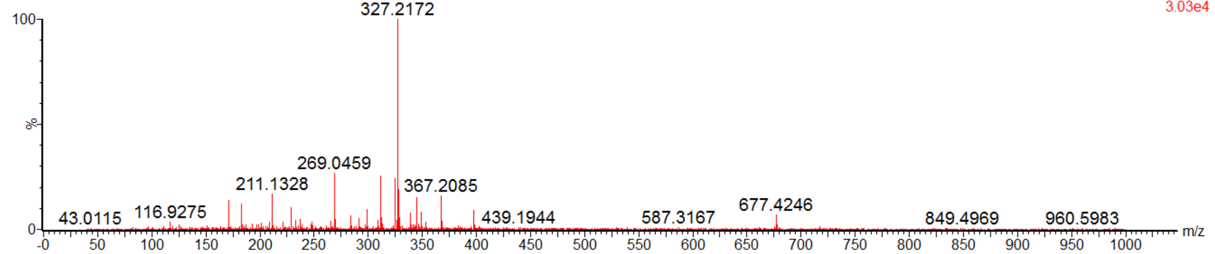


TU_CPUT_3

TU_CPUT_170710_7 3122 (24.291) Cm (3111:3122-3062:3076) 1: TOF MS ES- 1.43e5



TU_CPUT_170710_7 3120 (24.279) Cm (3113:3120-3066:3080) 2: TOF MS ES- 3.03e4



Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

114 formula(e) evaluated with 6 results within limits (all results (up to 1000) for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	N.	O
327.2168	327.2171	-0.3	-0.9	3.5	C18 H31 O5	28.63	18	31		5
	327.2113	5.5	16.8	12.5	C25 H27	9.75	25	27		
	327.2019	14.9	45.5	-0.5	C14 H31 O8	5.35	14	31		8
	327.2324	-15.6	-47.7	7.5	C22 H31 O2	19.83	22	31		2
	327.1960	20.8	63.6	8.5	C21 H27 O3	28.61	21	27		3
	327.2383	-21.5	-65.7	-1.5	C15 H35 O7	7.82	15	35		7

TU_CPUT_3

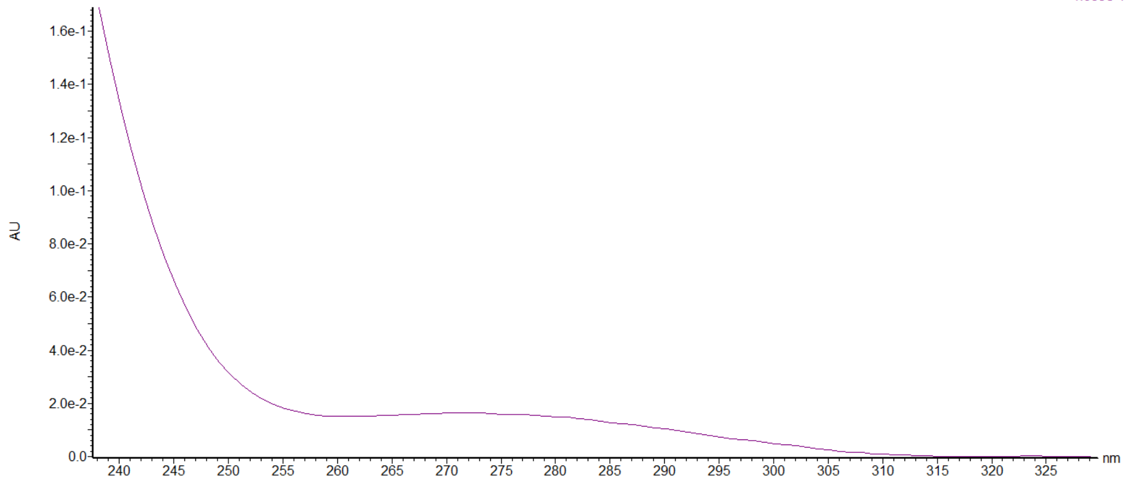
TU_CPUT_170710_7 3122 (24.291) Cm (3111:3122-3062:3076) 1: TOF MS ES- 1.43e+005

100
%
0
327.2168
325.1976
328.2203
269.0446
215.1277
199.1330
116.9275
397.2246 417.2113
503.2156 517.2290
627.2783
655.4426
677.4233
713.4398
747.4349
834.5673 872.5229
916.5341
991.5738
m/z
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000

For Help, press F1

TU_CPUT_3

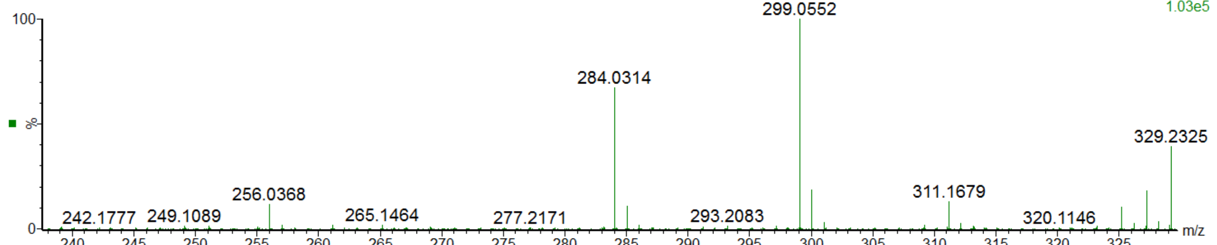
TU_CPUT_170710_7 28855 (24.049) Cm (28717:28968) 4: Diode Array 1.689e-1



TU_CPUT_3

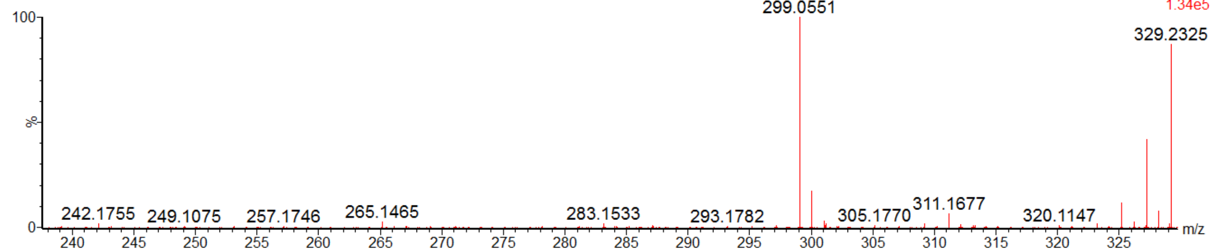
TU_CPUT_170710_7 3125 (24.317) Cm (3125:3136)

2: TOF MS ES-
1.03e5



TU_CPUT_170710_7 3127 (24.328) Cm (3126:3135-3068:3085)

1: TOF MS ES-
1.34e5



Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

98 formula(e) evaluated with 8 results within limits (all results (up to 1000) for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	N	O
299.0551	299.0556	-0.5	-1.7	11.5	C16 H11 O6	99.97	16	11		6
	299.0497	5.4	18.1	20.5	C23 H7 O	0.00	23	7		1
	299.0614	-6.3	-21.1	2.5	C9 H15 O11	0.00	9	15		11
	299.0462	8.9	29.8	-1.5	C5 H15 O14	0.00	5	15		14
	299.0403	14.8	49.5	7.5	C12 H11 O9	0.00	12	11		9
	299.0708	-15.7	-52.5	15.5	C20 H11 O3	0.01	20	11		3
	299.0344	20.7	69.2	16.5	C19 H7 O4	0.01	19	7		4
	299.0767	-21.6	-72.2	6.5	C13 H15 O8	0.01	13	15		8

TU_CPUT_3

TU_CPUT_170710_7 3127 (24.328) Cm (3126:3135-3068:3085)

1: TOF MS ES-
1.34e+005

100 % 0

299.0551 329.2325 327.2167 300.0582 325.1943 301.0607 309.1886 311.1677 313.1642 320.1147 323.1860 242.1755 249.1075 253.1422 257.1746 259.1919 265.1465 269.0451 271.1517 281.1430 283.1533 287.1495 293.1782 297.1592

