

**Effects of *Momordica charantia* on the Kidney following
antiretroviral therapy in Male Diabetic and Non-diabetic animal
model**

By

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Doctor of Philosophy in Anatomy**

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Preface

The study described in this thesis was carried out in the Discipline of Clinical Anatomy, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa from February 2016 to April 2018, under the supervision of Prof. O.O Azu and Dr. E.C.S. Naidu.

Declaration

I, Mr. OFFOR, Ugochukwu declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or other tertiary institution for purpose of obtaining an academic qualification, whether by me or any other party.
2. That my contribution to the project was as follows:
 - I was involved in the design and submission of the proposal for ethics approval by the University Animal Research Ethics Committee.
 - I was wholly responsible for carrying out the experiments, data and sample collection.
 - I performed all sample analyses, collated and did all the analyses
 - I was responsible for the writing of all the manuscripts and the thesis.
3. This thesis does not contain other person's writing, data, pictures, or other information, unless specifically acknowledged as being sourced from other persons or researchers. Where other written sources have been quoted then:
 - Their words have been re-written but the general information attributed to them has been referenced.
 - Where their exact words have been used, then it has been properly referenced in the reference section.



.....
Signed

20-11-2019

.....
Date

Dedication

To the Almighty God for his enduring mercies, love and care.

To my parents Mr. and Mrs. Collins Amaefule Offor.

To my Uncle Prof. Ozo-Mekuri Ndimele.

To my Supervisor Prof. Onyemaechi Okpara Azu.

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Abbreviations

AKI	Acute kidney injury
AMPK	Activated protein kinase
ApoC-III	Apolipoprotein C-III
ART	Antiretroviral therapy
ATP	Adenosine Tri-Phosphate
BUN	Blood Urea Nitrogen
cAMP	Cyclic adenosine monophosphate-dependent
CD4	Cluster of differentiation 4
CKD	Chronic kidney disease
CTL	Cytotoxic CD8 ⁺ T-lymphocytes
DM	Diabetes Mellitus
DN	Diabetic nephropathy
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FPG	Fasting Plasma Glucose
GLUT4	Glucose transporter isoform 4
HAART	Highly active antiretroviral therapy
HLA	Human leukocytes antigen
IDDM	Insulin Dependent Diabetes Mellitus
IFN- γ	Interferon gamma
IGF-I and IGF-II	Insulin like Growth Factor I and II

IRS	Insulin receptor substrate
LDL-c	Lipoprotein- cholesterol
MAP	Mitogen activated protein kinase
MAP-30	<i>Momordica</i> Anti-HIV Protein
MODY	Maturity Onset Diabetes of the Young
NADPH	Nicotinamide adenine dinucleotide phosphate
NDI	Nephrogenic diabetes insipidus
NF- κ B	Nuclear factor kappa B
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NNRTIs	Non- nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
OGTT	Oral Glucose Tolerance Test
PIs	Protease inhibitors
PLWHA	People living with HIV/AIDS
PKC	Protein kinase C
ROS	Reactive Oxygen Species
SREBP	Sterol regulatory element-binding protein
STZ	Streptozotocin
TDF	Tenofovir disoproxil fumarate
TNF- α	Tumor necrosis factor-alpha
UNAIDS	Joint United Nations on HIV/AIDS

Research output

Published articles:

- **Ugochukwu Offor**, Edwin Coleridge Naidu, Oluwatosin Olalekan Ogedengbe, Ayoola Isaac Jegede, Aniekan Imo Peter, Onyemaechi Okpara Azu (2018). **Nephrotoxicity and Highly active antiretroviral therapy: Mitigating action of *Momordica charantia***. *Toxicological report*, 5, 1153-1160.:
- **Ugochukwu Offor**, Edwin Coleridge Stephen Naidu, Oluwatosin Olalekan Ogedengbe, Ayoola Isaac Jegede, Peter Imo Aniekan, and Onyemaechi Okpara Azu (2019). **Renal histopathological and biochemical changes following adjuvant intervention of *Momordica charantia* and antiretroviral therapy in diabetic rats**. *Iranian journal of basic medical science*.

Manuscripts:

- **Ugochukwu Offor**, Simeon Eche, Edwin Coleridge Naidu, Onyemaechi Okpara Azu. **Gene expression profile of NGAL, KIM-1 and TNF- α in non-diabetic and diabetic rat model treated with *Momordica charantia* and highly active antiretroviral therapy**.
- **Ugochukwu Offor**, Edwin Coleridge Naidu, Onyemaechi Okpara Azu. **An ultrastructural investigation of the kidney following treatment with highly active antiretroviral therapy and *Momordica charantia* in non-diabetic and diabetic animals**.

Conference Presentations:

- **(Oral): Offor U**, Naidu ECS, Azu OO. Possible attenuating influence of *M. charantia* in Diabetic Nephropathy following triplavar: Any glimmer of hope? **“22nd International Conference on Prevention of Diabetes and Complications October 12-13, 2017 London, UK”**.
- **(Poster): Ugochukwu Offor**, Edwin Coleridge Naidu, Onyemaechi Okpara Azu. An ultrastructural investigation of the kidney following treatment with highly active antiretroviral therapy and *Momordica charantia* in non-diabetic and diabetic animals. **“19th Congress of the International Federation of Associations of Anatomists (IFAA) August 9-11, 2019 Excel, London, UK”**.

Abstract

Introduction

Management of HIV/AIDS has been successful with the use of antiretroviral therapy (ART). Consequently, the introduction of highly active antiretroviral therapy (HAART) has further increased the life expectancy of people living with HIV/AIDS and this has become a standard regimen in clinical practice. However, discordant views have been reported regarding its effects on the kidney; with a dearth of literature on the impact of HAART in a diabetic comorbid state on the renal morphology and the possible role of plant-based adjuvant. This study investigated the effect of *mormodica charantia* (*M. charantia*) on the kidney following HAART regimen (triplavar) and its impact in diabetic nephropathy (DN) in streptozotocin (STZ) induced diabetic rats.

Materials and Methods

78 adult male Sprague-Dawley rats were divided into non-diabetic and diabetic groups. Rat models of diabetes were successfully established by intraperitoneal injection of STZ (45 mg/kg body weight). Animals were administered an adjuvant treatment of *M. charantia* and HAART regimen (triplavar) according to protocols. On the 10th week, animals were euthanized with an overdose of halothane and kidney tissues were harvested and processed for light microscopy and transmission electron microscopy (TEM). Blood samples were obtained via cardiac puncture and centrifuged to collect the serums for biochemical analysis. Urine samples were collected at 3weeks interval during the 10 weeks experimental period for analysis of renal function test. Body weight and blood glucose levels (BGL) were measured once a week during the 10 weeks treatment.

Results

In the non-diabetic group, HAART alone treated rats showed renal dysfunction which were characterized by raised levels of blood urea nitrogen (BUN) and serum creatinine (Scr), microalbuminuria and gross electrolyte disturbances (Sodium and Potassium) as well as urea retention. Also, levels of oxidative stress (superoxide dismutase-SOD, catalase-CAT and glutathione peroxidase-GPx) were significantly decreased in these groups together with an increased levels of thiobarbituric acid reactive substances (TBARS) resulting in free radical formation via auto-oxidation. More so, the histopathological results displayed severe glomerular capillary abnormalities with inflammatory cellular infiltrations. This correlated with TEM analysis that showed swollen mitochondrial in the endothelium and thickness of the basement membrane with overexpression of extracellular matrix. Furthermore, there were upregulation of circulating neutrophil gelatinase associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1) and tumor necrosis factor-alpha (TNF- α) following HAART alone treatment.

In the diabetic groups consistent raised levels of blood glucose which remained peaked from the 5th week of experiment were seen in the diabetic control and HAART treated group. There were increased levels of both BUN and Scr. Renal function test showed leakage of albumin, retention of renal electrolytes (sodium and potassium) and high concentration of urea in the urine of diabetic control and HAART treated group. Activities of antioxidative enzymes (SOD, CAT) and levels of GSH were markedly decreased with an increased level of Malondialdehyde (MDA). Significant ($p<0.05$) upregulation of the gene expression profiles (NGAL, KIM-1 and TNF- α) were also seen.

Qualitative light microscopic result using hematoxylin and eosin (H and E) stains showed glomerular capillary abnormalities and tubular epithelial damage. These findings correlated with other special stains (PAS and MT) which showed high proportion of glycogen, glycoproteins as well as mild deposition of collagen fibers and hyaline substances respectively. TEM analysis displayed an abnormally increased thickness of basement membrane which reflects the existence of endothelial damage (diabetic control). By contrast, following adjuvant treatment with *M. charantia*, (low and high dose) these abnormalities were significantly reduced thus suggesting a protective effect of *M. charantia* on the kidney.

Conclusion

M. charantia extract administration improved blood glucose levels, maintained renal electrolytes (Sodium and Potassium), reinstated renal function (BUN and Scr) restored histoarchitectural and ultrastructural patterns and prevented DN development in an STZ-induced diabetic rat model.

Keywords: HAART, Nephrotoxicity, Diabetic nephropathy, TEM, *M. charantia*, Sprague-Dawley rats, Histoarchitecture.

Isifingqo (Isizulu Abstract)

Isingeniso

Ukuphathwa kwegciwane lesandulela ngculazi nengculaza kuye kwaphumelela ngokusetshenziswa kwe-antiretroviral therapy (ART). Ngenxa yalokho, ukwethulwa kwe-antiretroviral therapy (HAART) okwakhulisa kakhulu kuye kwandisa isikhathi sokuphila kwabantu abaphila negciwane lesandulela ngculaza nengculaza futhi lokhu sekube ngumgomo ojwayelekile emisebenzini yomtholampilo. Kodwa-ke, ukubukwa okungahambisani kahle kuye kwabikwa mayelana nemiphumela yaso ezinsoweni; kanye nokushisa kwezincwadi emthethweni we-HAART esimweni se-comorbid yesifo sikashukela ku-morphology ye-renal kanye nendima engenzeka ye-adjuvant esitshalo. Lolu cwaningo luhlolisise umphumela we-mormodica charantia (*M. charantia*) ezinsoweni ezilandela uhlelo lwe-HAART (triplavar) kanye nomthelela walo ku-nephropathy yesifo sikashukela (DN) ku-streptozotocin (STZ) ehoxise amakhomikhali ayisifo sikashukela.

Izimpahla nezindlela

78 Amadoda amadala aseSprague-Dawley ahlukaniswe abe amaqembu angewona wesifo sikashukela nesifo sikashukela. Amanothi omuthi wesifo sikashukela asungulwe ngempumelelo ngomjovo we-intraperitoneal we-STZ (45 mg / kg isisindo somzimba). Izilwane zanikwa ukwelashwa kwe-adjuvant ka-*M. charantia* kanye ne-HAART regimen (triplavar) ngokwezinhlelo. Ngeviki le-10, izilwane zazixhunyaniswe nokudlula ngokweqile kwama-halothane nama-tissue tissu zavuna futhi zenziwa ukukhishwa kwe-microscopy elula kanye ne-transmission electron microscopy (TEM). Ama-sampuli wegazi atholakala nge-cardiac puncture futhi ahloswe ngokuyinhloko ukuqoqa izinsimu zokuhlaziywa kwamakhemikhali. Amasampula e-urine aqoqwe ngesikhathi sokugcina ama-3weeks ngesikhathi samaviki angu-10 wokuhlola ukuhlaziywa kokuhlolwa komsebenzi wezintambo. Isisindo somzimba namazinga weglucose wegazi (BGL) kulinganiswa kanye ngesonto phakathi nokuphathwa kwamasono angu-10.

Imiphumela

Esikhathini esingeyona isifo sikashukela, ama-HAART kuphela aphantsi amantombazane abonisa ukungasebenzi komzimba okwakubonakala ngamazinga aphakanyisiwe wegazi urea nitrogen (BUN) kanye ne-serum creatinine (Scr), i-microalbuminuria kanye nokuphazamiseka okukhulu kwe-electrolyte (Sodium and Potassium) kanye nokugcinwa kwe-urea. Futhi, amazinga wokucindezeleka okwenziwe nge-oxidative (superoxide dismutase-SOD, i-catalase-CAT ne-glutathione peroxidase-GPx) yehle kakhulu

kulezi ziqhema kanye namazinga okwanda wezinto ze-thiobarbituric acid ezisebenzayo (TBARS) eziholela ekubunjweni okukhulu kwamahhala nge-oxidation ngokuzenzakalela. Ngaphezu kwalokho, imiphumela ye-histopathological yabonisa ukungavamile okukhulu kwe-capillary glomerular nge-infiltrations yama-cellular inflammatory. Lokhu kuqhathaniswa nokuhlaziywa kwe-TEM okwakubonisa i-mitochondrial evuvukale ekupheleni kwe-endothelium kanye nokuqina kwesimbatho sendawo esezansi nokugqithisa okukhulu komthamo we-extracellular. Ngaphezu kwalokho, kwakukhona ukuhanjiswa kwe-neutrophil gelatinase ehlobene ne-lipocalin (NGAL), i-kidney injection molecule 1 (KIM-1) nesifo se-necrosis factor-alpha (TNF- α) elandela ukwelashwa kwe-HAART kuphela.

Lezi zinkinga eziphazamisekile ezitholakala emaqenjini angewona wesifo sikashukela ama-rats aphantswe nge-HAART kuphela ayekhona kakhulu (i-HAART yodwa + yesifo sikashukela) kanye (namagundane angasetshenziswanga nesifo sikashukela). Amazinga aphakanyisiwe egazi egazini elalilokhu evezwe kusukela evikini lesihlanu lokuhlolwa aboniswa. Lokhu kuhambisana nokukhubazeka okunamandla okwenyuka kwama-renal okwakubonisa amazinga okwandisa kokubili i-BUN kanye ne-Scr nokungalingani kwamandla e-electrolyte kanye nokugcinwa kwe-urea ezinso. Imisebenzi ye-enzymes e-antioxidative (i-SOD, i-CAT ne-GSH) yehla ngokuphawulekayo ngezinga elengeziwe le-peroxidation ye-lipid. Ukuguqulwa okuphawulekayo kwamaphrofayli angama-gene (NGAL, KIM-1 no-TNF- α) nawo atholakale ku-serum elandelayo ukwelashwa (i-HAART yodwa + yesifo sikashukela) kanye (namagundane angasetshenziswanga nesifo sikashukela).

Umphumela omuhle wokukhanya osebenzayo usebenzisa ama-hematoxylin ne-eosin (H and E) amaconsi abonisa ukungalingani kwe-capillary kwe-capillary, ukusoka kwe-capillary kanye nokuphazanyiswa okuvamile kwamalokisi e-capillary. Ukuvuvukala kwamangqamuzana, umonakalo we-epithelial we-tubular kanye ne-necrosis eqinile edongeni lwamasongo elibhekene nokusetshenziswa kwe-hypercellularity nawo waphawula. Lezi zihambisana nezinye izinsizakalo ezikhethekile ezibonisa izinga eliphezulu lama-carbohydrate afana ne-glycogen kanye ne-glycoproteins kanye nokufakwa okuncane kwamakhofi ama-collagen nama-hyaline. Ukuhlaziywa kwe-TEM kuboniswe ukuqina okwejwayelekile kwesimbatho sendawo esezansi kanye nokuvuvukala kwe-mitochondrial okubonisa ukuthi kukhona khona umonakalo wokuphela. Ngokuphambene nalokho, ukulandela ukwelashwa kwe-adjutant no-M. charantia, (isilinganiso esincane nesiphakeme) lezi zinto ezingavamile zancishiswa kakhulu.

Isiphetho

UMnu charantia ukukhipha ukuphathwa ngcono ama-blood glucose amazinga, i-electrolyte yama-renal (i-Sodium ne-Potassium) eyabuyiselwayo, yabuyiselwa umsebenzi we-renal (BUN ne-Scr) iphinde

ibuyiselwe amakhemikhali akhethekile kanye nezindlela zokuvimbela ukuvimbela ukuthuthukiswa kwe-DN kwisimo sokulinganisa isifo sikashukela se-STZ.

Amagama angukhiye: Ukwelashwa kwe-antiretroviral ephezulu, i-Nephrotoxicity, i-nephropathy yesifo sikashukela, i-Ultrastructure, M. charantia, amagundane e-Sprague-Dawley.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background

In 2016, an estimated number of 36.9 million people globally were already living with the Human immunodeficiency virus (HIV), and 1.5 million people have died from acquired immune deficiency syndrome (AIDS) (UNAIDS, 2016a). Over 12.2% of the South African populations are living with HIV/AIDS and also an estimated 280,000 South Africans died of HIV/AIDS. Approximately 2 million South Africans are on antiretroviral therapy (HIV/AIDS, 2015, Shisana et al., 2014). While the HIV/AIDS pandemic has received tremendous global support in research and treatment evidenced by the improved morbidity and mortality, the challenge of the over 2 million people living with HIV/AIDS (PLWHA) in South Africa receiving antiretroviral therapy (ART) still remains the issues of drug toxicities and side effects (Shisana et al., 2014).

The introduction of ART for the management of HIV and AIDS significantly increased the life expectancy among HIV-infected individuals with unprecedented changes in disease progression and mortality (Azu et al., 2014). The U.S Food and Drug Administration (FDA) approved the combination of 2 or 3 active antiretroviral agents known as highly active antiretroviral therapy (HAART) (Azu et al., 2014). HAART involves combining two nucleoside reverse transcriptase inhibitors (NRTIs) or a combination of one medication from either non- nucleoside reverse transcriptase inhibitors (NNRTIs) or protease inhibitors (PIs) class (Azu et al., 2014). This has become a standard regimen for the management of HIV positive patients.

The therapeutic goal of HAART is to achieve and maintain viral suppression and improve the immune function of patients. Despite this have diminished the enthusiasm generated by HAART (Jegade et al., 2017, Hagmann, 2003). The most common and troublesome toxicities of HAART includes hepatotoxicity, nephrotoxicity linked to mitochondrial damage and metabolic disorders such as diabetes and hypertension (Lee, 2003, Izzedine et al., 2009, Offor et al., 2017b, Yedla et al., 2015). Nephrotoxicity is characterized by tubular necrosis with glomerular hypertrophy resulting from mitochondrial dysfunction induced by NRTIs since they inhibit nuclear or mitochondrial deoxyribonucleic acid (DNA) polymerase from host cell along with inhibition of reverse transcriptase of HIV (Sanz et al., 2018). NRTIs affect DNA polymerase of mitochondria with subsequent deficits in mitochondrial DNA encoded enzymes of the mitochondrial respiratory chain (Sanz et al., 2018). Additionally, HAART may increase

the risk of other metabolic complications such as diabetes mellitus leading to impaired kidney function. This has remained a concern for PLWHAs undergoing treatment.

Diabetes Mellitus (DM) is considered as one of the five leading causes of death in the world and it is a major global health concern with a projected rise in prevalence from 171 million in 2000 and may reach 366 million in 2030 (Guariguata et al., 2014). Diabetes remains the most common reason for progressing to end stage renal diseases. The clinical presentation of antiretroviral-associated diabetes is consistent with that of type 2 diabetes (T2DM). Many HIV/AIDS patients receiving HAART become insulin resistance and glucose intolerance resulting in them becoming diabetic (Hulgan, 2018, Mutimura et al., 2007).

Insulin resistance occurs when normal insulin levels are inadequate to stimulate glucose uptake in the insulin-signaling pathway. The insulin resistance manifests as hyperinsulinemia, hyperglycemia, and hypertriglyceridemia (Petersen and Shulman, 2018). Both insulin resistance and insulin deficiency plays significant role in the pathogenesis of T2DM (Ozougwu et al., 2013). Insulin resistance has been described in 61% of patients treated with PIs and impaired glucose intolerance was observed in 35% of HIV patients using HAART (Tsiodras et al., 2000, Brown et al., 2005). NRTIs were found to contribute to the disturbance of glucose metabolism (Blümer et al., 2008). Antiretroviral regimens like stavudine, didanosine, tenofovir and lamivudine are considered to be most potent to induce DM risk (Calmy et al., 2007). In clinical studies of HIV-infected human subjects, antiretroviral drugs were shown to acutely inhibit insulin-stimulated glucose disposal (Noor et al., 2002, Noor et al., 2006). The effect on glucose disposal is both rapid suggesting a direct blockade of glucose uptake through the glucose transporter isoform 4 (GLUT4) (Noor et al., 2006). Disturbance in glucose-insulin homeostasis through hyperglycemia, insulin resistance, and impaired β -cell function by PIs was reported in animal models also (King, 2012, Mastan and Kumar, 2010). The resulting insulin resistance and glucose intolerance may predispose to diabetes and further long- term complications of diabetes such as diabetic nephropathy.

Diabetic nephropathy, which is a microvascular complication of diabetes has generated a lot interest lately, due to significantly high mortality rate characterised by molecular, biochemical, structural and functional deficits, a complex process leading up to chronic kidney failure in diabetic patients (Gnudi et al., 2016, Duran-Salgado and Rubio-Guerra, 2014). Chronic kidney failure is one of the major end-points of diabetic nephropathy. In diabetic nephropathy, hyperglycemia adversely alters biochemical and molecular characteristics ultimately resulting in structural and functional deficits of the mitochondrial leading to endothelial thickening and consequently progresses to arterial hyalinosis of the afferent and efferent arterioles and glomerular hyperfiltration (Nishikawa et al., 2015, Gnudi et al., 2016). The

underlying pathophysiological processes leading to the biochemical, structural and molecular deficits in the kidney of diabetic patients are varied and complex. Some of the identified mechanisms including impaired insulin signaling, mitochondrial dysfunction, alteration of glucose utilization and increased fatty acid oxidation are known precursors ultimately leading to chronic kidney failure (Gnudi et al., 2016, Duran-Salgado and Rubio-Guerra, 2014).

Hyperglycaemia appears to be an important driver of diabetes-associated kidney diseases through the generation of oxidative stress, promoting adverse events in diabetic nephropathy (Miranda-Díaz, 2016 et al., Sagoo and Gnudi, 2018). Mitochondrial dysfunction, increased fatty acid oxidation and impaired insulin signaling are known contributors to increased oxidative stress found in diabetic nephropathy (Giacco and Brownlee, 2010, Vincent et al., 2004).

Oxidative stress disrupt endothelial homeostasis through apoptosis and increased generation of reactive oxygen species (ROS), depletion of scavenger antioxidant enzymes, activation of mononuclear cells, and suppression of associated maladaptive signalling cascades (Asmat et al., 2016). These abnormalities underline the development of nephrotoxicity and functional deficits associated with diabetic nephropathy.

It is important to identify potential therapeutic agents that may help in the prevention or halt the progression of diabetic nephropathy and nephrotoxicity associated with most antiretroviral therapies. Currently, treatment strategies are directed at controlling glycaemia, and glycemic control remains the focal target in the treatment of diabetic nephropathy. Antioxidants have been proposed to hold promise as adjuvant therapy to the synthetic therapeutic strategies known for glycemic control in the prevention of the development and progression of chronic renal failure associated with diabetes mellitus. Furthermore, it has been proposed that strategies that either reduce ROS or augment renal antioxidant defence mechanisms might possess therapeutic efficacy in improving renal function and alleviating nephrotoxicity in diabetes mellitus (Singh et al., 2013). Therefore, compounds with demonstrated antioxidant and hypoglycaemic effects may provide potential therapeutic benefits in ameliorating or reversing the diabetic nephropathy and nephrotoxicity associated with most antiretroviral regimens.

Momordica charantia (*M. charantia*), has been known to possess antioxidant, hypoglycemic, and other phytochemical properties. The fruit, stems, leaves and roots of *M. charantia* have all been used in traditional medicine to help treat ailments such as hyperlipidemia, digestive disorders and microbial infections amongst the indigenous populations of Asia, South America, India and Southern Africa (Ahmad et al., 2016, Raish, 2017). However, there is dearth of literature on the antidiabetic effects of *M. charantia* on the renal changes of rats induced by streptozotocin following treatment with HAART. Based

on this, this work investigates the adjuvant use of *M. charantia* and antiretroviral therapy in a non-diabetic and diabetic condition using rodent model.

1.2 HIV and AIDS

Human immunodeficiency virus (HIV) is a retrovirus, one of a unique family of viruses that consist of genetic material in the form of ribonucleic acid (RNA), surrounded by a lipoprotein envelope (Bates, 2016). HIV cannot replicate on its own and instead relies on the mechanisms of the host cell to produce new viral particles. HIV infects helper T cells by means of a protein embedded in its envelope called gp120. The gp120 protein binds to a molecule called CD4 on the surface of the helper T cell, an event that initiates a complex set of reactions that allow the HIV genetic information into the cell (Bates, 2016, Skarbinski et al., 2015).

Entry of HIV into the host cell also requires the participation of a set of cell surface proteins that normally serve as receptors for chemokines (hormone-like mediators that attract immune system cells to particular sites in the body) (Amini et al., 2018). It appears that the binding of gp120 to CD4 exposes a region of gp120 that interacts with the chemokine receptors. This interaction triggers a conformational change that exposes a region of the viral envelope protein gp41, which inserts itself into the membrane of the host cell so that it bridges the viral envelope and the cell membrane. An additional conformational change in gp41 pulls these two membranes together, allowing fusion to occur. After fusion the viral genetic information can enter the host cell (Amini et al., 2018). See figure 1.1.

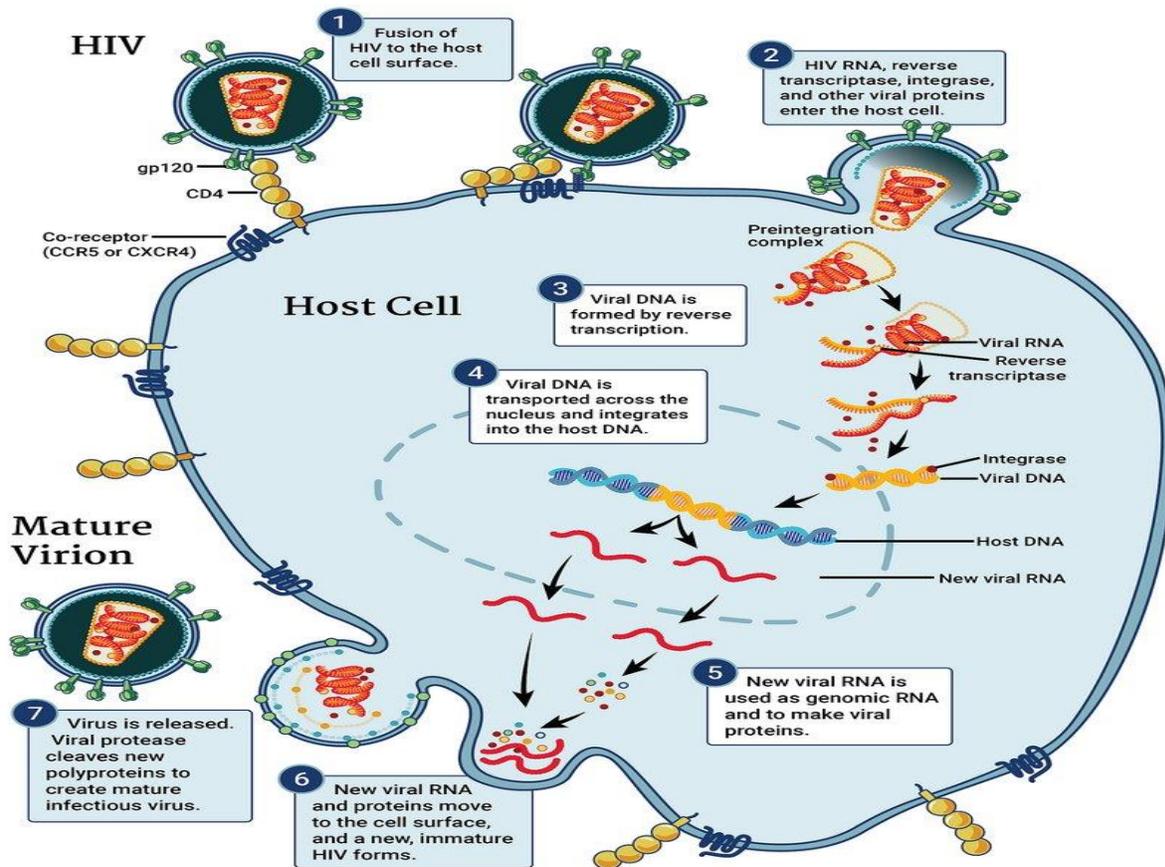


Figure 1.1: Overview of HIV replication cycle. **Source:** <https://www.niaid.nih.gov/diseases-conditions/hiv-replication-cycle>. Content last reviewed on June 19, 2018.

The main cellular target of HIV is a special class of white blood cells critical to the immune system known as helper T lymphocytes, or helper T cells. Helper T cells are also called CD4+ T cells because they have on their surfaces a protein called CD4 (Perreau et al., 2013). Helper T cells play a central role in normal immune responses by producing factors that activate virtually all the other immune system cells. These include B lymphocytes, which produce antibodies needed to fight infection; cytotoxic T lymphocytes, which kill cells infected with a virus; and macrophages and other effector cells, which attack invading pathogens (Perreau et al., 2013).

HIV is the etiological agent of acquired immune deficiency syndrome (AIDS) and is one of the most devastating pathogens worldwide. Acquired immunodeficiency syndrome (AIDS) results from the loss of most of the helper T cells in the body (Perreau et al., 2013). AIDS is the final stage of a chronic infection with HIV. There are two types of this virus: HIV-1, which is the primary cause of AIDS worldwide, and

HIV-2, found mostly in West Africa (Hemelaar, 2012). Once introduced into the body system, HIV enters cells of the immune system, especially white blood cells known as T cells. These cells orchestrate a wide variety of disease-fighting mechanisms. Particularly vulnerable to HIV attack are specialized "helper" T cells known as CD4 cells. When HIV infects a CD4 cell, it commands the genetic tools within the cell to manufacture new HIV virus. The newly formed HIV virus then leaves the cell, destroying the CD4 cell in the process (Hemelaar, 2012). No existing medical treatment can completely eradicate HIV from the body once it has infected human cells. The loss of CD4 cells endangers health because these cells help other types of immune cells respond to invading organisms. The average healthy person has over 1,000 CD4 cells per micro liter of blood. In a person infected with HIV, the virus steadily destroys CD4 cells over a period of years, diminishing the cells' protective ability and weakening the immune system. When the density of CD4 cells drops to 200 cells per micro liter of blood, the infected person becomes vulnerable to AIDS-related opportunistic infections which take advantage of the weakened immune defenses to cause disease (Perreau et al., 2013, Hemelaar, 2012).

1.2.1 Global Prevalence of HIV and AIDS

It is estimated that about 36.9 million people are living with the HIV worldwide in 2016, (See Table 1.1) and an estimated 1.5 million people have died from acquired immune deficiency syndrome (AIDS) since the first cases were reported in 1980s. Most of these deaths are due to inadequate access to HIV prevention, care and treatment services (UNAIDS, 2016a, UNAIDS, 2016b). Adolescents aged between 15 to 49 years account for an estimated 12 per cent of HIV infections globally (UNAIDS, 2016b). Since its discovery in 1983, the infection has quickly developed into a worldwide epidemic, affecting virtually every nation. In 1999, about 26.2 million people were living with the virus. By the end of 2009, it was estimated that 33.3 million people were living with the disease, corresponding to a 27%. By 2005, more than 1.2 million people had died of HIV and AIDS related diseases (WHO and UNICEF, 2013).

More than two-thirds of HIV infections, roughly 24.7 million persons, were in Sub-Saharan Africa which also accounted for close to 80% of women and 90% of children living with HIV (De Cock et al., 2012). Asia and the Pacific accounted for approximately 4.8 million HIV infected persons, Western and Central Europe and North America, accounted for 2.3 million, (Table1.1). Despite the decline in the new infections, South Africa, in the Sub-Saharan Africa region, still bears the highest burden of HIV epidemic globally (Poku, 2017). These are staggering data that impacts negatively on the socio-economic indices in these countries.

Table 1.1: Prevalence of HIV infection and estimated death rates by regions of the world (Ages 15-49).

World Region	Prevalence of HIV infection	Estimated death rates	Prevalence Rate (%)
Global Total	36.9 million	1.5 million	0.8
Sub-Saharan Africa	24.7 million	1.1 million	4.7
Asia and the Pacific	4.8 million	250000	0.2
Western and Central Europe and North America	2.3 million	27000	0.3
Latin America	1.6 million	47000	0.4
Eastern Europe and Central Asia	1.1 million	53000	0.6
Caribbean	250,000	11000	1.1
Middle East and North Africa	230,000	15000	0.1

Source: UNAIDS, 2016a. Global AIDS update 2016. *Geneva, Switzerland: World Health Organization Library* (UNAIDS, 2016a)

1.2.2 Prevalence of HIV and AIDS in Africa

The HIV pandemic has continued to challenge the development and economy of many countries in Africa and has overwhelmed the health-care systems, increased the number of orphans and caused life expectancy to reduce. It is one of the public health issues threatening the survival of millions of infected people in sub-Saharan Africa (Poku, 2017).

Its impact has been most severe in some of the poorest countries in Africa. At the end of 2009, there were 9 countries in Africa where more than one tenth of the adult population aged 15-49 years was infected with HIV (Youde, 2016). For instance, in some countries in the southern part of the continent, including Botswana, Lesotho, Swaziland, Zimbabwe and South Africa, about 30 percent of the populations have HIV infection or AIDS (Poku, 2017). In Kenya, about 2.3 million people live with HIV/AIDS and each year, approximately 200,000 Kenyans develop the AIDS syndrome (Luginaah et al., 2005).

HIV/AIDS pandemic affect all regions and communities and it impacts negatively on households and the economic growth of nations. Sub-Saharan Africa is more heavily affected by HIV/AIDS than any other region of the world. In 2008, it was home to two thirds (60%) of all PLWHAs and nearly three quarters (72%) of AIDS-related deaths occurs in this region (Kalipeni et al., 2004). It has ‘sapped’ the population

of young men and women in their productive years who form the foundation of the labor force (Morrell et al., 2009). Health care problems have already reached crisis proportions in some parts of the world already burdened by war, political upheaval, or unrelenting poverty.

1.2.3 Prevalence of HIV and AIDS in South Africa

The toll of HIV/AIDS continues to be harsh in South Africa. In 2013, the country accounted for the vast majority of people living with AIDS (Johnson et al., 2017). In 2017, it is estimated that 12.2% of the South Africa's population of 54.95 million had HIV/AIDS. The rising prevalence rate increased from 10.6% in 2007 to 12% in 2008, and in 2010, an estimated 280,000 South Africans died of HIV/AIDS (Atujuna et al., 2018). The Human Sciences Research Council, a South African institution, estimates that between 42% and 47% of all deaths among South Africans were caused by HIV/AIDS (Angotti et al., 2018). Currently, approximately 6.1 million South Africans are living with HIV/AIDS with over 2 million on antiretroviral treatment (Shisana et al., 2014). Provincial data on HIV prevalence is shown in table 1.2 below. The results show substantial variation of HIV prevalence by province; KwaZulu-Natal continues to lead South Africa in HIV prevalence. In most provinces, HIV prevalence in women; aged 15 years and above, remains significantly higher than in men (Shisana et al., 2014). See table 1.2

Table 1.2: Overall HIV Prevalence by Province in South Africa

Province	Prevalence Rate (%)
KwaZulu-Natal	16.9
Mpumalanga	14.1
Free State	14.0
North West	13.3
Gauteng	12.4
Eastern Cape	11.6
Limpopo	9.2
Northern Cape	7.4
Western Cape	5.0

Source: Shisana et al., 2014

1.2.4 Management of HIV and AIDS

Whereas no medical treatment can cure AIDS, ART was developed for the management of HIV and AIDS to reduce viral load and thus improve longevity. The primary goal of ART is suppression of plasma viral load, preservation and restoration of immunologic function, improvement of quality of life and reduction of HIV related morbidity and mortality (Fourie, 2006). Early in the 1980s when the HIV/AIDS epidemic began with no visible medical therapy known to reduce viremia, people with AIDS were not likely to live longer than a few years (Fourie, 2006). UNAIDS estimated that a total of 2.5 million deaths have been averted in low and middle-income countries since 1995 due to the roll out of antiretroviral therapy (HIV/AIDS, 2013). In other words, HIV/AIDS is no longer a ‘death penalty’ for victims.

The therapy entails the use of antiretroviral medications that attack the virus itself plus other non-antiretroviral medications to prevent and treat opportunistic infections (OIs) that can occur when the immune system is compromised by the virus (WHO and UNICEF, 2009). Counseling and support mechanisms are also done to help infected people deal with emotional and traumatizing repercussions by encouraging them to accept living with a disabling and potentially fatal disease. This has proven to unlock the declines in transmission of the virus (WHO and UNICEF, 2009).

ART consists of drugs from five classes; they are nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion or entry inhibitors and integrase inhibitors which are approved by the United States of America Food and Drug Administration (FDA) (FDA, 2012). Table 1.3 shows the various classes of antiretroviral drugs approved by the FDA.

Table 1.3: Classification of antiretroviral drugs in use.

Drug Class	Mechanism of action	Generic Name (Other names and acronyms)	Brand Name	FDA Approval Date	Manufacturers name
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	Inhibit reverse transcription by being incorporated into the newly synthesized viral DNA and preventing its further elongation.	Abacavir (ABC)	<u>Ziagen</u>	December 17, 1998	GlaxoSmithKline
		<u>Didanosine</u> (ddI, ddI EC)	<u>Videx</u>	October 9, 1991	Bristol Myers- Squibb
			<u>Videx EC</u> (enteric- coated)	October 31, 2000	Bristol Myers- Squibb
		<u>Emtricitabine</u> (FTC)	<u>Emtriva</u>	July 2, 2003	Gilead Sciences
		<u>Lamivudine</u> (3TC)	<u>Epivir</u>	November 17, 1995	GlaxoSmithKline
		<u>Stavudine</u> (d4T)	<u>Zerit</u>	June 24, 1994	Bristol Myers- Squibb
		<u>Tenofovir disoproxil fumarate</u> (TDF)	<u>Viread</u>	October 26, 2001	Gilead Sciences
		<u>Zidovudine</u> (ZDV)	<u>Retrovir</u>	March 19, 1987	GlaxoSmithKline
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Inhibit reverse transcriptase directly by binding to the enzyme and interfering with its function.	<u>Delavirdine</u> (DLV)	<u>Rescriptor</u>	April 4, 1997	Pfizer
		<u>Efavirenz (EFV)</u>	<u>Sustiva</u>	September 17, 1998	Bristol Myers- Squibb
		<u>Etravirine</u> (ETR)	<u>Intelence</u>	January 18, 2008	Tibotec Therapeutics
		<u>Nevirapine</u> (NVP)	<u>Viramune</u> (Immediate Release)	June 21, 1996	Boehringer Ingelheim

			<u>Viramune XR</u> (Extended Release)	March 25, 2011	Boehringer Ingelheim
		<u>Rilpivirine</u> (RPV)	<u>Edurant</u>	May 20, 2011	Tibotec Therapeutics
Protease Inhibitors (PIs)	Target viral assembly by inhibiting protease enzyme used by HIV to cleave nascent proteins for final assembly of new virions.	<u>Atazanavir</u> (ATV)	<u>Reyataz</u>	June 20, 2003	Bristol-Myers Squibb
		<u>Darunavir</u> (DRV)	<u>Prezista</u>	June 23, 2006	Tibotec, Inc.
		<u>Fosamprenavir</u> (FPV)	<u>Lexiva</u>	October 20, 2003	GlaxoSmithKline
		<u>Indinavir</u> (IDV)	<u>Crixivan</u>	March 13, 1996	Merck
		<u>Nelfinavir</u> (NFV)	<u>Viracept</u>	March 14, 1997	Agouron Pharmaceuticals
		<u>Ritonavir</u> (RTV)	<u>Norvir</u>	March 1, 1996	Abbott Laboratories
		<u>Saquinavir</u> (SQV)	<u>Invirase</u>	December 6, 1995	Hoffmann-La Roche
		<u>Tipranavir</u> (TPV)	<u>Aptivus</u>	June 22, 2005	Boehringer Ingelheim
Fusion or entry Inhibitors	Prevent HIV from binding to or entering human immune cells.	<u>Enfuvirtide (T-20)</u>	<u>Fuzeon</u>	March 13, 2003	Hoffmann-La Roche & Trimeris
		<u>Maraviroc</u> (MVC)	<u>Selzentry</u>	August 6, 2007	Pfizer
Integrase Inhibitors	Inhibit integrase enzyme needed by HIV to insert its genetic material into human cells.	<u>Dolutegravir</u> (DTG)	<u>Tivicay</u>	August 13, 2013	GlaxoSmithKline
		<u>Elvitegravir</u> (EVG)	Vitekta	September 24, 2014	Gilead Sciences
		<u>Raltegravir</u> (RAL)	<u>Isentress</u>	October 12, 2007	Merck & Co., Inc.

Source: <https://aidsinfo.nih.gov/understanding-hiv-aids/fact.../21/.../fda-approved-hiv-medicine>. Last reviewed: June 24, 2019

1.3 Highly Active Antiretroviral Therapy

Reports from clinical trials are indicating that single therapies are not completely effective in combating HIV virulence and this is the basis for the introduction of combination antiretroviral therapy, mostly called highly active antiretroviral therapy (HAART) (See Table 1.4) (Sendi et al., 2001). HAART has been effective in suppressing the viral load to undetectable levels resulting in decreased HIV RNA levels and CD4 T cell increases in the vast majority of patients (Andrade et al., 2017, Viswanathan et al., 2015). HAART has dramatically decreased the number of hospital admissions and PLWHA have achieved an impressive improvement in the quality of life (Andrade et al., 2017). Many people affected by the virus are now living with a manageable chronic condition. With increasing global awareness campaign and continuous roll out of HAART coupled with effective prophylaxis for opportunistic infections, the number of infection and HIV mortality rates has dramatically declined (Viswanathan et al., 2015, WHO and UNICEF, 2013).

Table 1.4: Possible combination antiretroviral therapy- FDA approved.

Drug Class	Generic Name (Other names and acronyms)	Brand Name	FDA Approval Date	Manufacturers name
Multi class combination products	Lamivudine, Zidovudine and Nevirapine (3TC,/ZDV, NVP)	Triplavar	September 12, 2017	Cipla Medpro
	<u>Abacavir and Lamivudine</u> (ABC / 3TC)	<u>Epzicom</u>	August 2, 2004	GlaxoSmithKline
	<u>Abacavir, Dolutegravir, and Lamivudine</u> (ABC / DTG / 3TC)	Triumeq	August 22, 2014	ViiV Health care and Shionogi & Co Ltd.
	<u>Abacavir, Lamivudine, and Zidovudine</u> (ABC / 3TC / ZDV)	<u>Trizivir</u>	November 14, 2000	GlaxoSmithKline
	<u>Atazanavir and</u>	Evotaz	January 29,	Bristol-Myers

<u>Cobicistat</u> (ATV / COBI)		2015	Squibb
<u>Darunavir and Cobicistat</u> (DRV / COBI)	Prezcobix	January 29, 2015	Jassen Therapeutics
<u>Efavirenz, Emtricitabine, and Tenofovir disoproxil fumarate</u> (EFV / FTC / TDF)	<u>Atripla</u>	July 12, 2006	Bristol-Myers Squibb and Gilead Sciences
<u>Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir disoproxil fumarate</u> (EVG / COBI / FTC / TDF)	<u>Stribild</u>	August 27, 2012	Gilead Sciences
<u>Emtricitabine, Rilpivirine, and Tenofovir disoproxil fumarate</u> (FTC / RPV / TDF)	<u>Complera</u>	August 10, 2011	Gilead Sciences
<u>Emtricitabine and Tenofovir disoproxil fumarate</u> (FTC / TDF)	<u>Truvada</u>	August 2, 2004	Gilead Sciences, Inc.
<u>Lamivudine and zidovudine</u> (3TC / ZDV)	<u>Combivir</u>	September 27, 1997	GlaxoSmithKline
<u>Lopinavir and Ritonavir</u> (LPV / RTV)	<u>Kaletra</u>	September 15, 2000	Abbott Laboratories

Source: <https://aidsinfo.nih.gov/education.../fact.../fda-approved-hiv-medicines>. Last reviewed: June 24, 2019

1.3.1 HAART- related side effects

Reduction in morbidity and mortality has been the most outstanding achievement in the global fight against HIV/AIDS in both low and middle-income countries, but despite this achievement, there are concerns about associated side effects of HAART, which have increased more recently (Viswanathan et al., 2015, Subbaraman et al., 2007). HAART- related side effects may be transient or may persist throughout therapy, and have been attributed to severe deleterious consequences mediated through different pathophysiological pathways and eventually a major reason for the discontinuation of therapy or change in treatment strategy, thereby limiting patients adherence and consequently, virologic effectiveness (Cihlar and Fordyce, 2016).

