

UNIVERSITY OF KWAZULU-NATAL

**The Effects Of Plant-Derived Oleanolic Acid
On Kidney Function In Male Sprague-
Dawley Rats And, In Cell Lines Of The
Kidney And Liver**

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**The effects of plant-derived oleanolic acid on kidney
function in male Sprague-Dawley rats and, in cell lines of the
kidney and liver**

By

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DECLARATION

I, **Hlengiwe Pretty Madlala** hereby declare that the dissertation entitled:

“The effects of plant-derived oleanolic acid on kidney function in male Sprague-Dawley rats and, in cell lines of the kidney and liver”

is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

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LIST OF ABBREVIATIONS

A	Absorbance
A	Alpha
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotrophic hormone
ADH	Anti-diuretic hormone
AGEs	Advanced glycation end-products
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	One-way analysis of variance
ARR	Aldosterone to renin ratio
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
B	Beta
b.wt	Body weight
BRU	Biomedical Research Unit
BSC1	Bumetanide sensitive cotransporter
BUN	Blood urea nitrogen
C	Carbon
°C	Celsius
C ₂ H ₅ OH	Ethanol
Ca ²⁺	Calcium
C _{Cr}	Creatinine clearance
CH ₃ OH	Methanol
CHCl ₃	Chloroform

Cl ⁻	Chloride
cm	Centimeters
CO ₂	Carbon dioxide
DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DV	Dual view
EA	Ethyl acetate
EAS	Ethyl acetate soluble
ECF	Extracellular fluid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMEM	Earle's minimum essential medium
ENaC	Epithelial sodium channel
ESRD	End-stage renal disease
FE	Fractional excretion
g	Grams
GGT	Gamma-glutamyl transferase
GFR	Glomerular filtration rate
h	Hour
H ⁺	Hydrogen
H ₂ O ₂	Hydrogen peroxide
HCO ₃ ⁻	Bicarbonate ions
HEK293	Human embryonic kidney cell line
HEPES	N-(2- hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)

HepG2	Hepatocyte-derived human transformed cell line
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
iNOS	Inducible nitric oxide synthase
K ⁺	Potassium
K ₂ HPO ₄	Dipotassium phosphate
KCl	Potassium chloride
Kg	Kilogram
l	Litre
L-FABP	Liver-type fatty acid-binding protein
Li ⁺	Lithium
Ltd	Limited
M	Molar
MAP	Mitogen-activated protein
MAP	Mean arterial blood pressure
MDBK	Madin-Darby bovine kidney cell line
MEM	Minimum essential medium;
Mg ²⁺	Magnesium
MHz	Mill hertz
m	Milli
mmHg	Millimeters of mercury
mol	Mole
mRNA	Messenger ribonucleic acid
MTT	3-4,5 dimethylthiazol-2-yl 2,5diphenyltetrazolium bromide
Na ⁺	Sodium
NaCl	Sodium chloride

NaHCO ₃	Sodium bicarbonate
NaOH	Sodium hydroxide
NaPi2	Sodium-phosphate cotransporter type 2
NCC	Sodium-chloride cotransporter
NF-κβ	Nuclear factor-kappa beta
NH ₄ ⁺	Ammonium
NHE3	Sodium-hydrogen exchanger type 3
NKCC2	Sodium-potassium-chloride cotransporter 2
nm	Nanometres
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
NSW	New South Wales
OA	Oleanolic acid
p.o.	per os (orally)
PCR	Polymerase chain reaction
PKC	Protein kinase C
ppm	Parts per million
Pty	Property
RAAS	Renin-angiotensin-aldosterone system
RAGE	Receptor for advanced glycation end products
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
SEM	Standard error of means
SGLT	Sodium-glucose cotransporter
SHR	Spontaneously hypertensive rats

STZ	Streptozotocin
TGF- β	Transforming growth factor beta
TLC	Thin layer chromatography
TP – 72	3,11-dioxooleanl, 12-dien-28-oic acid
TP- 69	3,12-dioxoolean-1-en-28-oic acid
TZD	Thiazolidinediones
μ	Micro
UA	Ursolic acid
UK	United Kingdom
UKZN	University of KwaZulu-Natal
USA	United States of America
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
γ	Gamma

TABLE OF CONTENTS

Page no.

Declaration	i
Acknowledgements	ii
Abbreviations	iii
Table of contents	viii
List of Tables	xi
List of Figures	xii
List of Appendices	xv
Abstract	xvi
CHAPTER 1- Introduction/ Literature review	1
1.0. Preamble	1
1.1. Introduction	2
1.1.1. Kidney function	2
1.1.2. Kidney structural and functional relationship	2
1.2. Importance of Na ⁺ handling	6
1.2.1. Transport processes in the proximal tubule	6
1.2.2. Processes in the loop of Henle, distal tubule and collecting ducts	7
1.2.2.1. Hormonal influence	8
1.3. Kidney Disease	10
1.3.1. Diabetic nephropathy	11
1.3.1.1. Metabolic Factors	11
1.3.1.1.1. Effects of advanced glycation end products (AGEs) on kidney function	12
1.3.1.2. Haemodynamic factors	14
1.3.2. Kidney function and blood pressure	15
1.3.3. Hypertension	16

1.4. Management of renal disorders	16
1.4.1 Traditional (indigenous/folk) medicine	17
1.4.1.1. <i>Syzygium aromaticum</i>	18
1.4.1.1.1. Triterpene OA	21
1.5. Conventional method used to assess kidney functional status	21
1.5.1. Plasma and urine biomarkers	21
1.5.2. Lithium Clearance	23
1.5.3. Cell Culture tests	24
1.5.3.1. Hepatocyte-derived human transformed cell line (HepG2)	25
1.5.3.2. Human embryonic kidney cell line (Hek293)	26
1.5.3.3. Madin Darby bovine kidney cell line (MDBK)	26
1.6. Justification and aim of the present study	26
1.6.1. Objectives	27
CHAPTER 2 - Materials and methods	28
2.1. Materials	28
2.1.1. Drugs and chemicals	28
2.2. Methods	28
2.2.1. Isolation of oleanolic acid	28
2.3. Animal studies	29
2.3.1. Ethical consideration	29
2.3.2. Animals	30
2.4. Experimental Design	30
2.5. Renal studies	30
2.5.1. Anaesthetized animals	30
2.5.1.1. Renal clearance studies	30
2.5.2. Conscious animals	31
2.5.2.1. Terminal studies	32

2.6. Laboratory analysis	33
2.6.1. Biochemical measurements	33
2.6.2. Lithium measurements	33
2.6.3. Calculations	34
2.7. Cell culture studies	34
2.7.1. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay	35
2.7.2. Single cell gel electrophoresis (comet) assay	36
2.8. Analysis of data	37
CHAPTER 3 - Results	38
3.0. General	38
3.1. OA structure	38
3.2. OA effects on body weight, food and water intake	40
3.3. Renal fluid and electrolyte handling	41
3.3.1. Anaesthetized animals	41
3.3.2. Conscious animals	44
3.4. Effects of OA on mean arterial blood pressure	47
3.4.1. Anaesthetized animals	47
3.4.2. Conscious animals	48
3.5. Effects of OA on glomerular filtration rate	49
3.5.1. Anaesthetized animals	49
3.5.2. Conscious animals	50
3.6. Renal clearance measurements	51
3.7. Cell culture studies	53
3.7.1. Cell viability	53
3.7.2. DNA integrity	55

CHAPTER 4 - Discussion	59
4.1. General	59
CHAPTER 5 - Conclusions	63
5.0. Conclusions	63
5.1. Limitations and future studies	63
CHAPTER 6 - References	64
APPENDICES	99

LIST OF TABLES

Table number		Page no.
Table 1:	Medicinal plants that possess hypoglycaemic and reno-protective properties	20
Table 2:	Comparison of the effects of various doses of OA on food intake, water intake and body weight changes in comparison with control animals	40
Table 3:	Comparison of the effects of OA on plasma biochemical parameters in male Sprague-Dawley rats which were administered OA twice every third day for five weeks with control animals	50

LIST OF FIGURES

Figure number		Page no.
Figure 1:	<i>S. aromaticum</i> leaves	19
Figure 2:	<i>S. aromaticum</i> -derived OA ^1H and ^{13}C - NMR spectroscopic spectra	39
Figure 3:	Comparison of urinary Na^+ excretion (A) rates of control rats with animals administered OA	42
Figure 4:	Comparison of urine flow (A), K^+ (B) and Cl^- excretion (C) rates of control rats with animals administered OA	43
Figure 5:	Comparison of the effects of various doses of OA treatment on 24 h urine flow (A) and Na^+ excretion (B) rates with control animals	45
Figure 6:	Comparison of the effects of various doses of OA treatment on K^+ (A) and Cl^- excretion (B) rates with control animals	46
Figure 7:	Comparison of MAP of control rats with animals administered OA	47
Figure 8:	Comparison of the effects of various doses of OA administration on MAP with control animals	48
Figure 9:	Comparison of GFR of control rats with animals administered OA	49
Figure 10:	Comparison of $\text{FE}_{\text{Na proximal}}$ and $\text{FE}_{\text{Na distal}}$ (A) and FE_{Li} (B) of control rats with animals administered OA	52

Figure 11:	The effects of OA on the viability and/or metabolic activity of HepG2 (A), HEK293 (B) and MDBK (C) cells <i>in vitro</i> after exposure to various concentrations of OA for 24, 48 and 72 h	54
Figure 12:	The effects of OA on DNA integrity of HepG2 cells <i>in vitro</i> after exposure to various concentrations of OA for 24, 48 and 72 h	56
Figure 13:	The effects of OA on the DNA integrity of HEK293 cells <i>in vitro</i> after exposure to various concentrations of OA for 24, 48 and 72 h	57
Figure 14:	The effects of OA on DNA integrity of MDBK cells <i>in vitro</i> after exposure to various concentrations of OA for 24, 48 and 72 h	58

LIST OF APPENDICES

	Page No.
APPENDIX I: Ethical clearance A	99
APPENDIX II: Ethical clearance B	100
APPENDIX IV: Conference presentations	
Madlala HP , Masola B, Singh M, Musabayane CT. The effects of <i>Syzygium aromaticum</i> -derived oleanolic acid in kidney and liver cell lines, and on kidney function of male Sprague-Dawley rats. Physiological Society of Southern Africa, 39 th Congress, 28-31 August 2011.	101

ABSTRACT

Adverse effects and increasing cost of therapeutic drugs have renewed an interest in the use of medicinal plant products for the treatment of a variety of chronic disorders. One such bioactive plant-derived compound is a pentacyclic triterpenoid, oleanolic acid (3 β -hydroxy-olea-12-en-28-oic acid, OA) present in herbs. OA possesses a variety of pharmaceutical activities and of interest in this study are the anti-diabetic properties. Diabetes is associated with disorders grouped as microvascular (retinopathy and nephropathy) and macrovascular (atherosclerotic) complications. Accordingly, this study further investigated the potential of OA in diabetes management by studying the effects of this triterpene on kidney function as well as proximal tubular Na⁺ handling in an effort to identify the site of action of OA. Furthermore, the study evaluated the effects of OA in kidney and liver cell lines to establish whether this triterpene exhibits any toxicity in these organs.