240.0 245.0 250.0 255.0 260.0 265.0 270.0 275.0 280.0 285.0 290.0 295.0 300.0 305.0 310.0 315.0 320.0 325.0 330.0 m/z

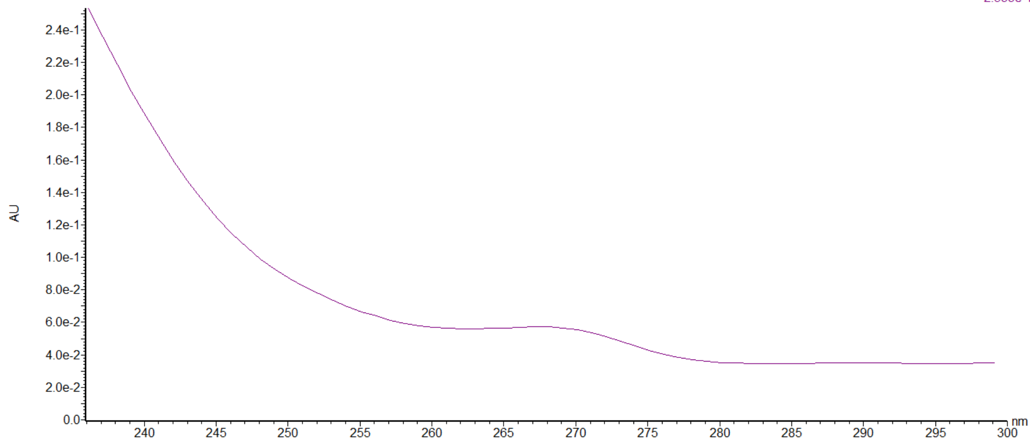
For Help, press F1

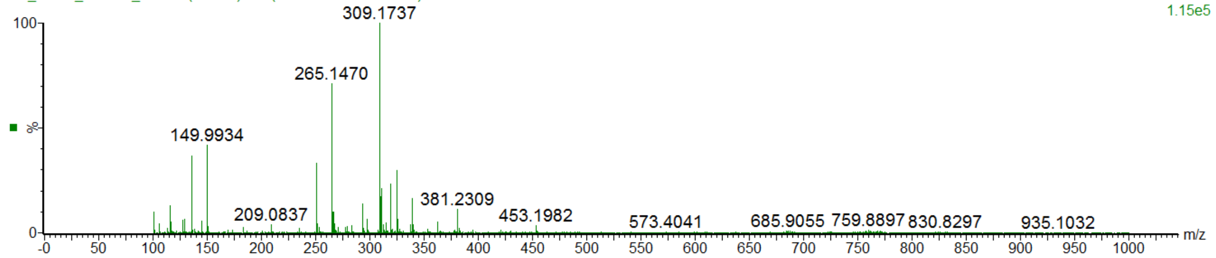
Hispidulin

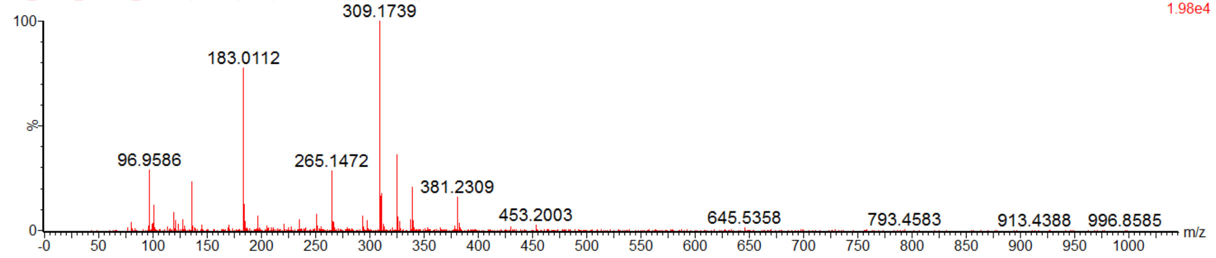
TU_CPUT_3

TU_CPUT_170710_7 29093 (24.247) Cm (29039:29168)

4: Diode Array
2.533e-1







Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

106 formula(e) evaluated with 6 results within limits (all results (up to 1000) for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	N	O
309.1737	309.1913	-17.6	-56.9	0.5	C14 H29 O7	22.63	14	29		7
	309.1549	18.8	60.8	1.5	C13 H25 O8	22.44	13	25		8
	309.1702	3.5	11.3	5.5	C17 H25 O5	18.49	17	25		5
	309.1491	24.6	79.6	10.5	C20 H21 O3	13.96	20	21		3
	309.1855	-11.8	-38.2	9.5	C21 H25 O2	13.02	21	25		2
	309.1643	9.4	30.4	14.5	C24 H21	9.45	24	21		

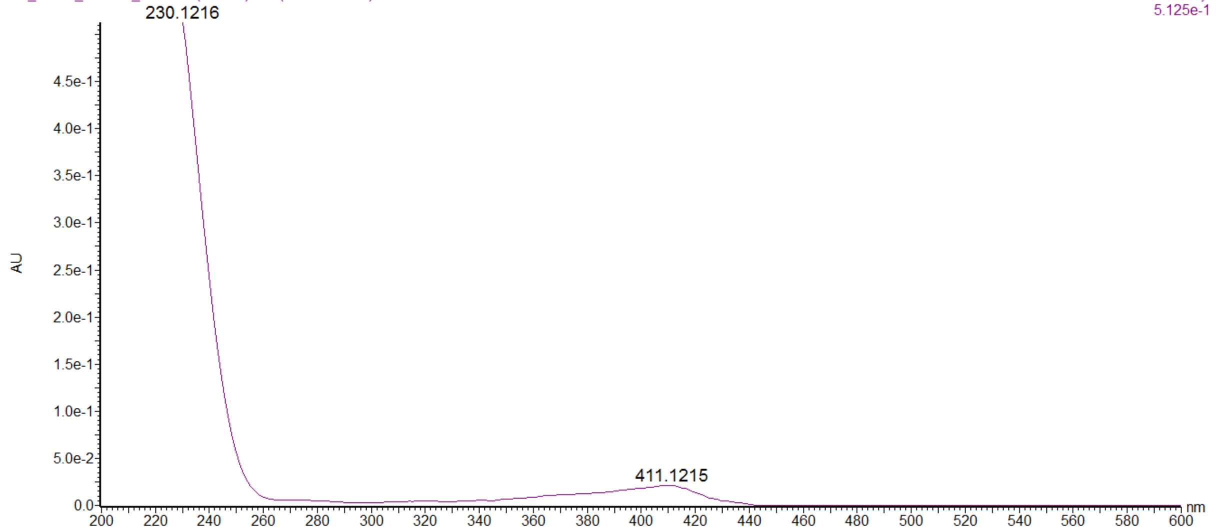
TU_CPUT_3

TU_CPUT_170710_7 3338 (25.964) Cm (3326:3346-3412:3426)

1: TOF MS ES-
1.15e+005

309.1737 265.1470 149.9934 251.0945 325.1841 339.1999 115.9195 381.2309 150.9952 209.0837 362.9685 382.2351 453.1982 471.3324 541.2311 573.4041 637.4334 685.9055 745.9025 759.8897 772.8314 830.8297 875.0128 935.1032 954.8338