Each drug in the HAART combination has its own side effects; however, the three common side effects of HAART are diarrhea, nausea and fatigue (Carr and Cooper, 2000). Toxicities like lactic acidosis, hepatic steatosis, progressive neuromuscular weakness, dyslipidemia and lipodystrophy also occurs after the drugs had been in use for years (Manfredi and Chiodo, 2001). Other increasing side effects caused by HAART ranges from mild to severe organ toxicity such as hepatotoxicity, testicular injury, nephrotoxicity and many others of which have been well documented in several studies (Mocroft et al., 2005).

1.3.2 HAART-related kidney Injury

The kidneys have important physiological functions including maintenance of water and electrolyte balance, secretion of hormones and excretion of waste products from the body. In addition, the kidneys plays an important role in the metabolism and excretion of drugs and hence may be exposed to high concentrations of drug metabolites making it liable to drug toxicity such as acute kidney injury (AKI), tubulopathies, chronic kidney disease (CKD) and end-stage renal disease that may often require renal replacement therapy (Pannu and Nadim, 2008, Tiwari et al., 2018). It is therefore not a surprise that drug-induced kidney injuries contribute up to 25% of all cases of acute renal failure (Pannu and Nadim, 2008). Drug-induced kidney injuries may cause cumulative dose-dependent toxicity or idiosyncratic dose-independent toxicity at any time during therapy (Pannu and Nadim, 2008). Renal injury may occur in various renal compartments such as the renal vascular supply, the glomerulus, the collecting ducts, the tubulointerstitium where extensive tubular-peritubular capillary exchange of solutes takes place (Humphreys, 2018).

Clinically, HAART causes various kidney injuries including various electrolyte and acid-base disorders as well as lactic acidosis. These injuries occur via mechanisms such as direct tubular toxicity, allergic reactions and precipitation of insoluble drug crystals within renal tubular lumens (Yedla et al., 2015).

HAART nephrotoxic effects accounted for 14% of late-onset of AKI episodes, occurring 3 months after initiating HAART (Mehdi et al., 2016). Combined therapy with tenofovir disoproxil fumarate (TDF) and protease inhibitors (PIs) such as ritonavir appears to increase renal toxicity, severe immunosuppression (CD4 count, <200 cells/mm³) and opportunistic infections (LaFleur et al., 2018). Approximately, 70% of the published cases of TDF-induced nephrotoxic effects are observed with concomitant use with ritonavir (LaFleur et al., 2018).

An interaction between lopinavir- ritonavir combination therapy and TDF, which manifests as a decrease in the renal clearance of TDF, has been identified (Jotwani et al., 2016). A recent study by the Columbia University group demonstrated that TDF nephrotoxicity is manifested as toxic acute tubular necrosis targeting proximal tubules and manifests in distinctive light microscopic and ultra-structural features of mitochondrial injury (Offor, 2015, Kalyesubula and Perazella, 2011). Exposure to TDF showed adverse serious renal events and graded elevations in serum creatinine of patients, and it has also been linked to the development of proximal tubular dysfunction including Fanconi syndrome (FS), AKI, nephrogenic diabetes insipidus (NDI), and severe hypokalemia (Kalyesubula and Perazella, 2011). FS caused by tenofovir-induced nephrotoxicity is characterized by generalized proximal tubular dysfunction resulting in glycosuria, phosphaturia, uricosuria, aminoaciduria, and tubular proteinuria (Fernandez-Fernandez et al., 2011, Elias et al., 2014). Lamivudine, stavudine, abacavir and didanosine have also been implicated in case reports of FS and NDI (Kalyesubula and Perazella, 2011, Izzedine et al., 2004). Acute interstitial nephritis (AIN), impaired kidney function and hyperlactemia have all been implicated with classes of nucleoside reverse transcriptase inhibitors (NRTIs) and non- nucleoside reverse transcriptase inhibitors (NNRTIs) and increased the propensity of leading to chronic kidney disease (Perazella and Markowitz, 2010, Kalyesubula and Perazella, 2011).

1.3.3 HAART- related comorbid conditions

Many patients taking HAART achieve a complete lifelong virologic suppression, and thus, AIDS-related conditions are becoming less common, with a dramatic drop in overall morbidity and mortality. However, despite the apparently full control of HIV infection by HAART, clinical evidence has demonstrated that HAART does not completely restore health in HIV-infected patients, who are affected by an increased risk of several non-AIDS complications, also known as comorbid conditions (Gazzola et al., 2010, Pallela et al., 1998). The HAART-related comorbid conditions are many and includes metabolic abnormalities such as; dyslipidemia, high blood pressure or hypertension, cardiovascular diseases, lipodystrophy, glucose intolerance and insulin resistance, these abnormalities are together referred to as metabolic syndromes (Gazzola et al., 2010).

The potential long-term consequences of HAART-associated dyslipidemia are not completely understood, several pathophysiological ways have revealed that dyslipidemia in HIV-infected patients involves interactions between the virus and antiretroviral therapies and host factors. (Green, 2002, Mooser and Carr 2001).

Dyslipidemia is characterized by hypertriglyceridemia and hypercholesterolemia which are features of defective lipoprotein metabolism. Although abnormal lipid profiles are reported in HIV infected individuals before the onset of HAART, however, hypertriglyceridemia and hypercholesterolemia becomes both more prevalent and severe during treatment with HAART (Feeney and Mallon, 2011, Van Wijk and Cabezas, 2012). According to Calza et al., dyslipidemia occurs in up 70%-80% of HIV- infected individuals receiving HAART and can be associated with the entire available PIs (Calza et al., 2004, Calza et al., 2016). As a result, risk factors for long-term adverse effects have become increasingly important in therapeutic decisions regarding HIV/AIDS patient care.

Reduction in mortality rate as a result of antiretroviral therapy in HIV/AIDS population comes with increased significance of other risk factors such as cardiovascular diseases. Compelling evidence suggests that HIV/AIDS patient is at an increased risk for atherosclerotic cardiovascular disease. This appears to promote atherogenesis by sensitizing the arterial wall to various pathological processes (Dimala and Blencowe, 2017, Dimala et al., 2016b, Zanetti et al., 2018).

The role of inflammation in the pathologic process of atherogenesis has been established. The initiation of fatty streaks, the precursors of atheromatous plaques, is now known to be mediated by an inflammatory response with the migration of monocytes into the subintimal space of the artery. The inflammatory response results form a cascade of events triggered by noxious stimuli to the endothelium of the artery. This consequently leads to atherosclerotic cardiovascular disease (Libby et al., 2013, Gerszten et al., 1999).

With a heightened attention of cardiovascular disease associated with antiretroviral therapy, management of HIV and AIDs has now become a double edge sword. Although it is likely that cardiovascular disease risk in the HIV patients may be accounted for by other pathological conditions linked to HIV infection in interaction with mediating processes such as inflammatory cytokines, it appears that protease inhibitors and NNRTIs medications may exacerbate the cardiovascular disease to enhance the risk. This is particularly relevant in North America and Europe, where an increasing portion of the HIV-infected population comprises middle-aged men, and receiving treatment with PIs, shows significant development of cardiovascular disease (Dimala et al., 2016b, Dimala and Blencowe, 2017).

There is also compelling evidence that hypertension is an important risk factor for cardiovascular disease and it is a multifactorial disease arising from the combined action of many environmental, behavioral and genetic variants especially in PLWHAs, as well as oxidative stress and inflammation which may result from drug use (Dimala et al., 2016b, Lombo et al., 2015). Literature reveal an increasing frequency of hypertension among HIV-infected patients on HAART especially in combinations which includes Protease Inhibitors (PI) (Seaberg et al., 2005).

In countries with unlimited access or delayed roll-out of HAART, HAART-associated cardiovascular disease and hypertension has become one of the major causes of death. The pathogenesis of hypertension is clearly complex. The pathophysiology usually involves increase in cytokines release which will induce imbalance of the endothelium and endogenous vasodilators and constrictors (Bigna et al., 2015) . While a portion of this risk appears to be related to antiretroviral therapy, there is also evidence that traditional history of cardiac risk factors could play a role in the development of hypertension and other cardiovascular diseases in PLWHA. Therefore, management of cardiac risk will be important in the treatment of HIV/AIDS.

With the recent advances in antiretroviral therapy, management of HIV/AIDS infection has also become an important consideration for the development of other complications such as lipodystrophy. Lipodystrophy which includes both lipoatrophy and lipohypertrophy are common in HIV-infected participants and typically start to manifest after 6–12 months of HAART therapy (Cerrato et al., 2015). Lipoatrophy denotes a decrease in adipose tissue volume while lipohypertrophy denotes the opposite, and it commonly occurs in visceral adipose tissue and in the upper trunk. This unusual fat redistribution has an implication in the body physiology and anatomy which may eventually lead to CVD risk (Cerrato et al., 2015, Pereira et al., 2015).

The HAART associated lipodystrophy is multi-factorial in etiology, affecting both lipid/glucose metabolism and insulin sensitivity, altering the activity of glucocorticoid receptors in several tissues (Cerrato et al., 2015). Fat redistribution may also promote insulin resistance and impaired glucose tolerance through altered secretion of adipokines and other inflammatory markers (such as ILs 6, 8 and 10, and macrophage chemotactic protein-1) (Gustafson et al., 2015). Impaired glucose tolerance (IGT) is a pre-diabetic state of dysglycemia that is associated with insulin resistance and increased risk of cardiovascular pathology. Impaired glucose tolerance and diabetes mellitus can occur in HIV patients receiving HAART through various pathophysiological mechanisms.

1.4 Diabetes Mellitus

Diabetes mellitus (DM) is a group of chronic disorders of carbohydrate, proteins and fat metabolism characterized by hyperglycemia arising from defects in insulin secretion, insulin action or both (Association, 2013). Two major types of diabetes mellitus are recognized on the basis of absolute or relative deficiency of insulin (Table 1.5). DM associated with absolute insulin deficiency (Type I DM or T1DM) has previously been described with terms such as Insulin Dependent Diabetes Mellitus (IDDM) or juvenile onset diabetes mellitus. T1DM is an autoimmune disease triggered by environmental factors in genetically susceptible individuals and its pathogenesis is characterized by destruction of beta cells of the islets of the pancreas, thus making them dependent on exogenous insulin (Atkinson et al., 2014). (Type II DM or T2DM) on the other hand is associated with relative deficiency of insulin, either due to insufficient amount (defect in secretion) or insulin resistance (defect in insulin action) even in presence of sufficient insulin levels. It was previously referred to as Non-Insulin Dependent Diabetes Mellitus (NIDDM) (Atkinson et al., 2014).

Apart from these major types of diabetes mellitus, other recognized types of diabetes include Maturity Onset Diabetes of the Young (MODY), an autosomal dominant genetic defect of β -cell function characterized by early onset of hyperglycemia by the age of 25 years due to an impairment of insulin secretion with minimal or no loss of insulin action (Asmat et al., 2016). Others include gestational diabetes mellitus; a condition defined by presence of impaired glucose tolerance in pregnancy and has been associated with increased incidence of complications in pregnancy including intra-uterine fetal death (IUFD), stillbirths and macrosomic babies (Asmat et al., 2016).

Recently, type 3 diabetes mellitus (T3DM) was described, it is characterized by brain tissue resistance to insulin action, histopathological, molecular and biochemical features of Alzheimer's disease and good responses to insulin sensitizers (Kandimalla et al., 2017, Suzanne, 2014).

Table 1.5: Classification of Diabetes Mellitus

Class of Diabetes Mellitus	Mechanism of Diabetes Development
1. Type 1 Diabetes Mellitus	Pancreatic beta-islet cell destruction; Absolute insulin deficiency
2. Type 2 Diabetes Mellitus	Insulin resistance (obesity), relative insulin deficiency
3. Gestational Diabetes	Insulin resistance
4. Others	A. Genetic defects in B-cell dysfunction B. Genetic defects in Insulin action C. Disease of exocrine pancreas D. Endocrinopathies E. Drugs or chemical Induced F. Infection G. Immune mediated defects H. Genetic syndromes associated with DM

Source: Adapted from the report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes care*, 1997; 20: 1183-1197.

1.4.1 Epidemiology of Diabetes Mellitus

Human, social and economic costs imposed globally by diabetes are enormous, and this is as a result of the increasing incidence and prevalence of diabetes world-wide. Currently, 382 million adults have diabetes mellitus and a further 361 million are estimated to have impaired glucose tolerance. By 2035, it is estimated that 471 million adults will have diabetes mellitus (Mejnikman et al., 2016, Cos et al., 2015). The prevalence of diabetes mellitus which stood at 4% globally in 1995 has dramatically risen in less than two decades to 8.3 % in 2013 and is expected to rise further in the years to come (Shaw et al., 2010). The majority of the 382 million people with diabetes mellitus are between the ages of 40-59 and greater than 75% of these live in low- and middle-income countries (Mejnikman et al., 2016). T2DM accounts for 90-95% of all diabetes cases while T1DM accounts for only 5-10% (Dabelea et al., 2014). The prevalence rate of diabetes in Africa is expected to rise from 3.8% in 2013 to 4.7 % by the year 2035 (Guariguata et al., 2014). In South Africa, it is estimated that about 10 million people aged between 20-79 years have one form of diabetes or the other with a prevalence rate ranging between 9-12 % and this is expected to rise significantly in the future (Guariguata et al., 2014).

1.4.2 Clinical features of Diabetes Mellitus

DM is a multi-systemic disease with biochemical and structural consequences. The pathological hallmark of DM is marked hyperglycemia which is responsible for most of the symptoms and complications. DM classically presents with symptoms including polyuria, polydipsia, polyphagia, weight loss (cachexia), fatigue, nausea and blurred vision (Figure 1.2) (Association, 2014). Hyperglycemia by increasing the plasma osmolality leads to dehydration that fuels increased water intake. Furthermore, excess circulating glucose predisposes to increased glomerular filtration of glucose resulting in glycosuria and further fluid loss worsening dehydration and contributing to weight loss (Guariguata et al., 2014). In T1DM there is increased catabolic activity characterized by increased ketosis, hyperglucagonemia, protein catabolism to generate precursors needed in gluconeogenesis, and increased lipolysis which also explains weight loss observed in diabetic patients (Katsarou et al., 2017). In humans, T1DM can occur at any age but it is most common in the young (juveniles) although adults in their third and fourth decades of life are also at risk (Katsarou et al., 2017). T1DM unlike T2DM is not associated with obesity and is commonly diagnosed for the first time in patients with diabetic ketoacidosis due to their dependency on exogenous insulin for survival (Katsarou et al., 2017).

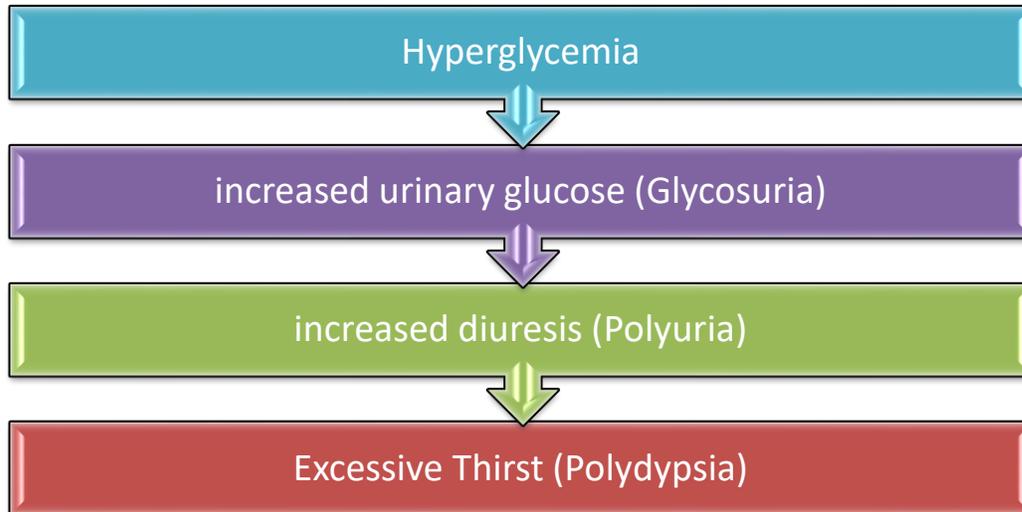


Figure 1.2: Schematic representation of major symptoms of diabetes mellitus.

1.4.3 Diagnosis of Diabetes Mellitus

Diagnosis of diabetes mellitus is based on blood glucose criteria, and two tests most often indicated are Fasting Plasma Glucose (FPG) and or 2-hour postprandial plasma glucose levels (2-HRPP) after glucose load in the Oral Glucose Tolerance Test (OGTT) (Association, 2017). Designated diagnostic cut off points of FPG values ≥ 7.0 mmol/l or 2-HRPP values ≥ 11.1 mmol/l after a 75-g oral glucose or random plasma glucose ≥ 11.1 mmol/l in an individual with classic symptoms of hyperglycemia or hyperglycemic crisis are regarded as diabetic (See Table 1.6) (Association, 2017). The International Expert Committee appointed by American Diabetic Association, European Association for the Study of Diabetes and International Diabetes Association recommend plasma HbA1c assay as a diagnostic method in individuals without classic symptoms of T1DM (Association, 2017).

Table 1.6: Diagnostic Criteria for Diabetes Mellitus

Stage	Diagnostic Test	
	Fasting Blood Glucose (FBG)	2-Hour Glucose Tolerance Test (GTT)
Normal	<6.1 mmol/l	< 7.8 mmol/l
Pre-diabetes mellitus	≥ 6.1 mmol/L and < 7.0 mmol/l	≥ 7.8 mmol/L and < 11.1 mmol/l
Diabetes Mellitus	≥ 7.0 mmol/l	≥ 11.1 mmol/l

Source: Adapted from the diagnosis and classification of diabetes mellitus. *Diabetes care*; 33: Suppl. 1: S62-S69. The report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetic care* 2004; 27: S1-10.

1.4.4 Insulin synthesis and secretion

Insulin deficiency is the characteristic hallmark of T1DM and is responsible for the marked hyperglycemia that is central to the pathophysiology, clinical features and complications of T1DM (Kent et al., 2015). Insulin is a polypeptide hormone produced by the beta-islet cells of the pancreas, its deficiency in T1DM leads to increased gluconeogenesis and ketogenesis by the liver resulting in severe hyperglycaemia and hyperketonemia (Ali et al., 2017). Insulin is synthesized from amino acid precursors as pre-proinsulin which undergoes several enzymatic cleavages to yield pro-insulin (containing 86 amino acid residues), insulin (a 51-amino acid polypeptide) and c-peptide, a precise marker of endogenous insulin secretion due to its resistance to degradation by liver during the first pass metabolism (Hebrok and

Russ, 2017). Insulin and c-peptide are released from the pancreas in equimolar amounts into the portal circulation by glucose stimulated insulin secretion (Hebrok and Russ, 2017).

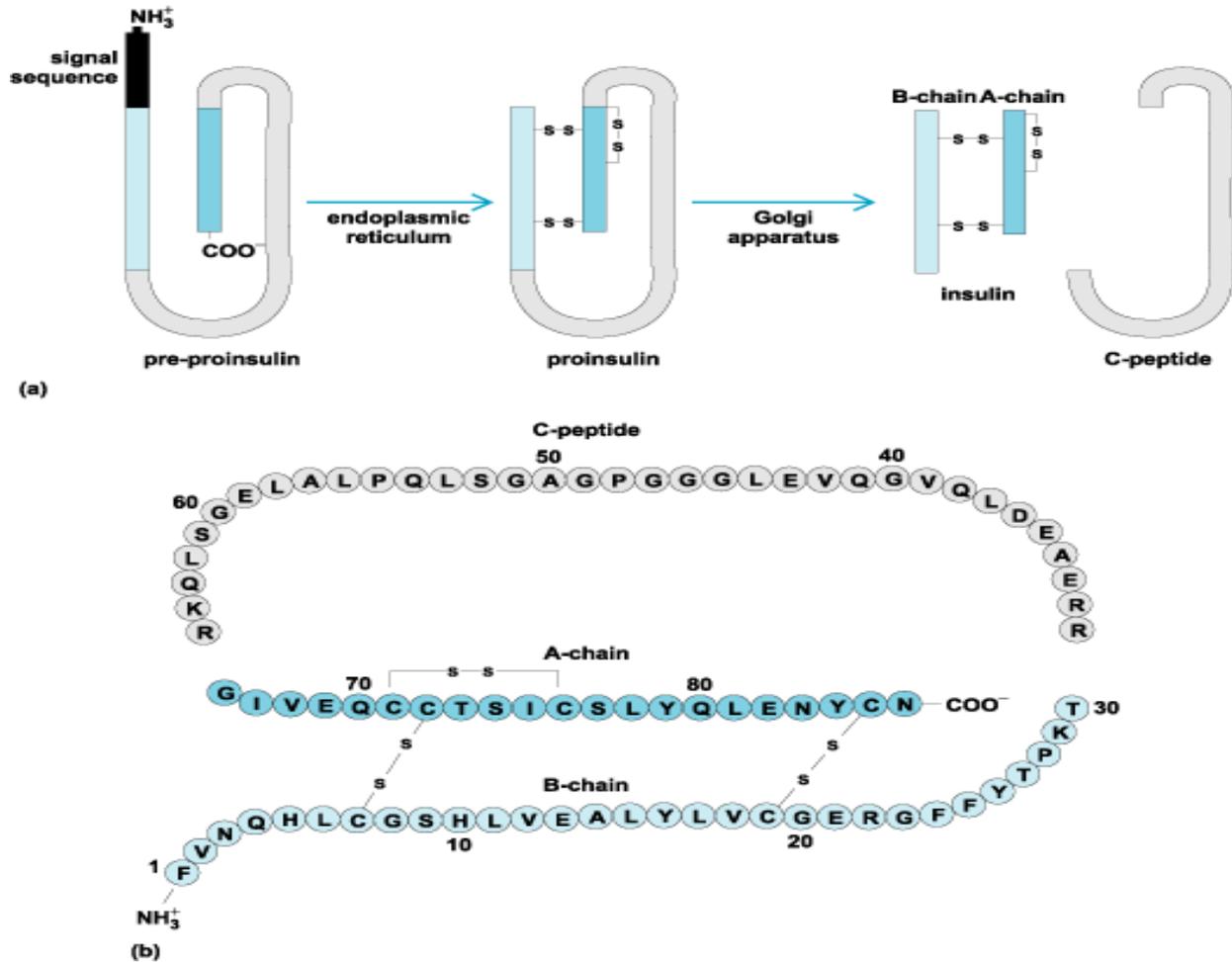


Figure 1.3: Insulin synthesis process.

Source: Adapted from Namrata Chhabra, Diabetes mellitus, Insulin chemistry and Function.

Insulin secretion by the pancreatic beta cells of the pancreas is regulated primarily and largely by the plasma glucose concentrations, amino acids (e.g. leucine), fatty acids and drugs (e.g. sulphonylureas, repaglinide and nateglinide) (Gunton and Girgis, 2012). Glucose enters β -pancreatic cells via facilitated transport through membrane associated specialized glucose transporters (GLUT-2) and is phosphorylated by glucokinase in the cytosol to glucose-6 phosphate, and subsequently metabolized through the glycolytic pathway to generate Adenosine Tri-Phosphate (ATP) (Hatting et al., 2018). The increase in cellular ATP levels causes a closure of the ATP-sensitive K⁺ channels present on the beta-cell

membranes; this leads to membrane depolarization, calcium entry through voltage-gated calcium channels and release of insulin into portal circulation by exocytosis (Gunton and Girgis, 2012).

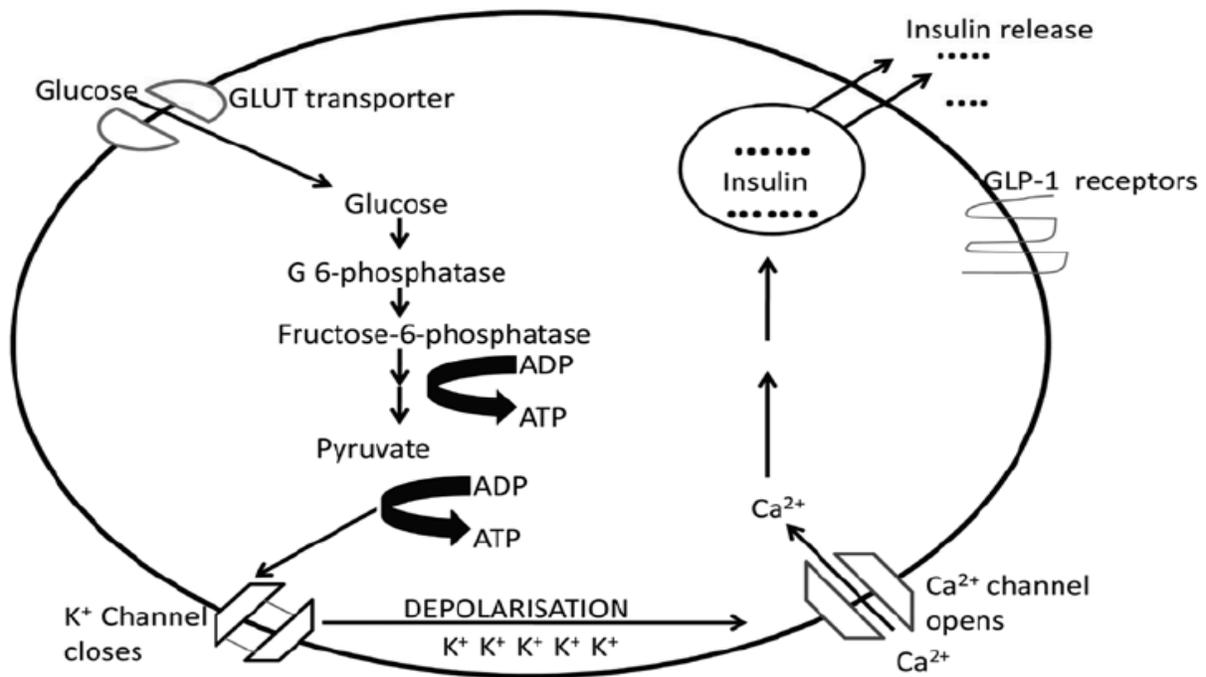


Figure 1.4: Insulin release from pancreatic beta cells in response to increased glucose.

Source: Adapted from Gunton and Girgis, 2012. Pancreatic beta-cell failure in the pathogenesis of type 2 diabetes.

1.4.5 Insulin action on peripheral tissues

The liver, muscle and adipose tissues are insulin sensitive tissues, on which insulin exerts its effects through interactions with heterodimeric insulin receptors (IR) (Petersen and Shulman, 2018). IR is a tyrosine kinase transmembrane receptor activated by a small group of endogenous ligands including insulin, Insulin like Growth Factor I and II (IGF-I and IGF-II). It consists of two α receptors that serve as the ligand binding domain located in the cell membranes and two intracellular β receptor domains that have intrinsic tyrosine kinase activity essential for insulin receptor function. The binding of insulin to the α -subunit causes an activation of the β -subunit tyrosine kinase, leading to the autophosphorylation of the receptor, phosphorylation (activation) insulin receptor substrate (IRS) proteins, (IRS1–IRS4) and subsequent activation of multiple downstream signaling cascades, including PI-3K and the MAP kinase

pathways, which mediate the metabolic and mitogenic activities of insulin in tissues (Petersen and Shulman, 2018, Haeusler et al., 2018, Boucher et al., 2014).

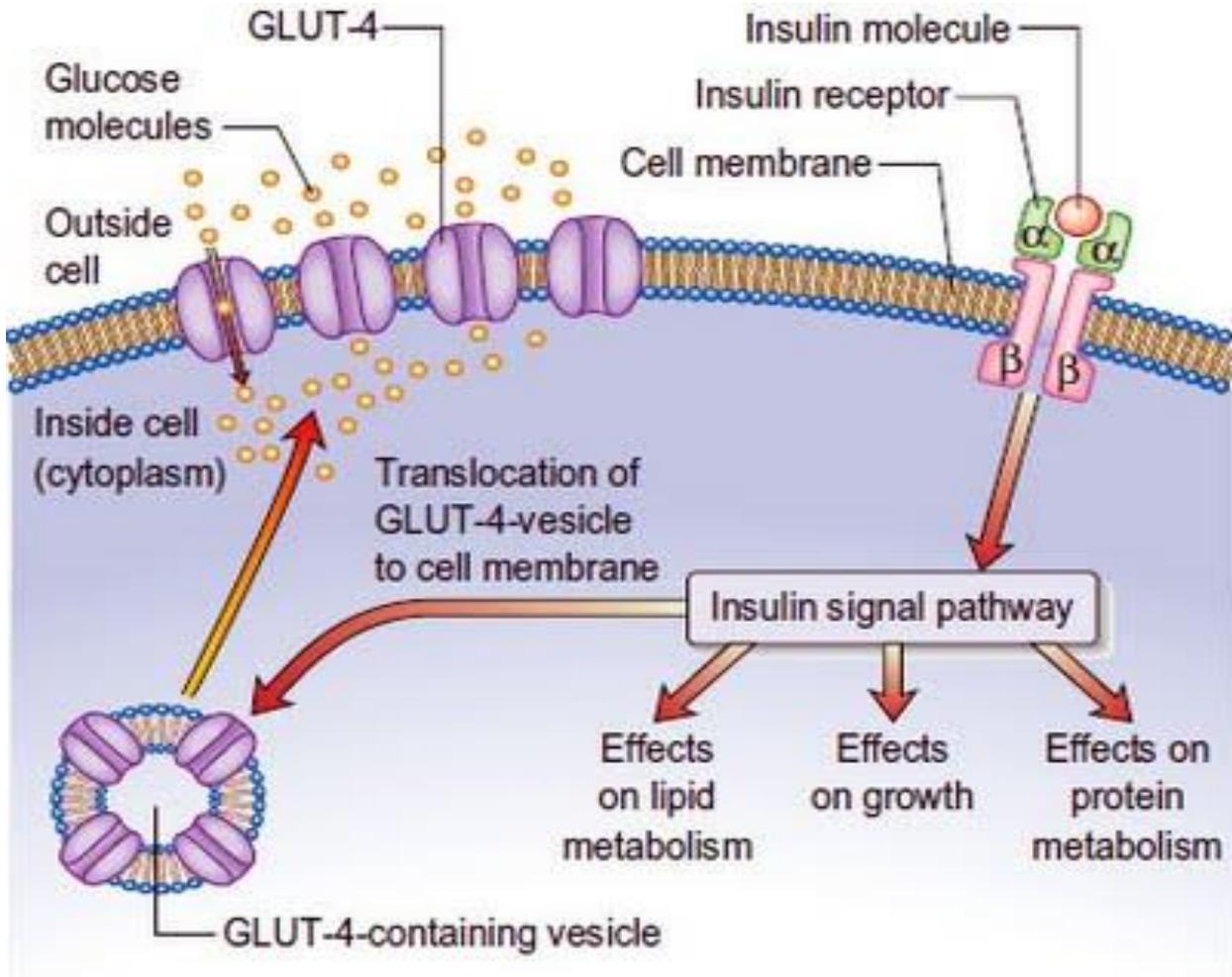


Figure 1.5: Mechanism of action of Insulin.

Source: Adapted from Namrata Chhabra, Diabetes mellitus, Insulin chemistry and Function.

In non-diabetics, insulin promotes hepatic glucose uptake and increased uptake of glucose by insulin-sensitive peripheral tissues including skeletal muscle and adipose tissue where glucose is converted to glycogen and triglycerides, respectively (Kotronen et al., 2017). Furthermore, insulin enhances positive nitrogen balance in tissue by facilitating uptake of amino acids in response to protein feeding, prevents protein breakdown and to a lesser extent stimulates protein synthesis (Abdulla et al., 2016). In the adipose tissues insulin accelerates triglyceride incorporation by stimulating lipoprotein lipase and simultaneously inhibiting hormone sensitive lipases which catalyzes the hydrolysis of stored triglycerides (Lambadiari et al., 2015). Insulin inhibits lipolysis, promotes triglyceride synthesis in adipose tissues, and suppresses endogenous production of glucose (gluconeogenesis and glycogenolysis) consequently promoting anabolism (Lambadiari et al., 2015). Deficiency of insulin in T1DM reduces glycogen synthesis allowing stimulatory effects of glucagon on glycogenolysis to predominate (Perry et al., 2014). Counter-regulatory hormones (glucagon, growth hormone, cortisol and catecholamines) are produced in response to marked insulin deficiency, stress and illnesses; they oppose the anabolic actions of insulin through endogenous glucose production (Ghorani et al., 2016). Glucagon is produced in the α -cell of the pancreas in response to hypoglycaemia, amino acids or autonomic nervous system activation and exerts its effects via cyclic adenosine monophosphate-dependent (cAMP) increases in glycogenolysis, gluconeogenesis and ketogenesis (Briant et al., 2016, Ravnskjaer et al., 2015). In T1DM, hyperglycaemia does not inhibit the secretion of glucagon from the α -cells as expected in normal individuals; this due to loss of insulin's restraining effect on α -cells which leads to a relative increase in portal glucagon levels (Perry et al., 2014). Furthermore, growth hormone levels in T1DM are inappropriately elevated secondary to increased circulating levels of insulin-like growth factor (IGF) arising from insulin deficiency, growth hormone promotes lipolysis and inhibition of glucose utilization (Møller and Jørgensen, 2009). Low portal insulin in T1DM allows for growth hormone-induced stimulation of gluconeogenesis contributing to an already detrimental hyperglycaemic state (Møller and Jørgensen, 2009).

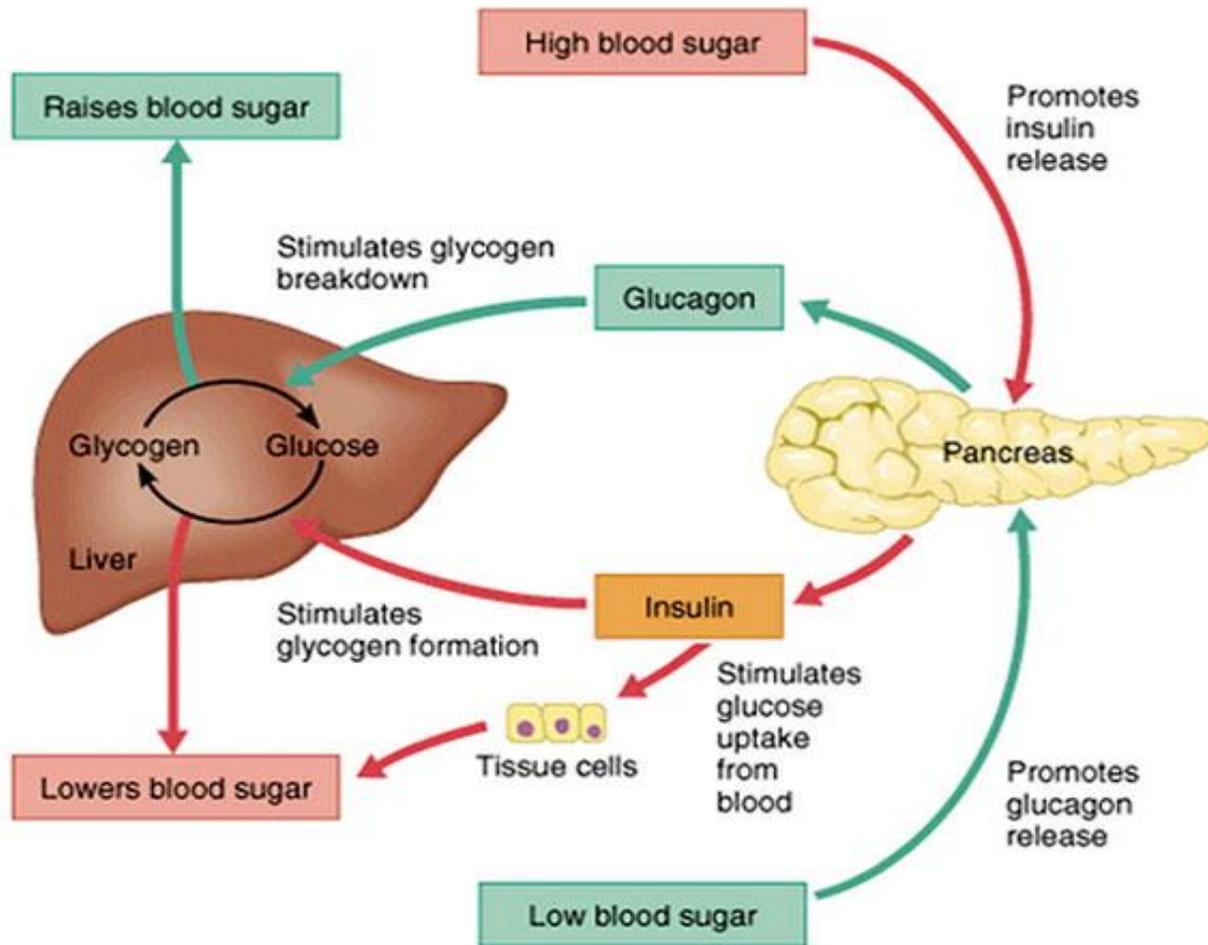


Figure 1.6: Insulin effects in the skeletal muscle, liver and adipose tissue.

Source: Adapted from Qaid, M.M. and Abdelrahman, M.M., 2016.

1.4.6 Etiology and Pathophysiology of Type 1 Diabetes Mellitus

T1DM is caused by profound pancreatic β -cell failure which results from an interplay of genetic, environmental and autoimmune factors that selectively destroy insulin producing β -cells (Figure 1.7) (Rahaman et al., 2015). Human leukocytes antigen (HLA) genes found on the short arm of chromosome 6 have been identified to be strongly associated with T1DM. Identical HLA genes in siblings increase the risk of developing T1DM, which appears to be less if they do not share HLA genes (Noble, 2015). Specific HLA haplotypes associated with T1DM include HLA DR3 or DR4 class II molecules which are expressed in 90-95% of T1DM patients (Noble, 2015). Other identified HLA haplotypes associated with T1DM disease susceptibility are the HLA DQ genes (DQ8 and DQ2) which are strongly linked with T1DM susceptibility in the Caucasian populations. Because of heterogeneity, genetic associations do not fully account for susceptibility to diabetes (Noble, 2015).

Environmental triggers such as toxins, viruses and diet are important contributors to the development of T1DM. Viral epidemics of mumps, congenital rubella and Coxsackie virus infections are associated with increased frequency of occurrence of T1DM and are as a result of autoimmune responses leading to the destruction of pancreatic beta cells (Ting et al., 2013, Beyan and Leslie, 2007). The mechanism of this beta cell destruction involves self-reactive cytotoxic CD8⁺ T-lymphocytes (CTL) and CD4⁺ lymphocytes, which recognize self-antigens expressed on β -cells leading to their apoptosis through different mechanisms including Fas receptor activation, perforin and granzyme-induced cell death (Espinosa-Carrasco et al., 2018, Trivedi et al., 2016).

Apart from CTLs, CD4⁺ T- cells contribute to the destruction of beta cells in T1DM, through the recruitment of CD8⁺ T-cells to infiltrate pancreatic islets tissue through the secretion of proinflammatory cytokines (TNF- α and IFN- γ). These cytokines also activate and recruit dendritic cells, natural killer cells and macrophages that propagate the destruction of β -cells of the pancreas (Espinosa-Carrasco et al., 2018, Trivedi et al., 2016).

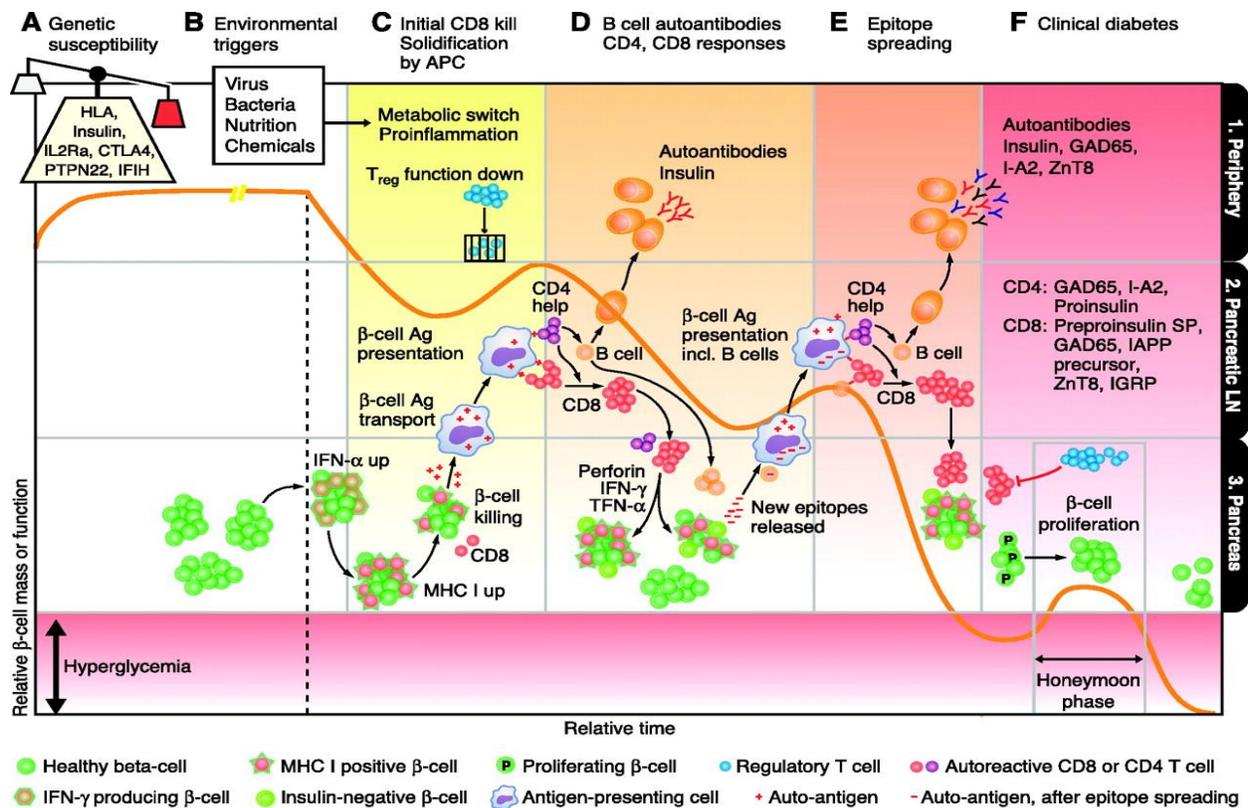


Figure 1.7: Pathophysiology of Type 1 diabetes mellitus integrating the genetic, environmental and autoimmune components. (Source: Van Belle TL, Coppieters KT and Von Herrath MG 2011, Type 1 Diabetes mellitus: Etiology, Immunity and Therapeutic strategies.

1.4.7 Etiology and Pathophysiology of Type 2 Diabetes Mellitus

T2DM is caused by a complex interplay of environmental, genetic and lifestyle factors. Lifestyle changes associated with the development of T2DM include excessive caloric intake (particularly energy dense foods), inadequate caloric expenditure (sedentary lifestyles) and obesity (weight greater than 120% of desirable weight) (Prasad and Groop, 2015, Murea et al., 2012). Other predisposing factors associated with increased incidence of T2DM include low birth weight, age greater than 45 years, family history (first degree relatives), history of impaired glucose tolerance or impaired fasting insulin, dyslipidemia, history of gestational diabetes or previous birth of macrosomic babies, hypertension, pre-hypertension and polycystic ovarian syndrome (Murea et al., 2012, Temelkova-Kurktschiev and Stefanov, 2012).

Development of T2DM involves combination of insulin resistance in the periphery and impaired insulin secretion resulting from pancreatic beta cell dysfunction (Zaccardi et al., 2016). Insulin resistance often occurs early in the evolution of T2DM before the onset of hyperglycemia and is associated with beta cell hyperfunction and hyperinsulinemia followed by beta cell failure at some point (Zaccardi et al., 2016, Keane et al., 2015). Obesity is an important contributing factor to insulin resistance in T2DM because it has a profound effect on tissue sensitivity to insulin and by extension glucose homeostasis. The mechanisms of insulin resistance in obese individuals involve interplay of elevated adipokine secretion, increased circulating free fatty acids and chronic inflammation of the adipose tissue (Keane et al., 2015). Insulin resistance results in decreased glucose transport into muscle cells, elevated hepatic glucose production, and increased breakdown of fat (Rosen and Spiegelman, 2006). Decreased insulin receptor affinity (arising from down-regulation, mutations and increased degradation) and impaired insulin receptor signaling are some of the mechanisms advocated to explain insulin resistance in the peripheral tissues (Khodabandehloo et al., 2016, Sharabi et al., 2015). Beta cell exhaustion in T2DM is due to a combination of compensatory failure and intrinsic beta cell defects (Kahn et al., 2014). Furthermore, in T2DM, a loss of the reciprocal relationship between glucagon α -secreting cells and the insulin secreting beta cells characterized by glucagon excess suggests that T2DM is an islet paracrinopathy (Zaccardi et al., 2016).