OA was extracted using a previously validated protocol in our laboratory. Briefly, dried flower buds of *Syzygium aromaticum* were soaked in dichloromethane overnight, thereafter in ethyl acetate to obtain ethyl acetate solubles which contained a mixture of OA/ursolic and maslinic acid (MA). OA/MA mixture was subjected to column chromatograph and pure OA was obtained through recrystallization in methanol. The absolute stereostructure of OA was elucidated using ¹H and ¹³C NMR spectroscopy and was comparable to previously reported data. In kidney function studies, various doses of OA (30, 60, 120 mg/kg, p.o.) were administered to male Sprague-Dawley rats twice (8h apart) every third day for five weeks. Rats administered deionised water served as controls. Measurements of body weight, food and water intake, blood pressure, Na⁺, K⁺, Cl⁻, urea and creatinine were taken 24 h from dosing. Renal clearance studies investigated the influence of OA on Na⁺ handling in the proximal tubule of anaesthetized rats using lithium clearance. Animals were given water with lithium (12mmol/l) for 48 hours following which they were anaesthetized and cannulated using a previously validated standard protocol that has been reported from our laboratories. After a 3½ h equilibration, animals were challenged with hypotonic saline for 4 h of 1 h control, 1½ h treatment and 1½ h recovery periods. OA was added to the infusate during the treatment period. *In vitro* effects of various OA concentrations (5, 10, 20, 40, 80 μ mol/l) were investigated in HEK293, MDBK and HepG2 cell lines. Cells were exposed to OA for 24, 48 and 72 h, thereafter,

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and single cell gel electrophoresis (comet) assays were conducted. All data are presented as means \pm SEM.

OA significantly ($p < 0.05$) increased urinary Na^+ output from week 2 until the end of the experimental period in a dose independent manner. However, this OA-evoked natriuresis was not reflected in plasma collected at the end of the experiment as there was no change in plasma Na^+ concentrations compared with control animals at the corresponding time. OA administration had no significant influence on K^+ and Cl^- excretion rates throughout the experiment. However, OA significantly ($p < 0.05$) reduced plasma creatinine concentration with a concomitant increase in glomerular filtration rate (GFR). Furthermore, OA administration significantly ($p < 0.05$) decreased mean arterial pressure from week 2 until the end of the experimental period. Intravenous infusion of OA at 90 $\mu\text{g}/\text{h}$ for 1 $\frac{1}{2}$ h induced a marked increase in urinary excretion rates of Na^+ . This increase was accompanied by concomitant increase in $\text{FE}_{\text{Na proximal}}$ and $\text{FE}_{\text{Na distal}}$ and FE_{Li} which persisted until the end of the experiment without any apparent changes in GFR. The cell viabilities of HepG2, HEK293 and MDBK cell lines were significantly increased after 24 h exposure, however, the viabilities of all the three cell lines dropped after 72 h exposure to values that did not achieve statistical significance in comparison to the respective controls. In addition, all OA-treated cells in the comet assay had intact DNA after exposure for 24, 48 and 72 h. Hence, the decrease in viability that was observed in the MTT assay after 72 h exposure could probably be attributed to the depletion of nutrients in the culture medium.

The results of the present study, apart from confirming our previous observations of the natriuretic effects of OA in rats, indicate that this effect is in part mediated via the inhibition of proximal tubular Na^+ reabsorption and increased Na^+ secretion. We speculate that this increased Na^+ secretion could have been due to increased tubular function and not to the toxicity of OA as indicated by MTT and comet assays. These findings suggest that OA does not exhibit toxicity in the kidney and the liver.

CHAPTER 1

Introduction / Literature review

1.0. Preamble

In recent years, there has been a renewed interest in the use of plant products for the treatment of a variety of chronic disorders. One such bioactive plant-derived compound is a pentacyclic triterpenoid, oleanolic acid (3 β -hydroxy-olea-12-en-28-oic acid, OA) present in herbs and dietary plants. OA possesses a variety of medicinal claims which include anti-inflammatory, anti-cancer, anti-stomach ulcer, anti-hyperlipidaemic, anti-hypertensive, cardiogenic, anti-dysrhythmic, anti-aggregation of blood platelets, hepato-protective and anti-diabetic effects. Of interest in our laboratory are the anti-diabetic properties of OA. Diabetes mellitus is characterized by hyperglycaemia which compromises kidney function. Reports indicate that the majority of morbidity and mortality in diabetes is caused by end stage renal disease (ESRD). Therefore, this study investigated the effects of OA proximal tubular Na⁺ handling. In addition, we evaluated the effects of OA in human embryonic kidney (HEK293) and Madin-Darby bovine kidney (MDBK) cell lines to establish whether OA has any toxic effects on the kidney. Furthermore, we evaluated the effects of OA in hepatocyte-derived human transformed (HepG2) cell lines since the liver is responsible for biotransformation of drugs. Chapter 1 describes the physiology of kidney function, adverse effects of diabetes, particularly on renal haemodynamics and kidney cell structure and how drugs can influence these adverse effects, and justification of the study.

1.1. Introduction

1.1.1. Kidney function

Kidneys play a vital role in maintaining the normal volume and composition of body fluids (DeFronzo, Davidson and Prato, 2011; Rosner and Bolton, 2006; Zhu and Wang, 2008; Edwards, 2010). They achieve this by working together with other body organs like the lungs and the liver to ensure that homeostasis is maintained. The kidneys maintain this homeostasis through filtration, reabsorption and secretion (DeFronzo *et al.*, 2011; Wagner, Kovacicova, Stehberger, Winter, Benabbas and Mohebbi, 2006). Tubular reabsorption of electrolytes and water is regulated by hormones and physical factors such as renal arterial pressure and glomerular filtration rate (GFR) (Rosner and Bolton, 2006; Edwards, 2010). However, in adverse conditions like diabetes mellitus, regulation of tubular reabsorption of electrolytes is disrupted due to structural and functional changes of the kidney caused by hyperglycaemia thereby resulting in the decline of renal function. This study focused on the potential of OA to avert the decline in renal function.

1.1.2. Kidney structural and functional relationship

The kidney is made up of the outer cortex and the inner medulla regions (Gloviczki, Glockner, Gomez, Romero, Lerman, McKusick and Textor, 2009; Dyson, Bezemer, Legrand, Balestra, Singer and Ince, 2011). The medulla has pyramids which have an apex that extend towards the renal pelvis to form a papilla. These renal pyramids have striations which appear to be the straight loop of Henle, collecting ducts and blood vessels (Moffat and Fourman, 1963; Yuan and Pannabecker, 2010). The basic functional unit of the kidney is a nephron (Prasad, Narra, Shah, Humphrey, Jagirdar, Catena, Dalrymple and Siegel, 2007) which is a thin tube with a blind glomerulus end and an open collecting duct end. The nephron is made up of a renal corpuscle and renal tubule; different sections of this structure are responsible for secretion and reabsorption of plasma constituents.

In the human kidney, approximately 180 litres of plasma containing sodium chloride, sodium bicarbonate, glucose and many other solutes are filtered through the renal corpuscle each day (DeFronzo *et al.*, 2011; Wright, Hirayama and Loo, 2007; Abdul-Ghani and DeFronzo, 2008). The kidney renal corpuscle comprises of the glomerulus surrounded by the Bowman's capsule which have specialized epithelial cells called podocytes (Prasad *et al.*, 2007; Navar, 2009). Podocytes, basement membrane and the endothelial cells form a filtration barrier which is semi-permeable to molecules entering the renal tubule (Neal, Muston, Njegovan, Verrill, Harper, Deen and Bates, 2007; Navar, 2009). However, the function of this glomerular filtration barrier becomes abnormal in diabetes due to glomerular hypertrophy, thickening of glomerular basement membrane and injury of the podocytes (Susztak, Raff, Schiffer and Böttinger, 2006). Other cells that line the renal corpuscle are juxtaglomerular apparatus composed of vascular smooth muscle cells, endothelial cells, mesangial cells, macula densa cells, and renin-secreting juxtaglomerular granular cells (Yao, Oite and Kitamura, 2009). The morphology of these kidney cells is altered due to injury caused by various substances which are products of hyperglycaemia. Accordingly, this study investigated the effects of OA on the viability and DNA integrity of isolated kidney cell lines to relate to *in vivo* effects of OA. In addition, the study investigated the effects of OA in liver cells since they play a vital role in the degradation of drugs.

The sequel of events in diabetes results in overproduction of nitric oxide (NO) which cause persistent vasodilation of the afferent and efferent arterioles which cause hyperfiltration (Tolins, Shultz, Raij, Brown and Mauer, 1993; Komers, Lindsley, Oyama, Allison and Anderson, 2000). Hyperfiltration increases the load of Na^+ delivered to the macula densa cells. Stimulation of the macula densa cells results in an enhanced tubulo-glomerular feedback mechanism that causes hyperactivation of the renin-angiotensin-aldosterone system (RAAS) which increases Na^+ reabsorption (Bickel, Knepper, Verbalis and Ecelbarger, 2002; Raij, 2005; Zerbini, Bonfanti, Meschi, Bognetti, Paesano, Gianolli, Querques, Maestroni, Calori, Maschio, Fazio, Luzi and Chiumello, 2006). This hyperfiltration may be reversed by synthetic drugs like L-arginine derivatives which inhibit NO synthesis resulting in increased renal vascular resistance (Bayliss, Harton and Engels, 1990; Ohishi and Carmines, 1995). In

addition, bioactive compounds isolated from various medicinal plants like triptolide from *Tripterygium wilfordii*, tetrandrine and related alkaloids from *Tetrandra Stephenia*, higenamine from *Aconitum coreanum*, tetramethyl pyrazine from *Ligusticum wallichii* and andrographolides from *Andrographis paniculata* have been shown to inhibit NO synthesis by inhibiting inducible nitric oxide synthase (iNOS) activity (Kondo, Takano and Hojo, 1993; Kang, Lee, Lee, Lee, Ryu, Yun-Choi and Chang, 1999; Wu, Liao, Chen and Yen, 1999; Chiou, Chen and Lin, 2000; Achike and Kwan, 2003; Wang, Ma, Tao and Lipsky, 2004). Of importance to this study was the observation that oleananes, 3,12-dioxoolean-1-en-28-oic acid (TP-69) and 3,11-dioxoolean-1,12-dien-28-oic acid (TP-72), derivatives of OA inhibit the expression of iNOS (Suh, Honda, Finlay, Barchowsky, Williams, Benoit, Xie, Nathan, Gribble and Sporn, 1998). Plant-derived active compounds like polyphenols which include flavonoids, tannins, lignans and catechins have been shown to decrease the formation of NO by either direct scavenging of NO or by inhibiting the expression of iNOS (van Acker, Tromp, Haenen, van der Vijgh and Bast, 1995; Ryu, Lee, Jeong, Ryu and Han, 1998; Wakabayashi, 1999; Son, Lee, Yun-Choi and Ryu, 2000; Chi, Cheon and Kim, 2001; Achike and Kwan, 2003).