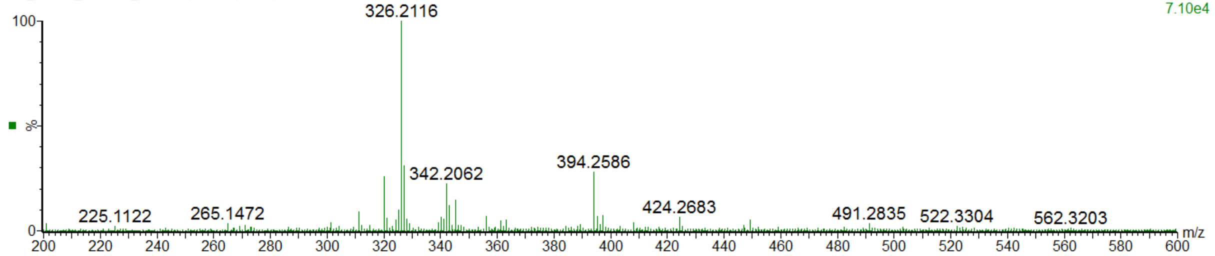
For Help, press F1



TU_CPUT_5

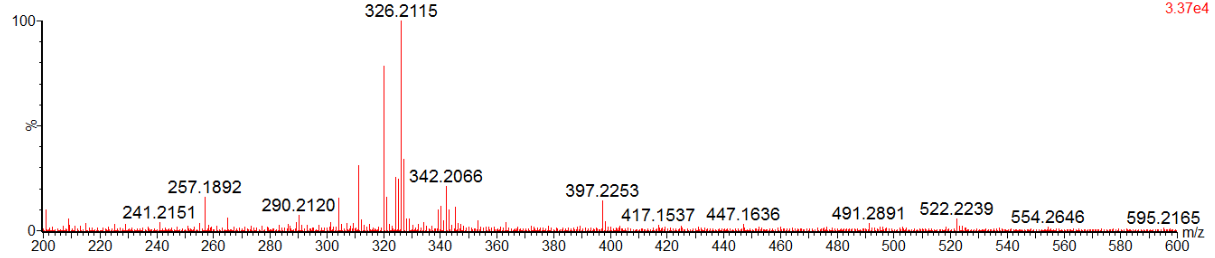
TU_CPUT_170710_9 3103 (24.149) Cm (3102:3114-3075:3080)

1: TOF MS ES-
7.10e4



TU_CPUT_170710_9 3104 (24.160) Cm (3102:3115-3081:3088)

2: TOF MS ES-
3.37e4



Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

110 formula(e) evaluated with 6 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 1-100 H: 1-100 N: 0-1 O: 0-100

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	N	O
326.2116	326.2120	-0.4	-1.2	8.5	C21 H28 N O2	21.05	21	28	1	2
	326.2179	-6.3	-19.3	-0.5	C14 H32 N O7	0.14	14	32	1	7
	326.1967	14.9	45.7	4.5	C17 H28 N O5	0.78	17	28	1	5
	326.1909	20.7	63.5	13.5	C24 H24 N	77.11	24	24	1	
	326.2331	-21.5	-65.9	3.5	C18 H32 N O4	0.86	18	32	1	4
	326.1815	30.1	92.3	0.5	C13 H28 N O8	0.06	13	28	1	8

TU_CPUT_5

TU_CPUT_170710_9 3103 (24.149) Cm (3102:3114-3075:3080)

1: TOF MS ES-
7.10e+004

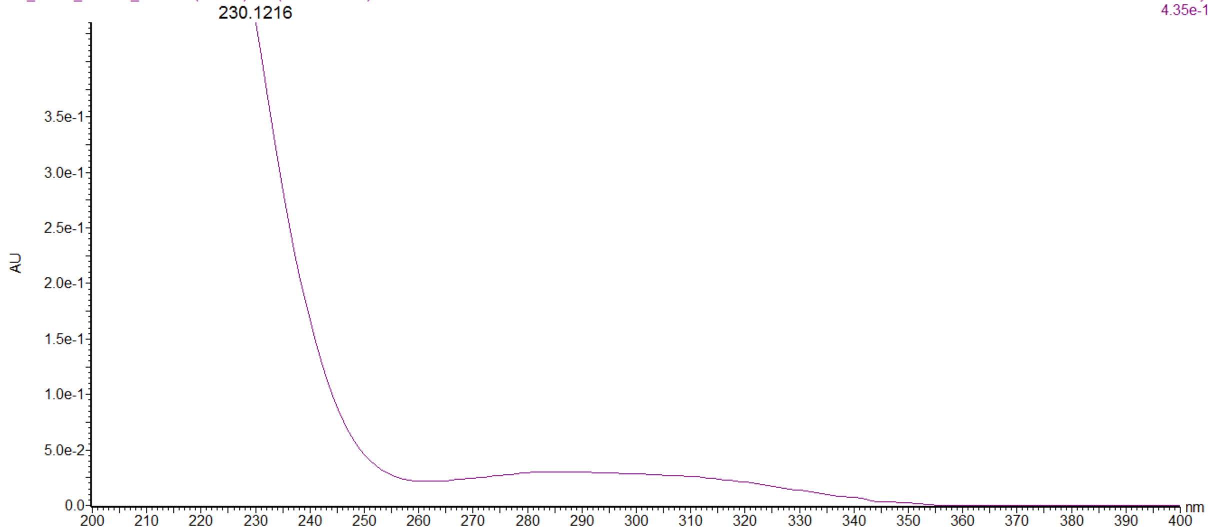
326.2116, 327.2154, 320.2215, 342.2062, 345.2268, 394.2586, 201.1125, 225.1122, 243.1223, 265.1472, 301.1999, 311.1673, 356.2211, 363.2372, 397.2238, 424.2683, 449.2762, 482.2766, 491.2835, 522.3304, 542.3264, 562.3203, 590.4769, 599.3447

For Help, press F1

TU_CPUT_5

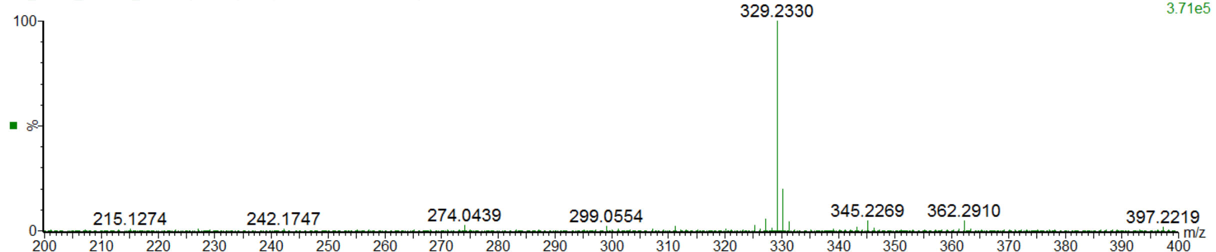
TU_CPUT_170710_9 28811 (24.012) Cm (28724:28907)

4: Diode Array
4.35e-1

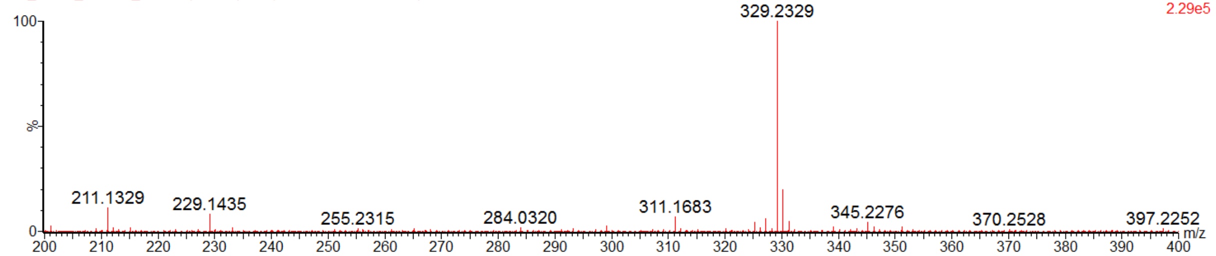


TU_CPUT_5

TU_CPUT_170710_9 3130 (24.351) Cm (3123:3137-3067:3077) 1: TOF MS ES- 3.71e5



TU_CPUT_170710_9 3129 (24.347) Cm (3122:3138-3213:3225) 2: TOF MS ES- 2.29e5



Elemental Composition

File Edit View Process Help

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

115 formula(e) evaluated with 5 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 1-100 H: 1-100 N: 0-1 O: 0-100