1.5 Diabetes Mellitus and Highly active antiretroviral therapy

The optimism generated by HAART has been forestalled by the recognition of an increasing array of adverse metabolic effects such as DM (Soepnel et al., 2017). Since the introduction of HAART in the mid-1990, abnormalities in glucose homeostasis have been reported with increasing frequency in persons with HIV (Brown et al., 2005). HIV-infected persons receiving HAART are at a 4-fold increased risk of developing diabetes compared with HIV-seronegative men (Brown et al., 2005). In

May 1997, The FDA received 83 reports of the exacerbation of diabetes/ hyperglycemia in patients receiving HAART and by August 1997, issued a public health advisory warning regarding this adverse effect (Mastan et al., 2009).

Studies have confirmed the association of hyperglycemia or DM with PI use (Chandwani and Shuter, 2008). Recently, NRTIs and NNRTIs, were found to contribute to the disturbance of glucose metabolism (Ledgerber et al., 2007, Brown et al., 2005). Early cases of DM were linked to PI use, with cases of hyperglycemia appearing within 7 months of drug commencement (Chandwani and Shuter, 2008). Several NRTIs and drug combinations were related to the development of DM, in particular, lamivudine-stavudine, didanosine-stavudine, and didanosine-tenofovir (Tesfaye et al., 2014, Dimala et al., 2016a). Didanosine and stavudine which are the most currently used NRTIs have been linked to cause mitochondrial toxicity by inhibiting mitochondrial DNA polymerase- γ . These two drugs are also strongly known for leading to DM (Brinkman and Kakuda, 2000, Kakuda, 2000). The mechanism behind diabetes risk due to HAART is complex and multifactorial (See figure 1.8).

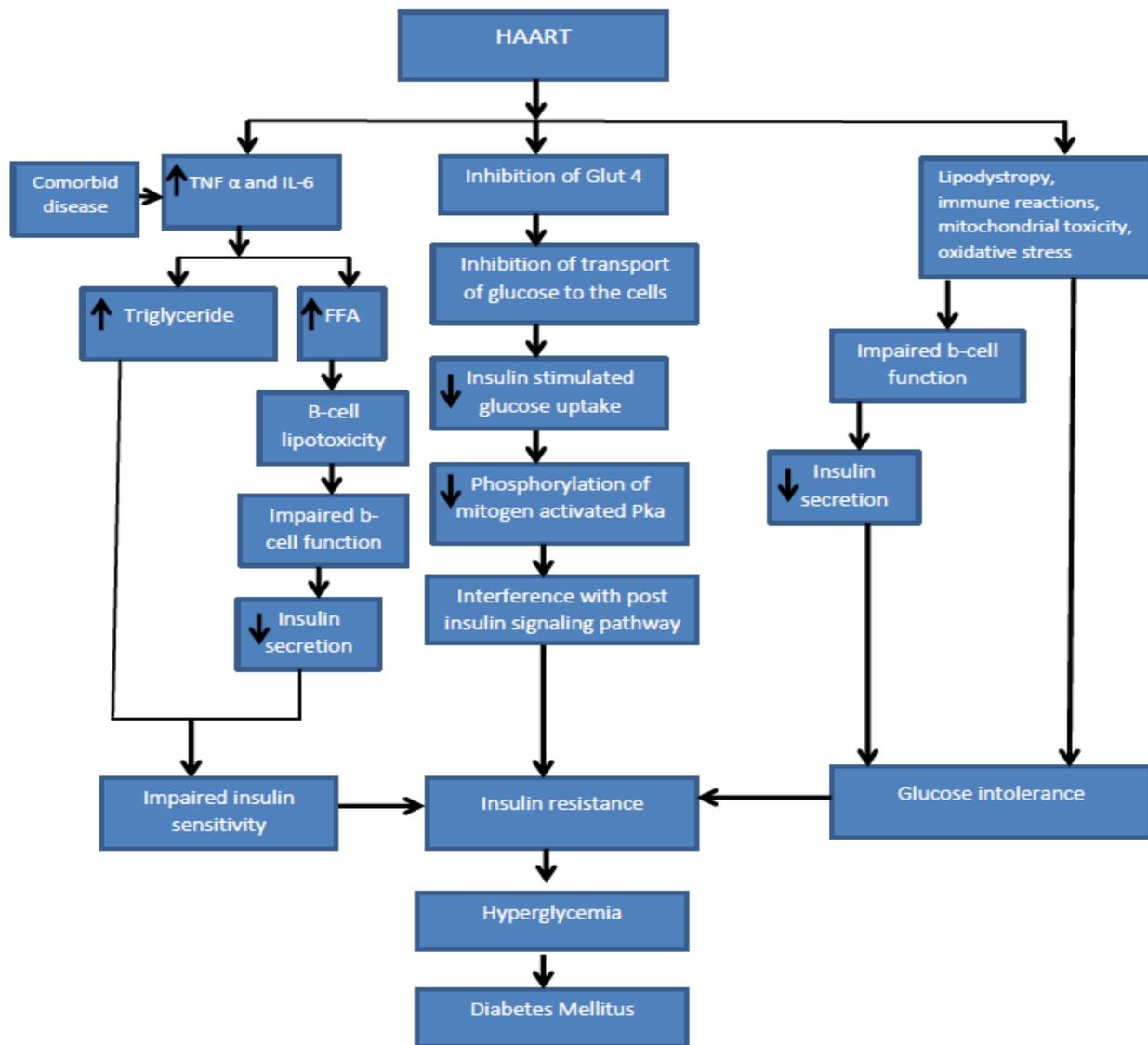


Figure 1.8: Possible mechanisms behind diabetes risk due to antiretroviral therapy: *Abbreviations:* *TNF*, Tumor necrosis factor; *IL-6*, Interleukin- 6; *GLUT-4*, Glucose transporter type 4; *FFA*, Free fatty acid. **Source:** Kumar, K.E., and Mastan, S. 2011. Diabetes Mellitus Risk with HIV infection treated with Antiretroviral therapy – An Overview.

1.6 Diabetic complications

Complications arising from DM are both microvascular (diabetic nephropathy, neuropathy, retinopathy, etc.) and macrovascular (coronary arterial disease, peripheral arterial disease, stroke, etc.) in nature. These

complications of DM are well-known and are associated with long-term damage and failure of various organ systems (Chawla et al., 2016).

The line of demarcation between the pathogenic mechanisms of microvascular and macrovascular complications of diabetes is blurred and these are reported to be the most prevalent contributors to morbidity and mortality on the global front (Chawla et al., 2016). It has been reported that diabetes induces pathognomonic changes in the microvasculature of the retina and the glomerular basement membrane by increasing their thickness, leading to diabetic microangiopathy. (Chawla et al., 2016). This glomerular basement membrane thickening will eventually lead to podocyte effacement, abnormal glomerular filtration rate as a result of damaged surface area and also narrowing of the vessels, inducing multiple clinical problems such as hypertension and tissue hypoxia (Chawla et al., 2016). Advanced glycation end products (AGE) and oxidative stress are accompanied with these changes and can lead to macrovascular complications (Asmat et al., 2016, Nowotny et al., 2015).

Oxidative stress, caused by the overproduction of reactive oxygen species (ROS) plays an important role in the activation of other pathogenic pathways involved in diabetic complications, including elevated polyol pathway activity, nonenzymatic glycation, and protein kinase C (PKC) levels which in turn lead to the development of micro and macrovascular complications (Asmat et al., 2016, Nowotny et al., 2015). Microvascular complications include retinopathy, nephropathy and neuropathy while macrovascular complications include cardiovascular diseases, cerebrovascular diseases and peripheral vascular diseases.

1.6.1 Diabetic nephropathy

Diabetic nephropathy (DN) is one of the causes of renal failure globally. Patients with early renal damage manifest with microalbuminuria which progresses into proteinuria (Kanwar et al., 2008). In renal tissue, advanced glycation end products can induce activities of transforming growth factor and raise expressions of various extracellular matrix mRNAs. These mediate hypertrophy of the glomeruli, thickening of basal membrane, and expansion of the mesangial extracellular matrix (Kanwar et al., 2008). The pathogenic mechanisms underlying DN involve generation of ROS, accumulation of advanced glycation end product (AGE), activation of intracellular signaling molecules such as protein kinase C (PKC) and over activity of the the hexosamine pathway (See figure 1.9) (Kanwar et al., 2008). These mechanisms are unified by the occurrence of overproduction of superoxide anions by the mitochondrial electron transport chain which is the principal pathophysiological mechanism of tissue damage in diabetes mellitus (Kanwar et al., 2008). Intracellular hyperglycaemia induces increased generation of ROS from the mitochondrial and ultimately predisposes to oxidative stress, a common denominator of diabetic nephropathy disease development and progression (Kanwar et al., 2008).

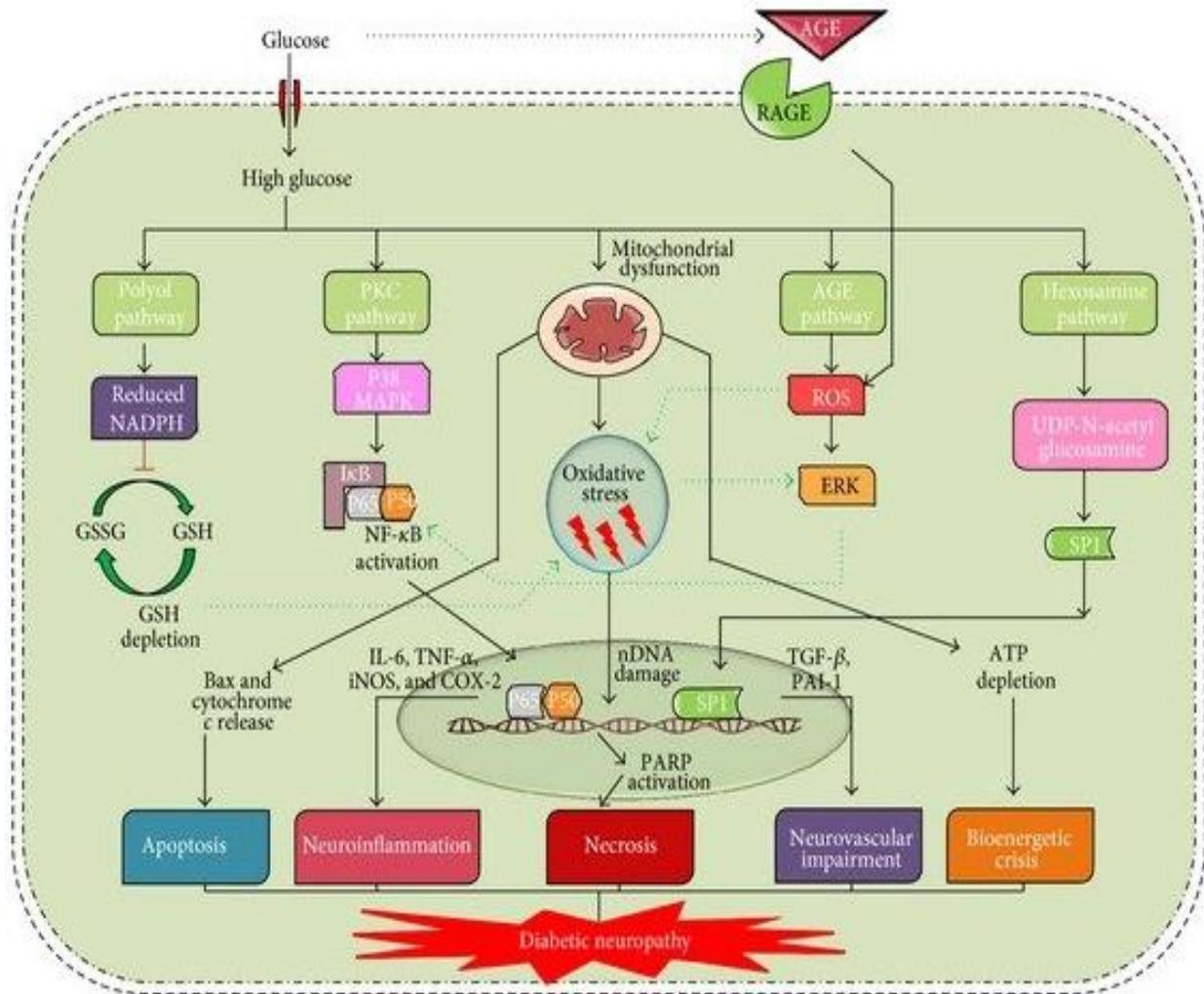


Figure 1.9: Schematic illustration for the pathogenesis of diabetic neuropathy. **Source:** Sandireddy et al., 2014. Neuroinflammation and oxidative stress in diabetic neuropathy: futuristic strategies based on these targets.

1.7 Oxidative stress

Oxidative stress is an imbalance between ROS production and ROS detoxification; oxidative damage to tissues occurs when the ROS production exceeds the ROS degrading enzymes detoxifying capacity or if there is a depletion of antioxidants in the presence of excess ROS (Sies, 2015, Schieber and Chandel, 2014). DM is strongly associated with increased oxidative stress which stems from increased ROS generation in tissues and a depletion of natural enzyme antioxidants expression and activity (Asmat et al., 2016). The development and progression of diabetic neuropathy are promoted by increased and uncontrolled generation of ROS and the reduction in scavenger antioxidant enzyme activity (Kanwar et

al., 2008). Increased ROS generation and depletion of natural antioxidant enzymes in T2DM have been previously reported (Panahi et al., 2017).

1.8 Reactive Oxygen Species

Reactive oxygen species (ROS) are highly unstable oxygen-based chemical species, these atoms are capable of independent existence and they contain one or more unpaired electrons in their outer orbit which makes them highly reactive entities. ROS include both free radicals [e.g. superoxide anions ($O_2^{\cdot-}$), Hydroxyl anions (OH^{\cdot}), peroxy (RO_2^{\cdot}) and alkoxy radicals (RO^{\cdot})] and chemicals capable of generating free radicals such as hydrogen peroxide (H_2O_2) (Knaus, 2018). ROS are generated in normal physiological processes (cellular respiration) in the mitochondria or pathological processes such as diabetes (Nishikawa et al., 2015). Although, mitochondria is a major source of ROS in diabetes, non-mitochondrial sources include Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme, xanthine oxido-reductase system and nitric oxide synthase pathway (Inoguchi et al., 2003).

Increased ROS production can oxidatively damage lipids, proteins and nucleic acids through interactions with redox-sensitive cysteine residues on proteins leading to the formation of sulfonic acids (-SOH) that form disulphide bonds (-S-S-) with neighbouring cysteine groups (Zorov et al., 2014). Furthermore, ROS interactions with polyunsaturated fatty acids in lipid membranes generate a series of reactive hydroperoxides that further damage membranes leading to structural and functional deficits (Sies, 2013). ROS through the formation of DNA adducts damages the DNA, causes loss of DNA repair mechanism and induce gene activation of oxidative-stress responsive maladaptive pathways thereby promoting apoptosis in glomerular cells which leads to characteristic morphological and functional abnormalities associated with the diabetic nephropathy (Reidy et al., 2014, Terryn and Devuyt, 2011).

1.8.1 Lipid Peroxidation

Both T1DM and T2DM are characterized by oxidative damage to macromolecules such as lipids, proteins and nucleic acids (Rani et al., 2016). Oxidative damage to lipids occurs by lipid peroxidation, a known biomarker of oxidative stress in tissues; it involves oxidative modification of lipids in tissues leading to deterioration of unsaturated lipids in plasma and organelle membranes (Frankel, 2014, Ayala et al., 2014). Peroxidation of membrane lipids involves the conversion of non-radical lipids into radicals, through a series of chain reactions (Repetto et al., 2012, Frankel, 2014). Lipids maintain cellular integrity through the abundant polyunsaturated fatty acids (PUFA) in biological membranes and are targets of peroxidation by free radicals (Ayala et al., 2014, Holman, 2009). Peroxidation of PUFA affects the structure and function of biological membranes through the alteration of their biophysical properties leading to loss of

fluidity and cellular integrity resulting in leakage of intracellular enzymes, failure of membrane pumps and impairment of cellular transport processes and activation of apoptosis (Ayala et al., 2014, Holman, 2009).

1.8.2 NADPH Oxidase

NADPH oxidase is a major source of intrarenal oxidative stress and is upregulated by metabolic factors leading to overproduction of ROS in podocytes, endothelial cells, and mesangial cells in glomeruli, which is closely associated with the initiation and progression of glomerular diseases (Wan et al.,2016). Membrane bound NADPH oxidases are important cellular sources of ROS which are pertinent to the pathophysiological processes in diabetic nephropathy (Wan et al., 2016, Fu et al., 2010). Diabetic nephropathy is often characterized by increased expression of NADPH oxidase isoforms, attenuated antioxidant defenses coupled with increased ROS generation (Jha et al., 2017, Zhang et al., 2017). Inhibition of NADPH oxidase-derived ROS generation mitigates diabetes- induced glomerular injury via reducing podocyte loss, proteinuria, glomerular hypertrophy, and mesangial matrix expansion (Wan et al.,2016, Asaba et al., 2007, Tojo et al., 2005).

Podocytes depletion due to apoptosis occurs early in DN and is characterized by effacement of the podocytes foot process and slit disruption as reported by Wan et al., 2016; Some researchers have highlighted the role of podocytes in DN pathogenesis and have revealed the upregulation of NADPH oxidase subunits expression in type 1 diabetic OVE26 mice and type 2 diabetic db/db mice (Wan et al., 2016 , Eid et al., 2009, Zhou et al., 2009) In vitro studies shows that high glucose induced the upregulation of NADPH oxidase expression, enhancement of NADPH oxidase activity, and apoptosis induction in podocytes at later time points (Wan et al., 2016, Susztak et al., 2006). (Eid et al., 2010) found that the increase of Nox4 expression was attributed to the inactivation of AMP-activated protein kinase (AMPK), and Nox4 promoted podocyte apoptosis via p53- dependent apoptotic pathway in high glucose condition.

In the diabetic nephropathy, NADPH oxidase is up-regulated and is a major source of ROS generation which contributes to complications of diabetes in tissues (Zhang et al., 2017). NADPH oxidase is activated in response to a variety of growth factors and protein regulators of cellular processes including angiotensin II, endothelin-1 (ET-1) and protein kinase C (Gill and Wilcox, 2006). NADPH oxidase activation in diabetes is dependent on hyperglycemia where excessive glucose is channeled into glycolytic pathways leading to the accumulation of diacylglycerol (DAG), a potent activator of protein kinase C (Zhu et al., 2015). PKC-dependent NADPH oxidase activation has been demonstrated to increase oxidative stress in the kidney (Yang et al., 2012, Zhu et al., 2015). Angiotensin II is also an

important growth factor that has been strongly linked with increased development of fibrosis, which is a prominent feature of diabetic nephropathy and its elevation due to hyperglycemia directly activates NADPH oxidase activity in diabetic tissues. This causes marked increase in ROS levels in kidney tissues contributing significantly to the development of fibrosis and hypertrophy (Garrido and Griendling, 2009).

1.9 Natural Enzyme Antioxidants

Maintenance of health is highly dependent on the fine balance between ROS production and its degradation by cellular antioxidants. Aerobic organisms possess sophisticated antioxidant defense mechanisms to detoxify ROS generated under physiological processes, such as protein-based enzyme antioxidants and non-enzymatic antioxidants which eliminate free radical intermediates in cells (Jiang and Xiong, 2016). Enzymatic antioxidants in kidney tissues include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). Hyperglycaemia is suggested to directly impair endogenous antioxidant enzymes through a mechanism that is still debatable (Bajaj and Khan, 2012).

1.9.1 Superoxide Dismutase

Superoxide dismutase (SOD) is an important metalloenzymes that catalyse the conversion of superoxides to hydrogen peroxide thus limiting intracellular levels of superoxide and attendant oxidative damage. Three isoforms of SOD are recognised based on their metal content and cellular location, Cu-Zn SOD (cytosol and intramembranous space of mitochondria), Mn-SOD (mitochondrial matrix) and Fe-SOD (extracellular matrix) (Azarabadi et al., 2017, Kim et al., 2015). Down-regulation or deletion of Mn-SOD genes in mice is associated with gross nephropathy and endothelial thickening, suggesting a protective role of MnSOD against mitochondrial superoxide-induced damage (Vara and Pula, 2014). Cu-Zn SOD protects against oxidative damages in the cytoplasm, nucleus and mitochondria. SODs in general, are therefore critical to the maintenance of oxidative balance in tissue since they detoxify superoxides from multiple sources such as NADPH oxidases, mitochondria respiratory chain and eNOS uncoupling (Vara and Pula, 2014). In diabetes, hyperglycemia impairs circulating and tissue (heart, renal, hepatic and brain) SOD expressions and activity, both in humans and experimental animals (Giacco and Brownlee, 2010).

1.9.2 Catalase

Catalase is another dismutase enzyme. It contains a heme moiety at the active site and converts two hydrogen peroxide molecules to oxygen and water. This reaction requires the presence of a small amount of hydrogen peroxide to bind at the active site in order to generate catalase compound, which reacts with a second molecule of hydrogen peroxide (Keilin and Hartree, 1945).

During the enzymatic reaction leading to H₂O₂ destruction, catalase is first oxidized to a hypervalent iron intermediate, known as compound I (Cpd I), which is then reduced back to the resting state by a second H₂O₂ molecule. The first reaction is characterized by the oxidation of the heme protein by a single H₂O₂ molecule leading to the formation of Cpd I, an oxoferryl porphyrin cation radical (Jones and Dunford, 2005). Once Cpd I is formed, it reacts rapidly with a second molecule of H₂O₂ to generate H₂O and O₂ in a two-electron redox process. This second reaction is particularly efficient in some catalases compared to other heme proteins such as myoglobin (Matsui *et al.*, 1999).

The first function assigned to catalase is the dismutation of H₂O₂ into oxygen and water without consumption of endogenous reducing equivalents, an important role in cell defense against oxidative damage by H₂O₂. Catalase may also have additional roles such as the detoxification or activation of toxic and anti-tumor compounds. For instance, catalase has been detected in mouse oocytes most likely to protect the genome from oxidative damage during meiotic maturation (Park *et al.*, 2016). Under stress conditions, the antioxidant enzyme catalase plays a major role by detoxifying H₂O₂. As consequences, a change of its activity or expression will lead to pathological processes.

1.10 Glutathione Peroxidase

Efficient and rapid removal of oxygen-derived free radicals (by-products of aerobic metabolism) by suitable antioxidant systems such as glutathione peroxidase (GPx) is necessary to prevent the accumulation of ROS which can decompose into more harmful products that can cause oxidative damage to tissues (Lubos *et al.*, 2011). Hydrogen peroxide and its decomposition product (hydroxyl ion (OH)) are generated in oxidative states and require an efficient removal system like GPx to prevent oxidative damage to tissue (Lubos *et al.*, 2011). GPx reduces hydrogen peroxide (H₂O₂) and lipid peroxides into water and lipid alcohols, using glutathione as a hydrogen donor, they are selenium containing enzymes with 4 isoforms (GPx1-4) localized in different sub-cellular locations within cells (Brigelius-Flohé and Maiorino, 2013).

GPx1 is expressed abundantly in the kidney tissue and is involved in most of the detoxification of H₂O₂ in the cytoplasm as well as organic peroxides such as cholesterol and long chain fatty acid peroxides (Crawford *et al.*, 2011). The protein expression of GPx1 has previously been reported to be reduced in diabetic rats *in vivo* leading to accelerated diabetes-induced atherosclerosis through the up-regulation of pro-inflammatory and pro-fibrotic mechanisms (Shanmugam *et al.*, 2011). GPx-2 is a tetrameric protein also localized in the cytosol and has 65% amino acid sequence identity with GPx-1 which explains their similar substrate specificities. GPx-3 is a glycoprotein found in plasma with extracellular functions unlike GPx-4, a monomeric protein found in different tissues with capacity to reduce phospholipid and hydrogen

peroxides. GPx protein levels are generally reduced in the kidney, brain and heart of diabetic patients and animals (Crawford et al., 2011).

1.11 Reduced Glutathione (GSH)

Glutathione is an important tripeptidethiol (γ -glutamylcysteinyl glycine) antioxidant composed of three amino acids, cysteine, glycine, and glutamate. It is an important tripeptidethiol antioxidant widely distributed in biological fluids and tissues of plants, animals, fungi, some bacteria and archaea (Flora et al., 2013). It is associated with many physiological functions, such as the transportation of amino acids, the synthesis of proteins and DNA, the stabilization of cell membranes and the detoxification of xenobiotics (Flora et al., 2013). Glutathione plays an important role in fighting against toxic effects brought by reactive oxygen species like free radicals, lipid peroxides, peroxides and heavy metals. The reduced glutathione/oxidized glutathione ratio (GSH/GSSG) is widely used in clinics for the evaluation of oxidative stress status in biological systems (Gil et al., 2011).

GSH is found in high concentrations in cellular systems and plays a major role in detoxification of various electrophilic compounds (Flora et al., 2013). GSH and GSSG work together with other redox-active compounds such as NADPH to maintain and regulate cellular redox status. GSH is quantitatively described as the redox potential. Because of its significant role, acting as an antioxidant in the organism, it is of great interest among pharmacologists to take GSH system as a possible target for medical interventions (Flora et al., 2013).

Deficiency of glutathione puts the cell at risk for oxidative damage. Besides its antioxidant defense and free radical scavenging, glutathione regenerates important antioxidants such as vitamins C and E. Glutathione maintains the redox state of critical protein sulfhydryl groups that are necessary for DNA repair (Gil et al., 2011). Besides neutralization of free radicals, glutathione also is suggested to chelate transition metals, thereby reducing their toxic ability (Flora et al., 2013). Glutathione is known to be both a carrier of mercury as well as an antioxidant with specific roles in protecting the body from mercury toxicity. Glutathione targets and binds methyl mercury, preventing it from binding to other cellular proteins, which results in organ damage. Glutathione also prevents mercury from entering the intracellular environment and becoming an intracellular toxin. It has been suggested that IV glutathione combined with EDTA chelation significantly increases cadmium excretion (Flora et al., 2013, Gil et al., 2011). It was also found that renal protection was augmented. However, this was conducted in a single patient, albeit in a controlled fashion. This suggests that glutathione could provide additional chelation in the proper setting, but needs verification in clinical trials (Gil et al., 2011).

1.12 Role of Reactive Oxygen Specie in the pathogenesis of Diabetic nephropathy

It has been reported that ROS play crucial role in the development of diabetic complications and oxidative stress is increased in diabetes with excessive production of ROS which is a direct consequence of hyperglycemia (Ha et al., 2008, Lee et al., 2007). Under hyperglycemic condition, various types of vascular cells including renal cells produces ROS (Ha et al., 2008). ROS mediate hyperglycemia- induced activation of signal transduction cascades and transcription factors leading to transcriptional activation of profibrotic genes in the kidney (Ha et al., 2008). The mechanisms underlying ROS induction signal transduction cascades and transcription factors involves oxidative damage to DNA and the mitochondria apparatus, leading to the activation of pro-apoptotic signalling events that culminate in cell death (Reidy et al., 2014). Commonly activated pro-apoptotic signalling molecules and factors include mitogen activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) 1/2 and p38 MAPK (Yang et al., 2014). MAPK are important signal transduction enzymes that are unique to eukaryotes and are involved in many cellular processes, including development, differentiation, proliferation and apoptosis (Manna and Stocco, 2011).

1.13 Streptozotocin model of Diabetes Mellitus

Streptozotocin (STZ)-induced diabetic model is an extensively used animal model in studies of human diabetes mellitus. STZ is a nitrosourea antibiotic synthesized by *streptomyces achromogenes* used in the induction of experimental diabetes. STZ selectively destroys pancreatic beta cells by alkylation of DNA through its nitrosourea moiety (Furman, 2015). STZ, a glucosamine, is taken up preferentially by the pancreatic beta cells via membrane glucose transporter (GLUT-2) to induce oxidative damage leading to toxicity and cell death ultimately manifesting as insulin deficiency (Skovsø, 2014). Intracellular metabolism of STZ leads to liberation of nitric oxide which mediates DNA damage and mitochondrial production leading to the release of superoxide ions (Skovsø, 2014). Studies of STZ-induced DM models have identified abnormalities that include decreased kidney function and efficiency, decreased mitochondrial energetics, increased lipid storage, increased fatty acid oxidation, reduced glucose oxidation, impaired calcium handling and increased oxidative stress which are noted abnormalities in diabetic mellitus (Wu and Yan, 2015). Furthermore, increased cellular ROS levels particularly enhanced superoxide generation, increased NADPH oxidase expression and decreased Glutathione disulfide /Gluthanione (GSSG/GSH) ratio (ratio of oxidized to reduced glutathione). All these have been reported by investigators of oxidative stress in STZ- induced diabetic nephropathy (Figgers et al., 2015). The STZ diabetic model has been used by many investigators as a research tool in the partial or whole destruction of pancreatic beta cells for the discovery of antidiabetic drugs or diabetic complications. Administration

of low dose of STZ partially destroys the pancreas and thus implicates T2DM condition that resulting to hyperglycemia due to insulin resistance (Furman, 2015).

Pancreatic beta cells are vulnerable to oxidative stress because of their particularly low quantity antioxidant enzymes. Damage to the pancreatic beta cells, would suppress glucose mediated insulin secretion. This effect could be mediated through increased oxidative stress (Figgers et al., 2015). Evidence from literature showed that adjunct therapy with antioxidants may ameliorate these deleterious consequences (El-Demerdash et al., 2018, Farzaei et al., 2015).

1.14 Plants based adjuvants as putative antioxidants

Plants based adjuvants have been shown to inhibit lipid peroxidation, chelate redox-active metals, and attenuate processes involving ROS in tissues (Offor et al., 2017a). Although the health benefits of natural plants are mostly attributed to their antioxidant or free radical scavenging properties, it is becoming apparent that they exert more specific effects on a variety of cellular or molecular processes including cell signalling (Ismail et al., 2018).

An estimated 80% of Africans are said to rely on plant based adjuvants for the management of various disease conditions. For the majority of this group of individuals, consultation with traditional health practitioners (THP) is the first call for health services (Azu et al., 2016). There has been increased interest in the role of plants as complementary alternative medicines (CAM) for the treatment of various acute and chronic diseases and previous studies support the use of plant-based adjuvants for disease management like diabetes, cancers, etc (Somanah et al., 2018, Kalantar-Zadeh and Fouque, 2017).

It has also been estimated that up to one-third of patients with DM use some form of medicinal plants. Medicinal plants and its products continue to be an important therapeutic aid for alleviating the ailments of human kind and South Africa is a country with a long history of use of indigenous plants by herbalists and traditional healers for the treatment of various diseases such as HIV, diabetes and diabetic complications etc (Offor, 2015).

Diabetic complications associated with HAART have attracted various studies designed to accommodate a holistic management approach including the use of medicinal herbal products. Thus, while the use of medicinal herbs, a principal component of CAM, predates the emergence of HAART, there has been a widespread increase in the consumption of herbal products as adjuvant in management of HIV (Offor, 2015, Nyamukuru et al., 2017, Haile et al., 2017). Of the various classes of plants, interest has focused on medicinal plants with pharmacological properties and *Momordica charantia* plant is known to possess a

wide range of pharmacological properties such as antioxidant, anti-diabetic, anti-inflammatory, antimicrobial, anticancer and anti-HIV properties (Gupta et al., 2011, Aljohi et al., 2018).

1.15 *Momordica charantia* Linn.

1.15.1 Plant description

The plant *Momordica charantia* (*M. charantia*) is a flowering vine in the family of cucurbitaceous, known variously as bitter gourd, balsam pear, bitter melon, karela, bitter cucumber and African cucumber (Tirakannanavar and Munikrishnappa, 2011, Basch et al., 2003). Although it has many culinary uses, especially in Asia countries, America and some east and Southern African countries particularly south Africa. It is also grown as an ornamental and is used extensively in folk medicine. The fruits are cooked with other vegetables, stuffed, stir-fried or added in small quantities to beans and soups to provide a slightly bitter flavor. However, for most food preparation, fruits are blanched, parboiled or soaked in salt water before cooking to reduce the bitter taste. In addition to frying or cooking, the fruits can be dehydrated, pickled, or canned. Fruits, flowers and young shoots are also used as flavoring agents in various Asian dishes (Gupta et al., 2011). It is a climbing perennial plant that usually grows up to 5m and bears elongated fruits with a knobby surface. It is useful in a useful medicinal and vegetable plant for human health and one of the most promising plant for diabetes mellitus (Gupta et al., 2011).



Figure 1:10: Photograph showing Plants of *momordica charantia* bearing fruits

Source: Gupta et al., 2011



Figure 1:11: Photograph showing leaves of *momordica charantia*

Source: Gupta et al., 2011



Figure 1:12: Photograph showing fruits of *momordica charantia*

Source: Gupta et al., 2011

1.15.2 Medicinal Uses

M. charantia has been used for centuries in the ancient traditional medicine of India, China, Africa and Latin America. *M. charantia* extracts possess antioxidant, antimicrobial, antiviral, antihepatotoxic and antiulcerogenic properties while also having the ability to lower blood glycaemia (Upadhyay et al., 2015, Raman and Lau 1996). These medicinal properties are attributed to an array of biologically active plant chemicals, including triterpenes, momordicine, charantin, polypeptide, vicine and steroids (Upadhyay et al., 2015). Ethnomedicine reports of *M. charantia* indicate that it is used in folkloric medicine for treatment of diabetes, various ulcers and infections (Upadhyay et al., 2015, Beloin et al., 2005). While the root decoctions have abortifacient properties, leaf and stem decoctions are used in treatment of dysentery, rheumatism and gout (Subratty et al., 2005). It is also known that juice of *M. charantia* which is gotten from fruit has traditionally been used for medicinal purposes globally (Upadhyay et al., 2015). Likewise, the extracted juice from leaf, fruit and even whole plant are routinely used for treatment of wounds, infections, parasites (e.g., worms), measles, hepatitis and fevers (Olivier et al., 2016, Behera et al., 2008).

1.15.3 Phytochemical constituents

Literatures have shown that *M. charantia* possesses so many phytochemical constituents. Few of this constituents arranged from A-Z are: alanine, alkaloids, ascorbigen, b-sitosterol-d-glucoside, charantin, cucurbitins, cucurbitacins, cucurbitanes, diosgenin, elasterol, elaeostearic acids, glutamic acid, guanylate cyclase inhibitors, lauric acid, linoleic acid, linolenic acid, lutein, lycopene, momorcharasides, momorcharins, momordenol, momordicilin, nerolidol, oleanolic acid, oleic acid, oxalic acid, peptides, petroselinic acid, polypeptides, ribosome-inactivating proteins, rosmarinic acid, rubixanthin, serine, spinasterol, steroidal glycosides, stigmasta-diols, stigmasterol, trehalose, trypsin inhibitors, uracil, v-insulin, vicine, zeatin ribosidezeinoxanthin amino acids-aspartic acid. Research has found that the leaves are nutritious sources of calcium, magnesium, potassium, phosphorus and iron; both the fruit and leaves are great sources of B-vitamins (Gupta et al., 2011, Joseph and Jini, 2013, Kumar et al., 2010).

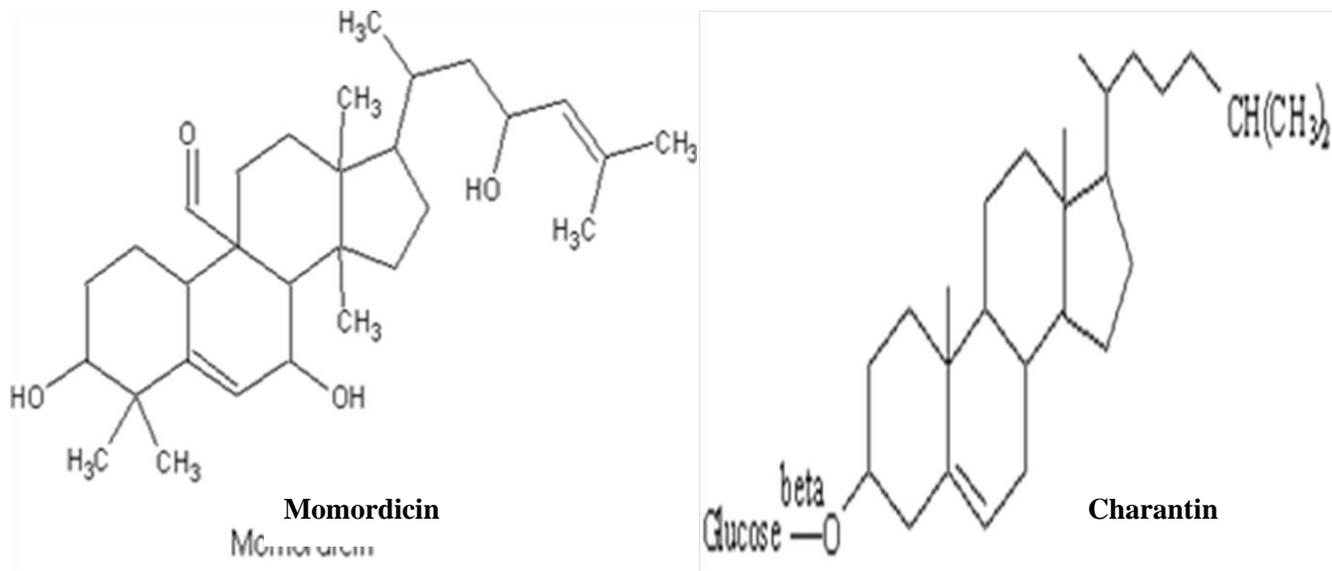


Figure 1:13: Phytochemicals: Momordicin and Charantin.

Source: Kumar et al., 2010. A medicinal potency of *Mormodica charantia*.

1.15.4 Selected bioactive compounds

Based on the plethora of medical conditions that *M. charantia* can treat, scientists are more interested in studying its bioactive compounds and their actions on the body. However, as many studies have reported, there has been substantial emphasis on the anti-diabetic compounds and hypoglycemic properties (Tan et al., 2016, Daniel et al., 2014). A number of reported clinical studies have shown that *M. charantia* extract from the fruit, seeds, and leaves contain several bioactive compounds that have hypoglycemic activity in both diabetic animals and humans (Joseph and Jini, 2013). Major bioactive compounds isolated from *M. charantia* and identified as hypoglycemic agents include charantin, polypeptide-p and vicine (Joseph and Jini, 2013, Chahar and Sharma, 2017).

1.15.4.1 Charantin

Charantin possesses antidiabetic properties and belongs to a typical cucurbitane- type triterpenoid in *M. charantia* (Patel et al., 2010, Joseph and Jini, 2013). (Dhiman et al., 2012), demonstrated that charantin could be used to treat diabetes and can potentially replace treatment known antidiabetic drugs. It is a mixture of two compounds namely, sitosterol glucoside and stigmasterol glucoside. Studies have reported that charantin is more effective than the oral hypoglycemic agent tolbutamide (Cousens 2007).

1.15.4.2 Polypeptide-p

Polypeptide-p also known as p-insulin is an insulin-like hypoglycemic protein, that has been reported by some researchers to cause hypoglycemia in gerbils, langurs and humans when injected subcutaneously

(Joseph and Jini, 2013, Lo et al., 2013). Polypeptide-p works by imitating the action of human insulin and may be used as plant-based insulin replacement in type-1 diabetic patient (Joseph and Jini, 2013, Lo et al., 2013). Recently (Wang et al., 2011), have cloned and expressed the 498 bp gene sequence coding for the *M. charantia* polypeptide-p gene and have also proved the hypoglycemic effect of the recombinant polypeptide in alloxan induced diabetic mice.

1.15.4.3 Vicine

Another major compound that has been isolated from the seeds of *M. charantia* is vicine. It is a pyrimidine nucleoside shown to induce hypoglycemia in non-diabetic fasting rats by intraperitoneal administration (Joseph and Jini, 2013, Zhang et al., 2004).

1.15.5 Selected Pharmacological properties of *M. charantia*

1.15.5.1 Antidiabetic activity

The phytochemical constituents of *M. charantia* such as charantin, vicine, glycosides and polypeptide-p are hypoglycemic in action and improve blood sugar levels by increasing glucose uptake and glycogen synthesis in the liver (Gupta et al., 2011). These constituents is also known to restore a damaged insulin-secreting beta cells and enhance insulin release from the pancreatic beta cells (Ahamad et al., 2017). Kumar et al., 2010 and Shain et al., 2015, reports that a phytochemical constituent, (polypeptide) particularly from the seeds have been shown to decreased and normalize the blood sugar level in rats. *M. charantia* also contains another bioactive component known as lectin which mimics insulin action. This compound is known to reduce blood glucose levels by exerting its action on peripheral tissues. This common principle is similar to insulin's effects in brain in trying to suppress appetite. Lectin is an important compound for balancing blood glucose levels and develops after taking *M. charantia* (Jha et al., 2018, Jia et al., 2017). Another important phytochemical constituent is charantin. This can be extracted by alcohol and possesses rich hypoglycemic agents which are composed of mixed steroids. Charantin as a compound is useful in treating diabetes as it rapidly lowers blood glucose levels (Kumar et al., 2010, Dhiman et al., 2012).

1.15.5.1.1 Possible mode of hypoglycemic action of *M. charantia* extracts

These components of *M. charantia* extracts are believed to exert their blood glucose lowering effects via different physiological, pharmacological and biochemical modes (Singh et al., 2011, Garau et al., 2003, Platel and Srinivasan, 1995). Possibly, there mode of actions are: stimulation of peripheral and skeletal muscle glucose utilization, inhibition of intestinal glucose uptake, inhibition of adipocyte differentiation,

suppression of key gluconeogenic enzymes, stimulation of key enzyme of HMP pathway, and preservation of islet β cells and their functions (Singh et al., 2011, Joseph and Jini, 2013, Nerukar et al., 2010).

According to (Kim and Kim, 2011), *M. charantia* suppressed the activation of mitogen- activated protein kinases (MAPKs) and the activity of nuclear factor kappa B (NF- κ B). The findings shows that *M. charantia* protects pancreatic β -cells through down regulation of MAPKs and NF- κ B in MIN6N8 cells (Kim and Kim, 2011). A similar study suggests that *M. chanratria* improves the serum and liver lipid profiles and serum glucose levels by modulating Peroxisome proliferator-activated receptor gamma (PPAR- γ) gene expression (Saad et al., 2017).

1.15.5.2 Antioxidant activity

Various parts of *M. charantia* have been used and are still in use to treat different diseases and to boost immunity in many parts of the world. Antioxidant activity of bitter melon has been reported by (Sathishsekar and Subramanian, 2005). There results showed that seeds of *M. charantia* normalized the impaired antioxidant status in streptozotocin induced- diabetic rats compared to the glibecamide treated groups (Sathishsekar and Subramanian, 2005).

1.15.5.3 Antiviral activity

Alpha-and beta- momorcharin which are two proteins that are extracted from the seeds, fruits and leaves of *M. charantia* have been establish as a potent inhibitor of HIV in an *in vitro* study. HIV infected cells treated with these proteins showed a nearly complete loss of viral antigen (Lee-huang et al., 1990). This novelty led the inventors of the chemical protein along with anti-HIV protein (MAP-30) to file a U.S. patent, stating that it was “useful for treating tumors and HIV infections”. In treating HIV infection, the protein can be administered alone or in conjunction with conventional AIDS therapies (Gupta et al., 2011). In another study, researchers also documented the *in vitro* antiviral activity against other viruses like *Epstein-Barr* and herpes viruses (Bourinbaiar and Leehuang, 1995, Lee-huang et al., 1995).

1.16 Kidney: Organ of study

The kidneys have important physiological functions including maintenance of water and electrolyte balance, secretion of hormones and excretion of waste products from the body. In addition, the kidneys also play a role in the excretion of drugs and xenobiotic, and hence may be exposed to high concentrations of drug metabolites making it liable to drug toxicity (Declodt and Maartens, 2011). It is therefore not a surprise that drug-induced kidney injury contributes up to 25% of all cases of acute renal

failure. Drug-induced kidney injury may cause cumulative dose-dependent toxicity or idiosyncratic dose-independent toxicity at any time during therapy (Decloedt and Maartens, 2011). Kidney injury may occur in various areas of the kidney such as the glomerulus, the collecting ducts and the tubulointerstitium where exchange of solutes and reabsorption takes place (Decloedt and Maartens, 2011).

During the process of metabolism, a lot of waste materials are produced in the tissues. Apart from these, the residue of undigested food, drugs, toxic substances and other pathogenic organisms like bacteria are also present in the body. All these substances must be removed to keep the body in a healthy condition. Although various organs in the body are involved in performing these functions, their excretory capacity is limited. The kidney has the maximum capacity of excretory function and so it plays the major role in homeostasis (Hall, 2015).

1.17 Embryology of the mammalian kidney

The mammalian kidney develops from intermediate mesoderm, proceeds through a series of three successive phases, each marked by the development of a more advanced pair of kidneys: the pronephros, mesonephros, and metanephros (Carlson, 2004).

The pronephros develops at the beginning of the fourth week of intrauterine life (IUL) and is represented by 7 to 10 solid cell groups in the cervical region. These groups form vestigial excretory units, called nephrotomes that regress before more caudal ones are formed. By the end of the fourth week of IUL, all indications of the pronephric system have disappeared (Sadler, 2018).