Increased Na⁺ reabsorption is associated with increase in blood pressure and conventional drugs used to manage elevated blood pressure include angiotensin converting enzyme inhibitors (ACE) and angiotensin II receptor blockers. In addition, extracts from *Irvingia gabonensi*, *Sclerocarya birrea* (A. Rich.) [Hochst], *Ekebergia capensis* (Sparrm) [Maliaceae], *Syzygium cordatum* (Hochst.) [Myrtaceae] and *Geranium macrorrhizum* (Geraniaceae) possess blood pressure lowering properties (Miliauskasa and van Beek, 2004; Gondwe, Kamadyaapa, Tufts, Chuturgoon and Musabayane, 2008; Kamadyaapa, Gondwe, Moodley, Ojewole and Musabayane, 2009; Mapanga, Tufts, Shode and Musabayane, 2009; Nosiri, Hussaini and Abdurahaman, 2011). Furthermore, extracts from *Schinus molle* (Molle), *Lepidium meyenii* Walp (Maca), *Cyclanthera pedata* (Caigua), *Zingiber officinale* (ginger), *Euphorbia hirta* and *Lamiaceae* species have been shown to have significant ACE inhibitory activities (Ivancheva, Manolova, Serkedjeva, Dimov and Ivanovska, 1992; Barbosa-Filho, Martins, Rabelo, Moura, Silva, Cunha, Souza, Almeida and Medeiros, 2006; Kwon, Vattem and Shetty, 2006; Ranilla, Kwon, Apostolidis and Shetty, 2010). Joining the glomerulus is the

proximal tubule which is divided into proximal convoluted and proximal straight tubules. The convoluted tubule has cuboidal columnar cells with a brush border of microvilli which has a large number of mitochondria for energy production required for active transport of solutes (Little, Brennan, Georgas, Davies, Davidson, Baldock, Beverdam, Bertram, Capel and Chiu, 2007; Yu, Carroll, Rajagopal, Kobayashi, Ren and McMahon, 2009). Therefore, transport processes in the straight tubule are highly developed compared to convoluted tubule which has fewer microvilli and mitochondria. Reports indicate that approximately 65% of Na^+ reabsorption occurs in the proximal tubule due to increased surface area attributed by the presence of the microvilli where the apical Na^+ -hydrogen exchanger type 3 (NHE3) and Na^+ -phosphate cotransporter 2 (NaPi2) are located (Girardi, Fukuda, Rossoni, Malnic and Reboucas, 2008, Lorenz, Schultheis, Traynor, Shull and Schnermann, 1999). Some studies indicate that angiotensin-converting enzyme inhibitor, captopril decreases proximal tubule Na^+ and water reabsorption by causing redistribution of these transporters out of the microvilli resulting in natriuresis and diuresis (Leong, Devillez, Sandberg, Yang, Yip, Klein and McDonough, 2006; Riquier-Brison, Leong, Pihakaski-Maunsbach and McDonough, 2010).

Extending from the proximal tubule is the thick and thin segments of the loop of Henle which have ascending and descending limbs. Cells of the thick limb are cuboidal, but do not have a brush border and those of the thin limb are flat and squamous (Prasad *et al.*, 2007). Following the loop of Henle is the distal convoluted tubule which joins the collecting ducts. Collecting ducts are lined by cuboidal cells which are impermeable to tubular contents and hence transport processes in this part of the tubule are influenced by hormones (Schuster, 1993; Yu *et al.*, 2009; Arroyo, Ronzaud, Lagnaz, Staub and Gamba, 2011). Understanding the structure of the nephron is essential since all the above mentioned segments are structurally adapted to the transport processes in the kidney which are being explained in details in paragraphs to follow.

1.2. Importance of Na^+ handling

Na^+ is the major cation that controls osmolality in the extracellular fluid (ECF) volume (DeFronzo, Cooke, Andres, Faloona and Davis, 1975) and hence Na^+ reabsorption is vital for maintenance of homeostasis in the ECF (DeFronzo *et al.*, 2001; Edwards, 2010). Since Na^+ is present in the highest concentration in the ECF, this cation is filtered and reabsorbed in the largest amount in the kidneys. Reabsorption of water and many other solutes is linked to Na^+ reabsorption as some substances depend on co-transport with Na^+ for their uptake (Hackenthal, Paul, Ganten and Taugner, 1990; Klar, Vitzthum and Kurtz, 2004). However, the normal ECF osmolality and volume brought about by Na^+ reabsorption is disturbed in diabetes since the long term effects of this disorder in the kidney results in increased Na^+ and water retention. Na^+ reabsorption consumes a tremendous amount of ATP and oxygen and this is why the kidney receives approximately 25% of cardiac output (Lewy, Quintanilla, Levin and Kessler, 1973; Ljungman, Laragh and Cody, 1990; Gomez, Warner, Haas, Bolterman, Textor, Lerman and Romero, 2009; Hallows, Mount, Pastor-Soler and Power, 2010). Two thirds of the filtered Na^+ is handled in the proximal tubule through transport processes explained below. Proximal tubular Na^+ handling can be quantitatively estimated through lithium clearance and hence this study employed this technique to establish OA effects on Na^+ handling in the proximal tubule.

1.2.1. Transport processes in the proximal tubule

The most important process in the proximal tubule is the reabsorption of two thirds of the filtered Na^+ by an active process using transport proteins like the apical NHE3 and NaPi2 and the basolateral Na^+ - K^+ ATPase, (Lorenz *et al.*, 1999; Ganz, Hawkins and Reilly, 2000; Aronson, 2002; Girardi *et al.*, 2008; Yingst, Araghi, Doci, Mattingly and Beierwaltes, 2009; Auriemma, Galdiero, De Martino, De Leo, Grasso, Vitale, Cozzolino, Lombardi, Colao and Pivonello, 2010). Diffusion of Na^+ into the proximal tubular cell is favoured by the negative intracellular potential and electrochemical gradient in relation to both luminal and peritubular fluids (Doucet, Favre and Deschênes, 2007). The entry of Na^+ into the tubular cell from the lumen occurs via simple diffusion, exchange for H^+ , co-transport with glucose and chloride (Cl^-) (Mahnensmith and Aronson, 1985; DeFronzo *et al.*, 2001; Aronson, 2002; Pannabecker and Dantzler, 2006, Pannabecker and Dantzler, 2007). Active extrusion of Na^+ from the

tubular cells into the lateral intercellular spaces accompanied by Cl^- leads to accumulation of NaCl in spaces and a local increase in osmotic pressure which causes osmotic entry of water into intercellular spaces (Layton, Layton, Dantzler and Pannabecker, 2009).

Na^+ reabsorption loosely links the active reabsorption of bicarbonate which also takes place in the proximal tubule and is accompanied by active secretion of H^+ ions into the tubular lumen (DeFronzo *et al.*, 2001; Aronson, 2002; Pannabecker and Dantzler 2006, 2007). Therefore, passive Na^+ entry into the intracellular space is favoured by both electrical and concentration gradients (Aronson, 1982; Funder, Wieth, Jensen and Ibsen, 1984; Mahnensmith and Aronson, 1985). Na^+ reabsorption is greatly increased in diabetes due to increase function of the sodium-glucose cotransporter (SGLT) and the NHE3 in the proximal tubule (Vallon, Richter, Blantz, Thomson and Osswald, 1999; Ganz, Hawkins and Reilly, 2000). This is accompanied by increased water retention. Diuretics are used to increase fluid excretion through inhibition of Na^+ transporters thereby causing diuresis and natriuresis. Some plant extracts like those from *Foeniculum vulgare* L. (fennel, Apiaceae) have been shown to increase water and sodium excretion (Bardai, Lyoussi, Wibo and More, 2001). The following paragraph briefly explains the transport processes that occur in the in the loop of Henle, distal tubule and collecting ducts.

1.2.1. Processes in the loop of Henle, distal tubule and collecting ducts

The most important process in the loop of Henle is the countercurrent multiplication system which preserves inner medullar interstitial solute gradient (Yuan and Pannabecker, 2010). The descending limb of the loop of Henle is highly permeable to water and relatively permeable to NaCl. Water extraction increases NaCl concentration in the tubule (Pannabecker, Dantzler, Layton and Layton, 2008; Layton *et al.*, 2009). Osmolality of the fluid in the lumen is increased by urea which is added to the medullary interstitium from the collecting duct by diffusion down a concentration gradient (Layton *et al.*, 2009). The ascending limb actively extrudes NaCl into the medullary interstitium through the bumetanide-sensitive cotransporter (NKCC2/BSC1) and the NHE3, but is impermeable to water hence the osmolality in the medullary interstitium is increased and that of the fluid in the ascending limb is decreased

(Pannabecker and Dantzer 2006, 2007). NKCC2/BSC1 mediates entry of 1Na^+ , 2Cl^- and IK^+ into the tubular cell across the luminal membrane in the thick ascending limb (Pannabecker and Dantzer 2006, 2007; Pannabecker *et al.*, 2008). Early distal tubule reabsorbs NaCl by a Na^+/Cl^- cotransporter (NCC) and is impermeable to water resulting in dilution of the luminal fluid (Arroyo *et al.*, 2010). The Na^+ that enters the cell is pumped out across the basolateral membrane by the neutral Na^+/H^+ which cause H^+ secretion (Bonventre and Leaf, 1982; Giebisch, 1998; Arroyo *et al.*, 2010).

The function of Na^+ transporters like NKCC2/BSC1, NHE3, NCC can be inhibited by drugs such as furosemide, bumetanide, thiazides, hydrochlorothiazide and bendroflumethiazide; potassium sparing, amiloride and carbonic anhydrase inhibitors, acetazolamide. This inhibition could be beneficial in diabetes since it results in natriuresis and diuresis. (Martinez-Maldonado and Cordova, 1990; Kim, 2004). Furthermore, aqueous extracts from the roots of *Carissa edulis* (forssk) vahl (Apocynaceae), *Ananas comosus* (Linn.) Merr. and *Carica papaya* Linn. (Pawpaw, Melon tree) have been shown to possess diuretic properties (Sripanidkulchai, Wongpanich, Laupattarakasem, Suwansaksri and Jirakulsomchok, 2001; Nedia, Mekonnena and Urgab, 2004). Transport processes occurring in the late distal tubules and collecting ducts explained above are mostly influenced by hormones.