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	N	O
329.2330	329.2328	0.2	0.6	2.5	C18 H33 O5	41.00	18	33		5
	329.2269	6.1	18.5	11.5	C25 H29	17.94	25	29		
	329.2481	-15.1	-45.9	6.5	C22 H33 O2	16.58	22	33		2
	329.2175	15.5	47.1	-1.5	C14 H33 O8	1.35	14	33		8
	329.2117	21.3	64.7	7.5	C21 H29 O3	23.12	21	29		3

TU_CPUT_5

TU_CPUT_170710_9 3130 (24.351) Cm (3123:3137-3067:3077) 1: TOF MS ES- 3.71e+005

100 % 0

329.2330

330.2360

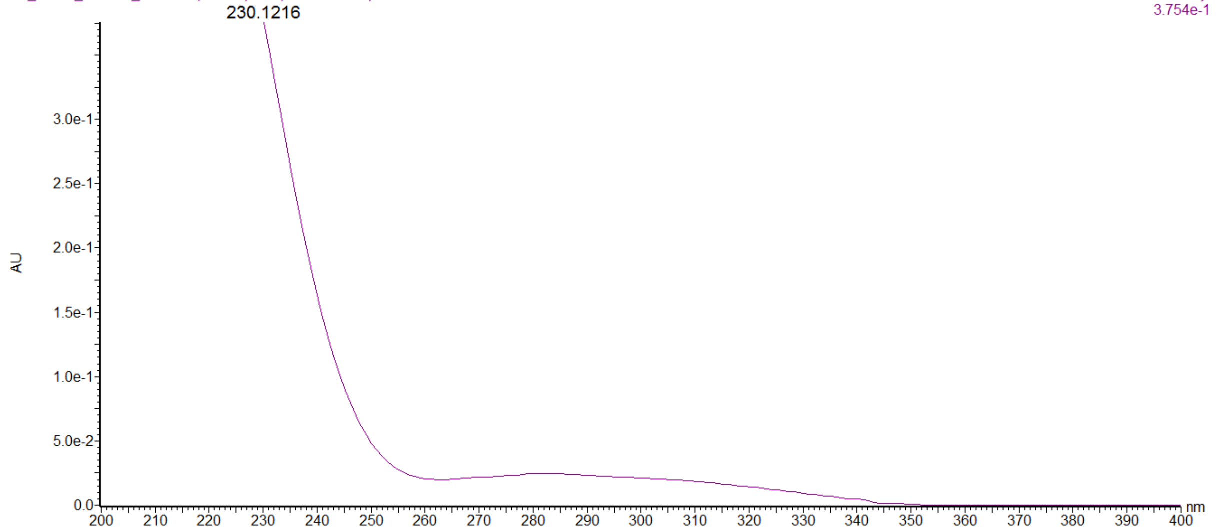
201.1119 215.1274 227.1277 242.1747 255.1572 265.1477 274.0439 287.1492 299.0554 311.1670 327.2176 331.2403 345.2269 362.2910 365.2110 373.2317 388.3058 397.2219

m/z 200 205 210 215 220 225 230 235 240 245 250 255 260 265 270 275 280 285 290 295 300 305 310 315 320 325 330 335 340 345 350 355 360 365 370 375 380 385 390 395

For Help, press F1

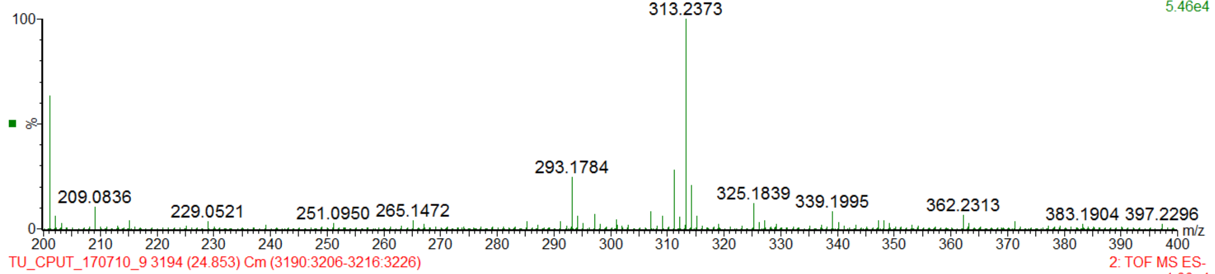
TU_CPUT_5

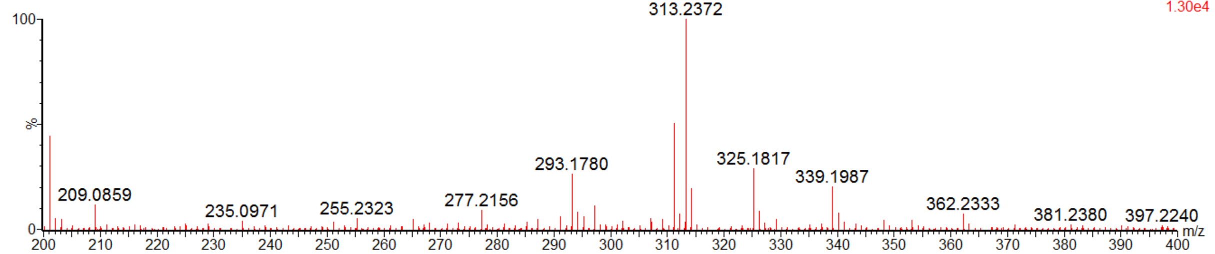
TU_CPUT_170710_9 28916 (24.100) Cm (28916:28988) 4: Diode Array 3.754e-1



TU_CPUT_5

TU_CPUT_170710_9 3196 (24.864) Cm (3191:3207-3231:3239)





Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

105 formula(e) evaluated with 4 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 1-100 H: 1-100 N: 0-1 O: 0-100

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	N	O
313.2373	313.2379	-0.6	-1.9	2.5	C18 H33 O4	27.95	18	33		4
	313.2226	14.7	46.9	-1.5	C14 H33 O7	8.07	14	33		7
	313.2531	-15.8	-50.4	6.5	C22 H33 O	28.61	22	33		1
	313.2168	20.5	65.4	7.5	C21 H29 O2	35.37	21	29		2

TU_CPUT_5

TU_CPUT_170710_9 3196 (24.864) Cm (3191:3207-3231:3239)

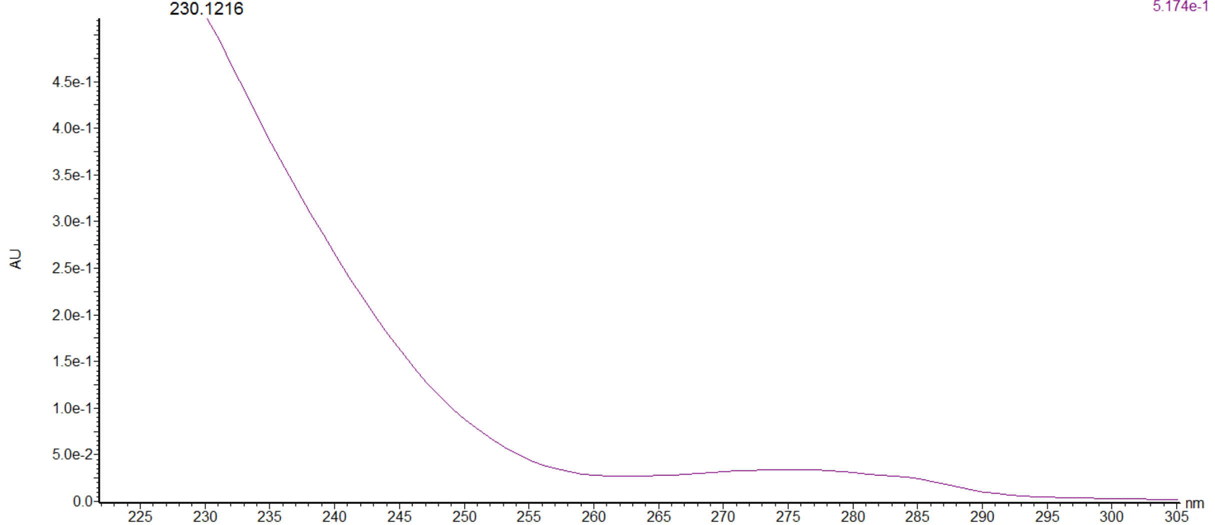
1: TOF MS ES-
5.46e+004

313.2373, 201.1120, 311.2219, 311.1682, 314.2409, 293.1784, 325.1839, 209.0836, 215.1646, 229.0521, 239.1633, 251.0950, 265.1472, 281.1731, 291.1929, 297.1532, 307.1699, 315.2495, 327.2125, 339.1995, 348.2134, 362.2313, 371.2438, 383.1904, 397.2296