The mesonephros and mesonephric ducts are derived from intermediate mesoderm from upper thoracic to upper lumbar segments. Early in the fourth week of IUL, during regression of the pronephric system, the first excretory tubules of the mesonephros appear. They lengthen rapidly, form an S-shaped loop and acquire a tuft of capillaries that will form a glomerulus at their medial extremity. Around the glomerulus the tubules form Bowman's capsule and together these structures constitute a renal corpuscle. Laterally the tubule enters the longitudinal collecting duct known as the mesonephric or Wolffian duct (Sadler, 2018).

The metanephros or permanent kidney appears in the fifth week of IUL. Its excretory units develop from metanephric mesoderm in the same manner as the mesonephric mesoderm. The collecting duct of the metanephros develops from the ureteric bud, an outgrowth of the mesonephric duct close to its entrance to the cloaca. The bud penetrates the metanephric blastema which is molded over its distal end as a cap. Subsequently the bud dilates forming the primitive renal pelvis and splits into cranial and caudal portions, the future major calyces. Each calyx forms two new buds while penetrating the metanephric tissue. These

buds continue to subdivide until 12 or more generations of tubules have formed. Meanwhile at the periphery more tubules form until the end of the fifth month of IUL. The tubules of the second order enlarge and absorb those of the third and fourth generations forming the minor calyces of renal pelvis. During further development, collecting tubules of the fifth and successive generations elongate and converge on the minor calyx forming the renal pyramid (Sadler, 2018).

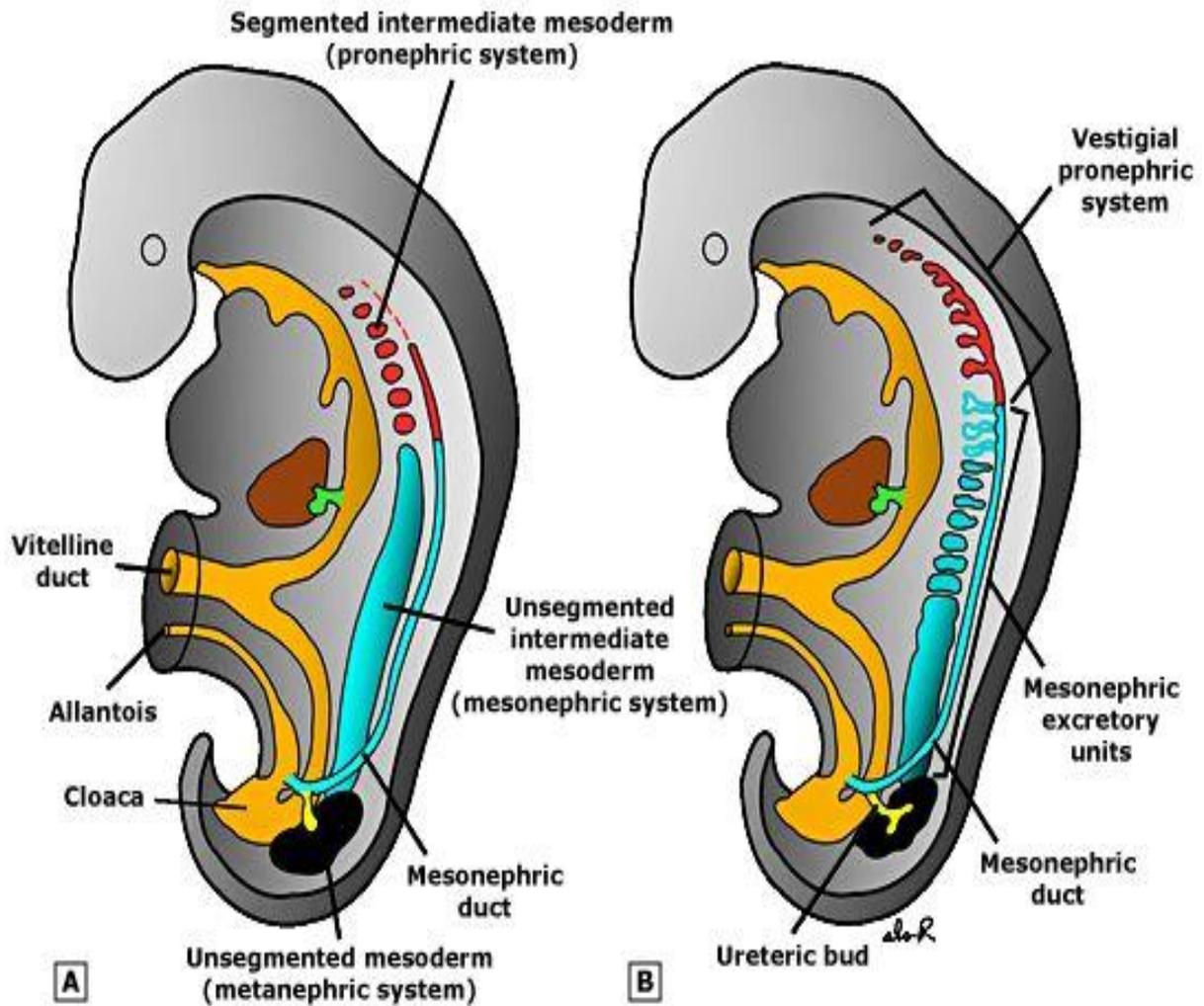


Figure 1.14: Picture showing embryology of the mammalian (human) kidney (Adopted from Medical embryology, Sadler 2018).

A. Relationship of the intermediate mesoderm of the pronephric, mesonephric, and metanephric systems. In cervical and upper thoracic regions intermediate mesoderm is segmented; in lower thoracic, lumbar, and sacral regions it forms a solid, unsegmented mass of tissue, the nephrogenic cord. The longitudinal collecting duct is formed initially by the pronephros but later by the mesonephros.

B. Excretory tubules of the pronephric and mesonephric systems in a 5-week old embryo.

1.18 Histology of the kidney

Microscopic anatomy of the kidney shows that it is composed of interstitial cells and functional units called the nephron. Each nephron consists of tufts of anastomosing capillaries called the glomeruli, formed from the afferent arteriole and draining into the efferent arteriole and a tubular system called the renal tubule. Epithelial cells called podocytes (or visceral epithelium of Bowman's capsule) invest the glomerulus.

The Bowman's capsule is the distended end of the tubular system and is invaginated by the glomerulus. The space between the glomerulus and Bowman's capsule is the urinary space. Extending from the capsule is the proximal tubule which is lined by cuboidal and columnar epithelial cells containing many mitochondria and a prominent brush border (Rouiller, C., 1969).

The proximal convoluted tubule is the longest portion of the tubular system and is made up of convoluted proximal and distal straight (pars recta) segments. The pars recta descend into the medulla where it forms the U-shaped loop of Henle. The loop of Henle re-enters the cortex within which it forms the straight and convoluted segments of the distal tubule (Rouiller, C., 1969).

The distal tubule runs close to the glomerular hilum and forms a specialized segment called the macula densa. The distal tubule is lined by cuboidal epithelium that lacks a brush border. The distal tubule empties into collecting tubules which in turn drain into collecting ducts. The collecting duct converges as they approach the medulla to form the collecting ducts of Bellini which run vertically through the medulla to the papillae. The collecting tubules and ducts are lined by pale-staining, cuboidal epithelial cells (Rouiller, C., 1969).

The interstitium is made up of the interstitial cells which consist of fibroblast-like cells, lipid-laden interstitial cells, macrophages, interstitial dendritic cells and perivascular cells. The fibroblast-like cells are mainly located in renal cortex and medulla, and have long cytoplasmic processes forming a reticular network between the tubules and capillaries (Rouiller, C., 1969).

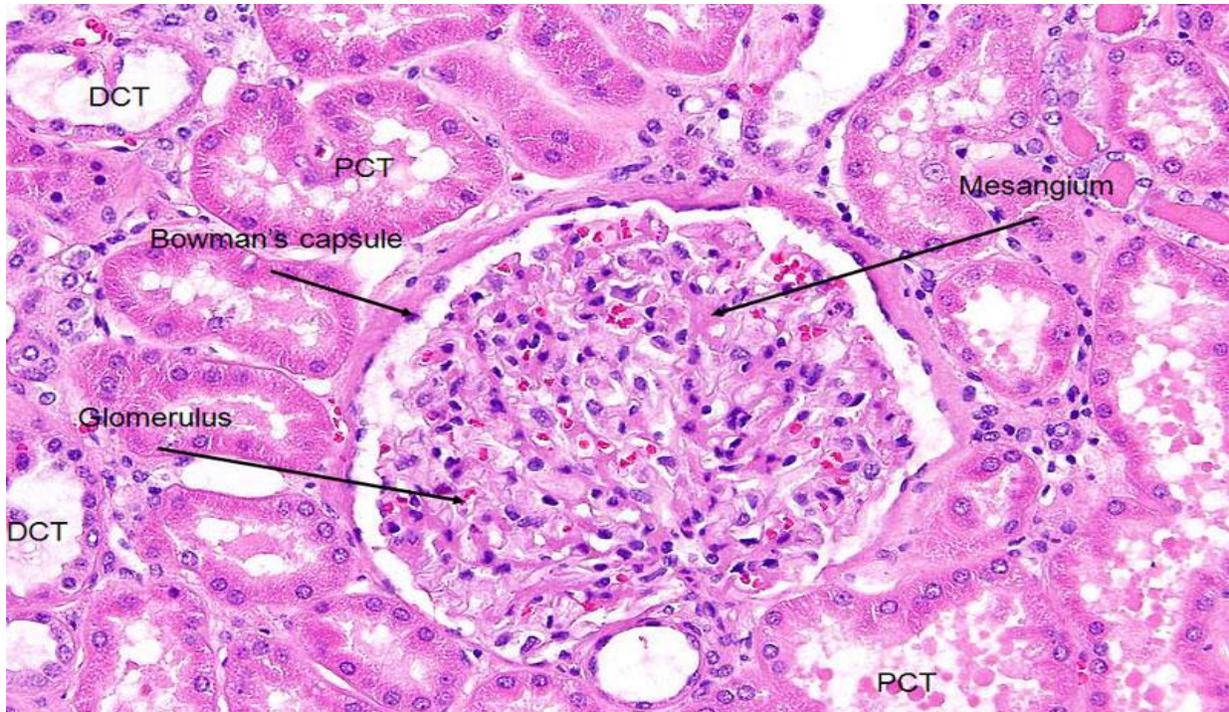


Figure 1.15: Photomicrograph of kidney tissue. Source: [https://www.auanet.org/education-products-and-resources/pathology-for-urologists/normal-histology-and-important-histo-anatomic-structures/kidney-renal-corpusele-\(glomerulus\)](https://www.auanet.org/education-products-and-resources/pathology-for-urologists/normal-histology-and-important-histo-anatomic-structures/kidney-renal-corpusele-(glomerulus)). Assessed on 18th May 2019.

1.19 Glomerular filtration barrier

The glomerular filtration barrier is made up of three layers of components which include: the fenestrated capillary endothelium, the intervening glomerular basement membrane and filtration slits between podocyte processes. The major component of the filter is formed by fusion of the basal laminae of a podocyte and a capillary endothelial cell (The McGraw-Hill Companies, Chapter 19. The Urinary System).

(Figure 1.16): TEM showing cell bodies of two podocytes (PC) and the series of pedicels on the glomerular basement membrane separated by the filtration slits (arrows). On the other side of the membrane is the thin lining of a capillary (C) endothelial cell, with fenestrations. Together these openings allow filtration of liquid from plasma into the urinary space (US) of Bowman's capsule (The McGraw-Hill Companies, Chapter 19. The Urinary System).

(Figure 1.17): At higher magnification, both the fenestrations (arrowhead) in the capillary endothelium (E) and the filtration slits (arrows) separating the pedicels (P) are better seen on the two sides of the fused basal laminae (BL). The endothelial fenestrations in glomeruli lack diaphragms, but very thin slit

diaphragms cross the space between pedicels and play an important role in filtration (The McGraw-Hill Companies. Chapter 19. The Urinary System).

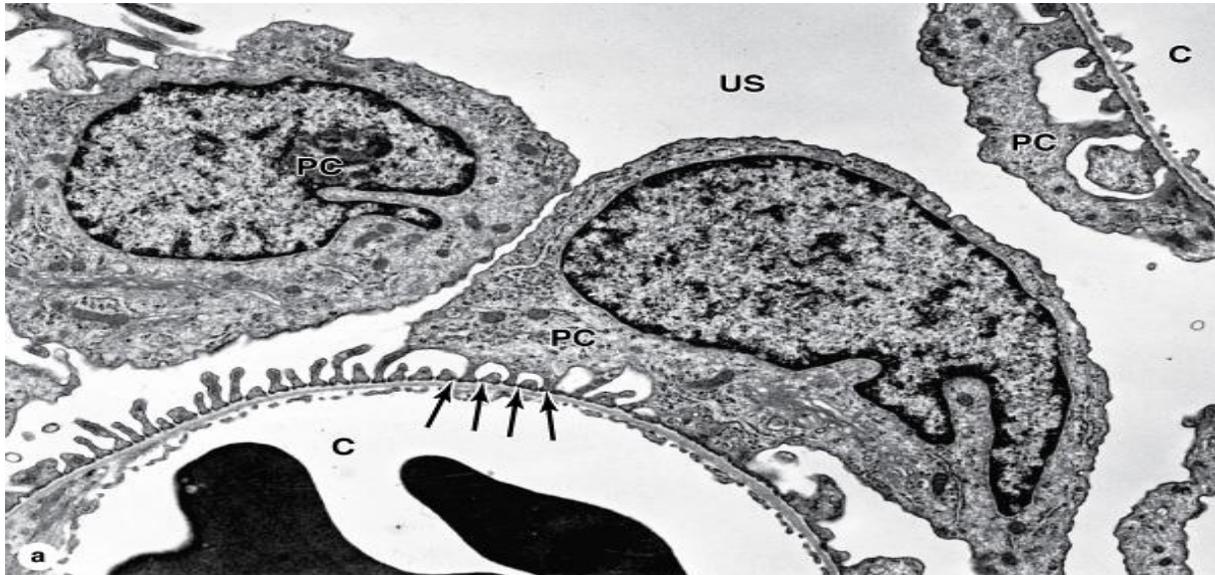


Figure 1.16: Ultrastructural micrograph of the kidney. **Source:** (Mescher, 2013). *Junqueira's basic histology: text and atlas*, (The McGraw-Hill Companies. Chapter 19. The Urinary System).



Figure 1.17: Ultrastructural micrograph of the kidney. **Source:** (Mescher, 2013) *Junqueira's basic histology: text and atlas*, (The McGraw-Hill Companies. Chapter 19. The Urinary System).

1.20 Gross anatomy of the mammalian kidney

The kidneys are bilateral, bean-shaped excretory organs in vertebrates. In humans, the kidneys are located on the posterior abdominal wall in retro-peritoneal position with the right kidney slightly lower than the left due to the asymmetry within the abdominal cavity caused by the liver. The right kidney sits just below the diaphragm and posterior to the liver while the left kidney is below the diaphragm and posterior to the spleen. Each adult kidney weighs between 125 and 170g in males and between 115 and 155g in females. The left kidney is usually slightly larger than the right kidney (Fenton and Praetorius, 2016).

Resting on top of each kidney is the adrenal gland. The upper parts of the kidneys are partially protected by the eleventh and twelfth ribs, and each whole kidney and adrenal gland are surrounded by two layers of fat (the perirenal and pararenal fat) as well being covered by the renal fascia (Fenton and Praetorius, 2016).

Internally, the kidney is divided into the outer cortex and inner medulla. The medulla further divides into triangular shaped structures called the renal pyramids. The tip of each pyramid empties urine into a minor calyx; minor calyces empty into major calyces, and major calyces empty into the renal pelvis, which becomes the ureter as shown in figure 1.18 (Mescher, 2013).

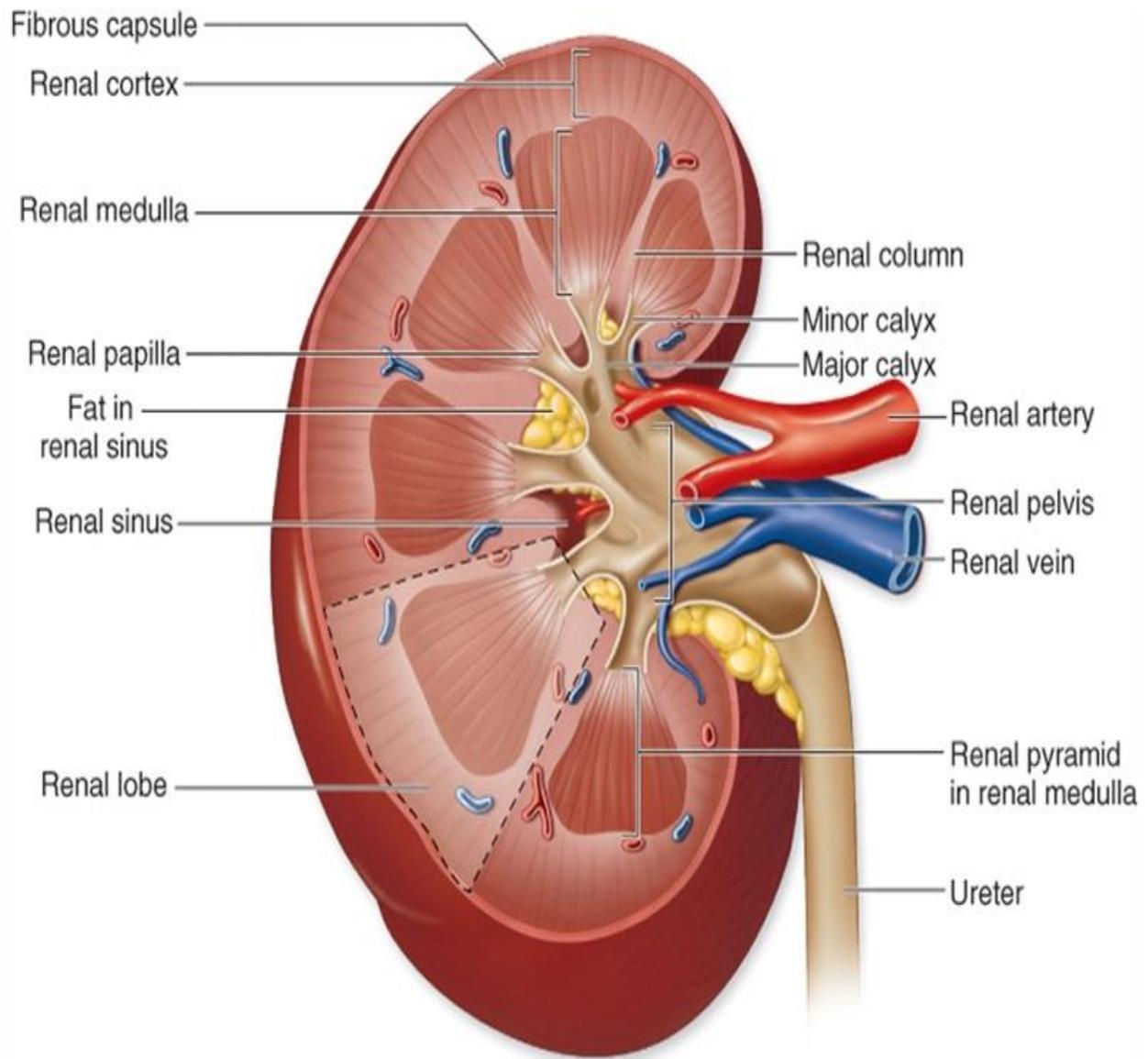


Figure 1.18: Picture showing kidney: Source: Anthony L Mescher: Junqueira's Basic Histology 2013: Text and Atlas 15th Edition. (The McGraw-Hill Companies. Chapter 19. The Urinary System).

1.21 Functions of the kidney

The kidney performs the function of osmoregulation and excretion of substances through the nephron by the following processes:

1. Filtration of most small molecules from blood to form an ultra-filtrate of plasma.
2. Selective reabsorption of most of the water and some other molecules from the ultra-filtrate, leaving behind excess and waste materials to be excreted.
3. Secretion of some excretory products directly from blood into the urine.
4. Maintenance of acid-base balance by selective secretion of H^+ ions into the urine

1.22 Kidney of other animals

The kidney of fish, amphibians, reptiles, birds and mammals show increasing sophistication in conservation of water and minerals. The kidneys of fish and amphibians are typically narrow, elongated organs, occupying a significant portion of the trunk. The collecting ducts from each cluster of nephrons usually drain into an archinephric duct (Romer and Parsons, 1977).

The kidneys of reptiles consist of a number of lobules arranged in a broadly linear pattern. Each lobule contains a single branch of the ureter in its center, into which the collecting ducts empty. Reptiles have relatively few nephrons compared with other amniotes of a similar size, possibly because of their lower metabolic rate (Romer and Parsons, 1977).

Birds have relatively large, elongated kidneys, each of which is divided into three or more distinct lobes. The lobes consist of several small, irregularly arranged, lobules, each centered on a branch of the ureter. Birds have small glomeruli, but about twice as many nephrons as similarly sized mammals (Romer and Parsons, 1977).

1.24 The rat kidney

The rat kidneys are located ventrolateral to the vertebral column in the retro-peritoneum just like in humans and other mammals. Each rat kidney has a convex and a concave surface. The renal hilum lies in the concave surface and is the point where the renal artery enters the kidney and the renal vein and ureter exit. Internally, the parenchyma of the rat kidney is divided into two areas: the outer cortex and the inner medulla. The cortex extends into the medulla dividing it into triangular shapes known as renal pyramids. The renal pyramids contain the renal corpuscles and the convoluted tubular segments (Yoldas, A., and Dayan, M.O. 2014).

Each adult rat kidney contains roughly 30000-35000 nephrons. The nephron begins in the cortex with the renal corpuscle. The corpuscle consists of a capillary tuft (glomerulus) which is pushed into a blind expansion of the Bowman's capsule. The tubular part of the nephron consists of the proximal convoluted tubule, the loop of Henle and the distal convoluted tubule. According to the location of the corpuscles in the cortex, the nephrons can be distinguished into three types: superficial, midcortical and juxtamedullary nephrons, and according to the length of the loop of Henle the nephrons of the rat kidney may be subdivided into two different types: nephrons with short loops and nephrons with long loops (Bachmann et al., 1986).

1.23 Problem statement

The metabolic disorder associated with HAART have become a public health problem contributing to more than 50% of kidney injuries, a fraction which may require immediate transplantation (Fortuny et al., 2015). It is anticipated that as HIV/AIDS patients continue to depend on HAART for their survival, HAART-related metabolic disorders would correspondingly increase, this will imply a huge economic cost and care. The cost of HIV care in the United States is a critical issue considering that there are approximately 1.2 million PLWHA. A recent study estimated that the lifetime medical costs for an individual who becomes infected with HIV at age 35 was \$326,500, with 60% of those expenses attributable to the costs of antiretroviral medication (Ritchwood et al., 2017, Schackman et al., 2015). This estimate was 3.3 times higher than the lifetime costs of medical care for high-risk individuals who remain uninfected and include the medical costs related to treating both HIV and non-HIV-related conditions. Between 2005 and 2015, HIV infections rose by 87% among young, African American. Additionally, in 2002, the costs associated with new HIV cases in the United States were estimated to be \$6.7 billion in direct medical costs and \$29.7 billion in indirect costs that result from productivity losses, which describes lost economic opportunities. The costs for lifetime treatment based upon the number of new diagnoses were estimated to be \$16.6 billion in 2009 (Ritchwood et al., 2017, Chen et al., 2006). Longer lifespans and the over-representation of members from vulnerable groups living with the virus have important implications for healthcare systems, particularly as it relates to resource utilization and allocation, and cost expenditures (Ritchwood et al., 2017, Fleishman et al., 2005).

DM is a major degenerative disease, found in all parts of the world and it is becoming the third most lethal disease of mankind and increasing rapidly (Joseph and Jini, 2013, Ogonnia et al., 2008). DN, which is one of the microvascular complications of diabetes characterized by overall decline in glomerular filtration rate and significant hypertrophy and hyperplasia of the kidney tissue, is a life threatening condition affecting large number individuals globally (Haneda et al., 2015). At present, DN

encompasses other diseases such as atheroembolic disease, ischemic nephropathy, and interstitial fibrosis that occur as a direct result of diabetes (Toth-Manikowski and Atta, 2015).

While diabetes incidence and nephrotoxicity is increasing in the general population of PLWHAs, there is very little describing the relationship between diabetes and nephrotoxicity among HIV-infected population under HAART in South Africa.

1.25 Justification for study

The problem of drug toxicity continues to attract the attention of researchers both in experiments and clinical trials. Evidence shows that animal models of drug toxicity represent a similar pattern in humans. For example, one-third of all drugs associated with nephrotoxicity in animals resulted in liver enzyme elevation in humans (Amacher, 1998).

Animal models of diabetes have provided understanding of pathogenesis and progression of diabetes mellitus in humans. Furthermore, animal models have allowed the evaluation of various therapeutic interventions which may be potentially used in man. Uses of animal models have made possible studies of disease characteristics in humans by providing genetic and immunological modifications that are not possible in humans (Van der Worp et al., 2010, Rees and Alcolado, 2005). In this study, the rat serves as a suitable model for investigating the mammalian kidney based on its similar structure and physiologic functions, while being easy to handle and generally considered less expensive.

The associated toxicities of HAART necessitate the need for safe and less costly adjuvants that can mitigate these effects. Plant-based extracts have the potential to fill this need due to its perceived effectiveness. An increasing reliance on the use of medicinal plants in industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants (Somanah et al., 2018).

The Southern African ecosystem is replete with medicinal plants that continue to drive improvement in indigenous knowledge systems. Estimates of about 80% of the general population in sub-Saharan Africa rely on African traditional medicines to treat various diseases (Azu et al., 2016). *M. charantia* falls into this category. The plethora of activities attributed to *M. charantia* extracts particularly its radical scavenging antioxidant and antidiabetic actions makes it a potentially good therapeutic candidate that may mitigate the effects of HAART and hyperglycaemia-induced oxidative damage in the kidney associated with diabetes mellitus. However, the scientific validation of these claims remains to be verified.

1.26 Contribution to knowledge

Results from this study would assist in the understanding of renal changes under diabetic condition with HAART hence provide elucidation of possible renal end-organ injuries. The result would shed further knowledge on the antioxidant, antidiabetic and anti-inflammatory properties of *M. charantia* in alleviating the associated toxicities of HAART. The publications that will emanate from this study will contribute to the existing body of knowledge on the potentials and usefulness of *M. charantia* as an adjuvant in mitigating nephrotoxicity and metabolic disorders.

1.27 Research questions

1. Does *M. charantia* ameliorate nephrotoxicity following HAART?
2. Does *M. charantia* have anti-diabetic effects?
3. Can *M. charantia* mitigate renal function impairment following HAART toxicity and diabetes in diabetic nephropathy?
4. Can the known toxicities of HAART on the renal histo-morphology and renal ultrastructural changes be mitigated by the plant based adjuvant *M. charantia*?
5. Can *M. charantia* improve mRNA expression profile of KIM-1, NGAL and TNF- α following HAART toxicity and diabetic nephropathy?

1.28 Aim

The overall aim of this study is:

1. To investigate the effects of *M. charantia* in the kidney following HAART in diabetic condition.

1.29 Specific Objectives

1. To determine the histomorphology of the kidneys using H&E, PAS and MT.
2. To weekly measure blood glucose levels
3. To evaluate urine samples every 3 weeks
4. To measure renal function test
5. To measure oxidative stress levels
6. To determine the ultrastructural changes of the kidney in diabetic and non-diabetic animals.
7. To determine the circulating mRNA expression profile of NGAL, KIM-1 and TNF- α in diabetic and non-diabetic animals.

1.30 Materials and Methods

Ethical approval:

The University of KwaZulu Natal Animal Research Ethics Committee gave full approval of the project with a reference number: **AREC/033/016D**.

Antiretroviral drug

Triplavar (*Cipla-Medpro*) containing Lamivudine 150 mg, Nevirapine 400 mg and Zidovudine 300 mg, was used for this study. The drug was obtained from Pharmed pharmaceuticals, Pty (Ltd) Durban, South Africa.

Preparation of *M. charantia* fruit ethanolic extract

Fifty kilogram of fresh mature unripe fruit of *M. charantia* was purchased from the local Durban markets between May-June 2016. Samples were authenticated at the herbarium unit of the Department of Life Sciences, University of KwaZulu-Natal, Durban, South Africa (voucher no. 4617). The fruits were cleaned, sliced into small pieces and the seeds separated out and discarded. The sliced green fruit was first weighed and then dried in shade for approximately 2 weeks. It was then weighed again to obtain the final dry weight before pulverizing into a fine power in a commercial grinder and stored at 5 °C until ready for extraction. The active ingredients were obtained by Soxhlet extraction using 100 % ethanol as the solvent. The solvent was evaporated in a rotary evaporator at 40°–50°C with a percentage yield of 85.25%. The wet residue was filtered through a whatman filter and the concentrated extract was stored at 4 °C ready for use.

Animal management and Experimental design

A total of seventy eight (78) adult male Sprague-Dawley rats weighing 178-232 grams were used for the study. The investigation was carried out at the Biomedical Research Unit Animal housing facility, University of KwaZulu-Natal, South Africa. The animals received humane care in accordance with the principle of laboratory animal care of the National Medical Research Council and the Guide and use of Laboratory animals of the National Academy of Sciences (National institute of health care 1985). All rats were housed in well ventilated plastic cages [3 rats per cage for the non-diabetic group (in 12 cages)] and [3 rats per cage for the diabetic group (in 14 cages)]. The dimensions of cages are (52 cm long × 36 cm wide and 24 cm high) and soft wood shavings served as beddings in the cages. Animals were maintained under standardized animal house conditions (temperature: 23–25 °C; light: approximately 12 h natural light per day) and were fed with standard rat pellets from (Meadow feeds a Division of Astral Operations Limited, Durban, South Africa) and given tap water ad libitum. The initial body weight of the animals

were recorded before treatment and randomly distributed to non- diabetic group (A-F comprising 6 animals per group) and diabetic group (G-L comprising 7 animals per group) as shown in the treatment schedules below.

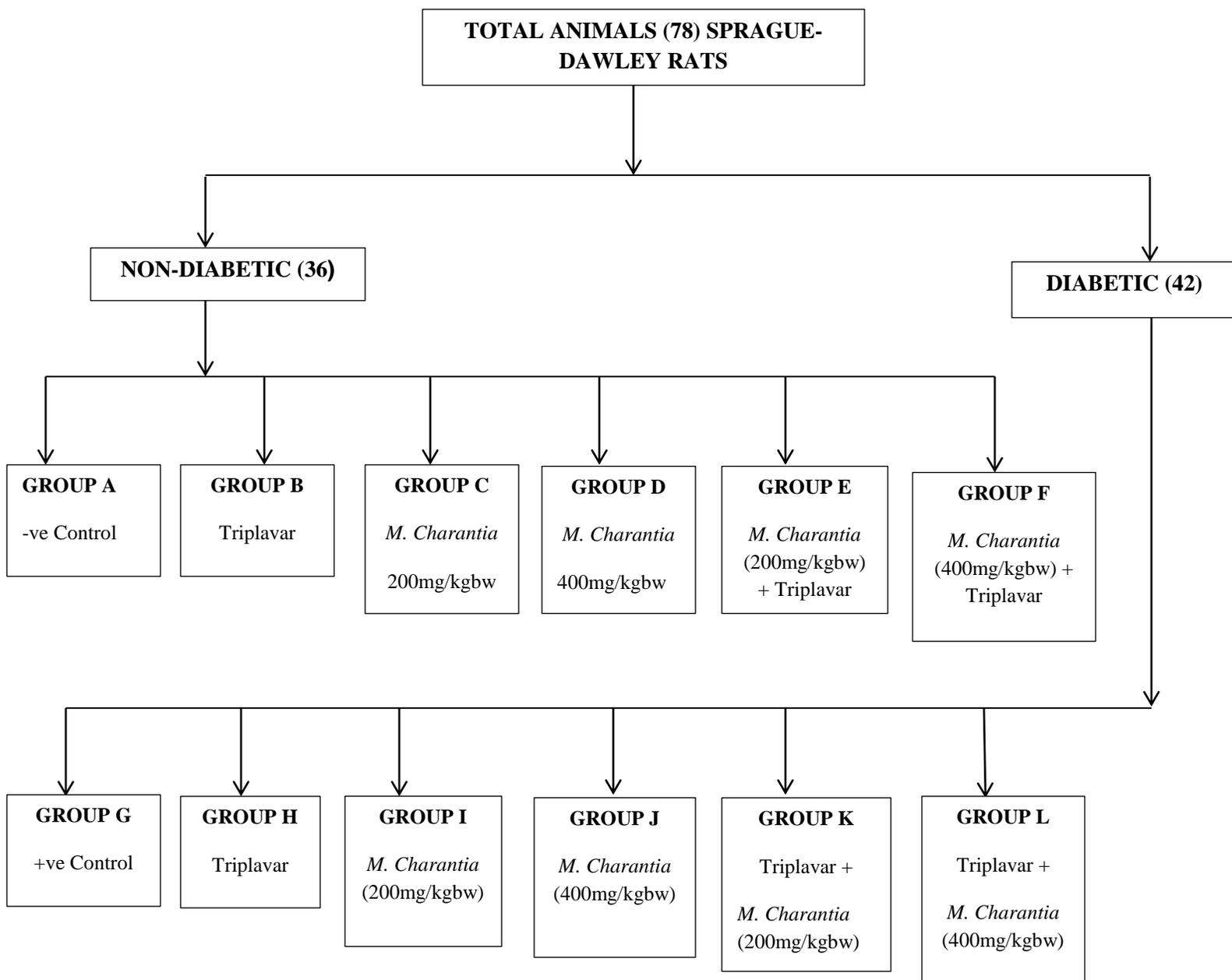


Figure 1.19: Flow charts showing groupings of animals and treatment schedules.

Induction of diabetes mellitus

All rats were placed on a 12 hours fast to obtain baseline fasting blood glucose levels (FBG). The experimental groups (B- G) were given intra-peritoneal streptozotocin (STZ) (Sigma-Aldrich Chemical Company, Missouri, St Louis, USA) at 45 mg/kg body weight dissolved in a citrate buffer (pH 4.5) (Etuk 2010). Successful induction of diabetes was determined by observation of polyuria and polydipsia and confirmed by a 72 hours post STZ FBG level ≥ 11 mmol/L.

Measurement of blood glucose

Blood samples were obtained from the tail using sterile needle prick. Glucose levels were measured once a week during the 10 weeks treatment using the one touch ultra-glucometer (Boehringer-Mannheim, Germany).

Measurements of body weight and collection of urine samples

All experimental animals were weighed weekly by a digital scale (Mettler-Toledo, 200). For collection of urine, the rats were placed in metabolic cages for 24 hour and provided with rat chow and water. The urine volume was measured and urine centrifuged to separate out debris. Urine samples were kept at -80°C until further analysis. This procedure was done at weeks 3, 6 and 9 during the 10 week experimental period.

Measurement of oxidative stress parameters and lipid peroxidation

Blood was collected in plain tubes via cardiac puncture and allowed to clot. It was then centrifuged at 3000 revolutions per minute (rpm) for 15 minutes and the serum decanted into eppendorf tubes and stored at -20°C for subsequent use. Serum was assayed for Lipid Peroxidation (LPO), Reduced Glutathione (GSH) level, Superoxide Dismutase (SOD) and Catalase activities (CAT).

Serum lipid peroxidation levels

This was measured using a complex formed from the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA) as described by (Hodges et al., 1999) . Into an assay mixture containing 200 μL of 8.1% sodium dodecyl sulfate (SDS), 750 μL of 20 % acetic acid (pH, 3.5), 2 mL of 0.25% TBA and 850 μL of distilled water, 200 μL of sample or MDA standard series (0, 7.5, 15, 22.5, and 30 μM) was added in a pyrex screw capped test tube. The mixture was heated at 95°C for 60 min in a sand bath, cooled down to room temperature and absorbance was read at 532 nm in a spectrophotometer (UVmini-

1240, Shimadzu Japan). Thiobarbituric acid reactive substances (TBARS) concentrations of samples were extrapolated from MDA standard curve.

Serum reduced glutathione concentration (GSH)

Reduced glutathione concentration was measured in serum according to methods modified from (Rahman et al., 2006). The sample was first precipitated with 10% Trichloroacetic acid (TCA) and then centrifuged at 2000 rpm for 10 min at 25 °C. The reaction mixture contained 400 µL of supernatant, 200 µL of 0.5 mM 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 1.2 mL of 0.2 M sodium phosphate buffer (pH, 7.8). Absorbance was measured at 415 nm after 15 min incubation at 25 °C and GSH concentrations of samples was extrapolated from standard curve of GSH.

Anti-oxidant enzyme activities

Superoxide dismutase

Superoxide dismutase (SOD) activity was assayed according to the method of (Weydert and Cullen 2010). A 15µL of 1.6 mM 6-hydroxydopamine (6-HD) was added to an assay mixture containing 170 µL of 0.1M diethylenetriamine – penta acetic acid (DETAPAC) in 50 mM sodium phosphate buffer (pH, 7.4) and 15µL of sample (serum) containing 0.1µg/µL of protein was used to start the reaction. The linear increase in absorbance was monitored at 490 nm for 5 min at 25 °C. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1µmol of 6-HD/min/µg protein.

Catalase

Catalase activity was measured using the method described by (Weydert and Cullen 2010). Into an assay mixture containing 340 µL of assay buffer (50 mM potassium phosphate buffer, pH 7.0) and 150 µL of 10 mM H₂O₂, 10 µL of sample containing 0.1µg/µL protein was added to start the reaction. The linear increase in absorbance was monitored at 240 nm for 5min at 25 °C. One unit of enzyme activity was expressed as the amount of enzyme needed to decompose 1mmol of H₂O₂ /min/µg protein.

Assessment of renal function

Serum was used for the estimation of blood urea nitrogen (BUN) and serum creatinine (CR-S) using Beckman Coulter Synchron® system(s) BUN and CR-S assay kit. Beckman Coulter Synchron® system BUN assay kit and Beckman Coulter Synchron® system CR-S assay kit were obtained from Global Viral Laboratory, Durban, South Africa

RNA extraction and cDNA Synthesis

Blood was collected in plain tubes via cardiac puncture and allowed to clot. It was then centrifuged at 3000 revolutions per minute (rpm) for 15 minutes and the serum decanted into Eppendorf tubes and stored at -20°C pending RNA extraction. RNA was isolated using the Zymo Research Quick-RNA™ Miniprep, CA-USA according manufacturer's protocol. The iScript cDNA synthesis kit (Bio-Rad, USA) was used to perform reverse transcription (RT). Manufactures protocol was followed in preparation of the reagents as well as volumes of reagents used.

Real –Time Polymerase Chain Reaction

The ROCHE light cycler SYBR Green I master mix was used to carry out PCR amplifications on ROCHE light cycler 96 machine. The Primer sequence used can be found in the table 1.7 below. PCR was done using the following cycling conditions: Pre-incubation for 10 min at 95°C , followed by 45 cycles of 95°C for 15 s, 60°C for 30s and 72°C for 30s. All samples were assayed in duplicate with a positive and negative control included in each run. The results were analysed using the $2^{-\Delta\Delta\text{Cq}}$ comparative method to compare Cq values of the treated groups to the control group. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used to normalise the Cq values of the treated groups and control groups. To calculate the fold change, a value of 1 was assigned to the controls (Anier et al., 2010).

Table 1.7: Gene primer sequence

GENE	FORWARD	REVERSE
TNF- α	5'-GACCCTCACACTCAGATCATCCTTCT-3'	5'-ACGCTGGCTCAGCCACTC-3'
KIM-1	5'-AACGCA GCG ATT GTG CAT CC-3'	5' -GTA CAC TCACCA TGG TAA CC-3'
NGAL	5'-GAT GAA CTGAAG GAG CGA TTC-3'	5'-TCG GTG GGA ACAGAG AAA AC-3'
GAPDH	5'-CCT GGA GAA ACCTGC CAA GTA T-3'	5'-AGC CCA GGA TGC CCT TTA GT-3'

Histopathological Examination of the rat kidneys

The rat kidneys were weighed and examined for any gross pathology. A phosphate buffer solution (PBS) was used to wash out blood before preparation for tissue fixation. Kidneys were fixed in 10% neutral buffered formalin for 24 hours. The samples were transferred to 70% ethanol solution. Ascending grades of alcohol (50%, 70%, 90%, 100%) were then used to dehydrate the samples and xylene was used as a clearing agent. The samples were immersed in molten paraffin wax at 58°C to 62°C . The prepared blocks

were cut into slices of 5 µm) using a microtome (Microtome HM 315, Walldorf, Germany) and stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and Masson's trichrome (MT).

Haematoxylin and Eosin (H&E) Staining

Slides were placed in staining jar and deparaffinized by submerging into three series of absolute xylene for 4 minutes followed by 100%, 100%, 95%, 90%, and 70% of ethanol for 4 minutes of each percentage. Next, slides were washed in running tap water for 2 minutes. Then, slides were submerged into Harris Hematoxylin (Sigma-Aldrich, GERMANY) for 2 minutes and then washed in running tap water for 2 minutes. The slides were then submerged into 1% acid alcohol for 3 dips to decolorize it and washed in running tap water for 2 minutes. After that, slides were submerged into Eosin for 2 minutes followed by washing in running tap water for 2 minutes. Stained slides were dried for 24 hours at 38°C. Before observation, slides were dipped into absolute xylene for 1 minute and finally mounted with cover slip using DPX mounting.

Periodic acid Schiff (PAS) Staining

PAS staining technique was used to detect the presence of polysaccharides (e.g., glycogen) and mucosubstances (e.g., glycoproteins, glycolipids, and mucins) in rat kidney tissues. Slides were placed in staining jar and deparaffinized by submerging into three series of absolute xylene for 4 minutes followed by 100%, 95%, 90%, and 70% of ethanol for 4 minutes of each percentage. Then slides were placed in distilled water for 3 minutes. Then, slides were submerged into Periodic Acid Solution for 5 minutes at room temperature (18–26°C). Next slides were rinsed well in several changes of distilled water. Thereafter slides were immersed in Schiff's reagent for 5-15 minutes at room temperature (18–26°C) until deep magenta. Next slides were washed in running tap water for 5 minutes and counterstained with haematoxylin solution for 1 minute. Next slides were rinsed in running tap water for 2 minutes. Slides were then dehydrated in graded series of alcohol of 70%, 80%, 95% and 100% for 1 minutes each percentage. Before observation, slides were dipped into absolute xylene for 1 minute and finally mounted with cover slip using DPX mounting.

Masson's Trichrome (MT) Staining

The MT staining is a three-color staining protocol used in histology for kidney pathologies. It is suited for distinguishing cells from surrounding connective tissue staining red keratin and muscle fibers, blue or green collagen and bone, light red or pink cytoplasm, and dark brown to black cell nuclei. It also differentiates between collagen and smooth muscle in tumors, and the increase of collagen in diseases such as cirrhosis. It is a routine stain for kidney biopsies.

Method was modified from Dries, (2008). Slides were placed in staining jar and deparaffinised by submerging into three series of absolute xylene for 4 minutes each followed by 100%, 95%, 90%, 80% and 70% of ethanol for 4 minutes in each percentage. The slides then were submerged in warmed Bouin's solution at 60°C for 45 minutes. Next, the slides were washed in running tap water until yellow colour in samples disappeared. To differentiate nuclei, slides were then immersed in modified Weigert's haematoxylin for 8 minutes, after that washed in running water for 2 minutes. In order to stain cytoplasm and erythrocytes, slides were submerged in anionic dyes, acid fuchsin (C.I. 42590, Merck, Germany) for 5 minutes; then again slides were washed with running tap water for 2 minutes. Next, slides were treated with phosphomolybdic acid solution for another 10 minutes as a mordant and immediately slides were submerged into methyl blue (C.I. 42780, Merck, Germany) solution for 5 minutes in order to stain fibroblast and collagen. After that, slides were washed in running water for 2 minutes and lastly treated with 1% acetic acid solution for 1 minute. Slides were then dehydrated into a series of alcohol of 70%, 80%, 95% and 100% for 1 minutes each percentage. Before observation, slides were dipped into absolute xylene for 1 minute and finally mounted with cover slip using DPX mounting.

The sections were viewed and photographed using an Olympus light microscope (Olympus BX, Tokyo, Japan) with an attached camera (Olympus E-330, Olympus Optical Co. Ltd., Tokyo,

Tissue preparation for electron microscopy

Ultrastructure of the kidney was studied using the transmission electron microscope. To accomplish this aim, small samples (about 1 mm) cut from different areas of the kidney were fixed by immersion method in glutaraldehyde at 4°C overnight. Subsequently, the specimens were washed (4 x 15 min) in 0.1 M cacodylate buffer (pH 7.2). All samples were post-fixed in 1% osmium tetroxide for 2 hr. Later, they were washed again (3 x 20 min) in 0.1 M cacodylate buffer (pH 7.2). After dehydration in a graded series of ethanol (50%, 70%, 90%, 100%) and propylene oxide (Merck, Darmstadt, Germany) the specimens were gradually embedded in Spurr's Low Viscosity Resin (Polysciences, Eppelheim, Germany). Briefly, samples were firstly placed (2 x 15 min) in propylene oxide (Merck, Darmstadt, Germany) then in propylene-Spurr's resin mixture (2:1) for 1 hr, in propylene-Spurr's resin mixture (1:1) overnight and finally in pure Spurr's resin overnight.