1.2.1.1. Hormonal influence

Late distal tubules and collecting ducts have principal and intercalated cells (Prasad *et al.*, 2007). Principal cells play a role in the reabsorption of Na^+ which is associated with secretion of K^+ . Na^+ reabsorption is increased by aldosterone, a mineralocorticoid hormone which exert its effects through Na^+ pumps and transporters (Abdallah, Schrier, Edelstein, Jennings, Wyse and Ellison, 2001; Tait, Tait and Coghlan, 2004; Hallows *et al.*, 2010; Arroyo *et al.*, 2010). This hormone is synthesised from cholesterol through a series of enzymatic reactions that occur in the zona glomerulosa of the adrenal cortex (Payne and Hales, 2004; Connell, MacKenzie, Freel, Fraser and Davies, 2008). Principal regulators of aldosterone biosynthesis are renin-angiotensin system (RAS), extracellular potassium concentration and adrenocorticotrophic hormone (ACTH) (Hackenthal *et al.*, 1990; Klar *et al.*, 2004; Connell *et*

al., 2008; Arroyo *et al.*, 2010).

The major action of aldosterone on epithelial cells is to regulate the reabsorption of Na^+ thereby also influencing the transport of water across the membrane (Hackenthal *et al.*, 1990; Klar, *et al.*, 2004; Arroyo *et al.*, 2010). Aldosterone-induced reabsorption of Na^+ in the distal tubule is mediated via Na^+/K^+ -ATPase and amiloride-sensitive epithelial sodium channel (ENaC) (Abdallah *et al.*; 2001). Reports indicate that in diabetes there are increased protein levels of ENaC subunits (α , β and γ) due to hyperactivation of RAAS mediated by the tubuloglomerular feedback mechanism (Klein, Rash, Sands, Ecelbarger and Tiwari, 2009). The mechanism in which aldosterone mediate Na^+ reabsorption is through modulating the expression of Na^+ channels in the plasma membrane or increasing their possibility to open (Hackenthal *et al.*, 1990; Ganz *et al.*, 2000; Abdallah *et al.*, 2001; Geering, Beguin, Garty, Karlsh, Fuzesi, Horisberger and Crambert, 2003; Klar *et al.*, 2004).

Increased Na^+ reabsorption stimulates anti-diuretic hormone (ADH) also known as arginine vasopress (AVP) which increases water permeability in the luminal membrane (Ecelbarger, Kim, Wade and Knepper, 2001; Floyd, Mason, Proudman, German, Marples and Mobasheri, 2007). The primary AVP receptor that controls water reabsorption is the G protein-coupled V2 receptor which, when activated, result in a cascade of events that lead to trafficking of aquaporin-2 (AQP2) water channels to the apical membrane (Perucca, Bichet, Bardoux, Bouby and Bankir, 2008; Bugaj, Pochynyuk and Stockand, 2009;). In the absence of AVP, the principal cells are virtually impermeable to water. In addition to V2 receptor, AVP has been shown to exert a natriuretic effect via V1 receptor in experimental animals (Lote, Thewles and Wood, 1989; Musabayane, Forsling and Balment, 1997).

Increased Na^+ reabsorption in diabetes is associated with water retention and synthetic drugs like aldosterone inhibitors are being used to inhibit this retention by increasing excretion. In addition, there are herbal extracts like those of *Zea mays*, *Imperata cylindrica*, *Plantago major* and *Orthosiphon stamineus*, *Spergularia purpurea*, *Rosmarinus officinalis* and *Centaurium erythraea* which have been shown to have diuretic properties hence may alleviate water

retention (Dat, Ham, Khac, Lam, Son, Dau, Grabe, Johansson, Lindgren and Stjernstron, 1992; Haloui, Louedec, Michel and Lyoussi, 2000; Jouad, Haloui, Rhiouani, El-Hilaly and Eddouks, 2001; Jouad, Lacaille-Dubois and Eddouks, 2001a; Jouad, Lacaille-Dubois, Lyoussi and Eddouks, 2001b; El-Hilaly, Hmammouchib and Lyoussi, 2003).

1.3. Kidney Disease

Sustained hyperglycaemia results in kidney dysfunction which ultimately results in ESRD. ESRD is responsible for the majority of global morbidity and mortality in diabetes, by 2030 it is estimated that more than 2million people will need dialysis or transplantation for kidney failure (Van Dijk and Berl, 2004; Atkins, 2005; Stevens, Coresh, Greene and Levey, 2006). The risk factors of compromised kidney function include hypertension, cardiovascular disease, age of more than 60 years, family history of the disease and diabetes (REF). Hyperfiltration results in the activation of the glomerulo-tubular feedback reflex which restores normal salt delivery to the juxtaglomerular apparatus (Bank and Aynedjian, 1990;). However, this causes increased intra-glomerular pressure which leads to renal hypertrophy and eventually to the development of diabetic nephropathy (Vervoort *et al.*, 2005). Agents which delay the progression of diabetic nephropathy may prevent this condition by increasing Na⁺ loss in the urine, thereby inhibiting the glomerulo-tubular feedback reflex (Bank and Aynedjian, 1990; Nelson, Bennett and Beck, 1996; Tuttle, Bruton, Perusek, Lancaster, Kopp and DeFronzo, 1991; Cooper, 1998).

1.3.1. Diabetic nephropathy

Diabetic nephropathy is responsible for the morbidity and mortality in diabetes as it is the most common cause of ESRD (Nathan, 1993; Cooper, 1998; Atkins, 2005; Vervoort *et al.*, 2005; Arya, Aggarwal and Yadav, 2010; Lin and Sun, 2011). Currently, there is no

therapeutic intervention that has been shown to stop the progress of diabetic nephropathy. Hence there is an increase in the prevalence of this condition and this will increase the financial health burden in affected countries (Nathan, 1993; The Diabetes Control and Complications Trial Research Group, 1993; Atkins, 2005). Initial symptoms of diabetic nephropathy include expansion of glomerular mesangial matrix which causes compression of capillaries resulting in decreased surface area for filtration and decreased GFR (Gnudi, Viberti, Rajj, Rodriguez, Burt, Cortes, Hartley, Thomas, Maestrini and Gruden, 2003; Mason and Wahab, 2003; Kumar and Shetty, 2008; Lin and Sun, 2011). Other symptoms of diabetic nephropathy include increase in renal collagen and creatinine, albuminuria, proteinuria, glomerulosclerosis and interstitial fibrosis (Sharma, McCue and Dunn, 2003; Atkins, 2005; Ritz and Dikow, 2006; Brosius, 2008; Mogensen, 2000). Diabetic nephropathy results in decreased kidney function which is associated with many complications such as anaemia, malnutrition, bone disease, decreased quality of life and hypertension (National Kidney Foundation, 2002). Diabetic nephropathy is caused by interaction between metabolic and haemodynamic factors altered by hyperglycaemia (Cooper, 1998; Gnudi, Thomas and Viberti, 2007; Kumar *et al.*, 2008).

1.3.1.1. Metabolic Factors

Kidney pathophysiology in diabetes may be as a result of the effects of excess glucose directly on kidney cells or indirectly through pathways activated within the kidney including oxidative stress, renal polyol formation and accumulation of advanced glycation end products (AGEs) (Dunlop, 2000; Arya *et al.*, 2010). AGEs cause complications in disorders like diabetes, inflammation, hypoxia and ischemia/reperfusion injury (Bucciarelli, Ananthakrishnan, Hwang, Kaneko, Song, Sell, Strauch C, Monnier, Yan, Schmidt and Ramasamy, 2008; Chang, Wendt, Qu, Kong, Zou, Schmidt and Yan, 2008; Arya *et al.*, 2010). The effects of AGEs on kidney function are explained in detail in the following paragraph.

1.3.1.1.1. Effects of advanced glycation end products (AGEs) on kidney function

AGEs are a group of compounds formed from a glucose dependant non-enzymatic reaction

between reducing sugars and amine residues found in proteins, lipids and nucleic acids (Arya *et al.*, 2010). These early glycosylation products accumulate on proteins and undergo a series of in vivo rearrangements to form irreversible and complex compounds which are resistant to proteolytic degradation (Arya *et al.*, 2010). AGEs exert their biological effects through receptor mediated mechanisms, with receptor for advanced glycation end products (RAGE) being the most important (D'Agati, Yan, Ramasamy and Schmidt, 2009; Arya *et al.*, 2010). RAGE is a signal transduction receptor which is expressed in podocytes and glomerular endothelial cells in the human and murine glomerulus (Wendt, Tanji, Guo, Kislinger, Qu, Lu, Bucciarelli, Rong, Moser, Markowitz, Stein, Bierhaus, Liliensiek, Arnold, Nawroth, Stern, D'Agati and Schmidt, 2003; Yan, Ramasamy, Bucciarelli, Wendt, Lee, Hudson, Stern, Lalla, Yan, Rong, Naka and Schmidt, 2004; D'Agati *et al.*, 2009).

Binding of AGEs to the RAGE receptor activates a number of pathways such as increased cytosolic reactive species formation, stimulation of protein kinase C (PKC) and nuclear transcription factors such as NF- κ B, activation and expression of transforming growth factor beta (TGF- β) and vascular endothelial growth factor (VEGF) which are implicated in the development of diabetic renal disease (Wendt *et al.*, 2003; Arya *et al.*, 2010). Indeed, inhibition of the formation of AGEs has been shown to ameliorate diabetic nephropathy (Arya *et al.*, 2010). AGEs formation inhibitors like amino guanidine and pyridoxamine have been shown to have reno-protective effects in diabetic animals (Lassila, Seah, Allen, Thallas, Thomas, Candido, Burns, Forbs, Calkin, Cooper and Jandeleit-Dahm, 2004; Hartog, Voors, Bakker, Smit and van Veldhuisen, 2007).

Various medicinal plant-derived compounds have been shown to prevent diabetic nephropathy through inhibition of the AGEs or RAGE formation like resveratrol, the extract from *Panax quinquefolium* (Linnaeus) [Araliaceae], phytoestrogen from *Vitis vinifera* (Linnaeus) [Vitaceae]; curcumin from *Curcuma longa* (Linnaeus) [Zingiberaceae] and glycosides from *Stelechocarpus cauliflorus* (Annonaceae) (Sheetz and King, 2002; Rahbar and Figarola, 2003; Kim, Kang, Yamabe, Nagai and Yokozawa, 2007a; Wirasathien, Pengsuparpa, Suttisria, Uedab, Moriyasub and Kawanishib, 2007). Furthermore, several plants like *Persea americana* (Miller) [Lauraceae], *Sclerocarya birrea* [(A. Rich) Hochst.] and *Ficus thonningii* (Blume)

[Moraceae] have been shown to possess reno-protective effects through amelioration of kidney function (Musabayane, Gondwe, Kamadyaapa, Chaturgoon and Ojewole, 2007; Gondwe *et al.*, 2008, 2008). However, the mechanisms through which these plant extracts ameliorate kidney function have not been fully evaluated. Therefore, this study attempted to elucidate the mechanism through which plant-derived OA ameliorate kidney function, in part, by targeting the proximal tubule since this is where most of Na⁺ reabsorption occurs. We used lithium clearance technique since lithium is regarded as the best available reabsorption marker for proximal tubular function (Skøtt, 1994).