For Help, press F1

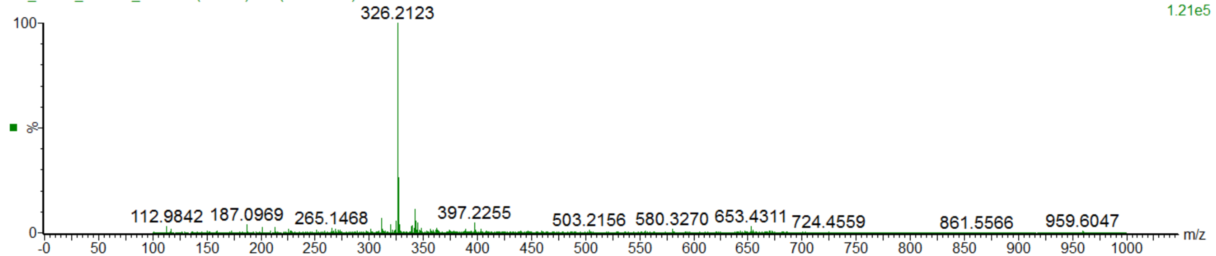
TU_CPUT_5

TU_CPUT_170710_9 29595 (24.666) Cm (29568:29644)

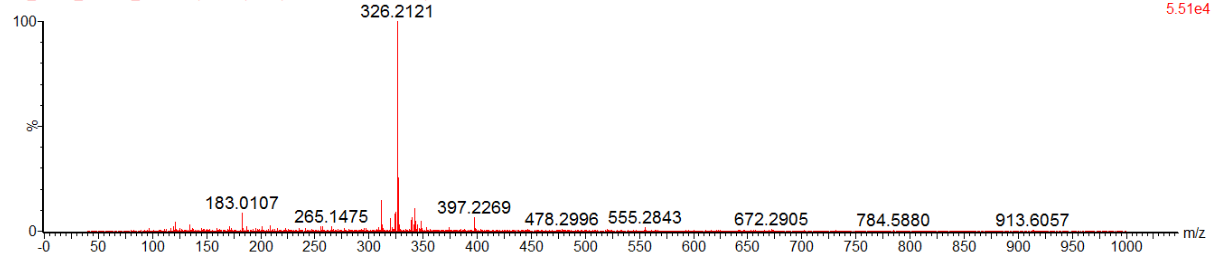


TU_CPUT_6

TU_CPUT_170710_10 3103 (24.148) Cm (3101:3112)   1: TOF MS ES- 1.21e5



TU_CPUT_170710_10 3105 (24.167) Cm (3102:3112-3070:3076)   2: TOF MS ES- 5.51e4



Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

110 formula(e) evaluated with 6 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 1-100 H: 1-100 N: 0-1 O: 0-100

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	N	O
326.2123	326.2120	0.3	0.9	8.5	C21 H28 N O2	19.68	21	28	1	2
	326.2179	-5.6	-17.2	-0.5	C14 H32 N O7	1.18	14	32	1	7
	326.1967	15.6	47.8	4.5	C17 H28 N O5	1.68	17	28	1	5
	326.2331	-20.8	-63.8	3.5	C18 H32 N O4	1.56	18	32	1	4
	326.1909	21.4	65.6	13.5	C24 H24 N	75.85	24	24	1	
	326.1815	30.8	94.4	0.5	C13 H28 N O8	0.04	13	28	1	8

TU_CPUT_6

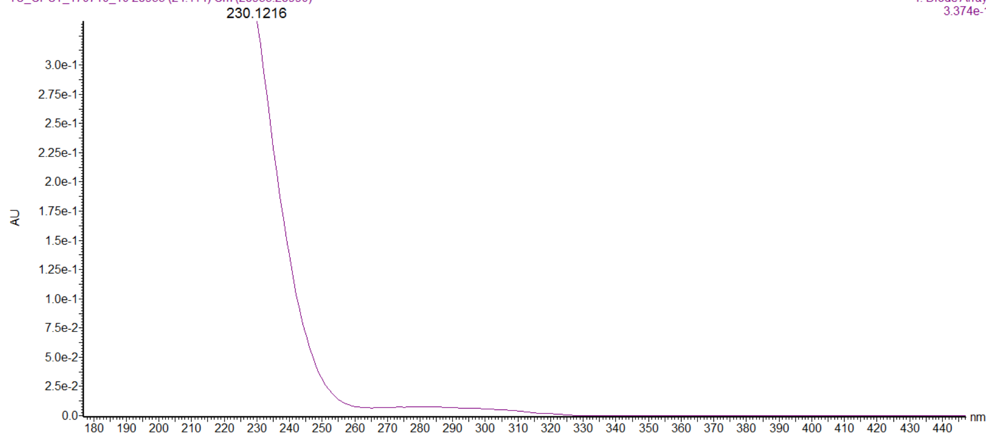
TU_CPUT_170710_10 3103 (24.148) Cm (3101:3112)   1: TOF MS ES- 1.21e+005

326.2123, 327.2154, 342.2072, 343.2118, 311.1693, 112.9842, 187.0969, 213.1126, 265.1468, 397.2255, 440.2052, 503.2156, 580.3270, 639.4061, 653.4311, 670.4302, 724.4559, 754.4600, 861.5566, 941.5440, 959.6047, 978.3611

For Help, press F1

TU_CPUT_6

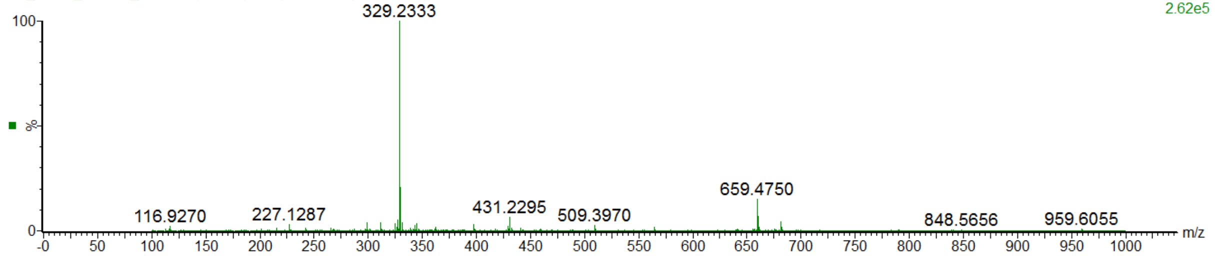
TU_CPUT_170710_10 28933 (24.114) Cm (28933:28990)   4: Diode Array 3.374e-1