Thereafter, kidney specimens were embedded in silicon molds (Plannet, Wetzlar) containing Spurr's resin and polymerized at 70°C for 8 hr. For general morphology, semi-thin sections (1µm) were cut using a Leica EM UC7 ultramicrotome (Leica, Germany) and stained with toluidine blue (Sigma-Aldrich Chemicals GmbH, Deisenhofen, Germany) for examination under light microscopy. Ultra-thin sections were then cut from selected blocks and mounted on copper grids (SSI, Science Services, Munich,

Germany) and routinely contrasted with uranyl-acetate and lead citrate prior to examination with a JEOL 2100 HR TEM (JOEL, Japan).

Statistical analysis

Analyses were carried out using one-way analysis of variance, (ANOVA) followed by Dunnet's multiple comparison post-hoc tests using Graph pad prism ® statistical software version 5.02. Values were expressed as mean ± standard deviation (SD) and all results tested for significance at the 95% confidence level ($p < 0.05$).

1.31 Article/Manuscripts from project

Table 1.8: List of article/Manuscripts from project:

S/no	Reference	Manuscript title	Journal	Status
1	Offor et al., 2018	Nephrotoxicity and Highly active antiretroviral therapy: Mitigating action of <i>Momordica charantia</i>	Toxicology report	Published 20 th September 2018
2	Offor et al., 2019	Renal histopathological and biochemical changes following adjuvant intervention of <i>Momordica charantia</i> and antiretroviral therapy in diabetic rats	Iranian Journal of Basic Medical Sciences	Published 15 th August 2019
3	Offor et al., 2019 (Unpublished)	Gene expression profile of KIM-1, NGAL and TNF in non-diabetic and diabetic rat model treated with <i>Momordica charantia</i> and highly active antiretroviral therapy		Manuscript
4	Offor et al., 2019 (Unpublished)	Ultrastructural perspective of the kidney treated with highly active antiretroviral therapy and <i>Momordica charantia</i> in non-diabetic and diabetic animal model		Manuscript

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BRIDGING

BETWEEN CHAPTER ONE AND CHAPTER TWO

The introduction and literature review from chapter one reveal the remarkable contribution of highly active antiretroviral therapy (HAART) towards the management of HIV/AIDS epidemic globally. However the success achieved is not without side effects which in many settings are not monitored. Antiretroviral agents are responsible for a wide range of toxicities, from low-grade intolerance that may be self-limiting, to life-threatening side-effects. As previously reported, plant-based adjuvants have been widely accepted and used for the management of various ailments with the potentials to fill the need for the management of comorbid disorders and toxicities emanating from the use of HAART. Thus, the next chapter evaluated the role of ethanolic extract of *Mormodica charantia* in the era of HAART on renal histoarchitecture and other biochemical parameters assessed in non-diabetic animals.

CHAPTER TWO

PUBLISHED ARTICLE

Nephrotoxicity and highly active antiretroviral therapy: Mitigating action of *Momordica charantia*

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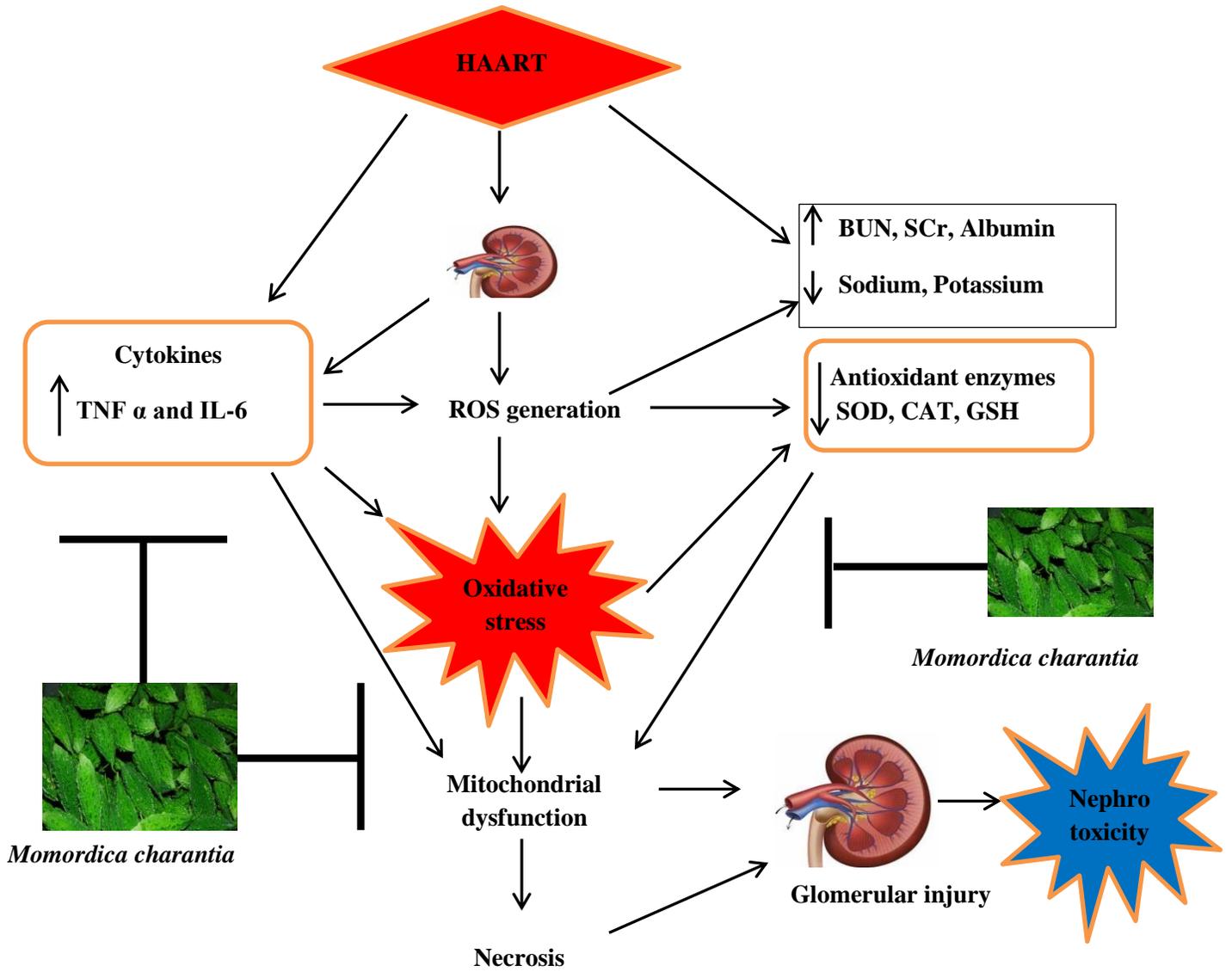
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Highlights

- Highly active antiretroviral therapy (HAART) associated nephrotoxicity is characterized by tubular necrosis with glomerular hypertrophy.
- Derangements of the cytoarchitectural patterns of the kidney were seen as a result of treatment with HAART regimen (triplavar).
- Co-administration of *M. charantia* and HAART mitigates the intensive histopathological changes of the rat kidney.
- Nephrotoxicity is associated with increased oxidative stress which stems from increased reactive oxygen species (ROS) generation in tissues.
- *M. charantia* possesses phytochemical properties which are powerful counter measures against oxidative stress tissue damage.

Graphical abstract





Nephrotoxicity and highly active antiretroviral therapy: Mitigating action of *Momordica charantia*



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ABSTRACT

Momordica charantia (*M. charantia*) is known for its antioxidant and antidiabetic properties. The aim of this study is to investigate the renoprotective effects of *M. charantia* in rats following treatment with highly active antiretroviral therapy (HAART) regimen triplavar. Adult male Sprague-Dawley rats weighing 178.1–220.5 g (n = 36) were divided into six groups (A–F) with each group comprising of six (n = 6) rats. The drugs and extract were administered via oral gavage. The therapeutic dose of triplavar was adjusted using the human therapeutic dose equivalent for the rat model. Animals were euthanized on the tenth week with kidneys removed for examination and blood obtained via cardiac puncture. Levels of oxidative stress enzymes (superoxide dismutase-SOD, catalase-CAT, and reduced glutathione-GSH) were significantly lowered in all groups not receiving *M. charantia*. The levels of thiobarbituric acid reactive substances (TBARS) were increased resulting in free radical formation via auto-oxidation. Renal parameters showed no albuminuria, normal blood urea nitrogen (BUN), serum creatinine (SCr) and electrolytes in groups treated with *M. charantia*. HAART treated (Group B) showed severe albuminuria, a significantly ($p < 0.05$) raised BUN and SCr and gross electrolyte disturbances. Blood glucose levels were significantly raised in groups not receiving the adjuvant *M. charantia* ($p < 0.05$). Histopathology in HAART treated animals showed glomerular capillary abnormalities and cellular infiltrations while *M. charantia* treated animals showed an essentially normal glomerular appearance with capillary loops and normal cytoarchitecture. In conclusion *M. charantia* extract administration improved blood glucose levels, restored renal histology, reinstate renal function, reduce body weight loss and restores hyperglycemia.

1. Introduction

The successful introduction of highly active antiretroviral therapy (HAART) for the management of human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) significantly increased the life expectancy among HIV-infected patients with

unprecedented changes in disease progression and mortality [1]. However, despite this full control, empirical evidence still demonstrates that nephrotoxicity is increasing rapidly among people living with HIV and AIDS (PLWHA) thus dampening the perceived impact of HAART. Nephrotoxicity results from mitochondrial dysfunction induced by nucleoside reverse transcriptase inhibitors (NRTIs) since they inhibit

Abbreviations: DTNB, 5, 5'-dithiobis-(2-nitrobenzoic acid); 6-HD, 6-hydroxydopamine; AIDS, acquired immune deficiency syndrome; ALB, albumin; ANOVA, analysis of variance; AREC, animal research ethics committee; BRU, Biomedical Resource Unit; BGL, blood glucose levels; BUN, blood urea nitrogen; BW, body weight; CAT, catalase; DNA, deoxyribonucleic acid; DETAPAC, diethylenetriamine – penta acetic acid; GSH, reduced glutathione; H and E, haematoxylin and eosin; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; KW, kidney weight; KWBR, kidney weight body ratio; LPO, lipid peroxidation; MDA, malondialdehyde; MT, Masson's Trichome; *M. charantia*, *Momordica charantia*; NRTIs, nucleoside reverse transcriptase inhibitors; PLWHA, people living with HIV and AIDS; PAS, Periodic Acid Schiff; PBS, phosphate buffer solution; ROS, reactive oxygen species; rpm, revolutions per minute; SCr, serum creatinine; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; SD, standard deviation; TBARS, thiobarbituric acid reactive substances; TGA, trichloroacetic acid; UKZN, University of KwaZulu Natal

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reverse transcriptase of HIV along with inhibition of mitochondrial deoxyribonucleic acid (DNA) polymerase from host cells with subsequent deficits in mitochondrial DNA encoded enzymes [2]. It is characterized by tubular necrosis with glomerular hypertrophy, which is instigated by mesangial cell proliferation and excessive accumulation of the extracellular matrix [3].

Nephrotoxicity is strongly associated with increased oxidative stress which stems from increased reactive oxygen species (ROS) generation in tissues and a depletion of natural enzyme antioxidants expression and activity [4]. ROS through the formation of DNA adducts damages the DNA causing loss of DNA repair mechanism and induce gene activation of oxidative-stress responsive maladaptive pathways thereby promoting apoptosis in glomerular cells which leads to characteristic morphological and functional abnormalities associated with nephropathy [4]. Strategies that reduce ROS and improve functional capacity of the kidney will provide therapeutic benefits in PLWHA under HAART.

One plant that has received so much attention for its array of phytochemical properties is *Momordica charantia* (*M. charantia*). *M. charantia* is an economically important medicinal plant belonging to the family *Cucurbitaceae* known as balsam pear or karela. It is a tropical vegetable which is a common food in Indian cuisine and has been used extensively in folk medicine as a remedy for a number of diseases and disorders. Isolated phytochemicals of *M. charantia* has been documented with in vitro antioxidant and antiviral activity against numerous viruses including *Epstein-Barr*, herpes and HIV viruses [5]. In an in vivo study, *M. charantia* leaf extract demonstrated the ability to increase resistance to viral infections as well as to provide an immunostimulant effect in humans and animals (increasing interferon production and natural killer cell activity). Two proteins known as alpha-and beta-momocharin (which are present in the seeds, fruit, and leaves) have been reported to inhibit the HIV virus in vitro [5].

But its efficacy in mitigating adverse biochemical defects and structural configurations of the kidney that arise as a result of antiretroviral therapy is unknown. Hence, the present investigation was postulated to explore the mechanism of renoprotective nature of *M. charantia* following antiretroviral therapy in adult male Sprague-Dawley rats.

2. Materials and methods

2.1. Materials

HAART regimen- Triplavar (*Cipla-Medpro*) containing Lamivudine 150 mg, Nevirapine 400 mg and Zidovudine 300 mg, was used for this study. The drug was obtained from Pharmed pharmaceuticals, Pty (Ltd) Durban, South Africa. Fifty kilograms of the fresh mature unripe fruit of *M. charantia* was purchased from the local Durban markets. Samples were authenticated at the herbarium unit of the Department of Life Sciences, University of KwaZulu-Natal, Durban, South Africa (voucher no. 4617). A total of thirty-six (36) adult male Sprague-Dawley rats weighing 178.1–220.5 grams were used for the study. Ethical approval was obtained from University of KwaZulu Natal (UKZN) animal research ethics committee (AREC) – ethics number AREC/033/016D. The study was conducted at the Biomedical Resource Unit (BRU) of UKZN.

2.2. Methods

2.2.1. Preparation of *M. charantia* fruit ethanolic extract

The fruits were cleaned, sliced into small pieces and the seeds separated out and discarded. The sliced green fruit was first weighed and then dried in shade for approximately 2 weeks. It was then weighed again to obtain the final dry weight before pulverizing into a fine powder in a commercial grinder and stored at 5 °C until ready for extraction. The active ingredients were obtained by Soxhlet extraction using ethanol as the solvent. The solvent was evaporated in a rotary

evaporator at 40°–50 °C with a percentage yield of 85.25%. The wet residue was filtered through a Whatman filter paper and the concentrated extract was stored at 4 °C until ready for use.

2.3. Experimental design

All rats were housed in well ventilated plastic cages [3 rats per cage (in 12 cages)] having dimensions of (52 cm long × 36 cm wide and 24 cm high) and soft wood shavings employed as beddings in the cages. They were maintained under standardized animal house conditions (temperature: 23–25 °C; light: approximately 12 h natural light per day) and were fed with standard rat pellets from (Meadow feeds a Division of Astral Operations Limited, Durban, South Africa) and given tap water ad libitum. The initial body weight of the animals was recorded before treatment and randomly distributed to the treatment groups with 6 animals per group.

Group A received Normal saline (Control)

Group B received Triplavar

Group C received *M. charantia* (200 mg/kg bw)

Group D received *M. charantia* (400 mg/kg bw)

Group E received Triplavar + *M. charantia* (200 mg/kg bw)

Group F received Triplavar + *M. charantia* (400 mg/kg bw)

The therapeutic dose of triplavar was adjusted using the human therapeutic dose equivalent for the rat model. Doses were administered via oral gavage daily for 6 days with 1 day rest over a 10 week period. Animals were euthanized on day 70 by bilateral pneumothorax under anesthesia with an overdose of halothane. Blood was collected by intracardiac puncture and tissues were harvested for preparation of light microscopy.

2.4. Measurements of body weight and collection of urine samples

All experimental animals were weighed weekly by a digital scale (Mettler-Toledo, 200). For collection of urine, the rats were placed in metabolic cages for 24 h and provided with rat chow and water. The urine volume was measured and urine centrifuged to separate out debris. Urine samples were kept at –80 °C until further analysis. This procedure was done at weeks 3, 6 and 9 during the 10-week experimental period.

2.5. Measurement of blood glucose concentration

Blood samples were obtained from the tail using sterile needle prick. Glucose levels were measured once a week during the 10 weeks treatment using the one-touch ultra-glucometer (Boehringer-Mannheim, Germany).

2.6. Measurement of oxidative stress parameters and lipid peroxidation

Blood was collected in plain tubes via cardiac puncture and allowed to clot. It was then centrifuged at 3000 rpm (rpm) for 15 min and the serum decanted into Eppendorf tubes and stored at –20 °C for subsequent use. Serum was assayed for lipid peroxidation (LPO), reduced glutathione (GSH) level, superoxide dismutase (SOD) and catalase activities (CAT).

2.7. Serum lipid peroxidation levels

This was measured using a complex formed from the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBARS) as described by [6]. Into an assay mixture containing 200 µL of 8.1% sodium dodecyl sulfate (SDS), 750 µL of 20% acetic acid (pH, 3.5), 2 mL of 0.25% TBARS and 850 µL of distilled water. 200 µL of sample of MDA standard series (0, 7.5, 15, 22.5, and 30 µM) was added in a pyrex screw-capped test tube. The mixture was heated at 95 °C for 60 min in a sand bath, cooled down to room temperature and absorbance was read

Table 1
Body weight and kidney weight of animals.

Groups	Initial BW	Final BW	Mean BW	BW difference	BW Diff in %	Mean KW	KBWR
A	178.1	351.5	264.8 ± 122.6	173.4	65.48	2.59 ± 0.19	0.98
B	220.5	348.3	284.4 ± 90.39	127.8	44.94*	1.96 ± 0.18	0.69
C	184.3	353.8	269.1 ± 119.8	169.5	62.98	2.04 ± 0.28	0.76
D	199.2	361.2	280.2 ± 144.6	169	60.31	2.16 ± 0.17	0.97
E	180.2	348.1	264.2 ± 118.7	167.9	63.55*	2.11 ± 0.17	0.08
F	186.8	347.7	267.3 ± 113.8	160.9	60.19	2.04 ± 0.15	0.76

Values are expressed as mean ± SD for each group and considered statistically significant at $p < 0.05^*$. BW is body weight of rats; KW is kidney weight of rats; KBWR is kidney weight body ratio. $KWBR = (Mean\ KW / Mean\ BW) \times 100$. $BW\ diff\ in\ \% = (BW\ diff / Mean\ BW) \times 100$.

at 532 nm in a spectrophotometer (UVmini-1240, Shimadzu Japan). Thiobarbituric acid reactive substances (TBARS) concentrations of samples were extrapolated from MDA standard curve.

2.8. Serum reduced glutathione concentration

Reduced glutathione (GSH) concentration was measured in serum according to methods modified from [7]. The sample was first precipitated with 10% Trichloroacetic acid (TCA) and then centrifuged at 2000 rpm for 10 min at 25 °C. The reaction mixture contained 400 µL of supernatant, 200 µL of 0.5 M 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 1.2 mL of 0.2 M sodium phosphate buffer (pH, 7.8). Absorbance was measured at 415 nm after 15 min incubation at 25 °C and GSH concentrations of samples were extrapolated from a standard curve of GSH.

2.9. Anti-oxidant enzyme activities

2.9.1. Superoxide dismutase

Superoxide dismutase (SOD) activity was assayed according to the method of [8]. A 15 µL of 1.6 mM 6-hydroxydopamine (6-HD) was added to an assay mixture containing 170 µL of 0.1 mM diethylenetriamine – penta acetic acid (DETAPAC) in 50 mM sodium phosphate buffer (pH, 7.4) and 15 µL of sample (serum) containing 0.1 µg/µL of protein was used to start the reaction. The linear increase in absorbance was monitored at 490 nm for 5 min at 25 °C. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of 6-HD/min/µg protein.

2.9.2. Catalase

Catalase (CAT) activity was measured using the method described by [8]. Into an assay mixture containing 340 µL of assay buffer (50 mM potassium phosphate buffer, pH 7.0) and 150 µL of 10 mM H₂O₂, 10 µL of a sample containing 0.1 µg/µL protein was added to start the reaction. The linear increase in absorbance was monitored at 240 nm for 5 min at 25 °C. One unit of enzyme activity was expressed as the amount of enzyme needed to decompose 1 mmol of H₂O₂ /min/µg protein.

2.9.3. Assessment of renal function

Serum was used for the estimation of blood urea nitrogen (BUN) and serum creatinine (SCr) using Beckman Coulter Synchron® system(s) BUN and SCr assay kit. Beckman Coulter Synchron® system BUN assay kit and Beckman Coulter Synchron® system SCr assay kit were obtained from Global Viral Laboratory, Durban, South Africa

2.9.4. Tissue preparation for light microscopy

Kidneys were weighed and examined for gross pathology. A phosphate buffer solution (PBS) was used to wash out blood before preparation for tissue fixation. They were sectioned at 4 µm thickness using Leica RM 2255 microtome. Tissue was stained with Haematoxylin and Eosin (H and E), Periodic Acid Schiff (PAS) and Masson's Trichrome (MT). Slides were digitally scanned using a Leica SCN 400 (Leica Microsystems GmbH, Wetzlar, Germany) and measurements were done

at 200 magnification using image analyzer Leica (DMLB) and Leica QWIN software.

2.10. Statistical analyses

Analyses were carried out using one-way analysis of variance, (ANOVA) followed by Dunnett's multiple comparison *post-hoc* tests using Graph pad prism® statistical software version 5.02. Values were expressed as mean ± standard deviation (SD) and all results tested for significance at the 95% confidence level ($p < 0.05$).

3. Results

3.1. Body weight and Organ (kidney) weight

While there was an overall increase in body weight (BW) in all groups, Groups, C and E recorded maximal significant increase in trend at $p < 0.05$ for BW gained with the percentage BW gain recording 62.98% and 63.55% respectively, but however, lower when compared to control. Group B recorded the least BW gain significant at $p < 0.05$ with percentage BW gain recording 44.94%. Mean kidney weight corresponds with the percentage BW gained across groups with the control group having an optimal mean kidney weight (Table 1).

3.2. Blood glucose levels

Baseline fasting blood glucose showed normal glycemia. However, from the 5th to 10th week, blood glucose levels (BGL) were significantly higher ($p < 0.05$) in triplavar treated group. Treatment with *M. charantia* alone and its concomitant use with triplavar maintained normal BGL and this was sustained between 4th and 6th week. (Fig. 1).

3.3. Urine parameters

Urine was collected at weeks (3, 6 and 9) for the 10 weeks duration of study for the measurement of urinary parameters (micro

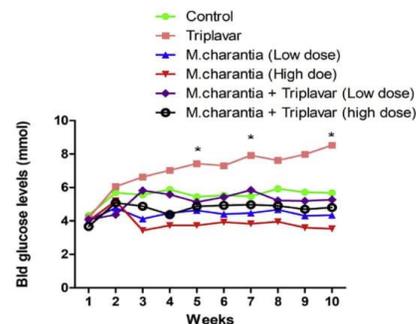


Fig. 1. Graphical representation of blood glucose levels.

Table 2
Urine Test (Week 3).

Groups	Alb (mg/L)	Creat (mmol/L)	Alb/crt ratio (mg/mmol)	Sodium (mmol/L)	Potassium (mmol/L)	Urea (mmol/L)
A	1.73 ± 0.28	9.60 ± 1.21	0.18 ± 0.05	279.30 ± 19.01	251.3 ± 13.43	411.4 ± 91.45
B	1.950 ± 5.18	7.56 ± 1.00	0.65 ± 1.80	261.33 ± 16.51	259.97 ± 22.25	340.4 ± 27.95
C	1.87 ± 0.23	8.96 ± 1.32	0.23 ± 0.06	268.0 ± 16.37	247.3 ± 8.95	358.9 ± 90.78
D	4.40 ± 0.46	8.53 ± 0.93	0.52 ± 0.08	265.7 ± 26.08	262.1 ± 28.86	361.2 ± 52.56
E	3.76 ± 1.10	8.23 ± 1.36	0.48 ± 0.22	268.3 ± 14.50	257.2 ± 14.70	409.8 ± 89.55
F	3.73 ± 0.77	8.83 ± 1.00	0.42 ± 0.05	258.0 ± 17.35	257.7 ± 11.71	471.8 ± 53.54

Values are expressed as means ± SD of each group. Alb is Albumin; Creat is Creatinine; Alb/crt ratio is Albumin creatinine ratio.

Table 3
Urine Test (Week 9).

Groups	Alb (mg/L)	Creat (mmol/L)	Alb/crt ratio (mg/mmol)	Sodium (mmol/L)	Potassium (mmol/L)	Urea (mmol/L)
A	1.80 ± 0.10	9.20 ± 1.87	0.19 ± 0.03	274.0 ± 1.00	290.0 ± 29.72	522.0 ± 44.54
B	11.63 ± 2.27*	1.53 ± 0.32*	7.70 ± 1.78	78.0 ± 10.15	78.33 ± 13.80	158.3 ± 32.87
C	2.13 ± 0.15	8.60 ± 2.76	0.26 ± 0.07	270.7 ± 35.02	264.0 ± 21.66	373.7 ± 216.6
D	1.53 ± 0.40	7.87 ± 2.73	0.21 ± 0.08	239.7 ± 30.99	256.0 ± 23.43	365.0 ± 114.0
E	1.43 ± 0.55	9.50 ± 2.36	0.15 ± 0.02	272.7 ± 24.11	311.0 ± 23.26	385.7 ± 82.31
F	1.20 ± 0.10	9.76 ± 1.68	0.12 ± 0.02	286.7 ± 9.71	308.0 ± 10.82	409.7 ± 33.65

Values are expressed as means ± SD of each group. Alb is Albumin; Creat is Creatinine; Alb/crt ratio is Albumin creatinine ratio.

albuminuria, creatinine, sodium, potassium and urea). From the result, there were no significant changes in all groups (Table 2). There were also no statistical changes observed in week 6 in all groups. However, from the 9th week, the urine parameters test for animals in group B showed significant changes ($p < 0.05$). It was observed at this stage a marked increase in albumin and poor creatinine clearance, the albumin-creatinine ratio was significantly higher at this stage, (Table 3). Sodium, potassium, and urea were also significantly ($p < 0.05$) reduced. Adjuvants treatment with *M. charantia* fruit extract in all weeks of monitoring seemingly showed normal urinalysis test results when compared with control (groups C, D, E, and F).

3.4. Blood urea nitrogen (BUN) and serum creatinine (SCr) levels

Group B exhibited a significantly higher SCr and BUN ($p < 0.05$) compared to control and other experimental groups (Table 4). Adjuvant uses of *M. charantia* at both low and high doses were found to be effective in lowering SCr and BUN in all groups receiving the extract. However, they did not show any significant difference when compared with controls.

3.5. Lipid peroxidation and oxidative stress

Levels of TBARS in group B were significantly ($p < 0.05$) elevated indicating disturbances in the normal redox state. Adjuvant treatments with *M. charantia* restored these disturbances to near normal. Activities of GSH, SOD and CAT were in all decreased in Group B (HAART-regimen). These activities were significantly raised ($p < 0.05$) in groups administered with *M. charantia* alone and its concomitant administration with HAART-regimen (triplavar). Concomitant administration of

Table 4
BUN and SCr levels.

Groups	BUN (mmol/L)	SCr (mmol/L)
A	1.00 ± 0.26	0.96 ± 0.25
B	9.20 ± 1.60	16.67 ± 3.51*
C	1.93 ± 0.32	2.10 ± 1.11
D	1.30 ± 0.10	2.23 ± 0.25
E	1.33 ± 0.73	1.86 ± 0.15
F	1.73 ± 0.85	2.20 ± 0.10

Values are expressed as means ± SD of each group. * $p < 0.05$ BUN is blood urea nitrogen; SCr is serum creatinine.

M. charantia and triplavar at a low dose in group E did not show any significant improvement in SOD activity (Fig. 2).

3.6. Histopathological findings

3.6.1. Haematoxylin and eosin (H and E) stains

Histology of the kidney of the control group was essentially normal without any cellular distortions. Tubular epithelium was distinct and adequately maintained with the distinct regular lumen. The kidneys of triplavar treated animals show obliteration of glomerular capillary loops and occlusion of the capillary walls with many indistinguishable tubules, inflammation of the interstitial, epithelial desquamation and extensive necrosis of the vascular wall. Groups treated with *M. charantia* extract and its co-administration with triplavar showed reversal of the degenerative changes observed with the triplavar alone treated groups. The glomeruli were intact with no inflammatory changes in the cytoarchitectural patterns (see Fig. 3). These were the characteristics findings of the H and E stains.

3.6.2. Periodic Acid Schiff (PAS) stains

Triplavar treated Groups showed vacuolation of tubules characterized by high proportion of carbohydrates such as glycogen and glycoproteins and mild deposition of polysaccharides while *M. charantia* treated groups showed an essentially normal glomerular appearance with normal cytoarchitecture comparable to control (see Fig. 4).

3.6.3. Masson's Trichome (MT) stains

MT stains showed deposition of collagen fibers in the triplavar treated animals (Group B). These changes were absent in all groups treated with *M. charantia* and showed restoration of the histological layout similar to control (see Fig. 5).

4. Discussion

The potential impacts of HAART cannot be underscored despite challenging pitfalls with therapy in the reduction of mortality and morbidity. However, people living with HIV and AIDS (PLWHA) require consideration of organ-specific toxicities (especially kidney and liver) that can emanate from HAART use. Medicinal plants rich in phytochemical components has been shown to hold promise as an alternative therapy for the alleviation of some of the toxicities of HAART [9]. *M. charantia* possesses large amounts of phytochemical properties which

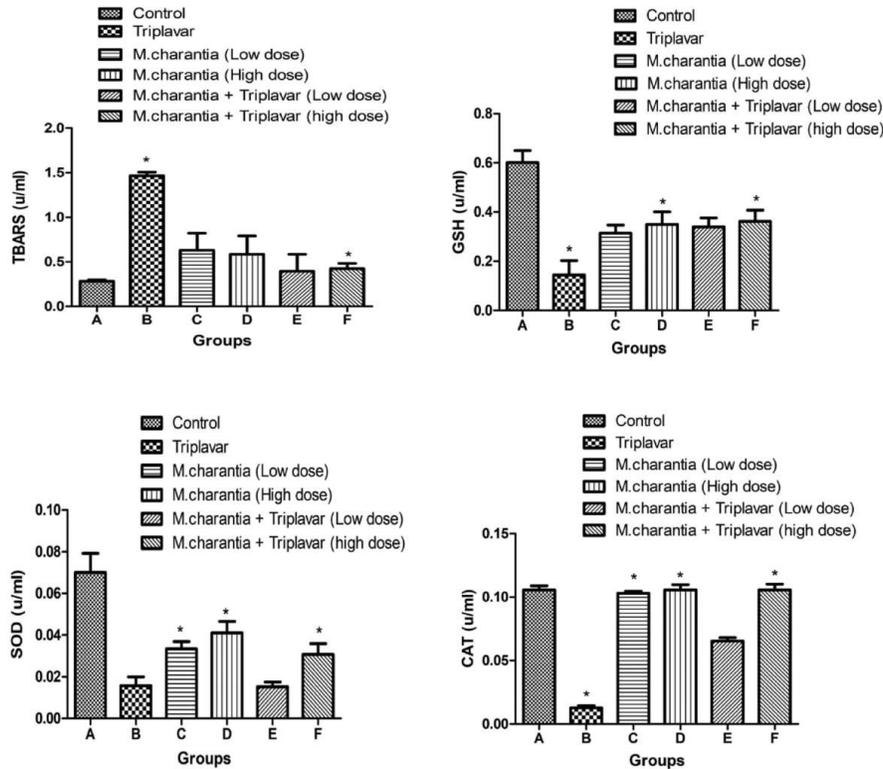


Fig. 2. Oxidative stress measurements: Bars indicate mean ± SD, *p < 0.05.

are powerful counter measures against oxidative stress tissue damage [10]. The study, therefore, demonstrates the possible protective role of *M. charantia* in the mitigation of HAART's ravages on the renal parameters.

From our study, derangements of the cytoarchitectural patterns of the kidney were seen as a result of treatment with HAART regimen (triplavar). These were characterized by tubular epithelial desquamation and glomerular capillary abnormalities (capillary wall occlusion and disruption of the capillary loops) with extracellular matrix accumulation which is suggestive that disturbances between the mesangial cell and glomerular capillaries may have been drastically altered. Glomerular abnormalities often result in a decrease in surface area available for filtration and a consequent decline in glomerular filtration rate and reduction in metabolic activity. Our observations in the H and E stains corresponded with PAS and MT staining intensities of the kidney sections and there is a supporting link. Photomicrographs of HAART- regimen (triplavar) showed vacuolation of tubules which are characterized by a high proportion of carbohydrates such as glycogen and glycoproteins and mild deposition of collagen fibers and hyaline substances.

These histological pathologies observed with HAART treated alone were ameliorated by *M. charantia* possibly due to its antioxidative properties as previously reported by [11,12]. Putatively, pathways for the generation of reactive oxygen species (ROS) leading to oxidative stress and toxicity of HAART relies on mitochondria-related perturbations that manifests in many side effects such as hepatic failure, testicular dysfunctions and renal disorders [10]. The 'mitochondrial dysfunction hypothesis' reviewed in [13] is believed to operate via energy

deprivation, mitochondrial oxidative stress and consequent mitochondrial DNA damage. Co-administration of *M. charantia* and HAART mitigates the intensive histopathological changes of the rat kidney in our study by restoring the interstitial and restitution of the normal epithelial lining of the tubules. Our result corroborates with the findings of [14] on the histological assessment of the kidney following *M. charantia* leaf extract which shows normal histological layout when compared to normal rats.

The severity of oxidative damage depends on the extent of disturbances in the normal redox state. Excess ROS must be promptly eliminated from cells and this is done by a variety of antioxidant defense mechanisms. From our result, there were reductions in SOD and CAT levels in the groups treated with HAART alone when compared with control rats. This decline may precipitate into an oxidative stress status with consequent damage to cells and membranes in the renal tissue. However, the reversed levels on treatment with a low and high dose of *M. charantia*, and its co-administration with triplavar indicate an antioxidative therapeutic effect against HAART induced oxidative stress via significant enhancement of enzymatic antioxidant activities [15]. Our result also showed that reduced levels of GSH observed in triplavar treated group may be due to inactivation caused by reactive oxygen species. Administration of *M. charantia* extracts increased the levels of GSH in rats. HAART toxicity amplifies free radical formation through auto-oxidation of unsaturated lipids in plasma. The free radical produced may react with polyunsaturated fatty acids in cell membranes leading to lipid peroxidation [16]. Lipid peroxide-mediated tissue damage has been observed in PLWHAs under HAART. The increased lipid peroxidation in PLWHAs undergoing treatment with HAART may be

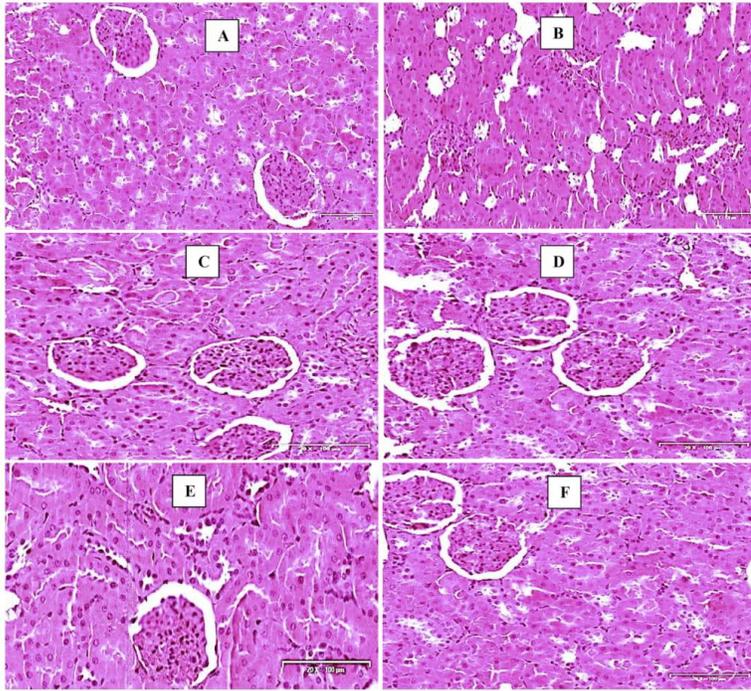


Fig. 3. Photomicrographs of the kidney (H and E stains). Scale bar $\times 200 \mu\text{m}$. (A) Control-normal structure of the kidney. (B) Triplavar treated- degeneration of the glomerulus and vacuolation of tubules (C) *M. charantia* extract Low dose- restoration of interstitium (D) *M. charantia* extract high dose- histoarchitecture essentially normal (E) *M. charantia* extract low dose and Triplavar- histoarchitecture essentially normal (F) *M. charantia* extract high dose and Triplavar- histoarchitecture essentially normal.

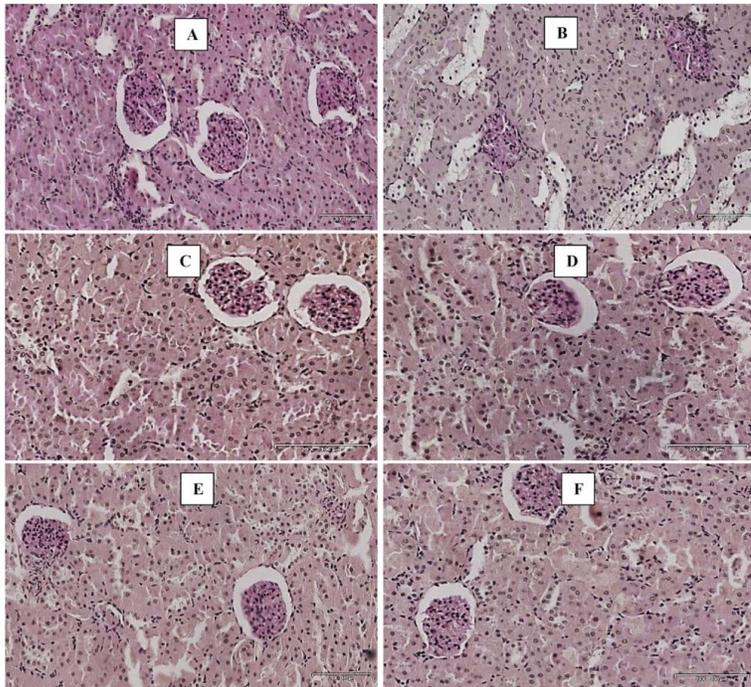


Fig. 4. Photomicrographs of the kidney (Periodic Acid Schiff stains). Scale bar $\times 200 \mu\text{m}$. (A) Control-normal structure of the kidney. (B) Triplavar treated- mucus substances and high proportion of carbohydrate macromolecules (glycogen, glycoproteins) (C) *M. charantia* fruit extract Low dose- histoarchitecture normal (D) *M. charantia* fruit extract high dose- histoarchitecture essentially normal (E) *M. charantia* extract low dose and Triplavar- histoarchitecture essentially normal (F) *M. charantia* fruit extract high dose and Triplavar- histoarchitecture essentially normal.

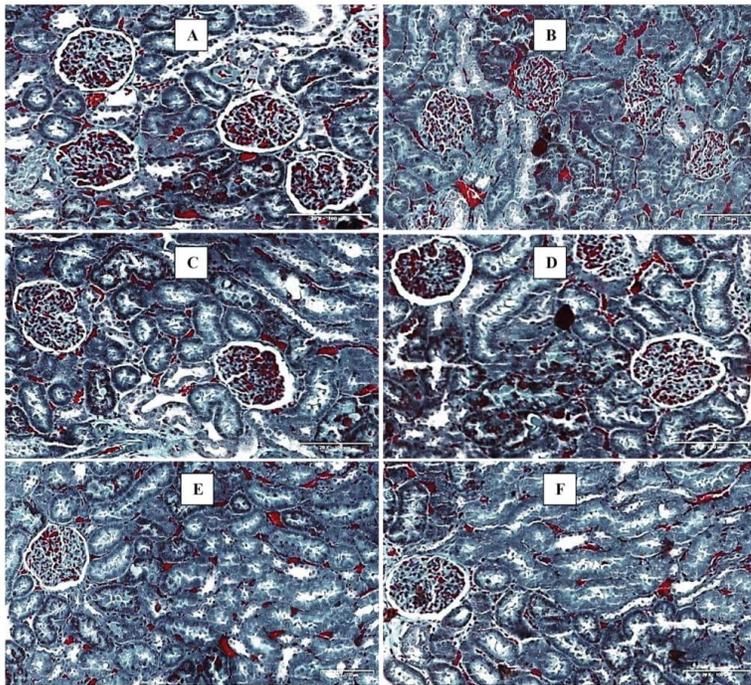


Fig. 5. Photomicrographs of the kidney (Massons Trichome stains). Scale bar $\times 200 \mu\text{m}$. (A) Control- normal structure of the kidney. (B) Triplavar treated- Presence of collagen fibers (C) *M. charantia* extract Low dose- histoarchitecture normal (D) *M. charantia* extract high dose- histoarchitecture essentially normal (E) *M. charantia* extract low dose and Triplavar- histoarchitecture essentially normal (F) *M. charantia* extract high dose and Triplavar- histoarchitecture essentially normal.

due to observed remarkable increase in the concentration of TBARS and hydroperoxides in the kidney [17]. In our study, TBARS levels in the kidney were significantly high in triplavar treated group, but however, treatment with *M. charantia* extract and its co-administration with triplavar significantly lowered the level of TBARS compared to control.

Retention of renal electrolytes (sodium and potassium) is a manifestation of a functional overload of the nephron and is likely to contribute to elevated pressure in the vascular wall of the kidney which will eventually lead to organ hypertrophy and a decline in glomerular filtration causing leakage of albumin, podocytes effacement and loss of surface area available for filtration [18]. In our study, we observed leakage of albumin and renal electrolytes retention in the urine of triplavar treated rats. Indicating that glomerular endothelial barrier may have been altered. Although this observation was mild and manifested from the sixth week of our study and continued till the end of our study. Adjuvant treatment with *M. charantia* extract at both low and high dose was able to mitigate the abnormalities and restored the functions of the kidney to normal. Our result also shows urea retention in the rats that were treated with triplavar. Low concentration of urea in the kidney suggests a reduced turnover of protein and thus reflects in glomerular filtration rate and worsens renal function [19], more so, increased level of urea in the blood suggests enhanced amino acids fueled gluconeogenesis which leads to increased nitrogen load to the liver where urea is formed [20]. Administration of *M. charantia* fruit extract was able to restore the urinary metabolites to normal when compared to control rats.

As markers of renal function, BUN and serum SCr routinely serve as indicators for normal biological, pathologic processes, or pharmacologic responses to a therapeutic intervention. Our study revealed marked impairment in renal function with significantly raised levels of BUN and SCr concentrations in triplavar treated rats and this is consistent with lower BUN and SCr clearance. These elevations in levels of

BUN and SCr concentrations might have resulted from remarkable leakage due to hypercellularity of the glomeruli and tubular degradation. Treatment with *M. charantia* fruit extract at both low and high dose prevented the development of nephrotoxicity by significantly lowering kidney injury markers such as BUN and SCr. The mitigation of kidney injuries due to increase in clearance of BUN and SCr by the kidney supports the report of [21] and further proves the biologically active components of the plant.

In our study we observed elevated levels of blood glucose in groups treated with HAART regimen (triplavar) indicating that HAART regimen suppresses glucose uptake by the tissues. This could occur as a result of complications arising from occlusion of the vascular wall and imbalance of endogenous vasodilators and constrictors [22]. HAART associated hyperglycemia is probably multifactor in its etiology, affecting glucose metabolism and insulin sensitivity, and altering the activity of glucocorticoid receptors in several tissues including the kidney. This promotes insulin resistance and impaired glucose tolerance through altered secretion of adipokines and other inflammatory markers such as interleukin 6, 8, 10 and macrophage chemotactic protein-1 [22]. Treatment with *M. charantia* extract significantly lowered blood glucose level. We speculate that the possible mechanism by which *M. charantia* decreases blood glucose level may be by potentiation of insulin effect by increasing either the pancreatic secretion of insulin from beta cells of islets of Langerhans. However, this claim is open for more studies to be conducted to provide any support.

Organ and body weight analysis offer insight on toxicity of test compounds in toxicology studies. In this study, there were significant decreases in body weight of rats treated with triplavar. These reductions may be attributed to morphological changes or protein wasting due to unavailability of carbohydrate for utilization of energy [23]. *M. charantia* extracts slowed the weight-gaining potential of the anti-retroviral regimen as depicted in the results. The overall implication of

this report agrees with the context of other results assessed in the present study. Although in our investigation, there was no significant difference in the weight coefficient (organ/body weight ratio) of kidney tissue in all groups. However, renal toxicity just like testicular toxicity can manifest in the form of organ hypertrophy.