Kidney complications in diabetes are not only caused by AGEs, but also by oxidative stress that is induced by hyperglycemia which results in increased production of reactive oxygen species (ROS). ROS cause peroxidation of cell membrane lipids, oxidation of proteins, renal vasoconstriction, DNA damage and excessive deposition of extracellular matrix (ECM) in the kidney which cause glomerular mesangial expansion and tubulointerstitial fibrosis (Arya *et al.*, 2010). Various plant extracts of *Salvia sclarea*, *Salvia glutinosa*, *Salvia pratensis*, *Lavandula angustifolia* (all Lamiaceae family), *Calendula officinalis*, *Matricaria recutita*, *Echinacea purpurea*, *Rhaponticum carthamoides* (all Asteraceae family), *Juglans regia*, (Juglandaceae), *Melilotus officinalis* (Fabaceae), *Geranium macrorrhizum* (Geraniaceae) and *Potentilla fruticosa* (Rosaceae) have been shown to possess anti-oxidant effects and can alleviate symptoms of diabetic nephropathy by scavenging the ROS radicals (Bolshakova, Lozovskaya and Sapezhinskii, 1998; Lionis, Faresjo, Skoula, Kapsokefalou and Faresjo, 1998; Tang and Yuan, 1997; Ohsugi, Fan, Has, Xiong, Tezuka, Komatsu, Namba, Saitoh, Tazawa and Kadota, 1999; Lei, Mehta, Berenbaum, Zangerl and Engeseth, 2000; Zupko, Hohmann, Redei, Falkay, Janicsak and Mathe, 2001; Miliauskasa and van Beek, 2004). In addition, reports suggest that the bioactive compound of interest in this study, OA isolated from the glossy privet fruit (*Ligustrum lucidum* Fructus), hawthorn fruit (*Crataegi pinnatifidae* Fructus), *Perilla ocymoides* L., mint (*Mentha piperita*), *Glycyrrhiza globora*, *Paeonia lactiflora* Pall, *Hibiscus sabdariffa* L., and *Mours alba* L. possesses anti-oxidant properties (Dinkova-Kostova, Liby, Stephenson, Holtzclaw, Gao, Suh, Williams, Risingsong, Honda, Gribble, Sporn and Talalay, 2005; Yin and Chan, 2007).

1.3.1.2. Haemodynamic factors

Haemodynamic changes in diabetic nephropathy are caused by activation of vaso-active hormone pathways such as the RAAS and endothelin which result in increased systemic and intraglomerular pressure (Hargrove and Wong, 2000). These haemodynamic pathways activate intracellular second messengers such as PKC, mitogen-activated protein (MAP kinase), nuclear transcription factors such as NF- κ B and various growth factors such as the pro-sclerotic cytokine, TGF- β and VEGF (Haneda, 1997; Arya *et al.*, 2010). Increased systemic and intraglomerular pressure decrease resistance in both the afferent and efferent arterioles of the glomerulus which results in glomerular hyperperfusion and hyperfiltration which are the early signs of diabetic nephropathy (Arya *et al.*, 2010). Factors like NO, prostanoids, VEGF, TGF- β 1, and the RAAS are implicated in this abnormal regulation of body fluid. These haemodynamic changes increase leakage of albumin from the glomerular capillaries, overproduction of mesangial cell matrix, thickening of the glomerular basement membrane and injury to podocytes (Arya *et al.*, 2010). Furthermore, these haemodynamic changes increase mechanical strain which cause localised release of some cytokines and growth factors (Wolf and Ziyadeh, 2007).

OA could be a promising inhibitor for the progression of diabetic nephropathy as it has been shown to possess hypoglycaemic properties and hence can prevent the formation of AGEs. OA has also been shown to ameliorate kidney function which is an added advantage for a potential anti-diabetic agent. This study was designed to investigate the effects of OA on proximal tubular Na⁺ handling to further elucidate the potential of OA in preventing kidney complications.

1.3.2. Kidney function and blood pressure

The kidney is responsible for regulation of blood pressure through stimulation of renin and aldosterone production (De'Oliviera, Price, Fisher, Allan, McKnight, Williams and Hollenberg, 1997; DeFronzo *et al.*, 2001; Edwards, 2010). Aldosterone to renin ratio (ARR) is

related to primary aldosteronism as substantial proportion of hypertensive patients were found to have elevated ARR (De'Oliviera *et al.*, 1999; Connell *et al.*, 2008). Inappropriate regulation of aldosterone accounts for 15% of hypertension cases and contributes to the development of cardiovascular damage (Mulatero, Stowasser, Loh, Fardella, Gordon, Mosso, Gomez-Sanchez, Veglio and Young, 2004; Rossi, Bernini, Caliumi, Desideri, Fabris, Ferri, Ganzaroli, Giacchetti, Letizia, Maccario, Mallamaci, Mannelli, Mattarello, Moretti, Palumbo, Parenti, Porteri, Semplicini, Rizzoni, Rossi, Boscaro, Pessina and Mantero, 2006; Connell *et al.*, 2008).

Indirect aldosterone production inhibitors like angiotensin-converting enzyme inhibitors and receptor blockers have an impact in declining the progression of chronic kidney disease and the risk of ESRD (Navaneethan, Nigwekar, Sehgal and Strippoli, 2009). Reports indicate that various medicinal plant extracts like *Marrubium vulgare* L. (horehound, Lamiaceae) and *Foeniculum vulgare* L. (fennel, Apiaceae) possess hypotensive properties. Additionally, herbal remedies from *Moringa oleifera*, *Terminalia superba* (Combretaceae), *Averrhoa carambola* L. (Oxalidaceae), *Phyllanthus acidus* (L.), *Tulbhagia violacea* (Alliaceae), *Herniaria glabra*, *Verbesina caracasana* and *Randia siamensis* have been shown to have anti-hypertensive effects and therefore may alleviate high blood pressure associated with diabetes (Faizi, Siddiqui, Saleem, Aftab, Shaheen and Gilani, 1998; Rhiouani, Settaf, Lyoussi, Cherrah, Lacaille-Dubois and Hassar, 1999; Botta, Carmignani, Volpe, Botta, Corelli and Delle Monache, 2003; Khwanchuea, Jansakul, Mulvany, Queiroz and Hostettmann, 2007; Mackraj, Ramesar, Singh, Govender, Baijnath, Singh and Gathiram, 2008; Leeya, Mulvany, Queiroz, Marston, Hostettmann and Jansakul, 2010; Soncini, Santiago, Orlandi, Moraes, Peloso, dos Santos, Alves-da-Silva, Paffaro, Bento and Giusti-Paiva, 2011; Tom, Demougeot, Mtopi, Dimo, Djomeni, Bilanda, Girard and Berthelot, 2011). Diabetes is associated with hypertension which is discussed briefly below.

1.3.3. Hypertension

The prevalence of hypertension was thought to be high only in Western countries such as North America and Western Europe. However, recent studies have revealed that complications of hypertension like stroke, heart failure and renal failure are reported in blacks

all over the World (Connell *et al.*, 2008). Hypertension is widely reported in rural Africa and is the common cause of cardiovascular disorders in the continent, and cardiovascular disorders are responsible for the majority of mortality in sub-Saharan Africa (Connell *et al.*, 2008). Hypertension can be defined as a condition of having elevated blood pressure and can be classified to three different categories. Primary hypertension, also referred to as essential hypertension is asymptomatic and therefore most people are not aware that they have this condition. The cause of primary hypertension has not yet been identified. However, it is believed that the kidney dysfunction plays a major role in development of hypertension. A study that was conducted in Okamoto demonstrated that transplantation of the kidney of spontaneously hypertensive rats (SHR) into a normotensive rat results in development of hypertension (Yen, Yu, Roeder and Willard, 1974; Grisk and Rettig, 2001). Similarly, when the kidney of an SHR was replaced with that of a normotensive rat, hypertension was observed to cease (Yen *et al.*, 1974; Grisk and Rettig, 2001). Kidney dysfunction may be the explanation for the development of secondary hypertension as this type of hypertension is caused by underlying conditions (Laragh, 2003). The last type of hypertension is gestational hypertension which is observed in pregnant women.

1.4. Management of renal disorders

Most synthetic drugs for management of renal disorders target the haemodynamic factors that alter GFR and also the pathways involved in the development of the diabetic nephropathy. The current methods of treating renal disorders include ACE inhibitors (Wing, Reid, Ryan, Beilin, Brown, Jennings, Johnston, McNeil, Macdonald, Marley, Morgan and West, 2003). Aldosterone antagonists like spironolactone block the action of aldosterone in the distal tubules hence result in natriuresis whilst the excretion of K^+ , H^+ , NH_4^+ ions is decreased (Nakao, Yoshimura, Morita, Takada, Kayano and Ideura, 2002; Wing *et al.*, 2003; Navaneethan *et al.*, 2009). Beta-blockers, alpha1-blockers, alpha2-adrenoceptor agonists and calcium channel-blockers also inhibit electrolyte reabsorption which prevents water retention (Wallin and Frisk-Holmberg, 1981; Heintzen and Strauer, 1994; Lasagna, 2000).

Other synthetic drugs used to manage renal disorders are diuretics which stimulate the kidneys to excrete extracellular fluid by inhibiting Na^+ reabsorption and increasing the urine flow. Carbonic anhydrase inhibitors like acetazolamide cause depression of the reabsorption of HCO_3^- in the proximal tubule (Swenson, 1998). However, these drugs also promote K^+ excretion hence cannot be used as a therapy associated with adrenocortical steroids as these patients are susceptible to K^+ loss (Brown, Nakamura, Ma, Donnert, Freeman, Vaughan and Fogo, 2000). Even though these medications are very effective in treating hormonal and physiological factors that cause kidney dysfunction, they have some limitations as each of them has various side effects such as systemic acidosis, drowsiness and disturbance of vision (Brown *et al.*, 2000). Furthermore, these medications are expensive and therefore are not easily accessible especially to the communities from poor socio-economic background, hence a renewed interest in the use of medicinal plant products for treatment of a variety of chronic disorders.