TU_CPUT_6

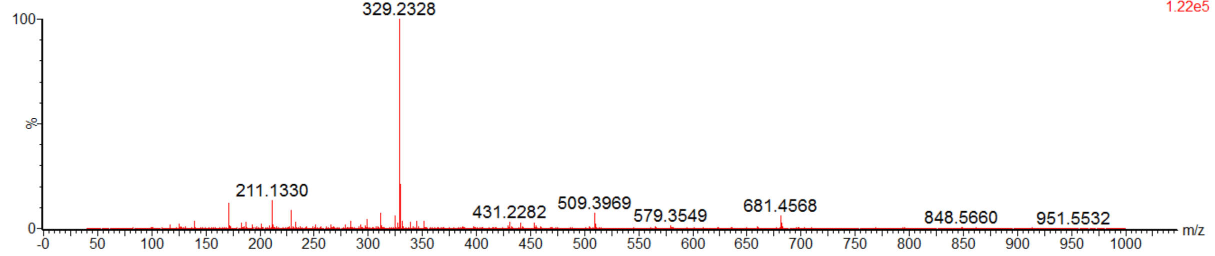
TU_CPUT_170710_10 3130 (24.350) Cm (3124:3136)

1: TOF MS ES- 2.62e5



TU_CPUT_170710_10 3129 (24.346) Cm (3123:3136-3109:3115)

2: TOF MS ES- 1.22e5



Elemental Composition

File Edit View Process Help

Tolerance = 100.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
115 formula(e) evaluated with 5 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 1-100 H: 1-100 N: 0-1 O: 0-100

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Fit Conf %	C	H	N	O
329.2333	329.2328	0.5	1.5	2.5	C18 H33 O5	32.96	18	33		5
	329.2269	6.4	19.4	11.5	C25 H29	12.88	25	29		
	329.2481	-14.8	-45.0	6.5	C22 H33 O2	19.71	22	33		2
	329.2175	15.8	48.0	-1.5	C14 H33 O8	0.59	14	33		8
	329.2117	21.6	65.6	7.5	C21 H29 O3	33.87	21	29		3

TU_CPUT_6
TU_CPUT_170710_10 3130 (24.350) Cm (3124:3136)
1: TOF MS ES- 2.62e+005

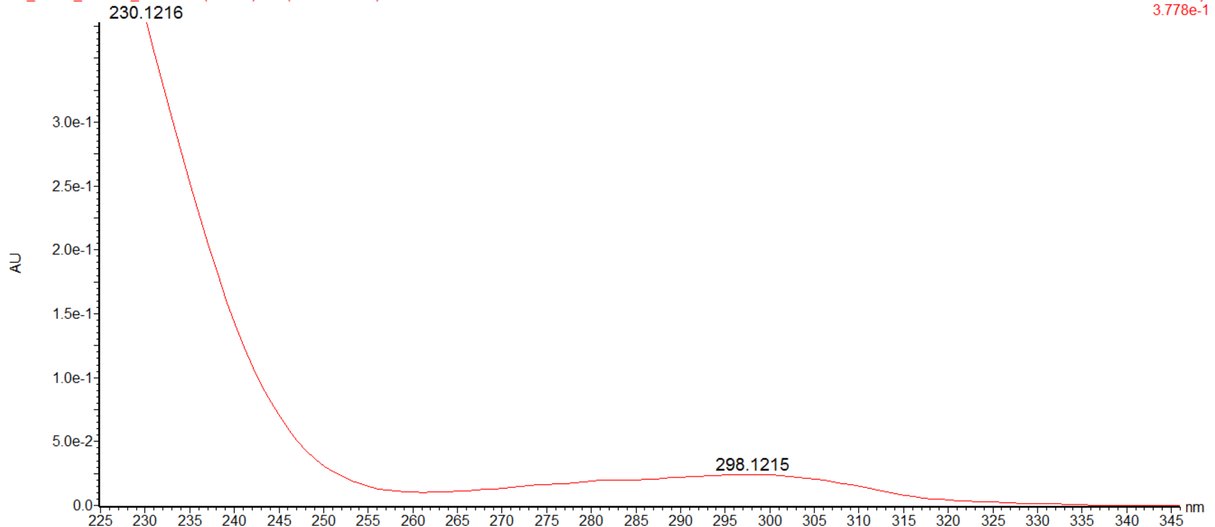
329.2333, 330.2364, 116.9270, 201.1129, 227.1287, 242.1753, 327.2175, 331.2409, 431.2295, 459.3104, 509.3970, 564.3322, 579.3519, 657.4589, 659.4750, 660.4783, 682.4612, 747.4254, 783.4496, 848.5656, 862.6138, 939.5827, 959.6055, 985.6065 (m/z)

For Help, press F1

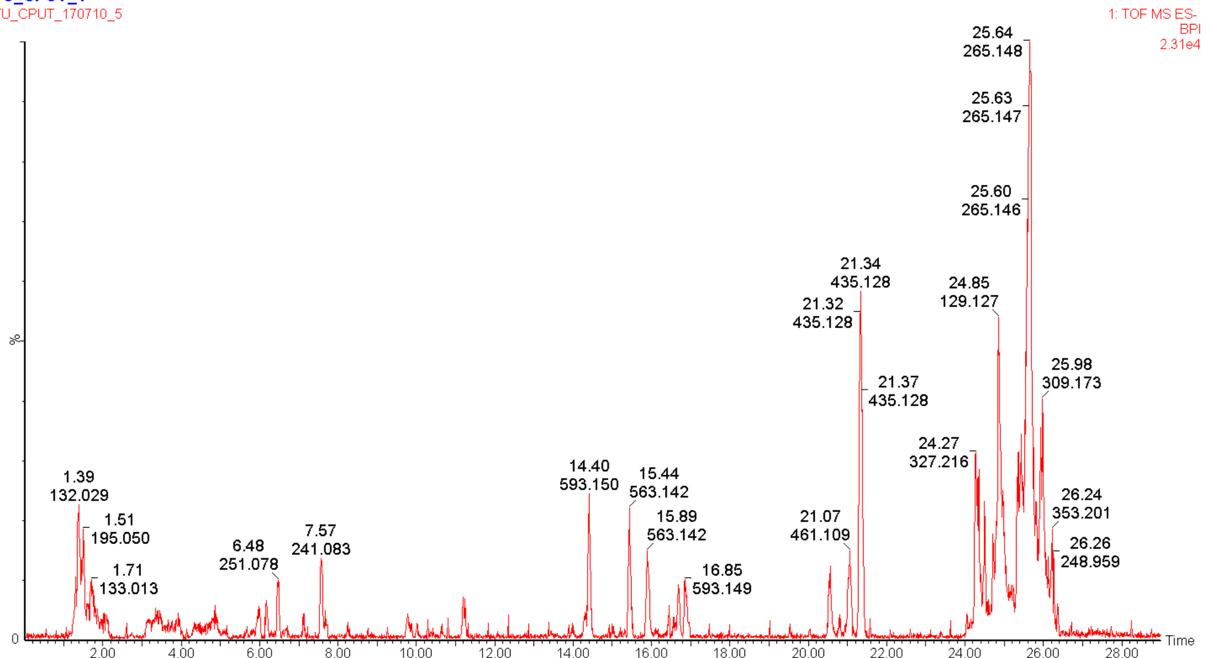
TU_CPUT_6

TU_CPUT_170710_10 28901 (24.087) Cm (28884:28917)