5. Conclusion

Based on the results assessed in our study, *M. charantia* fruit extract prevented the pathological changes observed to a moderate extent. This might be due to pronounced antioxidant properties and anti-lipid peroxidation activities. In conclusion we therefore inferred that HAART induces toxicity of the kidney and *M. charantia* fruit extract has appreciable potentials to prevent damage to the kidney.

Conflict of interest

The authors declared that there is no conflict of interest.

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BRIDGING

BETWEEN CHAPTER TWO AND CHAPTER THREE

The toxicity role of HAART on the kidney of non-diabetic Sprague-Dawley rats in chapter two of this study further supports previous researchers towards the untoward side effects of HAART. However, another major concern linked to HAART that requires management is the undesired metabolic comorbid disorder (diabetic nephropathy) with its accompanied nephrotoxicity. The next chapter therefore evaluated the impact of *Mormodica charantia* on the kidney in diabetic condition following the treatment with HAART.

CHAPTER THREE

PUBLISHED ARTICLE

Renal histopathological and biochemical changes following adjuvant intervention of *Momordica charantia* and antiretroviral therapy in diabetic rats

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Short running title: *M. charantia* mitigate HAART toxicity in diabetic rats

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Renal histopathological and biochemical changes following adjuvant intervention of *Momordica charantia* and antiretroviral therapy in diabetic rats

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ABSTRACT

Objective(s): Diabetic nephropathy (DN) is an important primary cause of end-stage kidney disease. This study explores the mechanisms of the reno-protective effects of *Momordica charantia* (*M. charantia*) in diabetic rats following treatment with highly active antiretroviral therapy (HAART) regimen triplavir.

Materials and Methods: Adult male Sprague-Dawley rats (n=48) were divided into 7 groups (A-G). Treatment groups (B-G) had 7 animals per group and control group (Group A) had 6 animals per group. Diabetes was induced with streptozotocin (STZ) by intraperitoneal injection (STZ 45 mg/kg body weight). The animals were euthanized on the tenth week with kidneys removed for examination and blood obtained via cardiac puncture.

Results: Key renal parameters showed no albuminuria, normal blood urea nitrogen (BUN), serum creatinine and electrolytes in all groups treated with *M. charantia*. Untreated diabetic (Group B) and HAART treated diabetic (Group C) showed severe albuminuria, a significantly raised BUN and serum creatinine ($P<0.05$) and gross electrolyte disturbances. Blood glucose levels were consistently and significantly raised in all groups not receiving the adjuvant *M. charantia* ($P<0.05$). Levels of oxidative stress enzymes Superoxide dismutase (SOD), Catalase and activities of Reduced Glutathione (GSH) and Malondialdehyde (MDA) were significantly lower in all groups not receiving *M. charantia*. Histopathology in untreated diabetic and HAART treated animals showed severe degenerative changes in the glomeruli and inflammatory cellular infiltration while *M. charantia* treated animals showed an essentially normal glomerular appearance with capillary loops and normal cytoarchitecture.

Conclusion: *M. charantia* extract administration improved blood glucose levels, reinstates renal function, reduces body weight loss and restores hyperglycemia.

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Introduction

The management of human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) have been highly successful with current antiretroviral regimens both in controlling HIV replication and restoring immunity. However, as a counterweight to this positive impact in restoring immunity, it has been reported that antiretroviral drugs carries along deleterious effects, which challenge the management of HIV- infected patients to a great extent. Metabolic disorders such as hypertension and diabetes mellitus have been linked to the continuous use of highly active antiretroviral therapy (HAART), thus dampening the perceived impact and making the management of the disease a double edge sword. Many HIV/AIDS patients receiving HAART become insulin resistant and glucose intolerant. The resulting insulin resistance and glucose intolerance often progresses to diabetes with long-term

complications such as diabetic nephropathy (1, 2).

Changes occurring in diabetic nephropathy can seriously compromise the integrity of the glomerulus. This often involves mesangial expansion seen on light microscopy and vascular dysfunction. This together with occlusion of glomerular capillaries results in a loss of surface area for filtration leading to a lower glomerular filtration rate (GFR) and declining renal function (3). Hyperglycemia in diabetic nephropathy adversely alters the biochemical milieu and severely compromises the molecular structure of the kidney tissue mainly through oxidative stress. This leads to mitochondrial dysfunction, increased fatty acid oxidation and impaired insulin signalling (1).

Oxidative stress further disrupts endothelial integrity and vascular homeostasis. Studies have demonstrated that antiretroviral drugs (ARVs) such as protease inhibitors (PI's) cause endothelial dysfunction by

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inhibition of nitric oxide synthase systems (NOS), induction of mitogen-activated protein kinases (4). There is a decrease in endothelial-dependent vasorelaxation and endothelial cell apoptosis from the increased generation of reactive oxygen species (ROS) with depletion of the scavenger antioxidant enzymes such as superoxide dismutase, catalase and reduced glutathione (5). Endothelial dysfunction is a known initiating and contributing event in HIV associated atherosclerosis with activation of mononuclear cells, and suppression of associated maladaptive signalling cascades (4). Adjuvant therapy with plants that have antioxidants properties such as *Hypoxis hemerocallidea* may ameliorate these deleterious effects of antiretroviral regimens (6).

Momordica charantia (*M. charantia*) hold promise as an adjuvant in the therapeutic strategy for glycemic control aimed at preventing the development and progression of chronic renal failure associated with uncontrolled diabetes mellitus (7). Studies have documented the hypoglycemic effects of *M. charantia* through physiological, pharmacological and biochemical mechanisms (7). It is believed to act via stimulation of peripheral skeletal muscle glucose utilization (8), inhibition of intestinal glucose uptake, (9) inhibition of adipocyte differentiation (10), suppression of key gluconeogenic enzymes, stimulation of key enzyme of hexose mono phosphate (HMP) pathway, and preservation of β cell islet and its functions (11). We therefore sought to investigate the therapeutic potential of *M. charantia* in treating diabetic nephropathy as a possible consequence of antiretroviral toxicity.

Materials and Methods

Ethical approval

Ethical approval was obtained from University of KwaZulu Natal (UKZN) Animal Research Ethics Committee (AREC) - ethics number AREC/033/016D. The study was conducted at the Biomedical Resource Unit (BRU) of UKZN.

Antiretroviral drug

Triplavar (*Cipla-Medpro*) containing Lamivudine 150 mg, Nevirapine 400 mg and Zidovudine 300 mg, was used for this study. The drug was obtained from Pharmed Pharmaceuticals, Pty (Ltd) Durban, South Africa.

Preparation of *M. charantia* fruit ethanolic extract

Fifty kilogram of fresh mature unripe fruit of *M. charantia* was purchased from the local Durban markets. Samples were authenticated at the herbarium unit of the Department of Life Sciences, University of KwaZulu-Natal, Durban, South Africa (voucher No. 4617). The fruits were cleaned, sliced into small pieces and the seeds separated out and discarded. The sliced green fruit was first weighed and then dried in shade for approximately 2 weeks. It was then weighed again to obtain the final dry weight before pulverizing into a fine powder in a commercial grinder and stored at 5 °C until ready for extraction. The active ingredients were obtained by Soxhlet extraction using 100% ethanol as the solvent. The solvent was evaporated in a rotary evaporator at 40–50 °C with a percentage yield of 85.25%. The wet

residue was filtered through a whatman filter and the concentrated extract was stored at 4 °C ready for use.

Experimental design

A total of forty eight (n=48) adult male Sprague-Dawley rats weighing 178-232 grams (219.31±36.17) were used for the study. Animals were housed in well ventilated plastic cages (4 rats per cage having dimensions of 52 cm long × 36 cm wide and 24 cm high with bedding of soft wood shavings). They were maintained under standardized animal house conditions (temperature: 23–25 °C; light: approximately 12 hr natural light per day) and were fed with standard rat pellets from Meadow feeds (Division of Astral Operations Limited, Durban, South Africa) and given tap water *ad libitum*. The initial body weights of the animals were recorded before treatment. The animals were randomly assigned to 7 groups (A-G). The treatment groups had 7 animals per group (B-G) and the control group (group A) had 6 animals per group.

Group A served as negative control

Group B served as positive control (Diabetic)

Group C received Triplavar (Diabetic)

Group D received *M. charantia* (200 mg/kgbw) (Diabetic)

Group E received *M. charantia* (400 mg/kgbw) (Diabetic)

Group F received Triplavar + *M. charantia* (200 mg/kgbw) (Diabetic)

Group G received Triplavar + *M. charantia* (400 mg/kgbw) (Diabetic)

The therapeutic dose of triplavar was adjusted using the human therapeutic dose equivalent for the rat model. Doses were administered via oral gavage daily for 6 days with 1 day rest over a 10 week period. Animals were euthanized on day 70 by bilateral pneumothorax under anesthesia with an overdose of halothane. Blood was collected by intra-cardiac puncture and tissues were harvested for preparation of light microscopy.

Induction of diabetes mellitus

All rats were placed on a 12 hr fast to obtain baseline fasting blood glucose levels (FBG). The experimental groups (B- G) were given intra-peritoneal streptozotocin (STZ) (Sigma-Aldrich Chemical Company, Missouri, St Louis, USA) at 45 mg/kg body weight dissolved in a citrate buffer (pH 4.5) (12). Successful induction of diabetes was determined by observation of polyuria and polydipsia and confirmed by a 72 hr post STZ FBG level ≥ 11 mmol/l.

Measurement of blood glucose

Blood samples were obtained from the tail using sterile needle prick. Glucose levels were measured once a week during the 10 weeks treatment using the one touch ultra-glucometer (Boehringer-Mannheim, Germany).

Measurements of body weight and collection of urine samples

All experimental animals were weighed weekly by a digital scale (Mettler-Toledo, 200). For collection of urine, the rats were placed in metabolic cages for 24 hr and provided with rat chow and water. The urine volume was measured and urine centrifuged to separate out

debris. Urine samples were kept at -80°C until further analysis. This procedure was done at weeks 3, 6 and 9 during the 10 weeks experimental period.

Measurement of oxidative stress parameters and lipid peroxidation

Blood was collected in plain tubes via cardiac puncture and allowed to clot. It was then centrifuged at 3000 revolutions per minute (rpm) for 15 min and the serum decanted into eppendorf tubes and stored at -20°C for subsequent use. Serum was assayed for lipid peroxidation (LPO), reduced Glutathione (GSH) level, superoxide dismutase (SOD) and catalase activities (CAT).

Serum lipid peroxidation levels

This was measured using a complex formed from the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA) as described by others (13). Into an assay mixture containing 200 μl of 8.1% sodium dodecyl sulfate (SDS), 750 μl of 20% acetic acid (pH, 3.5), 2 ml of 0.25% TBA and 850 μl of distilled water, 200 μl of sample or MDA standard series (0, 7.5, 15, 22.5, and 30 μM) was added in a pyrex screw capped test tube. The mixture was heated at 95°C for 60 min in a sand bath, cooled down to room temperature and absorbance was read at 532 nm in a spectrophotometer (UVmini-1240, Shimadzu Japan). Thiobarbituric acid reactive substances (TBARS) concentrations of samples were extrapolated from MDA standard curve.

Serum reduced glutathione concentration (GSH)

Reduced glutathione concentration was measured in serum according to methods modified from others (14). The sample was first precipitated with 10% Trichloroacetic acid (TCA) and then centrifuged at 2000 rpm for 10 min at 25°C . The reaction mixture contained 400 μl of supernatant, 200 μl of 0.5 M 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 1.2 ml of 0.2 M sodium phosphate buffer (pH, 7.8). Absorbance was measured at 415 nm after 15 min incubation at 25°C and GSH concentrations of samples was extrapolated from standard curve of GSH.

Antioxidant enzyme activities

Superoxide dismutase

Superoxide dismutase (SOD) activity was assayed according to the method of others (15). A 15 μl of 1.6 mm 6-hydroxydopamine (6-HD) was added to an assay mixture containing 170 μl of 0.1M diethylenetriamine-penta acetic acid (DETAPAC) in 50 mm sodium phosphate buffer (pH, 7.4) and 15 μl of sample (serum) containing 0.1 $\mu\text{g}/\mu\text{l}$ of protein was used to start the reaction. The linear increase in absorbance was monitored at 490 nm for 5 min at 25°C . One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of 6-HD/min/ μg protein.

Catalase

Catalase activity was measured using the method described by (15). Into an assay mixture containing 340 μl of assay buffer (50 mm potassium phosphate buffer, pH 7.0) and 150 μl of 10 mm H_2O_2 , 10 μl of sample containing 0.1 $\mu\text{g}/\mu\text{l}$ protein was added to start

the reaction. The linear increase in absorbance was monitored at 240 nm for 5 min at 25°C . One unit of enzyme activity was expressed as the amount of enzyme needed to decompose 1 mmol of H_2O_2 /min/ μg protein.

Assessment of renal function

Serum was used for the estimation of blood urea nitrogen (BUN) and serum creatinine (Scr) using Beckman Coulter Synchron® system(s) BUN and Scr assay kit. Beckman Coulter Synchron® system BUN assay kit and Beckman Coulter Synchron® system Scr assay kit were obtained from Global Viral Laboratory, Durban, South Africa.

Tissue preparation for light microscopy

Kidneys were weighed and examined for gross pathology. A phosphate buffer solution (PBS) was used to wash out blood before preparation for tissue fixation. They were sectioned at 4 μm thickness using Leica RM 2255 microtome. Tissue was stained with Haematoxylin and Eosin (H and E), Periodic Acid Schiff (PAS) and Masson's Trichrome (MT). Slides were digitally scanned using a Leica SCN 400 (Leica Microsystems GmbH, Wetzlar, Germany) and measurements done at 250 magnification using image analyzer Leica (DMLB) and Leica QWIN software.

Statistical analysis

Analyses were carried out using one-way analysis of variance, (ANOVA) followed by Dunnett's multiple comparison *post-hoc* tests using Graph pad prism @ statistical software version 5.02. Values were expressed as mean \pm standard deviation (SD) and all results tested for significance at the 95% confidence level ($P<0.05$).

Results

Body and kidney weights

In this investigation, the STZ induced type 2 diabetes in 80% of animals which showed characteristic signs of hyperglycemia, polyuria, glycosuria, weight loss and increased water intake. Diabetes was related to reduce body weight compared to normal controls. The changes in initial and final body weight (BW) for all groups are shown in Table 1. Rate of weight gain in the treatment groups was significantly higher in the treatment versus the diabetic group with weight gain in all groups significantly lower than the controls ($P<0.05$). Body weights differed significantly ($P<0.05$) across all groups when compared with the control. The final body weights were higher in all groups than the initial weight. However, there was a lower rate of body weight gain in the experimental groups compared to the control. This was particularly so in Group B and C.

M. charantia extract administration (200 and 400 mg/kg BW) showed a narrower range of weight change. The least weight gain was observed in Group D and F treated with the low dose *M. charantia*. The higher dose showed a greater increase in weight gain significant at $P<0.05$. This was observed in Groups E and G with the percentage body weight gain recorded at 34.9 % and 20.9% respectively.

There is a significant difference in the relative organ weights ($P<0.05$) between control and treated

Table 1. Body weight and kidney weight of animals

Groups	Initial BW	Final BW	Mean BW	BW difference	BW Diff in %	Mean KW	KBWR
A	178.1	351.5	264.8±122.6	173.4	65.48	2.59±0.19	0.98
B	227.1	255.3	240.1±18.28	28.2	11.74	2.17±0.31	0.90*
C	227.1	258.1	242.6±21.92	31.0	12.77	2.13±0.25	0.87*
D	212.6	258.0	235.3±32.12	45.4	19.29	2.08±0.37	0.88*
E	240.3	342.1	291.2±72.02	101.8	34.9	2.28±0.31	0.78
F	217.9	249.9	235.9±22.63	32.0	13.68	2.11±0.41	0.90*
G	232.1	286.3	259.2±38.32	54.2	20.9	2.24±0.26	0.86

Values are expressed as mean±SD for each group and considered statistically significant at $P<0.05$. BW= body weight of rats, KW= kidney weight of rats, KBWR= kidney weight body ratio. KBWR = (Mean KW/ Mean BW) x 100. BW diff in % = (BW diff/Mean BW) x 100

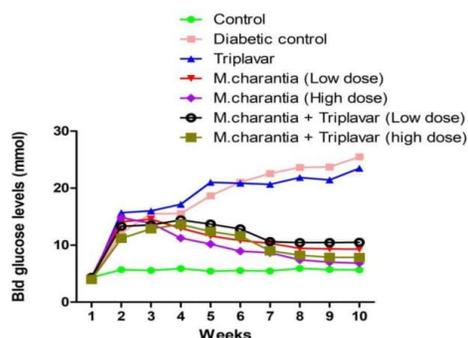


Figure 1. Graphical representation of blood glucose levels

groups (Table 1). The Mean kidney weight of Group D was significantly lower compared to control ($P<0.05$). However, the organ body weight ratios show a significant decreasing trend ($P<0.05$) for group B, and C, D and F.

Blood glucose levels

The blood glucose concentration for group B and C

was significantly higher than control ($P<0.05$) (Figure 1). This level was sustained throughout and was peaked between 6th to 10th weeks (Figure 1). Group D, E, F, and G showed significant mild hyperglycemia ($P<0.05$) compared to the control. From the 4th to 6th week of treatment, group D, E, F and G receiving *M. charantia* extract showed a significant lowering of blood glucose ($P<0.05$). Between weeks 8 to 10, groups F and G showed significant ($P<0.05$) reduction in blood glucose to near normal levels (Figure 1). This reduction of blood glucose level in Groups F and G shows in addition, a dose dependent mitigation of hyperglycemia.

Urine parameters

Urine was collected at weeks (3, 6 and 9) during the 10 weeks experimental period for the measurement of urinary parameters (micro albuminuria, creatinine, sodium, potassium and urea). Groups B and C showed significant ($P<0.05$) albuminuria with reduced creatinine clearance. Renal electrolytes (sodium, and potassium) were statistically reduced ($P<0.05$), in group B and C. Urea levels were significantly reduced ($P<0.05$) in group D (Table 2) but statistically elevated in week 9 (Table 3). Week 9 of the Urine output for groups D, E, F and G showed normal values from an initial increase in week 3.

Table 2. Evaluation of Urine test of control and experimental animals (Week 3)

ps	Alb (mg/l)	Creat (mmol/l)	Alb/creat ratio (mg/mmol)	Sodium (mmol/l)	Potassium (mmol/l)	Urea (mmol/l)
	1.73±0.28	9.60±1.21	0.18±0.05	279.30±19.01	251.3±13.43	411.4±91.45
	23.17±2.07*	1.10±0.17	21.20±1.65	59.33±16.01*	53.47±8.83*	148.9±23.68*
	17.33±5.42*	0.90±0.10	19.17±5.00	69.67±5.51*	49.87±7.41*	135.5±12.63*
	12.20±1.57	5.43±1.17	2.33±0.79	51.33±18.01	65.67±21.44	166.0±49.37*
	4.37±1.07	6.03±1.07	0.74±0.25	163.70±25.50	202.0±37.22	272.3±169.7
	7.33±2.51	4.93±0.32	1.44±0.61	162.7±24.42	155.1±13.67	138.2±24.20
	2.80±1.38	6.90±1.67	0.45±0.31	192.0±16.55	184.3±24.48	265.4±200.7

Values are expressed as mean±SD of each group. * $P<0.05$
Alb: Albumin; Creat: Creatinine

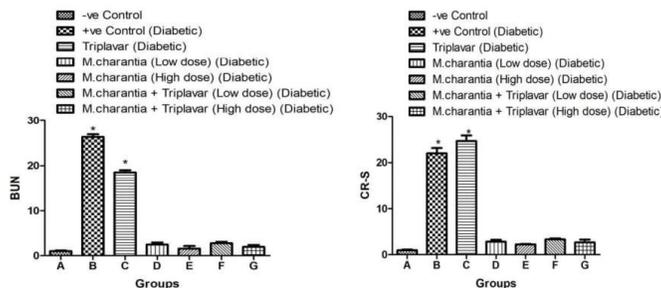


Figure 2. BUN and Scr levels: Bars indicate mean±SD, * $P<0.05$
BUN: Blood Urea Nitrogen; Scr: Serum creatinine

Table 3. Evaluation of Urine test of control and experimental animals (Week 9)

Groups	Alb (mg/l)	Creatinine (mmol/l)	Albumin/creatinine ratio (mg/mmol)	Sodium (mmol/l)	Potassium (mmol/l)	Urea (mmol/l)
A	1.80±0.10	9.20±1.87	0.19±0.03	274.0±1.00	290.0±29.72	522.0±44.54
B	22.07±0.30*	0.73±0.21	32.17±11.06*	61.67±26.35*	58.33±20.53*	152.0±36.35*
C	21.83±0.21*	0.80±0.10	27.57±3.58*	51.67±10.79*	44.00±4.36*	112.3±1.15*
D	0.86±0.45	9.70±2.25	0.09±0.06	243.3±11.02	251.3±10.12	338.3±30.62
E	1.27±0.80	8.80±0.79	0.14±0.09	244.7±11.02	236.3±14.15	361.3±64.08
F	1.27±0.153	10.90±1.05	0.08±0.05	242.7±9.45	243.0±5.57	330.3±6.03
G	1.23±0.57	10.37±2.21	0.12±0.03	250.7±22.03	237.7±19.09	361.7±82.50

Values are expressed as means±SD of each group. * $P < 0.05$

Alb: Albumin; Creat: Creatinine

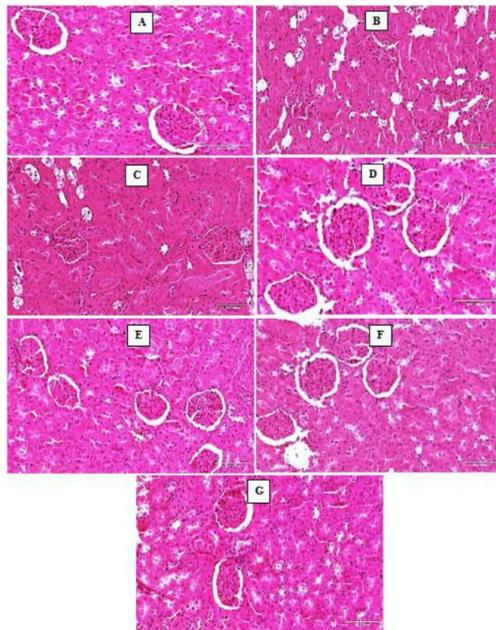


Figure 3. Photomicrographs of the kidney (H and E stains). Scale bar x 200 μm . (A) Control- normal structure of the kidney. (B) Diabetic untreated- degeneration of the glomerulus and vacuolation of tubules (C) Diabetic treated with triplavar- glomerular lesion with tubular necrosis (D) Diabetes treated with *Momordica charantia* extract low dose- restoration of interstitium (E) Diabetes treated with *M. charantia* extract high dose- histoarchitecture essentially normal (F) Diabetes treated with low dose of *M. charantia* extract and Triplavar- histoarchitecture essentially normal (G) Diabetes treated with high dose of *M. charantia* extract and Triplavar- histoarchitecture essentially normal

Blood urea nitrogen (BUN) and serum creatinine (CR-S) levels

Groups B and C exhibited a significantly higher CR-S and BUN ($P < 0.05$) (Figure 2). Adjuvant uses of *M. charantia* at both low and high doses were found to be effective in lowering CR-S and BUN in all groups receiving the extract. However they did not show any significant difference when compared with controls.

Oxidative stress parameters

Group B showed significant depletion of reduced

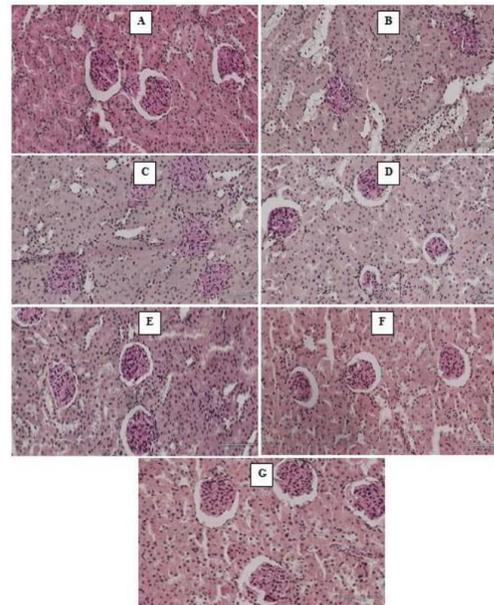


Figure 4. Photomicrographs of the kidney (Periodic Acid Schiff stains). Scale bar x 200 μm . (A) Control- normal structure of the kidney. (B) Diabetic untreated- mucus substances and high proportion of carbohydrate macromolecules (glycogen, glycoproteins) (C) Diabetic treated with triplavar- Mild deposition of polysaccharides (D) Diabetes treated with *Momordica charantia* fruit extract Low dose- histoarchitecture normal (E) Diabetes treated with *M. charantia* fruit extract high dose- histoarchitecture essentially normal (F) Diabetes treated with low dose of MC extract and Triplavar- histoarchitecture essentially normal (G) Diabetes treated with high dose of *M. charantia* fruit extract and Triplavar- histoarchitecture essentially normal

glutathione (GSH), decreased SOD and decreased catalase activities with a concomitant increase MDA ($P < 0.05$) indicating disturbances in the normal redox state (Table 4). Group C showed no significant difference in all antioxidative biomarkers compared to normal controls. However, administration of *M. charantia* at both doses led to significant increase ($P < 0.05$) in GSH level, SOD and CAT activities and depleted MDA level (D, E, F, and G). Administration of the higher dose of *M. charantia* significantly increased GSH levels and SOD activities compared to administration with the lower dose ($P < 0.05$).

Table 4. Oxidative stress measurements of control and experimental animals

Enzymes	Group A	Group B	Group C	Group D	Group E	Group F	Group G
GSH	0.35±0.09	0.43±0.07*	0.29±0.10	0.32±0.07*	0.49±0.07*	0.43±0.03*	0.33±0.01*
SOD	0.07±0.01	0.15±0.12	0.10±0.01	0.10±0.02	0.14±0.08*	0.11±0.01	0.09±0.02
CAT	0.15±0.02	0.47±0.11	0.38±0.14	0.27±0.15	0.24±0.13	0.35±0.05	0.38±0.25
MDA	0.42±0.10	2.02±0.21*	1.58±0.65	2.00±0.63*	0.98±1.27	0.89±1.04	1.07±0.70

Values are expressed as means±SD of each group. * $P < 0.05$. GSH: Reduced glutathione; SOD: Superoxide dismutase; CAT: Catalase; MDA: Malondialdehyde

Histopathological findings

Haematoxylin and Eosin (H and E) stains

Histology of the normal kidney (control group) showed normal glomeruli with Bowman's capsule. The proximal and distal convoluted tubules showed distinct regular lumen no inflammatory changes. The interstitial spaces were of normal architecture with no cellular infiltrations. The kidneys of untreated animals (Group B diabetic rats) and diabetic rats treated with triplavar (Group C) all showed degenerated glomeruli with inflammatory cell infiltration, glomerular capillary abnormalities characterized by capillary wall thickening, capillary occlusion and generalized disruption of capillary loops. Cellular inflammation, tubular epithelial damage and severe necrosis of the vascular wall characterized by hypercellularity were

also observed (Figure 3).

Periodic Acid Schiff (PAS) stains

Diabetic untreated groups (Group B) showed vacuolation of tubules characterized by high proportion of carbohydrates such as glycogen and glycoproteins. Diabetic treated with HAART regimen showed mild deposition of polysaccharides while *M. charantia* treated groups showed an essentially normal glomerular appearance with normal cytoarchitecture comparable to control (Figure 4).

Masson's Trichome (MT) stains

MT stains showed deposition of collagen fibers in the diabetic untreated animals and diabetic animals treated with HAART regimen (Group B and C). These changes were absent in all groups treated with *M. charantia* and showed restoration of the histological layout similar to the control (Figure 5).

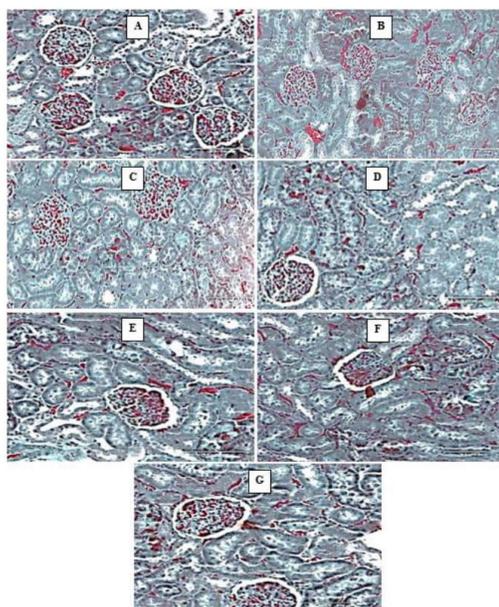


Figure 5. Photomicrographs of the kidney (Masson's Trichome stains). Scale bar x 200 μm . (A) Control- normal structure of the kidney. (B) Diabetic untreated- Presence of collagen fibers and intraluminal thrombosis (C) Diabetic treated with triplavar- Presence of collagen fibers and Interstitial edema, (D) Diabetes treated with *Momordica charantia* extract low dose- histoarchitecture normal (E) Diabetes treated with *Momordica charantia* extract high dose- histoarchitecture essentially normal (F) Diabetes treated with low dose of *M. charantia* extract and Triplavar- histoarchitecture essentially normal (G) Diabetes treated with high dose of *M. charantia* extract and Triplavar- histoarchitecture essentially normal

Discussion

The growing population of patients treated with HAART requires consideration of the potential metabolic disorders such as diabetic nephropathy (DN), cardiovascular diseases, hypertension that may accompany the use of HAART. DN, which is primarily a micro vascular complication of diabetes, has generated a lot interest due to the significantly high mortality and morbidity rates. These effects are characterised by complex molecular, biochemical, structural and functional derangements that progresses from chronic to end stage renal failure (16). DN is characterized by expansion of the mesangium, instigated by cellular proliferation and excessive accumulation of extracellular matrix. This was a key finding in the present investigation. Results of this study showed the glomeruli of the control rats were of normal architecture containing the usual complement of cells and intercellular matrix. The interstitial spaces showed no infiltrations and the capillaries showed a regular distinct lumen. The glomeruli from untreated diabetic rats and diabetic rats treated with triplavar were dramatically different in appearance. The characteristic histological findings include collapsing focal segmental glomerulosclerosis (17, 18). Treatment with *M. charantia* extract at both low and high doses showed remarkable partial reversal of the features of DN resulting in essentially normal histoarchitecture of the kidney with significant reduction of inflammatory cell infiltration. These results are in line with the findings of researchers which showed significant reno-vascular improvement following *M. charantia* leaf extract administration to diabetic rats with DM (19). This histopathological finding was related to the deleterious effects of oxidative stress.

It has been found that several renal cell types including endothelial cells, mesangial cells and tubular epithelial cells accumulate high levels of ROS under hyperglycemic conditions (20). The hyperglycemia contributes to the generation of an environment with high oxidative stress which can directly lead to micro vascular diseases such as seen in DN. Result of this study show depleted GSH, decreased SOD and catalase and increased MDA levels in the diabetic rats indicating the presence of high levels of ROS corroborating several other studies (21, 22). GSH is the first line of defense in the endogenous antioxidant system and regarded as a marker of oxidative stress at cellular level (22). The decreased SOD activities in the diabetic rats are consistent with reports on diabetic induced oxidative stress (22). SOD catalyzes the dismutation of O_2^- to H_2O_2 , which is further converted into oxygen and water by catalase. Furthermore the low catalase activity in diabetic rats result in high levels of OH, resulting from the continuous breakdown of H_2O_2 (23). The OH then attacks the lipid membrane to trigger peroxidative reactions leading to the production of highly reactive aldehydes such as MDA (23, 24). Thus administration of *M. charantia* which results in lower levels of the products of lipid peroxidation suggests a therapeutic effect in combating diabetes induced oxidative stress. This can be attributed to the reported phytochemical constituents of *M. charantia* (25, 26). The analysis of current data of these oxidative stress markers clearly demonstrates the antioxidative potential of the *M. charantia* extract in STZ-induced diabetic rat model. *M. charantia* extracts significantly reduced levels of MDA and replenished GSH, SOD and catalases and raising it towards essentially normal level.

The pathological events underlying the initial changes of DN are not well understood. However, it is proposed that significant pathological glomerular hypertrophy remains the underlying factor of DN. Electrolyte (sodium and potassium) retention is a manifestation of deranged renal function and is observed in diabetic patients before the onset of albuminuria (20). Our result shows significant retention of sodium and potassium in the kidney when compared with the control group. There was also high urea retention with reduced creatinine clearance in the untreated diabetic group and the diabetic group treated with triplavar, which is an indication of an impaired renal function. Treatments with high and low dose of *M. charantia* extract significantly normalized urinary parameters (urea, creatinine, Na) to near normal levels.

Microalbuminuria is a predictor of progression to DN in T2DM and is associated with an accelerated decline in glomerular filtration rate (2). The underlying mechanisms for microalbuminuria involves abnormalities of the glomerular endothelial barrier causing excessive filtration as well as reduction of renal tubular cell albumin degradation and reabsorption (2). Glomerular inflammation and oxidative stress worsen albuminuria and is associated with abnormalities of vasodilatation and the generation of ROS mediated by endothelial derived nitric oxide (NO). This suggests a link between vascular and metabolic abnormalities (3). Excessive albuminuria was seen in the untreated diabetic group as well as the diabetic group treated with triplavar. Treatment with *M. charantia* fruit extract and

both doses however, reversed the condition affirming its therapeutic potential.

As markers of renal function, BUN and serum CR-S routinely serve as indicators for normal biological, pathologic processes, or pharmacologic responses to therapeutic interventions. Our study revealed marked impairment in renal function with significantly raised levels of BUN and CR-S concentrations in untreated diabetic rats and diabetic rats treated with triplavar. This is consistent with low BUN and CR-S clearance observed. The capacity for glomerular filtration and tubular absorption may have been altered with resultant functional overload of nephrons and subsequent renal dysfunction. The treatment of STZ- induced diabetes rats with *M. charantia* extract at both low and high dose prevented the development of DN as evidence by lowering of kidney injury markers such as BUN and CR-S. The mitigation of renal injuries is consistent with other studies (10) on the progression of STZ- induced diabetic nephropathy and further supports the evidence for the antidiabetic properties of the plant.

Polyuria, polydipsia and polyphagia are the typical triad of diabetes, which is increased due to increased levels of blood glucose. Hyperglycemia leads to hyperosmolarity and reduction of intracellular water (27). It causes a negative energy balance and enhanced hunger in diabetic patients. The high blood glucose levels result in glucose spilling through the glomerular filtration apparatus resulting in an osmotic diuresis. This in turn leads to polyuria (27). Our result showed that the untreated diabetic and diabetic rats treated with triplavar all demonstrated the typical characteristics of diabetes mellitus such as polyuria, polydipsia and polyphagia. The *M. charantia* extract at low dose was found to effectively limit polyuria and polydipsia.

Reports from researchers in glibenclamide treated rats also demonstrated that dry *M. charantia* supplementation prevents polyuria and polydipsia in diabetic rats and this could be attributed to the high fiber content of *M. charantia*. Fibers slow the absorption of glucose along the gastrointestinal tract (28). *M. charantia* extract has also been reported by previous researchers to exhibit anti-hyperglycemic effect in the STZ or alloxan- induced diabetic rats (29). Our result corroborates reports from these researchers.

Organ weight analysis is an important endpoint for the identification of potentially harmful effects of test compounds in toxicology studies. Interestingly, in the untreated diabetic rats, a significant increase in the kidney weight was observed (30). It has been described that the kidney enlargement in DM is attributed to certain factors like glucose over-utilization and increased uptake, glycogen accumulation, lipogenesis and protein synthesis in the kidney tissue (31). In this study *M. charantia* extract successfully prevented renal enlargement.

Conclusion

M. charantia extract administration improved blood glucose levels, reinstates renal function, reduces body weight loss and restores hyperglycemia. In addition, *M. charantia* extract appears to diminish oxidative stress damage, restored the total antioxidant ability and up-regulated antioxidant enzymes. *M. charantia* prevented

enlargement of glomerular mesangium and tubular damage. The above data provide substantial evidence that treatment with *M. charantia* extract mitigates the progression and development of STZ induced DN in rats through suppression of oxidative stress damage.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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BRIDGING
BETWEEN CHAPTER THREE AND CHAPTER FOUR

In the previous chapters (Chapter two and three) treatment with HAART imposed varieties of ravages on the kidney histoarchitectural patterns ranging from tubular necrosis, glomerular capillary abnormalities and other biochemical results assessed. These were evident in the non-diabetic and diabetic induced animals. *Mormodica chanratia* was able to mitigate these adverse effects of HAART in both non-diabetic and diabetic state with restoration of the kidney histological structure and function owing to its plethora of phytochemical constituents. In other to understand the impact of *Mormodica charantia* at the subcellular level the study further investigated key components for determining renal integrity and function in the next chapter and also to explore other potential gaps in knowledge that may be needful in addressing the main issues in HIV medicine from the gene expression profiles.

CHAPTER FOUR

MANUSCRIPT

Gene expression profile of NGAL, KIM-1 and TNF- α in non-diabetic and diabetic rat model treated with *Momordica charantia* and highly active antiretroviral therapy.

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Abstract

Background: There is an overwhelming concern regarding the potential toxic effects of highly active antiretroviral therapy (HAART) on the renal intracellular milieu. Early detection of this renal damage is critical for diagnosis of kidney diseases.

Aim: The aim of the study is to determine the effect of *Momordica charantia* (*M. charantia*) on kidney biomarkers Such as neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1) and tumor necrosis factor (TNF- α) following HAART in diabetic and non-diabetic rats.

Materials and Methods: 78 adult male Sprague Dawley rats were divided into two groups (non-diabetic and diabetic) and treated according to protocols. Diabetes was induced with streptozotocin (STZ) by intraperitoneal injection (45 mg/kg body weight). The animals were euthanized on the tenth week and blood obtained via cardiac puncture and centrifuged to collect the sera used for examining NGAL, KIM-1 and TNF- α .

Results: Repeated administration of HAART regimen (triplavar) to both the non-diabetic and diabetic groups not receiving *M. charantia* resulted in upregulation of circulating mRNA expression of NGAL, KIM-1 and TNF- α , significant at $p < 0.05$. Adjuvant treatment with *M. charantia* in both non-diabetic and diabetic group prevented the upregulation of these kidney injury markers suggesting their potent pharmacological properties and attenuating influence.

Conclusion: The mechanisms whether expression of circulating mRNA expression of these biomarkers holds predictive value for nephrotoxicity and diabetic nephropathy warrants further investigations.

Keywords: HAART; Nephrotoxicity; Diabetes, Kidney; *Momordica charantia*; Gene expression, Sprague-Dawley rats.

Introduction

Highly active antiretroviral therapy (HAART) has been associated with an array of organ toxicities such as testicular toxicity, hepatotoxicity, nephrotoxicity and long term complications such as diabetic nephropathy even in animal models (Offor et al., 2017, Azu et al., 2016, Azu et al., 2014). Studies from our laboratory suggest that nephrotoxicity associated with HAART arise from cumulative mitochondrial dysfunction (Offor et al., 2018). Mitochondrial energy generation capacity is subsequently reduced due to impairment of oxidative phosphorylation and DNA inhibition within the mitochondrial. This increases reactive oxygen species formation and disrupt endothelial homeostasis which later leads to glomerular hyperfiltration (Begrache et al., 2011, Newsholme et al., 2007). Several factors may also lead to hyperfiltration such as hyperglycemia, and increased glucose reabsorption in the renal tubules. Hyperglycemia-mediated oxidative stress plays a key role in the advancement of metabolic disorders such as cardiovascular diseases, dyslipidemia and diabetes mellitus (Giri et al., 2018, Alam et al., 2014, Evans et al., 2002).

Plants based adjuvants have been shown to exhibit counter regulatory effects in the process of tissue damage and intracellular disruption. One plant that has received wide recognition for its role in mediating in intracellular disruption and pro-inflammatory cytokines suppression is *Momordica charantia* (*M. charantia*) (Yang et al., 2018, Chao et al., 2014, Bao et al., 2013). *M. charantia* is an economically important medicinal plant belonging to the family Cucurbitaceae and has been used extensively as a remedy for a number of diseases and disorders. *M. charantia* extracts also enhance cellular uptake of glucose, promote insulin release and potentiate its effect and increase the number of insulin producing beta cells in the pancreas of diabetic animals (Mahmoud et al., 2017, Patel et al., 2012, Singh et al., 2011). *M. charantia* contains three anti-HIV proteins which are alpha- and beta momorcharin, MAP-30 (*Momordica* Anti-HIV Protein) and charantin. MAP-30 alpha- and beta-momorcharin inhibit HIV replication in acutely and chronically infected cells and thus are considered potential therapeutic agent in HIV infection and AIDS (Lim, 2012, Puri et al., 2009). MAP30 antiviral agent is capable of inhibiting infection of HIV type 1 (HIV-1) in T lymphocytes and monocytes as well as replication of the virus in already-infected cells (Puri et al., 2009). The inhibition of HIV-1 integrase by MAP30 suggests that obstruction of viral DNA integration may play a key role in the anti-HIV activity of this protein.

Gene expression profile plays a pivotal role in the pathogenesis of various kidney diseases, and the degree of tubulointerstitial damage is strongly associated with renal prognosis (Ding et al., 2007). Thus, markers of kidney injury may be capable of reflecting the degree of sustained renal damage. Neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule (KIM-1) and tumor necrosis factor (TNF- α) are established biomarkers that have been reported as early detectors of acute kidney injury. Studies have

documented that upregulation of these biomarkers are characterized with tubular epithelial injury, interstitial fibrosis and a decline in glomerular filtration rate (GFR) in type 2 diabetic patients (Kishore et al., 2017, Sadar et al., 2016, Huang et al., 2014). There is dearth of literature explaining the attenuating influence of *M. charantia* on kidney injury associated with HAART. In this study we detected the expression of NGAL, KIM-1 and TNF- α by real time RT-PCR following treatment protocol in diabetic and non-diabetic rats.

Materials and Method

Materials

HAART regimen- Triplavar (*Cipla-Medpro*) containing Lamivudine 150 mg, Nevirapine 400 mg and Zidovudine 300 mg, was used for this study. The drug was obtained from Pharmed pharmaceuticals, Pty (Ltd) Durban, South Africa. Fifty kilogram of fresh mature unripe fruit of *M. charantia* was purchased from the local Durban market. Samples were authenticated at the herbarium unit of the Department of Life Sciences, University of KwaZulu-Natal, Durban, South Africa (voucher no. 4617).

Preparation of *M. charantia* fruit ethanolic extract

The fruits were cleaned, sliced into small pieces and the seeds separated out and discarded. The sliced green fruit was first weighed and then dried in shade for approximately 2 weeks. It was then weighed again to obtain the final dry weight before pulverizing into a fine power in a commercial grinder and stored at 5 °C until ready for extraction. The active ingredients were obtained by soxhlet extraction using ethanol as the solvent. The solvent was evaporated in a rotary evaporator at 40°–50°C with a percentage yield of 85.25%. The wet residue was filtered through a whatman filter and the concentrated extract was stored at 4 °C ready for use.

Experimental design

A total of seventy eight (78) adult male Sprague-Dawley rats weighing 178-232 grams were used for the study. Ethical approval was obtained from University of KwaZulu Natal (UKZN) animal research ethics committee (AREC) - ethics number AREC/033/016D. The study was conducted at the Biomedical Resource Unit (BRU) of UKZN.

All rats were housed in well ventilated plastic cages [3 rats per cage for the non-diabetic group (in 12 cages)] and [3 rats per cage for the diabetic group (in 14 cages)]. The dimensions of cages are (52 cm long \times 36 cm wide and 24 cm high) and soft wood shavings served as beddings in the cages. Animals were maintained under standardized animal house conditions (temperature: 23–25 °C; light: approximately 12 h natural light per day) and were fed with standard rat pellets from (Meadow feeds a Division of Astral Operations Limited, Durban, South Africa) and given tap water ad libitum. The initial

body weight of the animals were recorded before treatment and randomly distributed to non- diabetic group (A-F comprising 6 animals per group) and diabetic group (G-L comprising 7 animals per group). As shown in the treatment schedules below:

Non-diabetic group

Group A served as –ve control
Group B received HAART regimen (Triplavar)
Group C received *M. charantia* (200 mg/kgbw)
Group D received *M. charantia* (400 mg/kgbw)
Group E received Triplavar + *M. charantia* (200 mg/kgbw)
Group F received Triplavar + *M. charantia* (400 mg/kgbw)

Diabetic group

Group G served as +ve control
Group H received HAART regimen (Triplavar)
Group I received *M. charantia* (200 mg/kgbw)
Group J received *M. charantia* (400 mg/kgbw)
Group K received Triplavar + *M. charantia* (200 mg/kgbw)
Group L received Triplavar + *M. charantia* (400 mg/kgbw)

The therapeutic dose of triplavar was adjusted for animal weight using the human therapeutic dose equivalent. These substances were administered daily via oral route with orogastric tube. The study lasted for 10 weeks. Monitoring of the animals was carried out by the research team. Thereafter, the animals were euthanized at the 10th week and the tissues were harvested and prepared for observation.