1.4.1. Traditional (indigenous/folk) medicine

Reports indicate that 80% of the world's population uses herbal medicine for primary health care especially in developing countries. Approximately, between 12 and 15 million South Africans still use traditional remedies from as many as 700 indigenous plant species (Meyer and Afolayan, 1995; Grierson and Afolayan, 1999). Herbal drugs comprise traditional medicines which primarily use medicinal plant preparations for therapy. Traditional medicine has been used for hundreds of years because of their safety, affordability, efficacy, accessibility and lesser side effects (Kamboj, 2000; Ozsoy-Sacan, Karabulut-Bulan, Bolkent S, Yanardag and Ozgey, 2004; Gao, Li, Li, Liu, Fan, Liu, Zhao, Li and Han, 2009). Increased side effects of synthetic products, lack of curative treatment for several chronic diseases, high cost of new drugs and emerging diseases are some of the reasons for renewed public interest in alternative medicines (Patwardhan, Warude, Pushpangadan and Bhatt, 2005). Reports indicate that medicinal plants possess bioactive compounds which are responsible for many therapeutic effects (Shinawie, Singh and Padmavathi, 2000) and there are a number of conventional drugs that originate from plants such as aspirin (from willow bark), digoxin (from foxglove), quinine (from cinchona bark), and morphine (from the opium poppy)

(Shinawie, 2002). The plant bioactive compound of interest in this study was a pentacyclic triterpenoid isolated from *Syzygium aromaticum* (Hochst.) [Myrtaceae].

1.4.1.1. Syzygium aromaticum

S. aromaticum belongs to *Myrtaceae* family and is referred to as a clove tree (Figure 1). *S. aromaticum* is an evergreen tree that grows to a height of 15 to 30 meters tall and is native in North Moluccas (Indonesia), but is also grown in India, Jamaica, Brazil, and other tropical areas (Agbaje, Adeneye and Daramola, 2009). This plant has opposite; ovate leaves which are more than 12.7 centimeters long. When fully grown, its flowers are red and white, bell-shaped, and grow in terminal clusters. The brown, dried, unopened flower buds are called cloves. The dried flower bud is familiarly used in the kitchen and the fruit is a one- or two-seeded berry (Agbage *et al.*, 2009). The leaves are glabrous, with numerous oil glands on lower surface. *S. aromaticum* contains bioactive chemical compounds such as glycosides, tannins, polyphenols, triterpenes and saponins. *Syzygium spp*-derived OA has been shown to possess hypoglycaemic, hypotensive, reno- and cardioprotective effects (Musabayane *et al.*, 2005; Mapanga *et al.*, 2009; Musabayane *et al.*, 2010; Ngubane, Masola and Musabayane, 2011) in experimental animals. Table 1 below summarises medicinal plant extracts that have been shown to possess hypoglycaemic and kidney beneficial properties in human and animal studies.

A



B



Figure 1: *Syzygium aromaticum* leaves (A) and cloves (B)

Table 1: Medicinal plants that possess hypoglycaemic and renoprotective properties.

Scientific name	Part of plant used	Extract/active compound	Disease	Reference
<i>Adenopodia spicata</i> (E. Mey.) Presl.	leaves and roots	aqueous and ethanolic extracts	KD	Duncan <i>et al.</i> , 1999
<i>Allium sativum</i> L. (garlic) (Alliaceae)	roots	extracts of raw garlic	DM, KD	Al-Qattan <i>et al.</i> , 2008; Eidi <i>et al.</i> , 2005
<i>Cassia fistula</i> L. (Caesalpinaceae)	bark	ethanol and acetone extracts	KD	Somanadhan <i>et al.</i> , 1999
<i>Ficus thonningii</i> (Blume) [Moraceae]	stem-bark	ethanolic extract	DM, KD	Musabayane <i>et al.</i> , 2007
<i>Foeniculum vulgare</i> L. (fennel, Apiaceae)	fruits	aqueous extract	KD	El Bardai <i>et al.</i> , 2001; Ozbek <i>et al.</i> , 2003
<i>Fritillaria ussuriensis</i> Maxim. (Liliaceae)	bulb	aqueous extract	KD	Kang <i>et al.</i> , 2004
<i>Olea europaea</i> L., (Oleaceae)	leaves	aqueous extract flavonoids oleafricein oleanolic acid	DM, KD	Khayyal <i>et al.</i> , 2002; Komaki <i>et al.</i> , 2003; Somova <i>et al.</i> , 2003; Perrinjaquet-Mocchetti <i>et al.</i> , 2008; El and Karakaya, 2009
<i>Sclerocarya birrea</i> [(A. Rich) Hochst.] [Anacardiaceae]	stem-bark	ethanolic extract	DM, KD	Gondwe <i>et al.</i> , 2008
<i>Trigonella foenum-graecum</i> Linn (Fabaceae)	Seeds, leaves	alcoholic extract galactomannan	DM, KD	Vats <i>et al.</i> , 2002; Devi <i>et al.</i> , 2003; Jelodar <i>et al.</i> , 2005
<i>Viscum album</i> L (European mistletoe subspecies) (Loranthaceae)	leaves, young twigs, stems	ethanolic and aqueous extracts flavonoids, ursolic acid, kaempferol, quercetin, pectin	DM, KD	Ohiri <i>et al.</i> , 2003; Orhan <i>et al.</i> , 2005

Diabetes mellitus (DM), kidney disease (KD)

1.4.1.1.1. Triterpene OA

OA is a pentacyclic triterpenoid that widely exists in plants in the form of a free acid and has been isolated from more than 120 plant species (Wang and Jiang, 1992; Liu, 1995). OA has therapeutic importance due to its diverse pharmacological properties and lower systemic toxicities (Liu, 1995, Liu, 2005; Braga, Ayres-Saraiva, Gattass and Capella, 2007; Patil, Jadhav, Singh, Mundada and Patil, 2010). OA is the most abundant compound that is regarded as the major constituent of many African plant species used in traditional medicine and therefore is easily accessible. Multiple administrations of low doses of this triterpene do not cause any abnormalities in the heart, brain, and kidneys (Liu 1995). A study by Mapanga *et al.*, (2009) demonstrated that OA possesses kidney ameliorative properties in streptozotocin-induced diabetic rats. Hence this study employed the conventional methods explained below to further evaluate the effects of OA on the functional status of the kidney.

1.5. Conventional methods used to assess kidney functional status

1.5.1. Plasma and urine biomarkers

Kidney biomarkers are used to assess exposure to illness and to detect biological abnormalities, but are more often used to diagnose and measure a pathological condition (Madero, 2006; Endre and Westhuyzen, 2008; Tesch, 2010; Fassett, Venuthurupalli, Gobe, Coombes, Cooper and Hoy, 2011). There are a number of plasma and urine biomarkers that can be used to assess the functional status of the kidney and these biomarkers have to fulfill a certain criteria in order to be considered reliable. Preferred characteristic of a renal biomarker is that it should be measured easily, accurately and reproducibly. A renal marker should sensitively indicate renal injury or the kidney response to treatment and should provide useful and cost-effective clinical information. A renal marker should identify or differentiate specific types of renal injury or kidney disease and be applicable across a variety of populations (race, gender and age) (Tesch, 2010; Fassett *et al.*, 2011). Blood urea nitrogen (BUN) and creatinine clearance are well established biomarkers of renal function that can be measured cheaply and

easily using an enzyme/oxidation reaction assay and high performance liquid chromatography (HPLC) respectively (Mouton and Holder, 2006; Endre and Westhuyzen, 2008). Creatine clearance has been used by several authors to assess renal tubular function as a measure of GFR (Bursztyn, Ben-Ishay, Mekler and Raz, 1995; Travlos, Morris, Elwell, Duke, Rosenblum and Thompson, 1996; Girchev, Markova, Mikhov and Natcheff, 1998; Bertuzzi, Bensi, Mayer, Niebylski, Armario and Gauna, 2003; Gondwe *et al.*, 2008; Al-Ahmad, Rand, Manjunath, Konstam, Salem, Levey and Sarnak, 2001; Mapanga *et al.*, 2009). GFR can also be estimated using exogenous filtration markers like inulin, iothalamate, ethylenediaminetetra-acetic acid (EDTA), diethylene triamine penta-acetic acid and iohexol (Rosner and Bolton, 2006; Stevens *et al.*, 2006; Dharnidharka, Kwon and Stevens, 2001). The criteria for a good filtration marker requires the marker to be freely filtered, small and not bind to plasma proteins, to not be toxic or metabolized and to be present in the filtrate at the same concentration as in plasma (Endre and Westhuyzen, 2008). One of the effects of hyperglycaemia in diabetes is the expansion of mesangial matrix which causes compression of the renal capillaries and this has an effect on GFR. Accordingly, this study used creatinine clearance to assess the sub-chronic effects of OA on GFR.

A recently emerged, reliable alternative biomarker of renal function is cystatin-C which can be measured by an enzyme-linked immunosorbent assay (ELISA). Cystatin-C is a cysteine protease inhibitor that is constantly produced by nucleated cells and released into the blood, where it is normally reabsorbed and catabolized by kidney tubules without re-entering the blood stream (Curhan, 2005; Madero *et al.*, 2006; Mouton and Holder, 2006; Endre and Westhuyzen, 2008, Tesch, 2010). A potential biomarker of renal function in kidney diseases that are dependent on hypertension is urine angiotensinogen caused by increased uric acid which activates the renin-angiotensin system and can be measured by ELISA (Tesch, 2010). Biomarkers of renal oxidative stress are also useful in predicting onset of kidney diseases like diabetic nephropathy and these include presence of a DNA guanine product, 8-hydroxy-2-deoxyguanosine (8-OH-dG), 8-isoprostane, 4-hydroxy-2-nonenal 3-nitrotyrosine peptides caused by peroxidation of lipids and can all be measured by ELISA or liquid chromatography (Radabaugh, 2008; Tesch, 2010; Fassett *et al.*, 2011). Oxidative stress modifies the AGEs which deposit in the kidney and cause cellular dysfunction and renal damage as explained in

the paragraphs above. AGE pentosidine can be detected in both serum and urine using ELISA and can be used to evaluate early development of diabetic nephropathy (Tesch, 2010).

Glomerular filtration of albumin caused by injury of podocytes results in microalbuminuria which can be measured using ELISA and therefore is also a biomarker for renal damage (Tesch, 2010). Severely damaged podocytes can detach from the glomerular basement membrane and be detected in the urine sediment (Tesch, 2010; Fassett *et al.*, 2011). Analysis of the urine sediment by quantitative polymerase chain reaction (PCR) or ELISA can determine mRNA or protein levels of podocyte-specific molecules like nephrin, podocin and podocalyxin as markers of podocyte injury (Tesch, 2010). Increased urine sediment levels of nephrin and podocin have been detected in patients with diabetic nephropathy and active lupus nephritis (Wang, Lai, Lai, Chow, Li and Szeto, 2007; Wang, Lai, Tam, Li, Lai, Chow, Li and Szeto, 2007). N-acetyl-beta-D-glucosaminidase is a proximal tubular lysosomal enzyme which is released during damage to proximal tubules and increase urine levels are detected by enzymatic assays (Tesch, 2010; Fassett *et al.*, 2011). Liver-type fatty acid-binding protein (L-FABP) is another marker that is shed by proximal tubular cells in response to hypoxia (Tesch, 2010; Fassett *et al.*, 2011). Transforming growth factor- β 1 (TGF- β 1) and connective tissue growth factor are two of the major growth factors that promote renal fibrosis and can be measured in urine by ELISA (Tesch, 2010; Fassett *et al.*, 2011). In addition to creatinine clearance, the kidney function biomarker of interest in this study was lithium clearance which is used to quantify the amount of Na^+ output from the proximal tubule.