4: Diode Array 3.778e-1

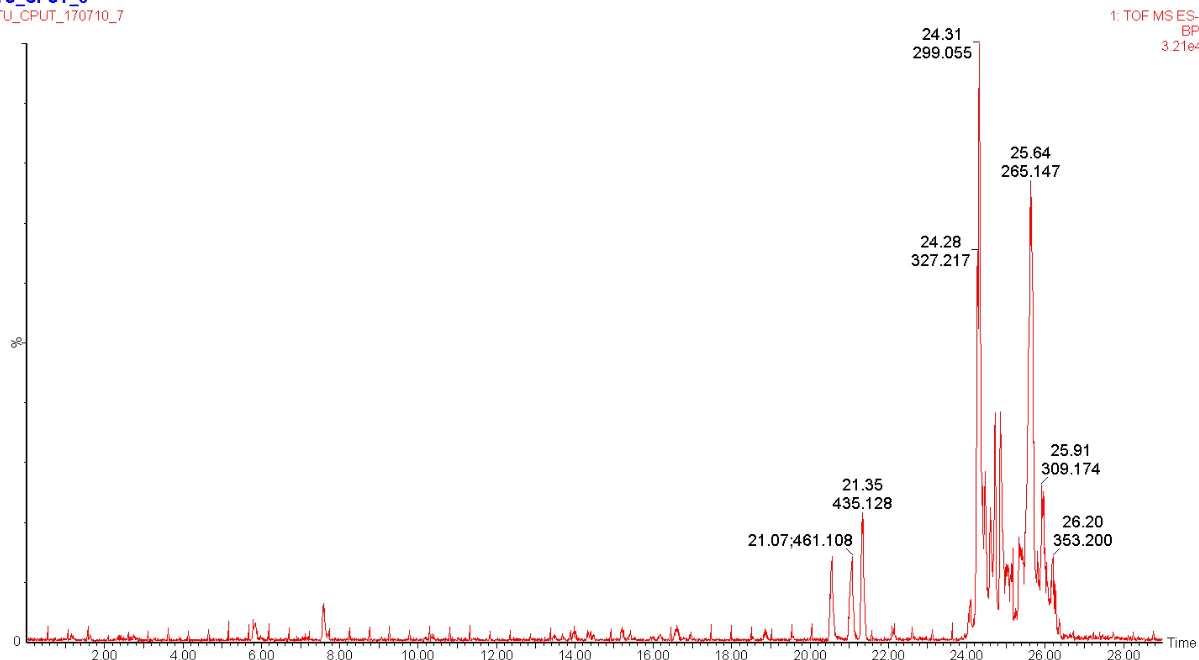


Aqueous Leaves

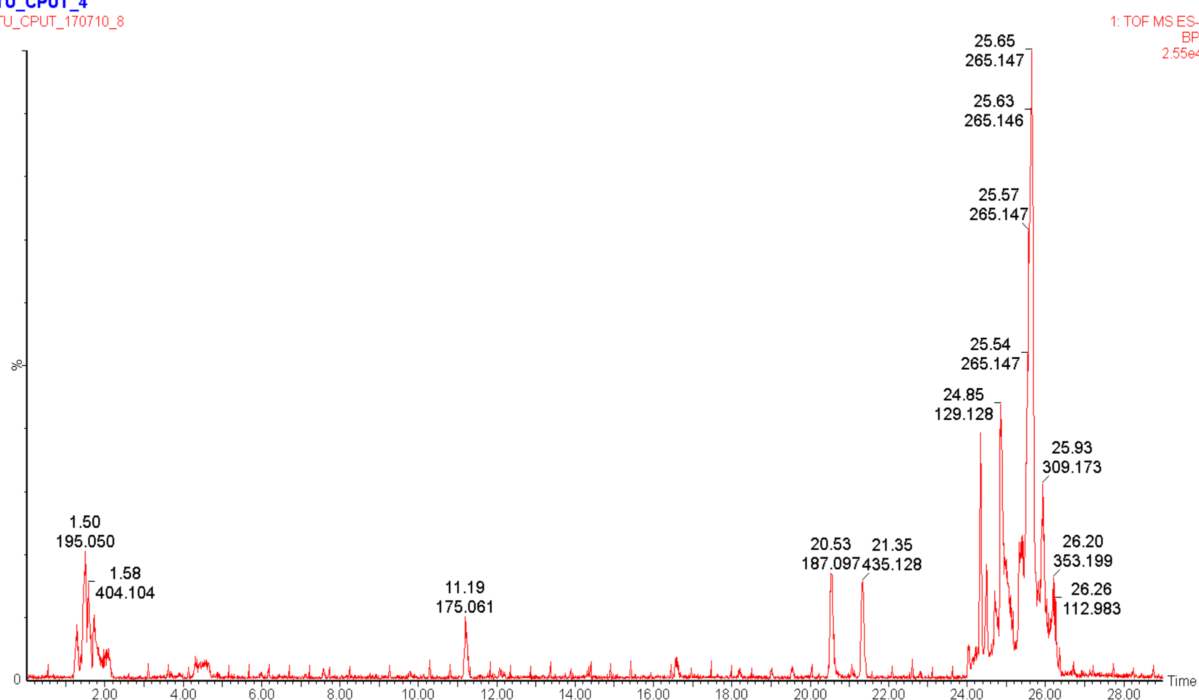


Ethanol Leaves

Ethyl Acetate Leaves

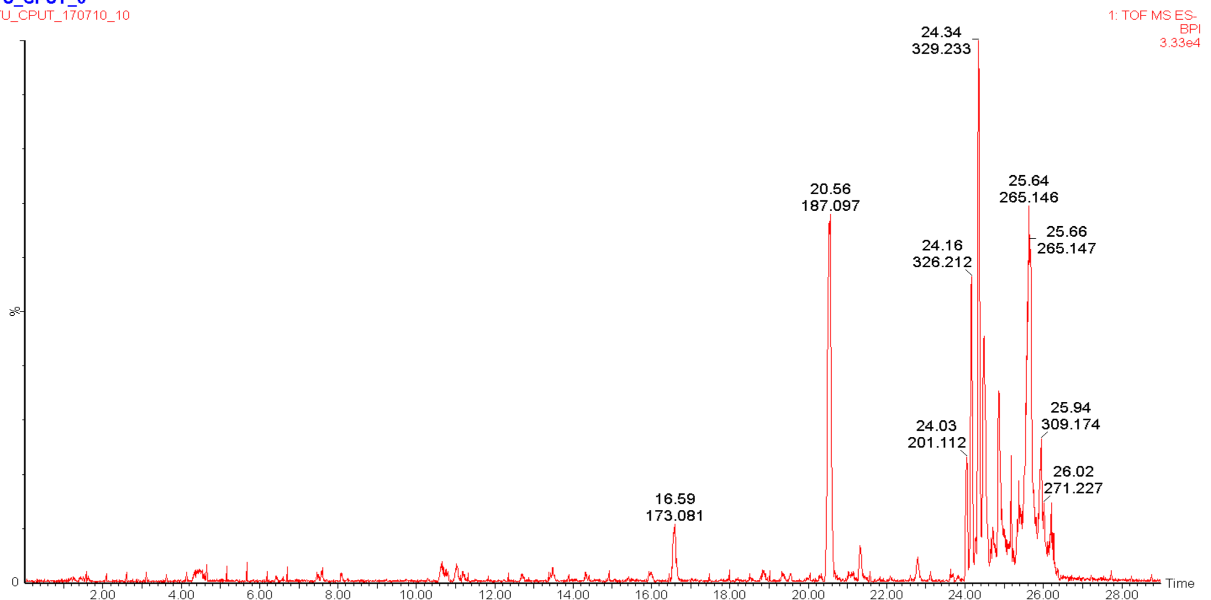


Aqueous Rhizome

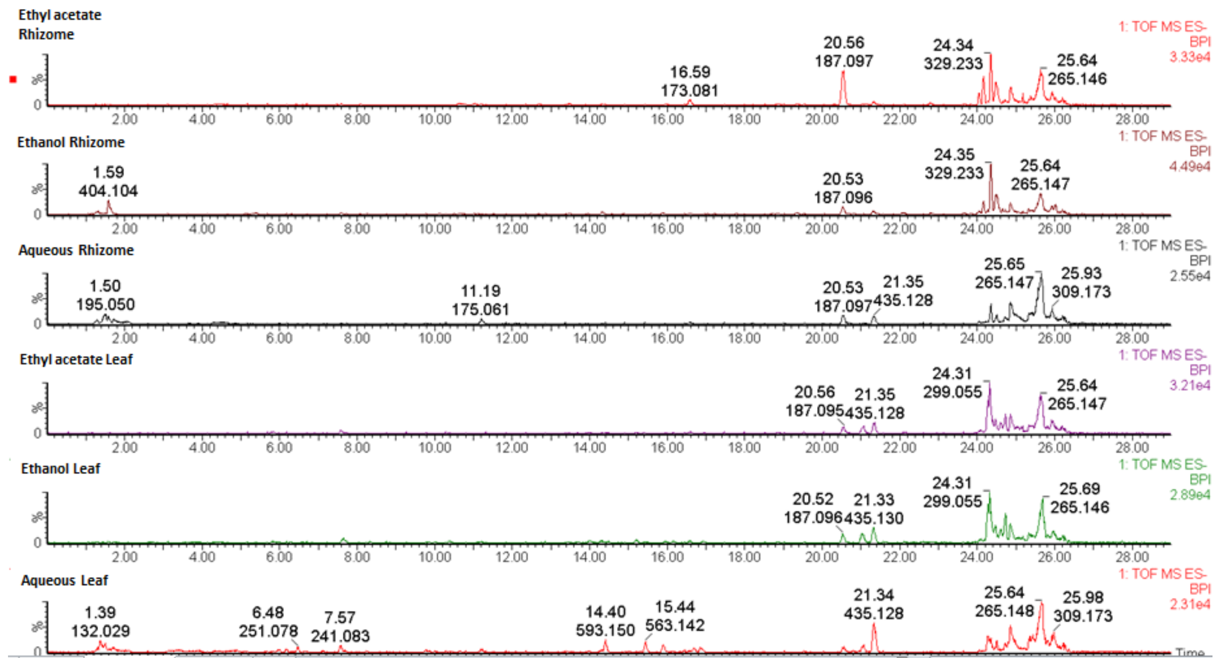


Ethanol Rhizome

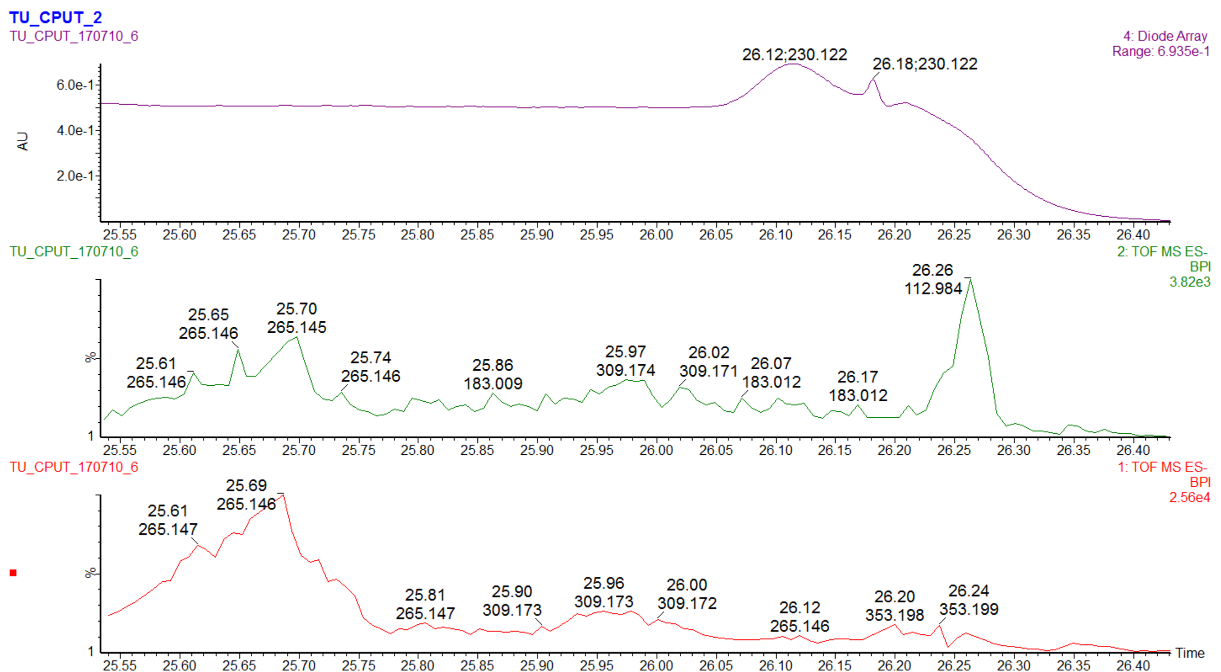
Ethyl Acetate Rhizome



Combined chromatogram for the six extracts from leaves and rhizome of AD



Chromatogram for other compounds likely present in AD



ADDENDUM 4: OVERVIEW OF THE *IN VITRO AND IN VIVO* ACTIVITIES *ANCHOMANES DIFFORMIS*

Table 1: Phytochemical characterization and antioxidant capacities of AD extracts

	ORAC	FRAP	TEAC	Polyphenols	Flavonols	Flavanols	Compounds
Aq Leaves	H*	H*	H*	H*	I	I	19
Aq Rhizome	L	L*	L	L	L*	L	19
E-OH Leaves	L*	I	I	H	H	H*	19
E-OH Rhizome	I	L	I	L	I	L*	20
Ethyl-A Leaves	I	L	L*	I	I	I	16
Ethyl-A Rhizome	I	I	I	I	H	I	15

Aq - aqueous, E-OH - ethanol, Ethyl-A - ethyl acetate, H- high, H*-highest, L-low, L*-lowest

Table 2: The effect of STZ and treatment with AD on antioxidant defense system, oxidative stress status and blood glucose in diabetes control and diabetes treated rats

	Nrf2	CAT	SOD	ORAC	FRAP	TBARS	tGSH	Blood glucose
Liver	_	↓	NS	↓	NS	_	↓	_
Heart	↑	↑	NS	↓	NS	NS	_	_
Kidney	NS	NS	NS	NS	NS	NS	_	_
Serum	_	_	_	↓	↓	↑	_	↑
After treatment with AD								
Liver	_	↑	NS	↑	NS	_	↑	_
Heart	Norm	NS	NS	NS	NS	NS	_	_
Kidney	NS	NS	NS	NS	NS	NS	_	_