Induction of diabetes mellitus

All rats were placed on a 12 hours fast to obtain baseline fasting blood glucose levels (FBG). The diabetic groups were given intra-peritoneal streptozotocin (STZ) (Sigma-Aldrich Chemical Company, Missouri, St Louis, USA) at 45 mg/kg body weight dissolved in a citrate buffer (pH 4.5) (Etuk, 2010). Successful induction of diabetes was determined by observation of polyuria and polydipsia and confirmed by a 72 hours post STZ FBG level ≥ 11 mmol/L.

Blood sample collection

Blood was collected in plain tubes via cardiac puncture and allowed to clot. It was then centrifuged at 3000 revolutions per minute (rpm) for 15 minutes and the serum decanted into Eppendorf tubes and stored at –20°C pending RNA extraction.

RNA extraction and cDNA Synthesis

RNA was isolated using the Zymo Research Quick-RNA™ Miniprep, CA-USA according manufacturer's protocol. The iScript cDNA synthesis kit (Bio-Rad, USA) was used to perform reverse transcription (RT). Manufactures protocol was followed in preparation of the reagents as well as volumes of reagents used.

Real –Time Polymerase Chain Reaction (qRT-PCR)

The ROCHE light cycler SYBR Green I master mix was used to carry out PCR amplifications on ROCHE light cycler 96 machine. The Primer sequence used can be found in the table 1 below. PCR was done using the following cycling conditions: Pre-incubation for 10 min at 95 °C, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30s and 72 °C for 30s. All samples were assayed in duplicate with a positive and negative control included in each run. The qRT-PCR results were analysed using the $2^{-\Delta\Delta Cq}$ comparative method to compare Cq values of the treated groups to the control group. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used to normalise the Cq values of the treated groups and control groups. To calculate the fold change, a value of 1 was assigned to the controls (Anier et al., 2010).

Table 1: Gene primer sequence

GENE	FORWARD	REVERSE
TNF- α	5'-GACCCTCACACTCAGATCATCTTCT-3'	5'-ACGCTGGCTCAGCCACTC-3'
KIM-1	5'-AACGCA GCG ATT GTG CAT CC-3'	5' -GTA CAC TCACCA TGG TAA CC-3'
NGAL	5'-GAT GAA CTGAAG GAG CGA TTC-3'	5'-TCG GTG GGA ACAGAG AAA AC-3'
GAPDH	5'-CCT GGA GAA ACCTGC CAA GTA T-3'	5'-AGC CCA GGA TGC CCT TTA GT-3'

Statistical Analysis

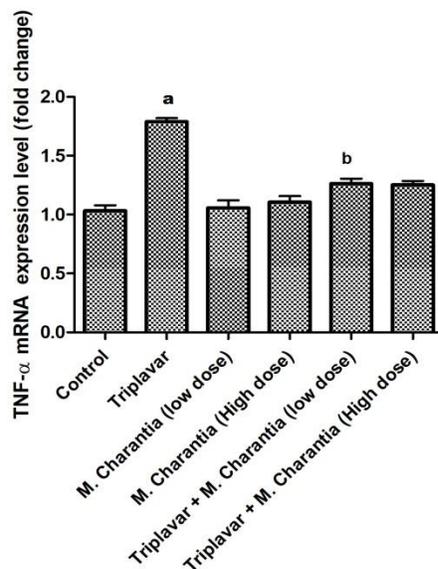
Statistical analysis was performed using the GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). Student t-test was used to test statistical significance of the obtained data. Results are expressed as Mean \pm Standard Deviation. In this study, a p-value ≤ 0.05 was considered to be statistically significant.

Results

Circulating TNF- α mRNA expression level in non-diabetic rats

The circulating TNF- α mRNA expression level in non-diabetic rats showed the triplavar treated group had an upregulated TNF- α mRNA level, this is higher than the level in the *M. charantia* low dose and *M. charantia* high dose groups respectively ($p < 0.05$). The circulating mRNA level in the group treated with high dose of *M. charantia* did not show any difference when the level was compared with that in the group treated with low of dose *M. charantia* ($p > 0.05$).

There was also no significant difference in the circulating level of TNF- α mRNA in the group treated with low dose of *M. charantia* and high dose of *M. charantia*. Although there was an increase in the TNF- α mRNA level in the groups treated with triplavar in combination with *M. charantia* (both low and high dose) but the levels in these groups respectively was significantly lower than in the group treated with only triplavar ($p < 0.05$).



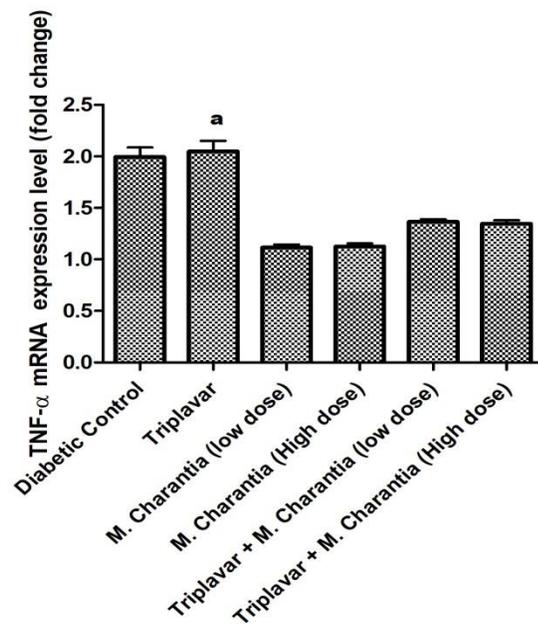
Circulating TNF- α mRNA expression level in non diabetic rats

(Bar indicate mean \pm SD, a= $p < 0.05$, b= $p > 0.05$)

Figure 1: Circulating TNF- α mRNA expression level in non-diabetic rats.

Circulating TNF- α mRNA expression level in diabetic rats

The circulating TNF- α mRNA expression level in diabetic rats showed that there was increase in the triplavar treated group, this was significantly higher than the level in the *M. charantia* low dose treated groups and *M. charantia* high dose treated groups respectively ($p < 0.05$). The circulating TNF- α mRNA level in the group treated with high dose *M. charantia* was not significantly different from the group treated with low dose *M. charantia*. Co-administration of triplavar and both doses of *M. charantia* (low and high dose) did not show any significant difference in the level of TNF- α mRNA.



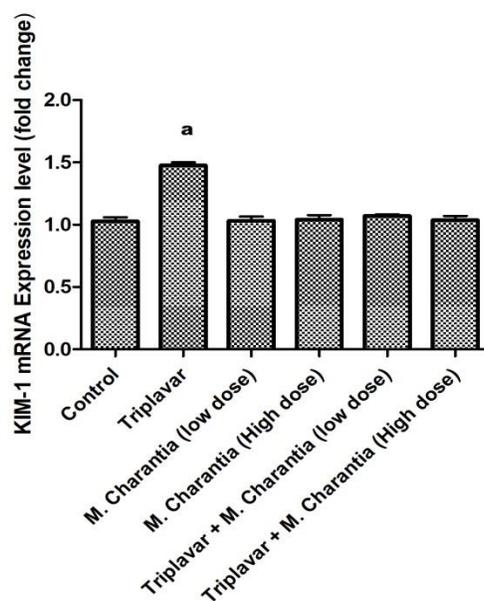
Circulating TNF- α mRNA expression level in diabetic rats

(Bar indicate mean \pm SD, $a = p < 0.05$)

Figure 2: Circulating TNF- α mRNA expression level in diabetic rats

Circulating KIM-1 mRNA expression level in non-diabetic rats

There was an upregulation in the circulating KIM-1 mRNA level in groups treated with triplavar alone and this was significantly higher ($p < 0.05$) when compared with groups treated with concomitant administration of triplavar and *M. charantia* (low and high dose). However, groups treated with low and high dose of *M. charantia* alone did not show any significant change in KIM-1 mRNA levels, as compared to control group.



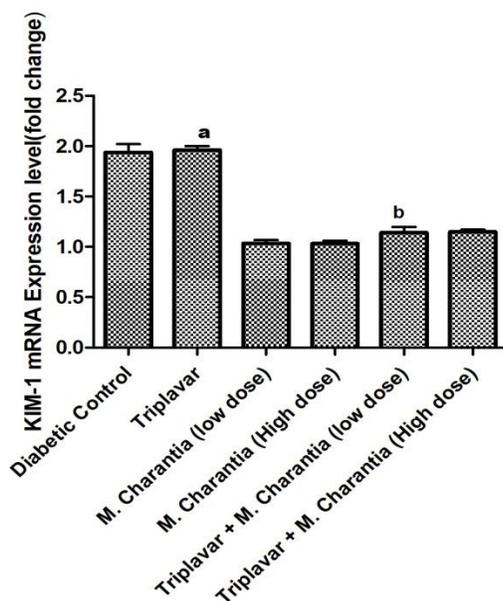
Circulating KIM-1 mRNA Expression level in non diabetic rats

(Bar indicate mean \pm SD, $a = p < 0.05$)

Figure 3: Circulating KIM-1 mRNA expression level in non-diabetic rats

Circulating KIM-1 mRNA expression level in diabetic rats

The circulating KIM-1 mRNA expression level in diabetic rats treated with triplavar alone were upregulated significantly ($p < 0.05$), and this was higher in comparison with adjuvant treatment of triplavar and *M. charantia* even at the two different doses (low and high dose). There was no difference in the circulating KIM-1 mRNA expression levels between animals treated with low dose of *M. charantia* and high dose of *M. charantia*. There was also a marked decrease in the circulating KIM-1 mRNA expression in groups of animals treated with concomitant use of triplavar + *M. charantia* (200 mg/kgbw and 400 mg/kgbw) significant at ($p > 0.05$).



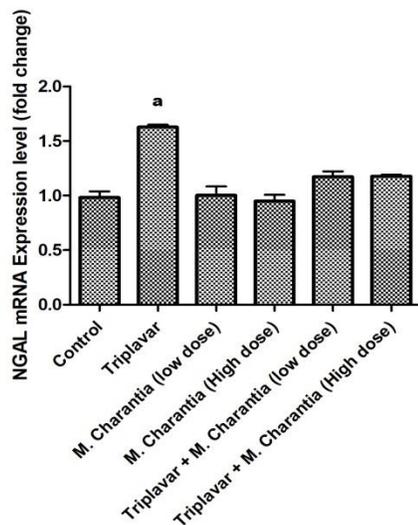
Circulating KIM-1 mRNA Expression level in diabetic rats

(Bar indicate mean \pm SD, a= $p < 0.05$, b= $p > 0.05$)

Figure 4: Circulating KIM-1 mRNA expression level in diabetic rats

Circulating NGAL mRNA expression level in non-diabetic rats

The circulating NGAL mRNA expression level in non-diabetic rats showed there was an upregulation in animals treated with triplavar alone. This expression was significantly higher ($p < 0.05$) than groups of animals treated with combination of triplavar and *M. charantia* at both low and high dose. The NGAL mRNA level in the *M. charantia* high dose group did not show any significant difference when compared with the level in the *M. charantia* low dose group. There was also no significant difference in the circulating NGAL mRNA level between the group treated with triplavar + low dose of *M. charantia* and triplavar + high dose of *M. charantia*.



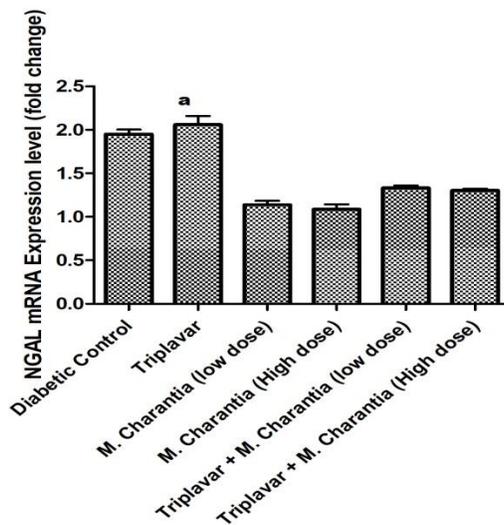
Circulating NGAL mRNA expression level in non diabetic rats

(Bar indicate mean \pm SD, $a=p < 0.05$)

Figure 5: Circulating NGAL mRNA expression level in non-diabetic rats

Circulating NGAL mRNA expression level in diabetic rats

The circulating NGAL mRNA expression level in diabetic rats showed there was a twofold upregulation in the triplavar treated group. The mRNA expression level in the triplavar treated group was significantly higher ($p < 0.05$) than the level in the *M. charantia* low dose, *M. charantia* high dose and in the groups treated with combination of triplavar and *M. charantia* (low and high dose). The NGAL mRNA level in the *M. charantia* high dose group did not significantly differ from the level in the *M. charantia* low dose group. There was a marked increase ($p < 0.05$) in the circulating NGAL mRNA level in the group treated with triplavar alone when compared to the level in the group treated with combination of triplavar and both doses of *M. charantia*.



Circulating NGAL mRNA expression level in diabetic rats

(Bar indicate mean \pm SD, $a = p < 0.05$)

Figure 6: Circulating NGAL mRNA expression level in diabetic rats

Discussion

The present study was designed to assess the value of tissue biomarkers for improved detection of kidney injury in rats using HAART as model compound in non-diabetic and diabetic animal model following treatment with *M. charantia*. HAART may accumulate in epithelial tubular cells causing a range of effects starting with loss of the brush border in epithelial cells and ending in overt tubular necrosis, activation of apoptosis and massive proteolysis (Tiong et al., 2014, Kim and Moon, 2012).

Our results show that HAART induced renal injury in triplavar treated groups both in non-diabetic and diabetic animals as reflected by an upregulation in the circulating NGAL, KIM-1 and TNF- α mRNA levels in rat serum. The increased expression of NGAL, KIM-1 and TNF- α in our study agrees with (Vaidya et al., 2008) that these three biomarkers reflect renal injury accurately. In a study by (Vaidya et al., 2010), changes in gene expression were found to correlate with the progressive histopathological alterations with significantly raised levels of traditional clinical parameters, (blood urea nitrogen and serum creatinine) indicative of impaired kidney function . These biomarkers are sensitive indicators of acute kidney injury caused by exogenous toxicants. Our reports agrees with (Vaidya et al., 2006) that changes in the circulating mRNA expression of NGAL and KIM-1 were one of the earliest effects observed in kidneys of animals exposed to noxious substances and correlated with the histopathological changes in epithelial tubular cells.

Interestingly, the circulating mRNA expression profiles of these biomarkers (NGAL, KIM-1 and TNF-a) were shown to differ considerably following concomitant treatment with *M. charantia* and HAART and *M. charantia* alone (low and high dose) in both non-diabetic and diabetic animals. The capacity of *M. charantia* treatments to reverse the over expression of the circulating levels of these kidney injury markers caused by HAART is an indication of its antioxidative properties. *M. charantia* possesses phytochemical properties which are powerful counter measures against oxidative stress tissue damage. Studies have documented the hypoglycemic effects of *M. charantia* through physiological, pharmacological and biochemical mechanisms. It is believed to act via stimulation of peripheral skeletal muscle glucose utilization (Kwatra et al., 2016), inhibition of intestinal glucose uptake (Abas et al., 2015), inhibition of adipocyte differentiation (Nerurkar et al., 2010), suppression of key gluconeogenic enzymes, stimulation of key enzyme of hexose monophosphate (HMP) pathway and preservation of β cell islet and its functions (Perumal et al., 2015).

These three kidney biomarkers (NGAL, KIM-1 and TNF- a) might also provide sensitive endpoints for in vivo toxicity. Few studies have investigated the relationship between these biomarkers and diabetes

pathogenesis. On the other hand no studies have evaluated the relationship between these markers on diabetes pathogenesis following antiretroviral treatment and the role of *M. charantia*.

Measuring these circulating expressions of these biomarkers may enable clinicians to make an early diagnosis of nephrotoxicity and diabetic nephropathy. Support for this notion comes from numerous clinical studies in which plasma KIM-1 and NGAL increased a few hours after induction of nephrotoxic drugs (Adiyanti and Loho, 2012).

Conclusion

Based on the results assessed in this study, *M. charantia* fruit extract restored the over expression of the circulating levels of these kidney injury markers caused by HAART to a moderate extent. This might be due to pronounced antioxidant properties. It is known that the pathogenic pathway for HAART-mediated injury has been associated with mitochondrial toxicity. HAART may exert a selective pressure that works on the mitochondrial population and destroys mitochondrial that cannot metabolize its components eventually resulting to progressive kidney injury. This may initiate a cascade of events starting with membrane deterioration, disruption of other energy-dependent process that eventually lead to necrosis and upregulation of circulating mRNA expression of NGAL, KIM-1 and TNF- α , as we have observed in our study. However, the putative mechanisms whether expression of circulating mRNA expression of these biomarkers holds predictive value for nephrotoxicity and diabetic nephropathy warrants further investigations.

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Conflict of interest

The authors declared that there is no conflict of interest.

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BRIDGING

BETWEEN CHAPTER FOUR AND CHAPTER FIVE

As the previous chapters have highlighted the toxicities of HAART both in the non-diabetic and diabetic condition looking at the light microscopic observation as well as other biochemical and metabolic parameters that were assessed which are actually the first lines of examination following the deleterious effects associated with HAART. The next chapter however shed more light on the ultrastructural changes that are accompanied with HAART treatment to further potentiate the previous observations.

CHAPTER FIVE

MANUSCRIPT

An ultrastructural investigation of the kidney following treatment with highly active antiretroviral therapy and *Momordica charantia* in non-diabetic and diabetic animals.

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Abstract

With the introduction of highly active antiretroviral therapy (HAART) in the management of HIV/AIDS, the prognostic indication has shifted the pandemic into a manageable chronic condition. Despite this, there is still an unmet medical need on the severe side effects coupled with micro-structural perturbations especially in the kidney that have debilitated patients. This study reports ultrastructural changes in the kidney following HAART and adjuvant treatment with *Momordica charantia* in an experimental model.

Following ethical approval from the University of KwaZulu Natal animal research ethics committee (AREC) - ethics number AREC/033/016D, 78 adult male Sprague-Dawley rats were grouped into non-diabetic and diabetic and diabetes was induced by intraperitoneal injection of STZ (45 mg/kg body weight). Animals were treated with *M. charantia* and HAART regimen (triplavar) according to protocols. On day 70, the animals were euthanized with Halothane and kidney tissues were harvested and processed for transmission electron microscopy (TEM).

In the non-diabetic groups, TEM images showed nuclear condensation and mitochondrial distortion marked in HAART-alone group compared to others.

In the diabetic groups, podocytes effacement and membrane disruptions were seen in the diabetic control as well as HAART treated group. Glomerular basement membrane surfaces were irregular. Treatment with *M. charantia* alone and as adjuvant with HAART mitigated some of these structural observations thus suggesting a protective effect of *M. charantia* on the kidney. *M. charantia* extract improved renal ultrastructure and reversed HAART-induced ultrastructural alterations.

Keywords: Highly active antiretroviral therapy, Ultrastructure, Basement membrane, Diabetic nephropathy, *M. charantia*, Sprague-Dawley rats.

Introduction

Reduction in morbidity and mortality has been the most outstanding achievement in the global fight against HIV/AIDS in both low and middle-income countries (Günthard et al., 2014, Hayes et al., 2014) but despite this achievement, there are concerns about associated side effects of highly active antiretroviral therapy (HAART), which have increased more recently (Trickey et al., 2017, Carr and Cooper, 2000). HAART-related side effects may be transient or may persist throughout therapy (Takaki et al., 2017, Montessori et al., 2004) and have been attributed as a major reason for the discontinuation of therapy or change in treatment strategy, thereby limiting patients adherence and consequently, virologic effectiveness (Gaida et al., 2016, Günthard et al., 2014).

HAART related kidney toxicity can present as acute or chronic kidney diseases that lead to changes in renal function, inducing metabolic changes and renal damage (Hou and Nast, 2018, Boswell and Rossouw, 2017). The pathophysiological mechanisms leading to HAART associated nephrotoxicity remains elusive. Although, evidence suggests that HAART nephrotoxicity is linked to mitochondrial DNA inhibition which disrupts endothelial homeostasis resulting to endothelial thickening (Milián et al., 2017, Yedla et al., 2015). Endothelial thickening progresses to arterial hyalinosis which later leads to glomerular hyperfiltration (Milián et al., 2017). Several factors may lead to this hyperfiltration such as hyperglycemia and increase glucose reabsorption in the renal tubules. This plays a key role in the advancement of diabetic nephropathy. (Offor et al., 2018).

With the heightened attention of nephrotoxicity associated with HAART, management of HIV and AIDS has now become a double edge sword with an increased risk of hyperglycemia and insulin resistance. Although, it is likely that hyperglycemia in HIV patients undergoing treatment with HAART may be accounted for by other pathological conditions linked to HIV infection in interaction with mediating processes such as inflammatory cytokines (Brown et al., 2005, Calza et al., 2004). However, in a study by (Montessori et al., 2004, Walli et al., 1998), protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) medications exacerbated the risk of hyperglycemia and insulin resistance. Disturbances in glucose-insulin homeostasis through hyperglycemia and insulin resistance predispose to type 2 diabetes mellitus and further microvascular complications of diabetes such as diabetic nephropathy (DN). The pathophysiology of DN includes increase in cytokines secretion which disrupts endothelial homeostasis with a subsequent decline in glomerular filtration rate (Wada and Makino, 2013, Navarro-González et al., 2011).

Since ancient times, medicinal plants have been a worthy source treating various ailments. One of these plants is *Momordica charantia* (*M. charantia*), also known as karela, or bitter melon, which belongs to

the *cucurbitacea* family, grows in tropical areas, like east and southern Africa, Asia, and the Caribbean. It is cultivated throughout these areas as a food and medicine (Dhiman et al., 2012, Grover and Yadav, 2004). The *M. charantia* contains anti-hyperglycemic constituents such as glycosides, saponins, alkaloids, fixed oils, triterpenes, proteins and steroids (Grover and Yadav, 2004). Fruit of the *M. charantia* has shown more pronounced anti-hyperglycemic activity. Several studies have reported the anti-diabetic effects of *M. charantia* on renal functional and histological changes in type 2 diabetes mellitus rats induced by streptozotocin (Patel et al., 2012, Kumar Shetty et al., 2005). However, there is dearth of literature on the antidiabetic effects of *M. charantia* on the renal changes of rats induced by streptozotocin following treatment with HAART. Based on this, this work investigates the adjuvant use of *M. charantia* and antiretroviral therapy in a non-diabetic and diabetic animal model.

Materials and Methods

Ethical approval

Ethical approval was obtained from University of KwaZulu Natal (UKZN) animal research ethics committee (AREC) - ethics number AREC/033/016D. The study was conducted at the Biomedical Resource Unit (BRU) of UKZN.

Materials

A total of seventy eight (78) adult male Sprague-Dawley rats weighing 178-232 grams were used for the study. HAART regimen- Triplavar (*Cipla-Medpro*) containing Lamivudine 150 mg, Nevirapine 400 mg and Zidovudine 300 mg, was used for this study. The drug was obtained from Pharmed pharmaceuticals, Pty (Ltd) Durban, South Africa. Fifty kilogram of fresh mature unripe fruit of *M. charantia* was purchased from the local Durban markets. Samples were authenticated at the herbarium unit of the Department of Life Sciences, University of KwaZulu-Natal, Durban, South Africa (voucher no. 4617).

Methods

Preparation of *M. charantia* fruit ethanolic extract

The fruits were cleaned, sliced into small pieces and the seeds separated out and discarded. The sliced green fruit was first weighed and then dried in shade for approximately 2 weeks. It was then weighed again to obtain the final dry weight before pulverizing into a fine power in a commercial grinder and stored at 5 °C until ready for extraction. The active ingredients were obtained by soxhlet extraction using ethanol as the solvent. The solvent was evaporated in a rotary evaporator at 40°–50°C with a percentage yield of 85.25%. The wet residue was filtered through a whatman filter and the concentrated extract was stored at 4 °C ready for use.

Experimental design

All rats were housed in well ventilated plastic cages [3 rats per cage for the non-diabetic group (in 12 cages)] and [3 rats per cage for the diabetic group (in 14 cages)]. The dimensions of cages are (52 cm long × 36 cm wide and 24 cm high) and soft wood shavings served as beddings in the cages. Animals were maintained under standardized animal house conditions (temperature: 23–25 °C; light: approximately 12 h natural light per day) and were fed with standard rat pellets from (Meadow feeds a Division of Astral Operations Limited, Durban, South Africa) and given tap water ad libitum. The initial body weight of the animals were recorded before treatment and randomly distributed to non- diabetic group (A-F comprising 6 animals per group) and diabetic group (G-L comprising 7 animals per group). As shown in the treatment schedules below:

Table 1: Showing groups and treatment schedules for non- diabetic animals

Non- diabetic groups	Treatment
A	-ve control group
B	Triplavar
C	<i>M. charantia</i> (200 mg/kg)
D	<i>M. charantia</i> (400 mg/kg)
E	Triplavar + <i>M. charantia</i> (200 mg/kg)
F	Triplavar + <i>M. charantia</i> (400 mg/kg)

Table 2: Showing groups and treatment schedules for diabetic animals

Diabetic groups	Treatment
G	+ve control group
H	Triplavar
I	<i>M. charantia</i> (200 mg/kg)
J	<i>M. charantia</i> (400 mg/kg)
K	Triplavar + <i>M. charantia</i> (200 mg/kg)
L	Triplavar + <i>M. charantia</i> (400 mg/kg)

The therapeutic dose of triplavar was adjusted for animal weight using the human therapeutic dose equivalent. These substances were administered daily via oral route with orogastric tube. The study lasted

for 10 weeks. Monitoring of the animals was carried out by the research team. Thereafter, the animals were euthanized at the 10th week and the tissues were harvested and prepared for observation.

Induction of diabetes mellitus

All rats were placed on a 12 hours fast to obtain baseline fasting blood glucose levels (FBG). The diabetic groups were given intra-peritoneal streptozotocin (STZ) (Sigma-Aldrich Chemical Company, Missouri, St Louis, USA) at 45 mg/kg body weight dissolved in a citrate buffer (pH 4.5) (Etuk, 2010). Successful induction of diabetes was determined by observation of polyuria and polydipsia and confirmed by a 72 hours post STZ FBG level ≥ 11 mmol/L.

Tissue preparation for electron microscopy

Ultrastructure of the kidney was studied using the transmission electron microscope (Tsuno, 2000). To accomplish this aim, small samples (about 1 mm) cut from different areas of the kidney were fixed by immersion method in formaldehyde-glutaraldehyde mixture at 4°C overnight. Subsequently, the specimens were washed (4 x 15 min) in 0.1 M cacodylate buffer (pH 7.2). All samples were post-fixed in 1% osmium tetroxide for 2 hr. Later, they were washed again (3 x 20 min) in 0.1 M cacodylate buffer (pH 7.2). After dehydration in a graded series of ethanol (50%, 70%, 90%, 100%) and propylene oxide (Merck, Darmstadt, Germany) the specimens were gradually embedded in Spurr's Low Viscosity Resin (Polysciences, Eppelheim, Germany). Briefly, samples were firstly placed (2 x 15 min) in propylene oxide (Merck, Darmstadt, Germany) then in propylene-Spurr's resin mixture (2:1) for 1 hr, in propylene-Spurr's resin mixture (1:1) overnight and finally in pure Spurr's resin overnight.

Thereafter, kidney specimens were embedded in silicon molds (Plannet, Wetzlar) containing Spurr's resin and polymerized at 70°C for 8 hr. For general morphology, semi-thin sections (1 μ m) were cut using a Leica EM UC7 ultramicrotome (Leica, Germany) and stained with toluidine blue (Sigma-Aldrich Chemicals GmbH, Deisenhofen, Germany) for examination under light microscopy. Ultra-thin sections were then cut from selected blocks and mounted on copper grids (SSI, Science Services, Munich, Germany) and routinely contrasted with uranyl-acetate and lead citrate (Reynolds, 1963) prior to examination with a JEOL 2100 HR TEM (JOEL, Japan).

Results

The TEM images shows various ultrastructural features of the kidney. Figure 1A shows intact internal lining of the glomerular capsule which is composed of epithelial cells called podocytes covering each capillary, forming filtration slits (blue arrows) between interdigitating processes called pedicels (shown in fig E and F). Filtrate is produced in the glomerulus when blood plasma is forced under pressure across the glomerular basement membrane (GBM) of the glomerular capillary wall and through the filtration slits (blue arrows) between the pedicles of podocytes processes.

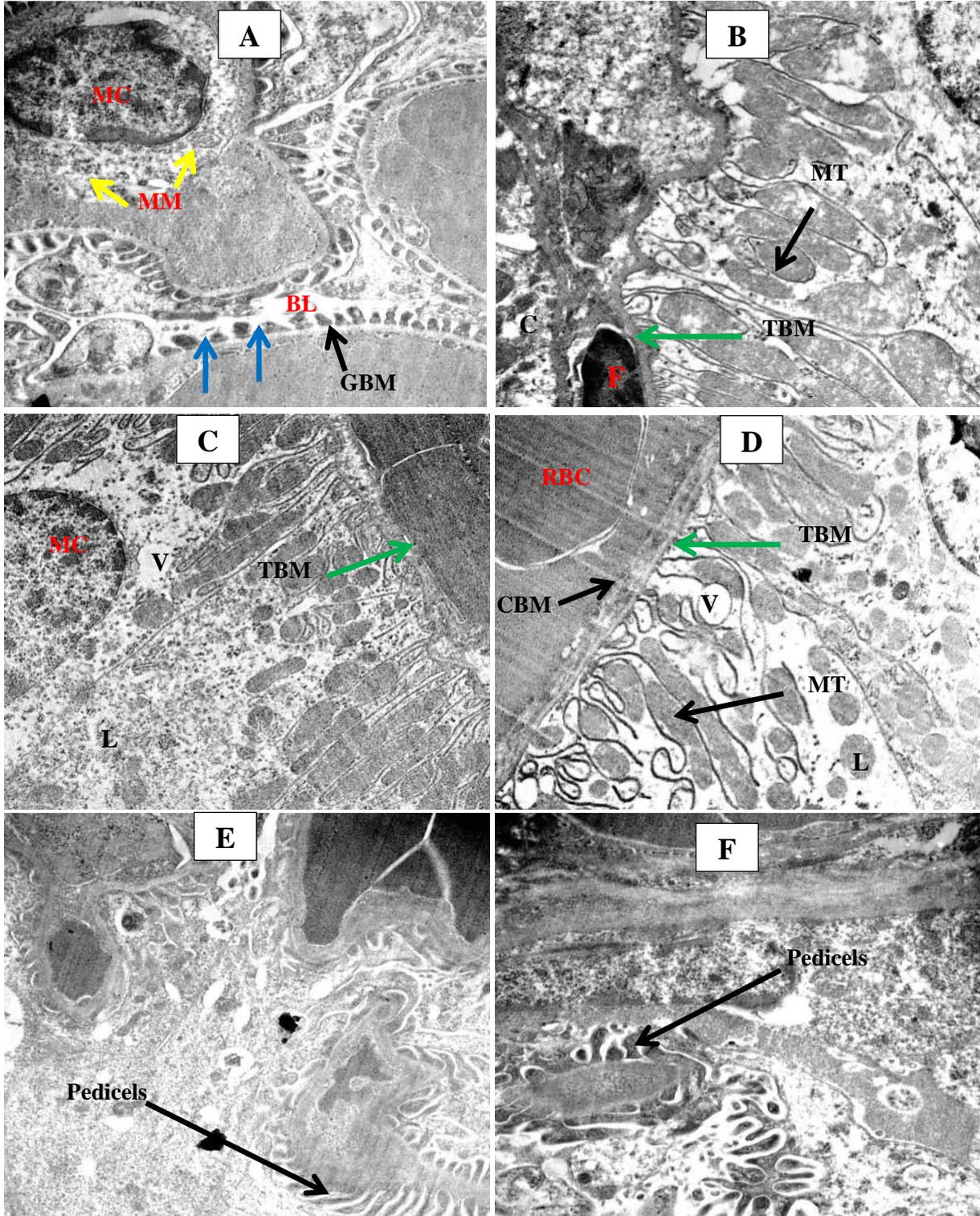
Also shown in Fig 1A are intact mesangial cells (MC) in the interstitial and are often stain more darkly. The Mesangial cells are enveloped by amorphous mesangial matrix (MM-yellow arrow) surrounding it which helps to support capillary loops.

Figure 1B showed wrinkling and folding of the tubular basement membrane (TBM-green arrow). Epithelial cells were markedly hypertrophied. The most striking ultrastructural feature was the finding of swollen mitochondria in the endothelium. The associated swelling of the mitochondria is an indication of degenerative nuclear change. Between the basement membranes of the tubule and the capillary is an extension of a fibroblast (F).

Fig 1 C and D shows important features of the tubules under the TEM. The abundant pinocytotic pits and vesicles (V) and the lysosomes (L). These features appear to be intact. Small proteins brought into the cells by pinocytosis are degraded in lysosomes and the amino acids released basally. The basolateral sides (Fig 1 C and D) are characterized by long invaginating folds of membrane along which many intact long mitochondria (MT) are situated. It is suggested that these folds provides an increased surface area for pumping of ions across the membrane.

Figure 1 E and F shows a distinct appearance of the podocytes foot processes or pedicels covering the glomerular capillaries. Podocytes have cell bodies which gives rise to several foot processes that embrace portions of the glomerular capillary. The pedicels interdigitate, creating elongated spaces called the filtration slits. The cell bodies of podocytes are not in contact to the GBM, but rather each pedicel is in direct contact with the GBM and this is the most substantial part of the filtration barrier preventing macromolecules and other negatively charged molecules from entering the barrier.

In Fig 1 G and H, the selective permeability of the GBM has been drastically altered indicating an increase in thickness of the GBM and podocyte effacement (red arrows). These ultrastructural abnormalities were not seen in fig 1 (I, J, K and L) which were treated with either *M. charantia* and/or its combination with HAART. The swollen mitochondria, thickening of GBM and podocytes effacement which were major features present in (figure B, G and H) were not conspicuous in other groups.



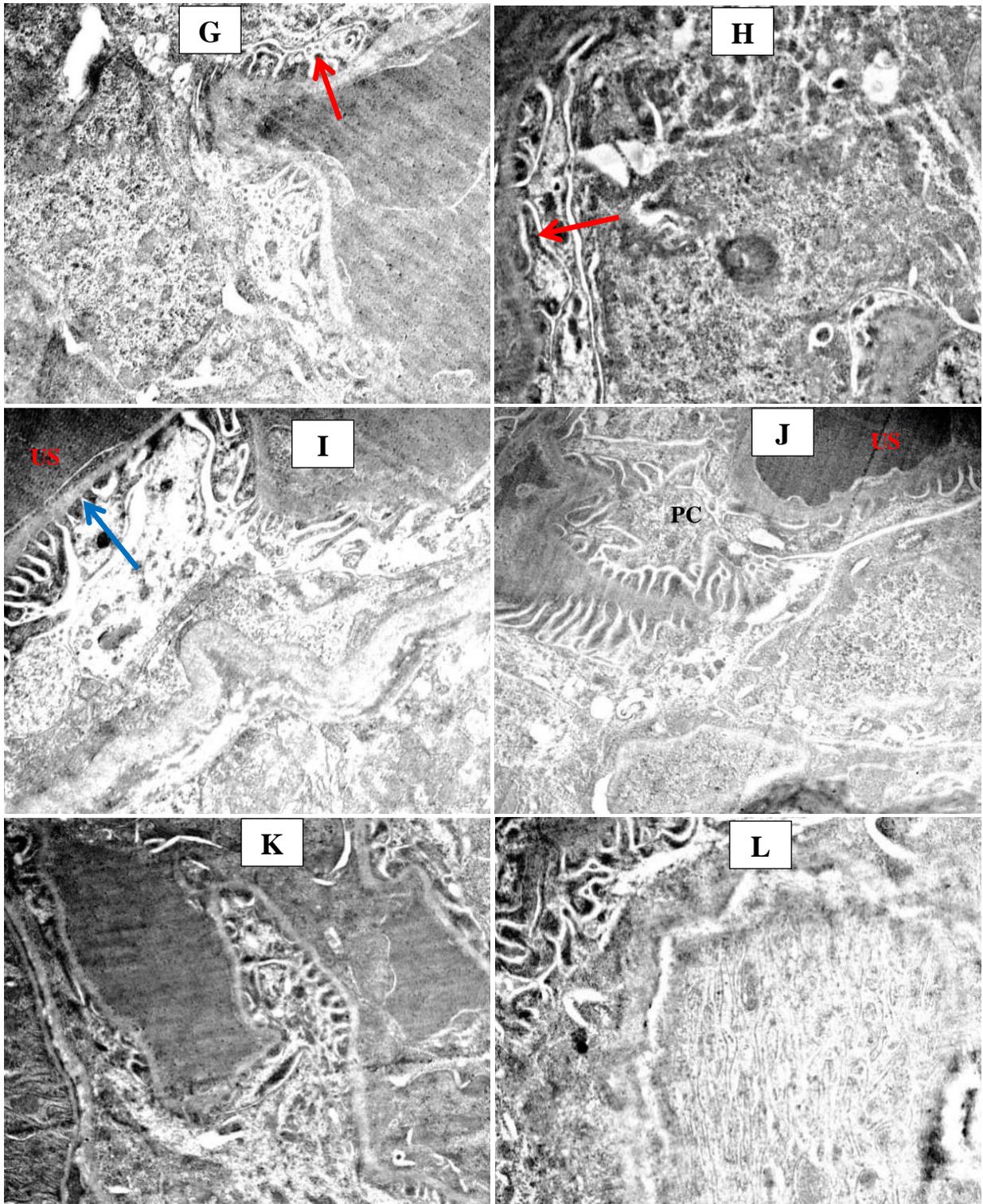


Figure 1. (A-L): TEM images of the rat kidney (Magnification 2,000,000 x).

Non diabetic (A-F) (A) –ve Control, (B) HAART alone. (C) *M. charantia* 200 mg/kg (D) *M. charantia* 400 mg/kg, (E) *M. charantia* 200 mg/kg + HAART (F) *M. charantia* 400 mg/kg + HAART.

Diabetic (G-L) (G) +ve Control (H) HAART alone (I) *M. charantia* 200 mg/kg (J) *M. charantia* 400 mg/kg, (K) *M. charantia* 200 mg/kg + HAART (L) *M. charantia* 400 mg/kg + HAART.

Discussion

Our results have shown an improvement in ultrastructural morphology of the kidneys, due to the concurrent administration of *M. charantia*, proving to provide protection against the toxic action of HAART. The improved restoration of the ultrastructural morphology of the kidney in the present study following concomitant administration with *M. charantia* supports our previous studies (Offor et al., 2018) under light microscopic observation where *M. charantia* prevented enlargement of glomerular mesangium and tubular damage. From our previous study (Offor et al., 2018), derangements of the cytoarchitectural patterns of the kidney were seen as a result of treatment with HAART regimen (triplavar). These were characterized by tubular epithelial desquamation and glomerular capillary abnormalities with extracellular matrix accumulation (Perazella and Moeckel, 2010, Arendshorst et al., 1975), which is suggestive that disturbances between the mesangial cell and glomerular capillaries may have been drastically altered. Glomerular abnormalities often result in a decrease in surface area available for filtration and a consequent decline in glomerular filtration rate and reduction in metabolic activity (Fioretto and Mauer, 2007). In diabetic kidney disorder, the glomerular filter is altered as a result of weakening basement membrane. This will create permeability to glucose and proteins with the subsequent release of protein into the urine.

In our current study, we found that HAART affects the endothelium by altering its basement membrane. Studies have shown that the basement membrane plays an essential role in sustaining the structural and functional integrity of the kidney (Miner, 1999). Impairment of the membrane will severely compromise the function of the kidney. In the present study, the renal ultrastructure of the HAART-treated rats displayed an abnormally, irregular arranged glomerular basement membrane (refer to figure 1G). This actually conforms to our previous work and another study indicating that HAART increases carbohydrate components and contributes to extensive fibrosis around the basement membrane (Ogedengbe et al., 2016, Azu et al., 2014).

As the entire basement membrane is made of extracellular matrix, overexpression or degradation of the extracellular matrix will contribute significantly to kidney failure. Accumulation of the extracellular matrix is a major cause of interstitial fibrosis, infiltration and a thickened basement membrane that results to glomerular abnormalities. Experimental animal studies on HAART have confirmed increased infiltration, thickened membrane, extensive necrosis and atrophy with disruption of the normal function of the kidney (Wyatt et al., 2009).

Antiretroviral drugs have been reported to give rise to several life-threatening complications such as mitochondrial dysfunction arising from altered mitochondrial DNA (mtDNA) replication and generation of reactive oxygen species (ROS) leading to oxidative stress (Deavall et al., 2012).

In this study, the damage to mitochondria is probably due to elevated ROS production leading to membrane damage with increased permeability to HAART. This is accompanied by disruption of normal oxidative phosphorylation and cytoplasmic swelling which eventually results in disruption of cristae, collapse of internal mitochondrial structure and energy deprivation (Lewis and Dalakas, 1995).

Remarkably, *M. charantia* was found to have protective effects on the kidneys in our study as it considerably restored the ultrastructural feature. The beneficial effect of *M. charantia* on the kidney is just a part of various pharmacological properties, particularly its potential as a free radicals scavenger. The antioxidant activity of *M. charantia* extracts mentioned in various studies (Leelaprakash et al., 2011, Wu and Ng, 2008, Sathishsekar and Subramanian, 2005), is considered to be exerted mainly by flavonoids. Taking into consideration that flavonoids extracted from other medicinal plants with renoprotective effects, have been described to inhibit nephrotoxicity induced by xenobiotics in some experimental animal models (Renugadevi and Prabu, 2009, Galati and O'brien, 2004). We can suggest that the renoprotective effects of *M. charantia* in our study could be described by a concurrent antioxidant and free radicals scavenging effect. This hypothesis is validated by our previous study (Offor et al., 2018) where we confirmed that *M. charantia* extracts was able to re-establish the kidney function of rats through its antioxidant activity.

The ultrastructural abnormalities in the kidney of diabetic rats, including the patchy thickening of the basement membrane, were evidently improved by *M. charantia* treatment. The STZ-induced diabetic rat model has been widely used in DN research. In the present study, we successfully established the rat model of diabetes according to previous description (King, 2012, Tesch and Allen, 2007). Generally, DN is functionally characterized by raised urinary protein and renal dysfunction. In the study by (Dronavalli et al., 2008), the DN group exhibited significant increase in renal functional parameters compared to control demonstrating that the STZ-induced diabetic rats were subjected to renal dysfunction. Renal dysfunction is usually caused by the alterations of structural/cellular basis in DN including extracellular matrix accumulation, basement membrane thickening, mesangial cell expansion and podocytes hypertrophy (Dronavalli et al., 2008, Ziyadeh, 1993). The present result agrees with (Dronavalli et al., 2008) that renal dysfunction is associated with renal morphological alterations and *M. charantia* could reduce the risk of renal dysfunction by preventing injuries to the structural/cellular basis.

Conclusion

The current study has shown that the treatment with *M. charantia* improved the kidney ultrastructural features. Our study, according to similar studies, has proven that the ultrastructural kidney alterations observed with HAART treatment in non-diabetic and diabetic condition were re-established by *M. charantia* treatment, concluding that *M. charantia* may have protective effects against morphological alterations of the kidneys. We suggest that *M. charantia* putative mechanisms to unravel the pathways by *M. charantia* could ameliorate/revert kidney abnormalities and other metabolic disorders following HAART treatment be explored.

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Conflict of interest

The authors declared that there is no conflict of interest.

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CHAPTER SIX

SYNTHESIS, CONCLUSION AND RECOMMENDATION

6.1 Synthesis

The introduction of highly active antiretroviral therapy (HAART) has led to a dramatic reduction in morbidity and mortality caused by HIV and AIDS. However, clinicians are increasingly faced with severe deleterious consequences associated with HAART which continue to undermine its success. These appear to result in damage to mitochondrial DNA mediated via oxidative stress mechanisms (Akay et al., 2014, Manda et al., 2011). These adverse effects of HAART which include various metabolic complications and multi-organ toxicities arising from long term use have been shown to severely compromise the quality of life of people living with HIV and AIDS (PLWHA). Due to cost of HAART majority of those affected are unable to afford or continue on these treatments on a consistent and sustainable basis making it difficult to meet the WHO plans to reduce the mortality caused by HIV and AIDS globally. Although, there has been steady progress in increasing accessibility to ART however, in resource-limited countries most patients have no access to this life-saving intervention due to lack of ART centers. Unfortunately, the poor and vulnerable segments of society are likely to miss out on accessing HAART (Natrass, 2006). The economic burden of sustaining adherence to HAART has posed significant challenges to PLWHA leading to settling for traditional remedies (Yang et al., 2018, Hughes et al., 2015). In Africa, traditional remedies have been considered a long-established form of health care and a way of life, and many of these products are generally safer than potent synthetic pharmaceuticals, locally available and cost-effective (Hughes et al., 2015).