1.5.2. Lithium Clearance

Renal clearance of lithium has been used widely in animal studies and clinical investigations as a means of assessing proximal tubular function in the mammalian kidney (Koomans, Boer and Dorhout-Mees, 1989; Thomsen, 1990, Walter and Shirley, 1991; Shirley and Walter, 1993; Boer, Fransen, Shirley, Walter, Boer and Koomans, 1995; Whiting, 1999). Lithium clearance measurements are based on the observation that lithium undergoes iso-osmotic reabsorption in the proximal renal tubule to the same extent as salt and water, hence is

regarded as the best available marker for proximal tubular reabsorption of Na⁺ (Thomsen, 1984; Walter and Shirley, 1991; Skott, 1994, Whiting, 1999). Consequently, lithium clearance techniques can be used in both experimental and clinical studies to assess glomerulo-tubular function and handling of Na⁺ by the proximal tubule in both health and disease (Whiting, 1999). Controversially, recent studies have shown that lithium reabsorption also takes place in the distal nephron, however, this was only observed in Na⁺/K⁺ deprived rats (Shalmi, Jonassen, Thomsen, Kibble, Bie and Christensen, 1998; Thomsen and Shalmi, 1997; Emamifar, Shalmi, Thomsen and Christensen, 2000). Clearance and micropuncture studies in rats have confirmed that distal Li⁺ reabsorption does not occur if it is used in low concentrations (Koomans *et al.*, 1989; Shirley, Walter and Sampson, 1992; Thomsen, 1990; Walter and Shirley, 1991; Shirley and Walter, 1993). Hence this study employed lithium clearance to investigate the effects of OA on proximal tubular Na⁺ handling since the effects of hyperglycaemia mostly affect this part of the nephron where most of Na⁺ reabsorption takes place. In addition, we evaluated the effects of OA on the functional state of the kidney using cell culture methods which test the effects of a drug on the viability and structural integrity of isolated cell lines.

1.5.3. Cell Culture tests

Cell culture is a process by which cells are grown under controlled conditions which can be easily manipulated and analysed (Li, Bode and Sakai, 2004). Cell culture studies are the precursor to whole animal studies and help to determine whether significant cytotoxicity exists for the given material (Li *et al.*, 2004). Culture of cell lines is essential for biomedical research which include medicine, vaccine development, genetics, HIV testing/treatment, reconstructive medicine, basic science, cell biology and drug discovery (Dunham and Guthmiller, 2008, Farkas and Tannenbaum, 2005). Therefore, understanding the toxicity of drugs and chemicals is essential for progress in the pharmaceutical industry, medical science and academic research (Farkas & Tannenbaum, 2005; Naesens, Kuypers and Sarwal, 2009). Cytotoxicity assays are widely used by pharmaceutical industries to screen for toxicity of their compounds (Galley, 2000; Li *et al.*, 2004). Accordingly, this study employed 3-4,5

dimethylthiazol-2-yl 2,5-diphenyltetrazolium bromide (MTT) assay which monitors cytotoxicity by measuring the reducing potential of the cell using a colorimetric reaction (Mosmann, 1983). In addition, the study used single cell gel electrophoresis commonly known as the comet assay which detects DNA damage in individual cells (Olive, Wlodek, Durand and Banath, 1992) to assess whether OA exhibit any toxicity in the kidney and the liver. In diabetes, the morphology and function of kidney cells is altered, and therefore this study elucidated the potential of OA in alleviating kidney damage. Furthermore, the effects of OA on cell viability and structural integrity were evaluated in liver cell lines since the liver is responsible for degradation of drugs.

1.5.3.1. Hepatocyte-derived human transformed cell line (HepG2)

HepG2 cells were derived from the liver tissue with a well differentiated hepatocellular carcinoma and are epithelial in morphology (Guillouzo, Corlu, Glaise, Morel and Guguen-Guillouzo, 2007). When cultured properly, HepG2 cells display strong morphological and functional differentiation with a formation of apical and basolateral cell surface domains that resemble the bile canalicular and sinusoidal domains respectively *in vivo* (van IJzendoorn, Zegers, Kok and Hoekstra, 1997; van IJzendoorn and Hoekstra, 2000). Because of their high degree of morphological and functional differentiation *in vitro*, HepG2 cells are a suitable model to study human liver diseases (Ohgaki, 2010). HepG2 cells and its derivatives are also used for studies of liver metabolism, toxicity of xenobiotics, detection of cytoprotective, anti-genotoxic and cogenotoxic agents, understanding hepatocarcinogenesis and for drug targeting studies (van IJzendoorn *et al.*, 1997; van IJzendoorn and Hoekstra, 2000). Of interest in this study, HepG2 cells were used to evaluate whether OA does not induce liver cell injury since the liver plays a vital role in the biotransformation of drugs.

1.5.3.2. Human embryonic kidney cell line (Hek293)

HEK cells are a specific cell line originally derived from primary human embryo kidney cells grown in tissue culture (Shaw, Morse, Ararat and Graham, 2002). The current commercially available source of HEK cell is from the original transformation by Graham, Smiley, Russell, and Nairn in 1977 (Thomas and Smart, 2005). These cells are characterized by properties found in early developmental stages of renal cell lines and are widely used for transfection studies. However, of interest in this study was to evaluate the effects of OA on the viability and structure of these cells since diabetes cause damage to kidney cells.

1.5.3.3. Madin Darby bovine kidney cell line (MDBK)

The MDBK cell line was derived from a kidney of a normal adult steer in 1957 by S.H. Madin and N.B. Darby (Nagama, Michiue and Sakurai, 1996; Haque, Mohamad, Uddin and Saeed, 2010). This epithelial cell line is used in laboratories around the world for a variety of applications in toxin research and growth of attenuated viruses for vaccine production. MDBK cells were chosen in this study to detect the cytotoxic effects of OA on their viability and proliferation. These cells are from the nephron which is the functional unit of the kidney and since diabetes results in injury of kidney epithelial cells, studying the effects of OA on MDBK cells can give an indication as to whether this triterpene may/or may not further damage the kidney cells when used as treatment for diabetes.

1.6. Justification and aim of the present study

Medicinal plants extracts have proven to be a potential alternative to manage diabetes mellitus despite the current use of conventional anti-diabetic drugs such as insulin. Of interest in this study were the effects of a plant-derived bioactive compound, oleanolic acid (OA), on promoters of a decline in renal function.

1.6.1. Objectives

The overall aim was to study the effects of OA on renal function and proximal tubular Na⁺ handling in an effort to identify the site of action for the previously reported OA-evoked

CHAPTER 1

Introduction / Literature review

1.0. Preamble

In recent years, there has been a renewed interest in the use of plant products for the treatment of a variety of chronic disorders. One such bioactive plant-derived compound is a pentacyclic triterpenoid, oleanolic acid (3 β -hydroxy-olea-12-en-28-oic acid, OA) present in herbs and dietary plants. OA possesses a variety of medicinal claims which include anti-inflammatory, anti-cancer, anti-stomach ulcer, anti-hyperlipidaemic, anti-hypertensive, cardiogenic, anti-dysrhythmic, anti-aggregation of blood platelets, hepato-protective and anti-diabetic effects. Of interest in our laboratory are the anti-diabetic properties of OA. Diabetes mellitus is characterized by hyperglycaemia which compromises kidney function. Reports indicate that the majority of morbidity and mortality in diabetes is caused by end stage renal disease (ESRD). Therefore, this study investigated the effects of OA proximal tubular Na⁺ handling. In addition, we evaluated the effects of OA in human embryonic kidney (HEK293) and Madin-Darby bovine kidney (MDBK) cell lines to establish whether OA has any toxic effects on the kidney. Furthermore, we evaluated the effects of OA in hepatocyte-derived human transformed (HepG2) cell lines since the liver is responsible for biotransformation of drugs. Chapter 1 describes the physiology of kidney function, adverse effects of diabetes, particularly on renal haemodynamics and kidney cell structure and how drugs can influence these adverse effects, and justification of the study.

CHAPTER 2

Materials and methods

2.1. Materials

2.1.1. Drugs and chemicals

Drugs and chemicals were sourced as indicated:

Dimethyl sulphoxide (DMSO), inactin (5-ethyl-5-(1'-methylpropyl)-2-thiobarbiturate), low and normal melting agarose, trizma base (tris), triton X-100, ethidium bromide, ethylenediaminetetraacetic acid (EDTA), sodium bicarbonate (NaHCO₃), sodium chloride (NaCl), potassium chloride (KCl), dipotassium phosphate (K₂HPO₄), sodium hydroxide (NaOH) and N-(2-hydroxyethyl) piperazine-N'-[2-ethanesulfonic acid] (HEPES) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Silica gel, ethyl acetate (EA), dichloromethane (DCM), ethanol (C₂H₅OH), methanol (CH₃OH), chloroform (CHCl₃) and hydrogen peroxide (H₂O₂) were purchased from Merck (Johannesburg, South Africa) and halothane was from Fluorothane[®] (AstraZeneca Pharmaceuticals, South Africa). Creatinine was purchased from BDH biochemicals Ltd (Poole, England). Earle's minimum essential medium (EMEM) was from Gibco BRL (Inchinnan, Scotland), foetal bovine serum, penicillin G and streptomycin sulphate were purchased from Whittaker Bioproducts (Walkersville, Maryland, USA).

All chemical reagents were of analytical grade.