Serum	_	_	_	Norm	NS	↓	_	↓

NS- not significant, Norm-normalized, ↓-decrease, ↑-increase

Table 3: The effect of STZ and treatment with AD on the immune response in diabetes control and diabetes treated rats

	NFkB	IL-1β	IL-6	IL-10	IL-18	MCP-1	TNF-α
Heart	↑	↓	↓	↓	↑	NS	NS
Kidney	NS	↑	↑	↑	↑	_	↑
After treatment with AD							
Heart	Norm	Norm	Norm	Norm	Norm	NS	NS
Kidney	NS	↓	↓	↓	NS	↓	NS

NS- not significant, Norm-normalized, ↓-decrease, ↑-increase

Table 4: The effect of STZ and treatment with AD on apoptosis in diabetes control and diabetes treated rats

	NFkB	Bcl2	Caspase-3
Heart	↑	NS	↑
Kidney	NS	↓	NS
After treatment with AD			
Heart	↓	NS	↓
Kidney	NS	Norm	NS

NS- not significant, Norm-normalized, ↓-decrease, ↑-increase

Table 5: The effect of STZ and treatment with AD on sperm functions in diabetes control and diabetes treated rats

	Sperm Conc	Sperm Viability	Sperm Morphology	Sperm Motility Indices	
				% motility	Velocity/kinematics
Diabetic control	↓	↓	↓	NS	↓
Diabetic treated	Norm	Norm	Norm	NS	↑

NS- not significant, Norm-normalized, ↓-decrease, ↑-increase

Table 6: The effect of STZ and treatment with AD on organ function in diabetes control and diabetes treated rats

	Liver				Kidney			Heart		Testis	Epididymis
	ALP	AST	ALT	TChol	Urea	Creat	Rwt	HFABP	Rwt	Rwt	Rwt
DC	↑	↑	↑	↑	↑		↑	↑	↑	↑	↓
DT	↓	Norm	Norm	Norm	↓	NS	NS	NS	↓	NS	Norm

NS- not significant, Norm-normalized, ↓-decrease, ↑-increase