Metabolic complications related to long-term exposure to HAART have remained a global problem in the management of HIV and AIDS patients. Since the introduction of HAART in the mid-1990, the risk of diabetes mellitus (DM) have been reported with increasing frequency in PLWHA due to long-term exposure to HAART (Lin et al., 2018). The incidence of diabetes mellitus has reached epidemic proportions worldwide, and it is expected to increase to over 300 million by 2025. The most frequently observed microvascular complications for diabetes mellitus is diabetic nephropathy (DN). DN has become an interesting area to researchers especially on the use of traditional herbal medicine to manage a chronic condition.

While the use of herbal products predates the emergence of synthetic therapeutic drugs, its consumption in the management of various ailments is a common practice in the African continent (Hughes et al., 2015). More recently, there has been an increase in the use of herbal products as an adjuvant for people living with HIV/AIDS in the era of HAART and this is high among adults in sub-Saharan Africa (Sibanda

et al., 2016, Langlois-Klassen et al., 2007). Therefore, this study explores possible protective role of *M. charantia* (traditionally available herbal plant) in the mitigation of HAART's effects on the kidney in non-diabetic and diabetic animal models with specific focus on histological and ultrastructural alterations, gene expression profiles, blood glucose levels and other biochemical changes.

This study also looked at the organ- body weight changes following the administration of HAART and how *M. charantia* could affect this parameter. Organ-body weight alterations offers significant insight in toxicology studies (Azu et al., 2016). The study revealed a significant decrease in body weight of animals treated with HAART. Although weight gain may be associated with the initiation of HAART possibly due to other metabolic issues related to lipid/sugar, the long term consequence is eventual weight loss (Azu et al., 2016). The investigation showed no significant difference in the weight coefficient (organ/body weight ratio) of the kidney tissue in the non-diabetic group. However in the diabetic group the organ body weight ratios show a significant decreasing trend ($p < 0.05$) for animals treated with HAART alone and animals treated with low and high dose of *M. charantia* alone (Groups C, D and E, table 1, chapter 3).

Nephrotoxicity just like testicular toxicity can manifest in the form of organ hypertrophy. The untreated diabetic rats and the diabetic rats treated with HAART displayed a significant increase in kidney weight as observed in the study (see table 1, chapter 3). Increase in kidney weight may be attributed to certain factors like accumulation of lipid droplets. Accumulation of lipids usually signifies dysfunctional mitochondrial and therefore indicates an unhealthy struggling cell and the beginning of necrosis (Begrliche et al., 2011). Mitochondrial dysfunction may initiate cascade of events starting with membrane deterioration, impairment of mitochondrial fatty acid β -oxidation, oxidative phosphorylation and the disruption of other energy-dependent processes that may eventually lead to necrosis of the glomerular endothelium. Adjuvant treatment with *M. charantia* successfully maintained kidney weight in this study.

Assessment of blood glucose level plays a critical role in evaluating functional capacity of the kidney. Elevated levels of blood glucose could occur as a result of occlusion of the vascular wall of the kidney (Rask-madsen, and King, 2013). This pathogenic pathway is complex, although it is suggested that there is an increase in cytokines secretion and an imbalance of the endogenous vasodilators and constrictors. In this study there was significant increase ($p < 0.05$) of the blood glucose levels in animals treated with HAART regimen (triplavar) alone which possibly suggest that HAART suppresses glucose utilization by tissues. The corresponding increase in blood glucose levels observed resulted to glucose spilling through the glomerular filtration apparatus that led to osmotic diuresis. The study also showed that untreated diabetic rats and diabetic rats treated with HAART regimen all demonstrated the typical triad of diabetes

mellitus (polyuria, polyphagia and polydipsia) as well as elevated blood glucose levels. Interestingly, treatment with *M. charantia* significantly lowered ($p < 0.05$) blood glucose levels in all animals receiving *M. charantia* extract and its concomitant treatment with HAART in both non-diabetic and diabetic animals. It is believed that *M. charantia* extracts exert their blood glucose lowering effects via different physiological, pharmacological and biochemical pathways (Singh et al., 2011, Garau et al., 2003, Platel and Srinivasan, 1995). Possibly, the mode of action could be stimulation of peripheral and skeletal muscle glucose utilization, inhibition of intestinal glucose uptake, inhibition of adipocyte differentiation, suppression of key gluconeogenic enzymes, stimulation of key enzyme of HMP pathway, and preservation of islet β cells and their functions (Singh et al., 2011, Joseph and Jini, 2013, Nerukar et al., 2010). We speculate that the mode of action of *M. charantia* in our study could be by preservation of islet β cells and their functions.

This study also evaluated renal electrolytes (sodium and potassium) and other renal functional indices such as albumin, urea, BUN and Scr. These parameters are helpful in understanding kidney function. Retention of renal electrolytes (sodium and potassium) can occur due to excessive pressure on the nephron and this may eventually lead to organ hypertrophy and a decline in glomerular filtration causing leakage of albumin, podocytes effacement and loss of surface area available for filtration (Rask-madsen, and King, 2013). In this study, there was leakage of albumin and electrolytes retention in the urine of HAART treated animals in non-diabetic and diabetic animals. These functional abnormalities (albumin, sodium, potassium, urea) were also present in the urine of untreated diabetic animals which is an indication that the glomerular endothelial barrier may have been altered. It is proposed that significant pathological glomerular hypertrophy and retention of renal electrolytes is a clear manifestation of deranged renal function and this remains the underlying factor for DN.

Microalbuminuria is a predictor of progression to DN in type 2 diabetes mellitus (T2DM) and is associated with an accelerated decline in glomerular filtration rate (El Din et al., 2017). The underlying mechanisms for microalbuminuria involves abnormalities of the glomerular endothelial barrier causing excessive filtration as well as reduction of renal tubular cell albumin degradation and reabsorption (Tojo and Kinugasa, 2012). Urinalysis revealed high concentration of urea in the urine of animals treated with HAART both in non-diabetic and diabetic state. Administration of *M. charantia* alone (low and high dose) and its concomitant treatment with HAART restored the urinary metabolites in the non-diabetic and diabetic animals studied.

BUN and Scr are routine indicators to monitor kidney function. These substances are normal metabolic waste products that are excreted by the kidneys. In kidney diseases such as diabetic nephropathy or

nephrotoxicity, these metabolic waste are not excreted normally and so they accumulate in the body thus causing an increase in blood levels of urea and creatinine serum. Accumulation of these metabolic wastes in the body eventually affects kidney function. Our study revealed marked impairment in renal function with significantly ($p < 0.05$) raised levels of BUN and Scr concentrations in HAART alone treated animals (diabetic and non-diabetic) and untreated diabetic animals. The functional capacity of the glomerulus in filtration and tubular absorption may have been impaired leading to elevations in levels of BUN and Scr concentrations as observed in our study. Adjuvant treatment with *M. charantia* at both low and high dose significantly ($p < 0.05$) restored these kidney function test to near normal by lowering the levels of BUN and Scr (chapter 2 table 4 and chapter 3 figure 2).

It is known that the structural basis of cell is easily destroyed by oxidative stress which has been verified to participate in the pathogenesis of nephrotoxicity and diabetic complications, including DN (Hakim and Pflueger, 2010). Oxidative stress refers to the imbalance between the antioxidative defense system and the oxidative system. It stimulates the excessive production of reactive oxygen species that are toxic to the cell, particularly the cell membrane in which these radicals interact with the lipid bilayer and produce lipid peroxides (Sharma et al., 2012, Halliwell, 2007). Lipid peroxidation is a metabolic process that results in the oxidative degradation of lipids by excess ROS formation leading to attacks by highly reactive free radicals. The oxidation of lipid membranes mediates the damage to cell membranes that can alter structure and function (Ghosh et al., 2017, Pisoschi and Pop, 2015). This further predisposes cellular organelles to functional impairment. Thus, interference with lipid metabolism can seriously compromise tissue integrity. Thiobarbituric acid reactive substance (TBARS) is a marker for determining the extent of lipid peroxidation in tissues (Ghani et al., 2017). In this study, TBARS levels in the kidney were significantly higher in HAART treated group compared to control and other treatment groups. Consequently treatment with *M. charantia* and its adjuvant use with HAART significantly lowered the level of TBARS.

HAART elicits a myriad of mitochondrial alterations which will results in the generation of ROS which are the chief arbiters of oxidative stress damage. Mitochondrial alterations due to oxidative stress arising from negative interactions of HAART will ultimately cause damage to tissues (Azu, 2012). Endogenous antioxidant enzymes (SOD and CAT) and GSH are responsible for the detoxification of deleterious ROS. In the present study, the activities of SOD, CAT and levels GSH in the glomeruli of HAART alone treated non-diabetic rats and HAART alone treated diabetic rats as well as untreated diabetic rats were evidently inhibited along with an increase in MDA levels. By contrast, following *M. charantia* treatment, the activities of all the enzymes displayed a significant increase accompanied with obvious decrease in MDA levels. These results demonstrate that *M. charantia* possesses antioxidant activity and could

improve the antioxidative defense system of the kidney. This may be attributed to its high phytochemical constituents (e.g. charantin, momordicin) contributing to its antioxidant capacity with a positive correlation on other biochemical results that was investigated in this research.

In this study, significant upregulation of circulating NGAL, KIM-1 and TNF- α mRNA levels were detected in the serum following treatment with HAART alone in both non-diabetic and diabetic rats. Blockade of the overexpression of these genes could prevent pathological alterations such as extracellular matrix (ECM) accumulation and possibly glomerular basement membrane (GBM) thickening (Vaidya et al., 2008). The increased expression of the circulating mRNA levels of NGAL, KIM-1 and TNF- α along with excessive oxidative stress in the kidney as a result of HAART treatment both in non-diabetic and diabetic animals provide evidence that oxidative stress may induce ECM accumulation through upregulation of gene expression. When *M. charantia* was used as an adjuvant with HAART in the experimental protocol, circulating NGAL, KIM-1 and TNF- α mRNA levels in the serum decreased, accompanied with the reduced oxidative stress and ECM accumulation.

The histopathological and ultrastructural examinations undertaken in this study reveal morphological alterations of the kidney in animals that were administered with HAART alone. Qualitative light microscopy data of kidney sections of HAART alone treated (non-diabetic rats) showed derangements of the histoarchitecture characterized by tubular epithelial desquamation and glomerular capillary abnormalities. There were many indistinguishable tubules, inflammation of the interstitium and extensive necrosis. These were findings observed with H&E stains (figure 3B, chapter 2). The PAS and MT staining also showed vacuolation of tubules and presence of polysaccharides and collagen fibres (figure 4B and figure 5B respectively, chapter 2). *M. charantia* treated groups showed an essentially normal glomerular appearance with normal cytoarchitecture comparable to control.

HAART alone treated (diabetic rats) and untreated diabetic rats showed inflammatory cell infiltrations and extracellular matrix accumulation which is suggestive that disturbance between the mesangial cell and glomerular capillaries may have been altered. Our study showed obliteration of glomerular capillary loops and occlusion of the capillary walls with many indistinguishable tubules, inflammation of the interstitial and epithelial desquamation. Cellular inflammation, tubular epithelial damage and severe necrosis of the vascular wall characterized by hypercellularity were also observed (see figure 3B and 3C, chapter 3). These were key findings in our study. Glomerular abnormalities often result in a decrease in surface area available for filtration and a consequent decline in glomerular filtration rate and reduction in metabolic activity.

Our observations with H and E stains corresponds with PAS and MT staining of the kidney sections showing high proportion of carbohydrates such as glycogen and glycoproteins and mild deposition of collagen fibers in HAART alone treated (diabetic rats) and untreated diabetic rats (see figure 4B, 4C and 5B, 5C respectively, chapter 3). The histopathological changes observed in the renal interstitium as a result of HAART in diabetic and non-diabetic rats is an indication that this causes a significant damage to the mitochondria most likely due to elevated ROS production. Increase in ROS production is suggested to disrupt normal oxidative phosphorylation that will result to cytoplasmic swelling and create an imbalance in the intracellular milieu (Ozbek, 2012). *M. charantia* mitigated these effects with a resultant restoration of the kidney histoarchitecture in both low and high dose of *M. charantia* (diabetic and non-diabetic animals).

The ultrastructural observations obtained also corroborate the light microscopic studies, showing several HAART abnormalities such as effacement of the podocytes foot process weakness of the glomerular basement membrane. The renal ultrastructure of the HAART- alone treated (non-diabetic) rats showed wrinkling and folding of the tubular basement membrane and hypertrophy of epithelial cells. There was also swollen mitochondrial in the endothelium (figure 1B, chapter 5). The swelling of the majority of the mitochondria seen in the section may suggest change in the permeability of the membrane and depicts and unusual degenerative nuclear change (Finsterer and Scorza, 2017). The prime target for HAART is the mitochondrial and the hypothesized mechanism is that it reduces the production of ATP that is needed for cellular energy. HAART alone treated (diabetic rats) and untreated diabetic rats showed an abnormally increased thickness of basement membrane and podocyte effacement which reflects the existence of endothelial damage (figure 1G and H, chapter 5). Podocyte effacement as observed in this study is thought to be due to a breakdown in the actin cytoskeleton of the foot processes, which is a complex contractile apparatus that allows podocytes to be dynamic in nature according to changes in filtration requirements (Mathieson, 2012).

The glomerular basement membrane also plays an essential role in giving structural support to the kidney and hence impairment of basement membrane will severely compromise tissue structure (LeBleu et al., 2007). As the basement membrane is made of extracellular matrix, overexpression or degradation of the extracellular matrix will contribute significantly to kidney dysfunction. Accumulation of the extracellular matrix is a major cause of interstitial fibrosis, infiltration and a thickened basement membrane that results to glomerular abnormalities. The swollen mitochondria, thickening of GBM and podocytes effacement which were major features present in figure 1B, G and H, (chapter 5) were not conspicuous in other groups treated with *M. charantia* adjuvant (diabetic and non-diabetic). *M. charantia* was found to have protective effects on the kidneys and may provide a naturally available plant-based adjuvant in ART.

6.2 Conclusion

There is no doubt that the roll out of HAART has been the most important progress in the management of HIV in the last two decades and has significantly improved survival of PLWHA. However, its associated organ toxicities as well as adverse effects have long been recognized to pose a serious clinical management dilemma with dire implications for global health. This calls for a more holistic approach in the management of HIV/AIDS; such as adjuvant use of plant-based therapy.

Results from this study have shown that HAART affected kidney function evidenced by leakage of albumin, electrolytes retention and significantly raised levels of BUN and Scr concentrations. This in addition to many other derangements as observed in the histological, ultrastructural, molecular and biochemical milieu. The use of *M. charantia* extract mitigates the untoward effects of HAART by significantly preserving kidney morphology and enhancing kidney function. In addition, the study has shown the worsening effects of HAART in the face of diabetes and the consequences are deleterious.

6.3 Recommendations

It is recommended that further studies should be conducted using specific active components of *M. charantia* to determine which of the components is responsible for protecting kidney morphology and maintaining its function. Precise explanations for the structural changes observed may be better explained using stereological techniques for a more detailed quantification. More so, further studies needed to be carried out using additional doses to ascertain if the protective effect of *M. charantia* is dose dependent that way an appropriate dose can be established.

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***Momordica charantia* mitigates hepatic injury following adjuvant treatment with antiretroviral drugs in diabetic animal models**

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Abstract

Momordica charantia (*M. charantia*) is a medicinal plant, used in traditional practice for treating diseases like hypertension and diabetes mellitus. This study investigated the possible hepato-protective effect of *M. charantia* following treatment with highly active antiretroviral therapy (HAART) in diabetic rats. 48 adult male Sprague Dawley rats were divided into seven groups (A–G) of 7 animals per group and treated according to protocols. Diabetes was induced with streptozotocin (STZ) by intraperitoneal injection (45 mg/kg body weight). The animals were euthanized on the 10th week with liver removed for examination and blood obtained via cardiac puncture and centrifuged to collect the sera. Blood glucose levels (BGL) were consistently and significantly raised ($p < 0.05$) in all groups not receiving the adjuvant *M. charantia*. Treatment with *M. charantia* reverses the increase in BGL to near normal. Markers of liver injury assayed showed significant increase ($p < 0.05$) in AST, ALP and ALT levels in groups not receiving *M. charantia*. Adjuvant HAART and *M. charantia* caused significant declines in the liver enzymes ($p < 0.05$). Serum GGT was not markedly altered. Treatment with *M. charantia* significantly restored liver enzymes elevations to near normal comparable to control. Histopathological observations ranged from severe hepatocellular distortions, necrosis and massive fibrosis following treatment of HAART in diabetic groups not receiving *M. charantia*. Treatment with *M. charantia* did not show any sign of hepatotoxicity as judged from the histological and biochemical observations.

Keywords Highly active antiretroviral therapy · Hepatotoxicity · Liver · Diabetes mellitus · *Momordica charantia* · Sprague-dawley rats

Introduction

Antiretroviral (ARV) drugs have been shown to suppress HIV replication while reducing morbidity and mortality. However, as a counterweight to this positive impact, ARV drugs carries along deleterious effects, which challenge the

management of HIV-infected patients to a great extent [1]. Some of these effects are nephrotoxicity, hepatotoxicity and other metabolic disorders such as hypertension and diabetes mellitus. These effects have been linked to the continuous use of ARV drugs [2].

ARV drugs were shown to inhibit insulin-stimulated glucose disposal by blocking the glucose uptake through glucose transporter isoform 4 (GLUT 4) [3]. Disturbances in glucose-insulin homeostasis through insulin resistance and impaired beta cell function predisposes to hyperglycemia and eventually diabetes mellitus and further complications of diabetes such as diabetic liver disease often called hepatogenous diabetes [4]. It is not clear how diabetic liver disease develop but however scientist have reported Porto systemic shunts and decreased overall liver mass through down- regulation of insulin receptors as a possible mechanism [3].

There are compelling evidences that increased consumption of fruits and vegetables reduces the risk of

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various pathological events, hence *Momordica charantia* L. (Cucurbitaceae) known as bitter melon, have been traditionally used worldwide, as an adjuvant in the therapeutic strategy for glycemic control aimed at preventing the development and progression of chronic liver failure associated with uncontrolled diabetes mellitus [5]. Studies have documented the hypoglycemic effects of *M. charantia* through physiological, pharmacological and biochemical mechanisms. It is believed to act via stimulation of peripheral skeletal muscle glucose utilization, inhibition of intestinal glucose uptake, [6], suppression of key gluconeogenic enzymes, stimulation of key enzyme of hexose mono phosphate (HMP) pathway, and preservation of β cell islet and its functions [5].

However, there is paucity of literature explaining the attenuating influence of *M. charantia* on the liver injury following treatment with ARV drugs and diabetic condition. Our study therefore sought to investigate the therapeutic potential of *M. charantia* in treating diabetic liver disease following antiretroviral treatment.

Materials and methods

Preparation of *M. charantia* fruit ethanolic extract

Fifty kilogram of fresh unripe green fruits of *M. charantia* was purchased from a local market in Durban South Africa and were authenticated in the Department of Life Science, Westville Campus, University of KwaZulu-Natal, with voucher no: 4617. The fruits of *M. charantia* was cleaned and sliced into small pieces and the seeds separated from it. The sliced green fruit was weighed and dried in shade and weighed again after drying to obtain the actual weight before pulverizing into fine powered in a grinder and stored at 5 °C until ready for extraction. Fruit powder was extracted with solvent (ethanol) by soxhlet extraction. Solvent was evaporated in a rotary evaporator at 40–50 °C with the percentage yield of 85.25%. The extract was filtered through whatman filter. The concentrated extract was stored at 4 °C until ready for use.

Drug and chemical

Highly active antiretroviral (HAART) regimen (Triplavar) (*Cipla-Medpro*) containing Lamivudine 150 mg, Nevirapine 400 mg and Zidovudine 300 mg, was used for this study. The drug was obtained from Pharmed pharmaceuticals, Pty (Ltd) Durban, South Africa. Streptozotocin (Sigma-Aldrich, St.

Louis, MO, USA) which was of analytical grade quality was purchased from Capital Lab Supplies, Durban, South Africa.

Animal management and experimental design

The University of KwaZulu Natal Animal Research Ethics Committee gave full approval of the project and assigned Reference Number: **AREC/033/016D**. A total of forty-eight ($n=48$) adult male Sprague-Dawley rats weighing 178–232 grams (219.31 ± 36.17) were used for the study. Animals were housed in well ventilated plastic cages (4 rats per cage having dimensions of 52 cm long \times 36 cm wide and 24 cm high with bedding of soft wood shavings. They were maintained under standardized animal house conditions (temperature: 23–25 °C; light: approximately 12 h natural light per day) and were fed with standard rat pellets from Meadow feeds (Division of Astral Operations Limited, Durban, South Africa) and given tap water ad libitum. The initial body weights of the animals were recorded before treatment. The animals were randomly assigned to the 7 treatment groups, (B–G) with 7 animals per group and 6 animals in the (–ve) control group A.

Group A served as –ve control

Group B served as +ve control (Diabetic)

Group C received Triplavar (Diabetic)

Group D received *M. charantia* (200 mg/kgbw) (Diabetic)

Group E received *M. charantia* (400 mg/kgbw) (Diabetic)

Group F received Triplavar + *M. charantia* (200 mg/kgbw) (Diabetic)

Group G received Triplavar + *M. charantia* (400 mg/kgbw) (Diabetic)

The therapeutic dose of triplavar was adjusted for animal weight using the human therapeutic dose equivalent for the rat model [7]. These substances were administered daily via oral route with orogastric tube. The study lasted for 10 weeks. Monitoring of the animals was carried out by the research team. Thereafter, the animals were euthanized at the 10th week and the tissues were harvested and prepared for observation.

Induction of diabetes mellitus

All rats were placed on a 12 h fast to obtain baseline fasting blood glucose levels (FBG). The diabetic groups were given intra-peritoneal streptozotocin (STZ) (Sigma-Aldrich Chemical Company, Missouri, St Louis, USA) at 45 mg/kg body weight dissolved in a citrate buffer (pH 4.5) [8]. Successful induction of diabetes was determined

by observation of polyuria and polydipsia and confirmed by a 72 h post STZ FBG level ≥ 11 mmol/L.

Measurement of blood glucose

Blood samples were obtained from the tail using sterile needle prick. Glucose levels were measured once a week during the 10 weeks treatment using the one touch ultra-glucometer (Boehringer-Mannheim, Germany).

Body and liver weight

Body weights of animals were recorded on the first day before treatment (initial), thereafter weekly during the 10 weeks treatment till the day of sacrifice (final). Liver weight (LW) was measured by an electronic balance (Mettler Toledo; Microsep (Pty) Ltd, Greifensee, Switzerland).

Assessment of liver function

Blood samples were collected through cardiac puncture and allowed to clot for 30 min and centrifuged for 15 min at 3000 revolutions per minute. The serum was decanted into Eppendorf tubes and prepared for biochemical analyses. Biochemical analyses of the serum enzymes for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) were spectrophotometrically determined by the method of Reitman and Frankel [9].

Histopathological examination of liver tissues

Liver were weighed and examined for gross pathology. A phosphate buffer solution (PBS) was used to wash out blood before preparation for tissue fixation. They were sectioned at 5 μ m thickness using Leica RM 2255 microtome. Tissues were stained with Haematoxylin and Eosin (H and E), for general assessment of liver structure. For histochemical studies, the tissues were stained with Masson's trichrome (MT) for the assessment of possible liver fibrous architecture [10]. The stained slides were scanned using a Leica SCN 400 (Leica Microsystems GmbH, Wetzlar, Germany) and measured at 200 magnification using image analyser Leica (DMLB) and Leica QWIN software.

Statistical analysis

Continuous variables (liver and body weights and liver function test), were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post-test using Graph pad prism® statistical software 6.02. The results are expressed as mean \pm SD (standard deviation). Values were considered significant at $p < 0.05$.

Results

Blood glucose levels

The blood glucose concentration for diabetic +ve control (group B) and diabetic treated with HAART-regimen (triplavar) alone was significantly high ($p < 0.05$) (Fig. 1). This level was sustained throughout and was peaked between 6th and 10th weeks. Treatment with *M. charantia* alone at both low and high dose as well as co-administration of *M. charantia* (low and high dose) and HAART showed significant mild hyperglycemia ($p < 0.05$) compared to normal control. From the 4th to 6th week of treatment, adjuvants treatment with *M. charantia* in all groups receiving *M. charantia* extract showed a significant lowering of blood glucose ($p < 0.05$). Between weeks 8 and 10, groups receiving *M. charantia* (low and high dose) together with HAART showed significant ($p < 0.05$) reduction in blood glucose to near normal levels (Fig. 1). This reduction of blood glucose level shows in addition, a dose dependent mitigation of hyperglycemia (Fig. 1).

Body weight and organ (liver) weight changes

While there was an overall increase in the final body weight (BW) in all groups, Groups, E and G recorded the highest increase in BW significant at $p < 0.05$ with the percentage BW gain recording 34.9% and 20.9% respectively, but however, lower when compared to control animals. Although there was a lower rate of body weight gain in all groups when compared to control, this was particularly so in Groups (B, C and F). Diabetes and HAART were related to lower rate of body weight gain as observed in Groups (B and C) with the percentage body weight

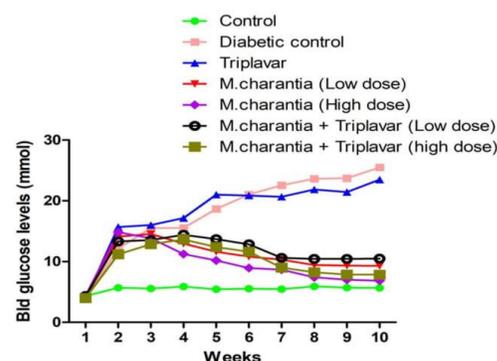


Fig. 1 Graphical representation of blood glucose levels

recording 11.74%, 12.77%. Groups D (administered with low dose (200 mg/kgbw) of *M. charantia* extract alone), and Group F (administered with low dose of *M. charantia* extract + HAART) showed a narrower range of weight gain when compared with Groups E (administered with high dose (400 mg/kgbw) and Group G (administered with high dose of *M. charantia* extract + HAART). Mean liver weight corresponds with the percentage BW gained across groups with the control group having an optimal mean liver weight (Table 1).

Liver function test

As shown in Table 2, the levels of AST, ALT and ALP were significantly decreased ($p < 0.05$) in animals treated with *M. charantia* alone as well as *M. charantia* + HAART (low and high dose) as seen in groups (D, E, F, G) when compared to groups (B and C). Treatment with HAART (group C) as observed showed significant increase in liver function parameters ($p < 0.05$). Similarly, the liver functional indices were elevated in the diabetic +ve control animals (group B).

Histopathological examination (H and E and MT)

The H&E stained sections of the liver tissues in control animals and animals treated with *M. charantia* alone and its co-administration with HAART revealed adequate preservation of hepatocellular architecture with the normal hepatocytes and no cytoplasmic vacuolation of liver cells and fine sinusoidal spaces (Fig. 2). The outline of hepatocytes and sinusoidal spaces were seen with no pathologies. Histopathological evaluation of groups treated with HAART alone as well as untreated diabetic group (B and C) showed various degrees of distortions and cytoarchitectural patterns ranging from extensive necrosis of hepatocytes with occlusion of sinusoidal spaces and cytoplasmic vacuolation.

Masson's trichrome stains fibrous tissue blue while the cytoplasm of hepatocytes are stained red and nuclei could be dark or red as well. Sections of liver in control group and groups treated with *M. charantia* alone and its co-administration with HAART showed normal red-stained hepatocytes. Photomicrographs from liver tissues of groups treated with HAART alone as well as untreated diabetic group showed extensive network of blue-stained fibrous components in a necrotized, disorganized hepatic tissue cord (Fig. 3).

Table 1 Body weight and liver weight of experimental and control groups

Groups	Initial BW (g)	Final BW (g)	Mean BW	BW difference	BW Diff in (%)	Mean LW	LWBR
A	178.1	351.5	261.7 ± 127.0	173.4	66.26	12.69 ± 1.41	4.85
B	227.1	255.3	240.1 ± 18.28	28.2	11.74	10.30 ± 1.79	4.29
C	227.1	258.1	242.6 ± 21.92	31.0	12.77	10.55 ± 1.22	4.35
D	212.6	258.0	235.3 ± 32.12	45.4	19.29*	10.07 ± 1.56	4.28
E	240.3	342.1	291.2 ± 72.02	101.8	34.9*	12.43 ± 1.89	4.27
F	217.9	249.9	233.9 ± 22.63	32.0	13.68	10.19 ± 1.58	4.36
G	232.1	286.3	259.2 ± 38.32	54.2	20.9*	11.34 ± 0.75	4.37

LWBR = (Mean LW/Mean BW) × 100. BW diff in% = (BW diff/Mean BW) × 100

BW body weight of rats, LW liver weight of rats, LWBR liver weight body ratio

*Values are expressed as mean ± SD for each group and considered statistically significant at $p < 0.05$

Table 2 Effect on serum AST, ALT ALP and GGT following treatment with HAART and *M. charantia*

Groups	AST (IU/L)	ALT (IU/L)	AST/ALT ratio	ALP (IU/L)	GGT (IU/L)
A	24.67 ± 3.05	27.67 ± 3.22	0.89	104.7 ± 29.37	4.33 ± 4.04
B	134.7 ± 31.50	104.0 ± 22.87*	1.29	558.7 ± 140.6*	19.00 ± 3.00
C	113.0 ± 44.54*	87.00 ± 22.27	1.29	225.3 ± 40.10	11.67 ± 2.52
D	32.33 ± 6.51*	33.67 ± 8.51	0.96	126.7 ± 14.19*	15.33 ± 6.81
E	31.67 ± 4.04	40.67 ± 6.03*	0.78	119.0 ± 26.63*	11.67 ± 6.51
F	29.33 ± 6.81	36.00 ± 12.53*	0.81	108.7 ± 27.79	9.00 ± 5.00
G	30.33 ± 5.13*	37.00 ± 4.58*	0.82	117.7 ± 14.57	4.33 ± 2.52

AST alanine amino aspartate, ALT alanine amino transferase, ALP alkaline phosphatase, GGT gamma-glutamyl transferase

*Values are expressed as mean ± SD for each group and considered statistically significant at $p < 0.05$

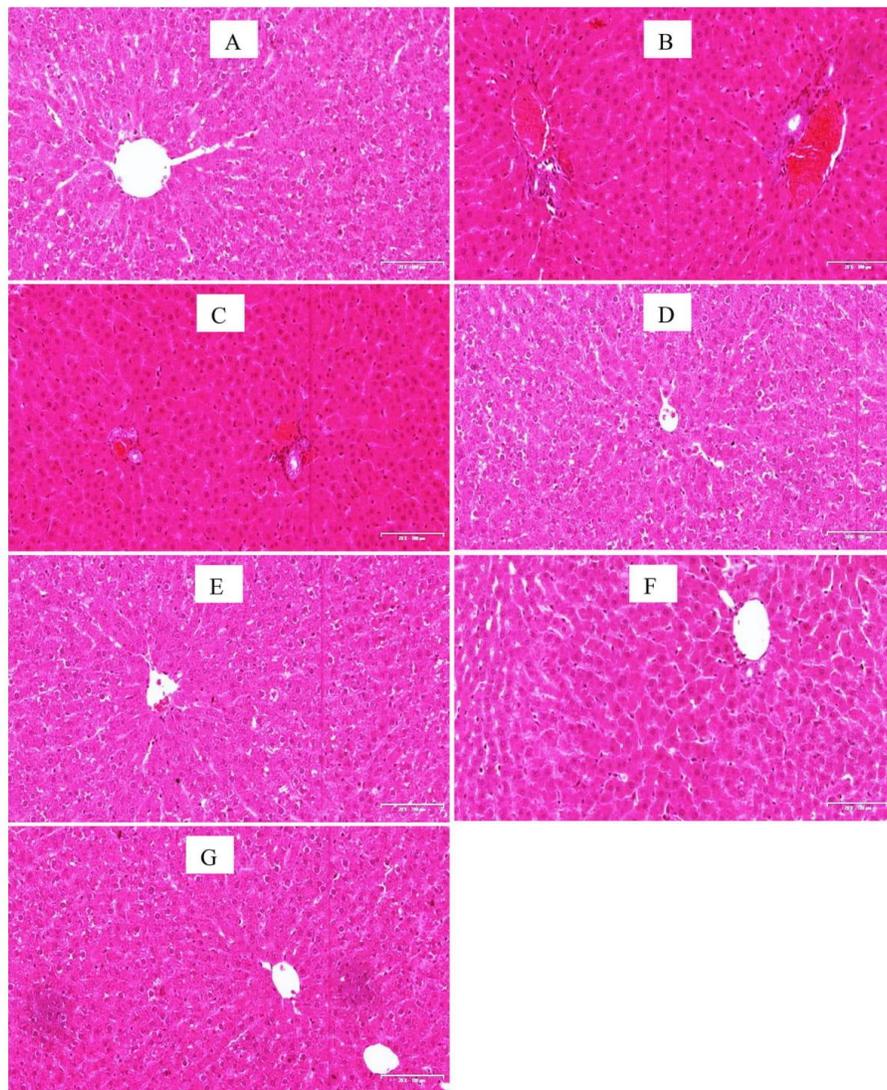


Fig. 2 Photomicrographs of liver sections (H &E stains). Scale bar 20×200 μm. Note the normal architecture of hepatocellular cords sinusoidal spaces and central vein in groups A, D, E, F and G. There are various degrees of distortion (mild to severe) in the radial arrangement of hepatic cords seen in groups B, extensive necrosis are

observed in groups C. **a** -ve Control, **b** Diabetic +ve Control **c** Diabetic + HAART **d** Diabetic + *M. charantia* 200 mg/kg **e** Diabetic + *M. charantia* 400 mg/kg, **f** Diabetic + *M. charantia* 200 mg/kg + HAART **g** Diabetic + *M. charantia* 400 mg/kg + HAART

Discussion

Despite the substantial benefit in the reduction of morbidity and mortality by HAART, organ toxicities (especially liver)

are frequently becoming a major concern for scientist and researchers ranging from mild side effects (elevated liver enzymes) to fatal complications (chronic liver failure, diabetic liver disease etc.) [11]. The liver performs numerous

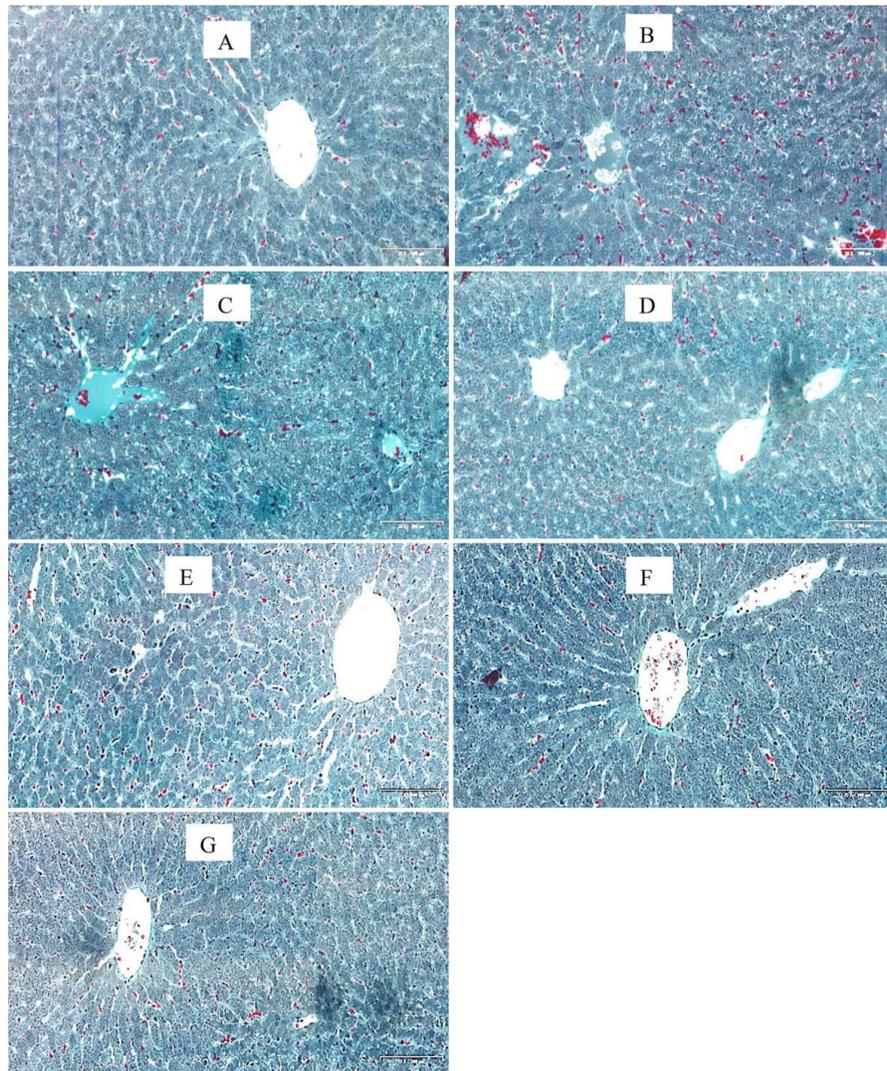


Fig. 3 Photomicrographs of liver sections (MT stains). Scale bar 20×200 μm. Note the normal architecture of the liver with intact hepatocytes stained red in groups A, D, E, F and G. Nuclei can be seen as dark red to black structures within cells and fibrous elements stained light blue. There is extensive necrosis of central veins, hepatic

cords and massive fibrosis in groups B, and C. **a** –ve Control, **b** Diabetic +ve Control **c** Diabetic+HAART **d** Diabetic+*M. charantia* 200 mg/kg **e** Diabetic+*M. charantia* 400 mg/kg, **f** Diabetic+*M. charantia* 200 mg/kg+HAART **g** Diabetic+*M. charantia* 400 mg/kg+HAART

vital metabolic activities, including synthetic and excretory functions of drugs/chemicals. Due to this central role, it is thus exposed to toxic injuries. In other to mitigate some of these toxicities, people living with HIV/AIDS (PLWHAs) in sub-Saharan Africa rely on medicinal plants rich in

phytochemical components as a complementary or alternative therapy for the alleviation of some of the toxicities of HAART [12].

In our study, we evaluated alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline

phosphatase (ALP) and gamma-glutamyl transferase (GGT) on the serum samples. These liver enzymes are helpful in understanding inflammatory and necrotic changes in the liver [13]. It has been reported that elevated levels of these liver enzymes are associated with complications of diabetes mellitus [14], and commencement of antiretroviral therapy [15]. Although this can be exacerbated by other co-infections like hepatitis B or C virus [16]. Our study revealed an elevation of these liver enzymes (ALT, AST and ALP) above normal levels in the diabetic +ve control animals and the diabetic animals treated with HAART. This elevations perhaps could reflect a major permeability problem or cell disruptions in the liver [17]. Elevations of these enzymes is a sensitive signal for liver injury and may worsen into severe complications of the liver with the continuation of therapy [15].

In the quest for better outcome and well-being of PLWHAs, there is increased utilization of alternative and complementary medicines in association with HAART, although many of these adjuvants have been implicated in drug-induced liver/other organ's injury [7]. From our study we observed lower levels of ALT, AST and ALP in diabetic rats treated with *M. charantia* alone and its concomitant treatment with HAART, indicating that consumption of *M. charantia* had a beneficial effect in suppressing elevation of liver enzymes levels. The decreased levels of these liver enzymes (especially following adjuvant HAART with *M. charantia*) in our study supports previous reports by [18] on effects of *M. charantia* on the serum and liver triglyceride levels in rats. While the levels of ALT, AST and ALP were reduced in our study, GGT showed normal levels in all groups. The tendency of *M. charantia* to suppress the rise in values of the liver enzymes provides substantial evidence that treatment with *M. charantia* extract could mitigate the progression of liver dysfunction.

Liver dysfunction is known to be associated with complications of diabetes and assessment of blood glucose level plays a critical role in evaluating the functional capacity of the liver following antiretroviral treatment in STZ induced diabetic rats. Elevated levels of blood glucose are associated with complications arising from occlusion of the vascular wall [19]. This pathogenesis is clearly complex and occurs as a result of cytokines secretion increase which induces dysregulation of endothelial and vascular smooth muscle cell growth [20, 21]. In the present study, elevated level of blood glucose level was observed when rats were treated with HAART regimen alone indicating that HAART regimen suppresses glucose uptake by the tissues. While blood glucose levels were elevated in animals treated with HAART alone, it was lowered when *M. charantia* was used as adjuvant to HAART. It is believed that *M. charantia* could exert their hypoglycemic properties via stimulation of peripheral skeletal

muscle glucose utilization [22, 23] inhibition of adipocyte differentiation [24], stimulation of key enzyme of hexose mono phosphate (HMP) pathway, and preservation of β cell islet and its functions [25].

We correlated our findings with the histopathological assessment of the liver tissues. HAART-treated groups revealed disorganized architecture and extensive network of fibrotic strands and hepatic structural injury. The pathogenic pathway for HAART-mediated hepatic injury has been associated with mitochondrial toxicity especially with NRTIs [26, 27]. HAART inhibit the mitochondrial DNA resulting in inhibition of normal mitochondrial replication which in turn decrease cellular respiratory chain and inhibits fatty acid β -oxidation pathway [26]. Studies have also concretely proved that interruption in electron transport chain results in the release of increased intracellular reactive oxygen species (ROS) leading to oxidative stress [28, 29]. These free radicals can cause lipid peroxidation of fatty acid present in the hepatocytes membrane consequently to release profibrogenic cytokines, and nuclear factor κ B to counteract the effect [30]. It is reasonable to believe that a perturbed antioxidant defense system could perhaps be a contributory arm towards the histopathological observations in our study.

Our observations in the H and E stains corresponded with the MT staining intensities of liver sections and there is a supporting link. Photomicrographs from liver tissues of groups B and C showed extensive network of blue-stained fibrous components in a necrotized, disorganized hepatic tissue cord possibly indicating low deposits of collagen fibres. These derangements could be as a result of low glucose metabolism and is supported by [31] involving PIs regimens. It is interesting to note that while all these deleterious effects were seen in HAART treated groups, *M. charantia* reversed the pathologies and showed good cyto-protective effects and maintained hepatic structural integrity. Although [7] reported that the pharmacokinetic interactions between antiretroviral therapy and plant based adjuvants can be unfavourable due to drug- drug interactions, the exact mechanistic pathway for *M. charantia* boosting in hepatic tissues in our study still remain a subject for further investigation.

Body and organ weight analysis offers insight on inflammatory changes and thus important in the identification of target organ by toxicants. In this study, HAART with *M. charantia* induced observational changes in the body weight of experimental animals. While loss of weight may be associated with induction of diabetes, initiation of HAART may as well contribute to the low pace of weight gain due to other metabolic activities relative to lipid and sugar [7]. Our study agrees with [7] that long term liver enzyme elevation in addition to other positive indices tilts the balance towards liver fibrosis. These perturbations were mitigated by *M. charantia* and its concomitant treatment with HAART.

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Compliance with ethical standards

Conflict of interest The authors declared that there is no conflict of interest.

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APPENDIX II

ETHICAL CLEARANCE



10 June 2016

Mr Ugochukwu Offor (213567926)
School of Laboratory Medicine & Medical Sciences
Nelson R Mandela School of Medicine

Dear Mr Offor,

Protocol reference number: AREC/033/016D

Project title: Effects of Momordica Charantia in the kidney following Antiretroviral Therapy in male diabetic and non-diabetic animals models

Full Approval – Research Application

With regards to your revised application received on 01 June 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 10 June 2017.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....
Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Dr Onyeamaechi O Azu
Cc Acting Dean & Head of School: Dr Musa Mabandla
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Jessica Light
Cc BRU – Dr Sanil Singh

Animal Research Ethics Committee (AREC)

Ms Mariette Snyman (Administrator)

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APPENDIX III

ETHICAL CLEARANCE RENEWAL LETTER



27 November 2019

Mr Ugochukwu Offor (213567926)
School of Laboratory Medicine & Medical Sciences
Nelson R Mandela School of Medicine

Dear Mr Offor,

Protocol reference number: AREC/033/016D

Project title: Effects of Momordica Charantia in the kidney following Antiretroviral Therapy in male diabetic and non-diabetic animal models

We acknowledge receipt of your correspondence dated 21 November 2019.

This is to confirm that you were compliant with AREC rules and regulations and that the study was approved on 10 June 2016. As data was collected by the end of December 2016, Renewal of the study was not required. Data analysis was done by using the archived animal tissue kept within Biomedical Research Unit.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....
Dr Sani Singh, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Dr Onyeamaechi O Azu

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