2.2. Methods

2.2.1. Isolation of oleanolic acid (OA)

The extraction of OA was performed in Chemistry laboratory at UKZN Pietermaritzburg campus. OA was isolated from *Syzygium aromaticum* [(Linnaeus) Merrill & Perry] [Myrtaceae] flower buds using a previously validated standard protocol that has been reported from our laboratories (Mapanga *et al.*, 2009; Musabayane *et al.*, 2010). Briefly, air-dried flower buds of *S.*

aromaticum (500 g) were sequentially extracted twice at 24h intervals with 1 L of dichloromethane (DCM) and ethyl acetate (720 ml) on each occasion. The resulting filtrate was filtered using a 30cm filter paper (Whatman, England). The filtrates were concentrated *in vacuo* $60 \pm 1^{\circ}\text{C}$ using a laboratory 4000 efficient rotary evaporator (supplied by Laboratory Consumables and Chemical Supplies, South Africa) to obtain DCM soluble (63 g) and ethyl acetate soluble (EAS, 85 g). Crude EAS were subjected to further purification since previous studies indicated that they contain mixtures of OA/UA and methyl maslinate/methyl corosolate (Somova, Nadar, Rammanan and Shode, 2003; Musabayane, Mahlalela, Shode and Ojewole, 2005). Column chromatography with silica gel was used for fractionation of a portion of EAS (10 g) using a step gradient of n-hexane-ethyl acetate increasing ratio from 9:10 – 7:30. Fractions collected were monitored and analysed by thin layer chromatography (TLC) on pre-coated aluminium plates using Merck Si gel F254. The TLC plate was developed with ethyl acetate/hexane (8:2) in a TLC tank. Analysed data collected from fractions was compared with authentic OA values. Eluates with similar TLC profiles to OA were combined and subjected to further chromatographic purification, a yield of OA was 2.5 – 3.0 g per 10 g EAS extraction. Pure OA was obtained by recrystallisation with methanol and its structure was confirmed by spectroscopic analysis using ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) technique. OA had the same physical properties as the OA purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and hence isolated OA was used to carry out all the experiments.

2.3. Animal studies

2.3.1. Ethical consideration

Ethical clearance for animal studies was obtained from the animal ethics committee of the University of KwaZulu-Natal (reference 077/10/Animal and 20/11/Animal, see Appendices I and II).

2.3.2. Animals

Male Sprague-Dawley rats (250-300g body weight) bred and housed in the Biomedical Resource Unit (BRU) of University of KwaZulu–Natal were used in this study. The animals were maintained under standard laboratory conditions of constant temperature (22 ± 2 °C), CO₂ (<5000 p.p.m), relative humidity ($55\pm 5\%$) and illumination (12 h light/dark cycles). The animals had free access to water and standard rat chow (Meadows, Pietermaritzburg, South Africa). All experiments were performed according to the University of KwaZulu-Natal Research Ethics Committee guidelines for experimental animals.

2.4. Experimental design

The studies were divided into *in vivo* and *in vitro* experiments. *In vivo* studies investigated the effects of OA on renal fluid and electrolyte handling assessed in anaesthetized and conscious male Sprague-Dawley rats. The effects of OA on Na⁺ handling in the proximal tubule were assessed in anaesthetized animals using lithium clearance. *In vitro* studies evaluated the effects of OA in kidney and liver cell lines to establish whether this triterpene exhibited any toxicity in these organs.

2.5. Renal studies

2.5.1. Anaesthetized animals

2.5.1.1. Renal clearance studies

Lithium clearance (C_{Li}) was used as a marker for the output of Na⁺ from the proximal tubules (Thomsen and Shirley, 1997). Male Sprague-Dawley rats were given water with lithium chloride (12 mmol/kg dry weight) for 48 h prior to experiment to raise plasma lithium to measurable concentrations. The rats were prepared for acute renal clearance studies and mean arterial blood pressure using previously validated standard protocol that has been reported from our laboratories (Musabayane *et al.*, 2007; Mapanga *et al.*, 2009). Briefly, animals were anaesthetized with intraperitoneal injection of 0.11 g/kg of inactin (5-ethyl-5-(1'-methylpropyl)-2-thiobarbiturate, Sigma-Aldrich, St Louis, MO, USA) and tracheotomy was performed. The right

jugular vein was cannulated with polyethylene tubing (internal diameter, (i.d.) 0.58 mm; external diameter, o.d. 0.96 mm; Portex, Hythe, Kent, UK) to allow intravenous infusion of 0.077M NaCl containing 0.013M creatinine at 9 ml/h using Harvard Apparatus Syringe Infusion Pump 22 (Harvard Apparatus, Holliston, Massachusetts, USA). The urinary bladder of each rat was cannulated via midline abdominal incision with polyethylene tubing (internal diameter, (i.d.) 0.86 mm; external diameter, o.d. 1.27 mm; Portex, Hythe, Kent, UK) to facilitate timed collection of urine samples. Body temperature was maintained by placing a rat in a thermally controlled heating table ($37\pm 1^{\circ}\text{C}$).

Following a 3½ h equilibration period, measurements were taken over the 4 h post-equilibration period of 1 h control, 1 h 30min treatment and 1 h 30min recovery periods. Control animals were continuously infused with 0.077M NaCl containing 0.013 M creatinine at 9 ml/h. In those animals in which the effects of OA were studied, OA was added to the infusate at 90 µg/h for 1.5 h (treatment period), the animals were returned to the infusate alone for the last 1.5 h (recovery period). Proximal tubular Na^{+} handling was assessed by determination of endogenous lithium in plasma and urine and by fractional excretion of lithium and sodium. For mean arterial blood pressure measurements, A heparinized cannula (Portex i.d. 0.58 mm; o.d. 0.96 mm, Hythe, Kent, UK) was inserted into the left carotid artery for continuous recording of arterial blood pressure at 30 min intervals via a pressure transducer (Statham MLT 0380, Ad Instruments, Bella Vista NSW, Australia), compatible with PowerLab System ML410/W (Bella Vista NSW, Australia). Blood samples (0.3 ml) were taken once per hour for measurements of electrolyte and clearance marker concentrations. Plasma and urine samples were stored at 4°C until assay of creatinine, urea and electrolytes was performed.

2.5.2. Conscious animals

Male Sprague-Dawley rats were divided into four groups of untreated and treated ($n = 6$) animals. Experimental groups were treated with various doses of OA (30, 60 and 120 mg/kg, p.o.) twice every third day for 5 weeks by means of a ball-tipped, 18-gauge gavage needle (Kyron Laboratories (Pty) LTD, Benrose, South Africa) attached to a 1 ml syringe (Naumann,

Weideman, Sarangapani, Hu, Dixit and Sargent; 2009). These doses were chosen because they have been shown to have hypoglycaemic effects in STZ- induced diabetic rats (Musabayane *et al.*, 2010). Rats were housed individually in Makrolon polycarbonate metabolic cages (Tecniplast, Labotec, South Africa) kept at the BRU. Quantitative measurements of urine volume and total urinary outputs of Na⁺, K⁺, Cl⁻, creatinine and urea determined after dosing at 09h00 were performed using Beckman Coulter (Synchron LX20 Clinical Systems, Fullerton, Carlifornia, USA) with reagent kits from Beckman Coulter (Synchron LX20 Clinical Systems, Dublin, Ireland). The type of bedding used for rat cages was wood shavings. Food, water consumption and body weight were recorded 24 h after dosing at 09h00. Untreated controls animals were given de-ionised water (3 ml/kg p.o). Mean arterial blood pressure was monitored every 3rd consecutive day for 5 weeks at 09h00 using non-invasive tail cuff method (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA) previously described (Musabayane *et al.*, 2007; Mapanga *et al.*, 2009). The unit utilizes IITC hardware system employing an automatic scanner pump, sensing cuff and amplifier to measure blood pressure in the animals' tail and the results are displayed on the computer screen. The equipment was calibrated before each day of mean arterial blood pressure measurements The animals were kept warm in an enclosed chamber (IITC Model 303sc Animal Test Chamber, IITC Life Sciences, Woodland Hills, California, USA) for 30 minutes at ±30°C before taking three blood pressure recordings (Gondwe *et al.*, 2008).

2.5.2.1. Terminal studies

At the end of a 5 week experimental period, animals were anaesthetized in a 2.3L anaesthetic chamber with 2.17 ml/L of halothane (Fluorothane®, Astra Zeneca 2002) pharmaceuticals (Pty) LTD for 3 minutes. The chamber relied on atmospheric air for vaposization of the halothane. Blood samples were collected from all groups of animals by a terminal cardiac puncture into individual pre-cooled heparinized containers 24 h after the last treatment. Blood was centrifuged in an eppendorf centrifuge (Fisher Scientific GmbH, Schwerte, Germany) for 15 minutes at 3500 rpm g x 100 at 4 °C to separate the plasma. Separated plasma was stored in a BioUltra freezer (Snijders Scientific, Tilburg, Netherlands) at -70 °C until assayed for Na⁺, K⁺, Cl⁻, creatinine and urea concentrations. Kidneys were collected and weighed gravimetrically (Mettler balance PC

180-instruments, Protea Laboratory Services, Johannesburg, South Africa) and snap frozen in liquid nitrogen.

2.6. Laboratory analysis

2.6.1. Biochemical measurements

Urine volume was determined gravimetrically using a balance (Mettler balance PC 180-instruments, Protea Laboratory Services, Johannesburg, South Africa). Na^+ , K^+ , Cl^- , urea and creatinine concentrations were determined by ion activity using the Beckman Coulter (Synchron LX20 Clinical Systems, Fullerton, California, USA). Creatinine estimation employed the reaction of creatinine and sodium picrate to form creatinine picrate. Urea estimation employed the hydrolytic degradation of urea in the presence of urease. The methods used reagent kits from Beckman Coulter, Dublin, Ireland.

2.6.2. Lithium measurements

A Perkin Elmer atomic absorption/flame emission spectrometer (Optima 2100 DV, New York, Waltham, USA) equipped with Li^+ hollow-cathode lamp as a radiation source and air-nitrous oxide-acetylene flame with a wavelength of 670.8 nm was used for determination of Li^+ concentrations in plasma and urine samples. The operating conditions were those recommended by the manufacturer. The sample, the acetylene flow rates and the burner height were adjusted in order to obtain the maximum absorbance signal while aspirating the analyte solution. Standard solutions of Li^+ ranging from 0.0004 - 0.0603 mmol/l were prepared from a stock solution of 0.1152 mmol/l and values of urinary and plasma Li^+ were extrapolated from a standard curve.

Technique: Photons of radiation from the analyte emission strike the photosensitive area of the detector where photoelectrons are produced in each pixel of the detector. The electrons are moved into the register where they accumulate as electric charge for the period selected for the migration time. At the end of the migration time, the charge is transferred out of the register to

the signal processing electronic. The pixels in each vertical row are binned into the register for the respective array. This instrument uses a 40-MHz free-running solid state generator to ionize the argon in the torch and excite the atoms of the liquid sample so that they emit energy at their atomic wavelength in the form of photons. The photons from the torch are detected optically and measured electronically in the spectrometer section of the Optima 2100 DV and displayed in terms of wavelength and intensity, which are converted to sample concentration (Bilhorn, Weedler J.V, Epperson and Denton, 1987; DoL, Knochen and Vieras, 1992, Leyssac and Christensen, 1994; Shalmi, Kibble, Day, Christensen and Atherton, 1994; Steinhduelin, Burnier, Magnin, Munafo, Buclin, Diezi and Biollaz, 1994; Shalmi *et al.*, 1998; Chen and Teo, 2001; Aliasgharpour and Hagani, 2009).

2.6.3. Calculations

Lithium clearance (C_{Li}) was used as a marker for the output of Na^+ from the proximal tubules (Thomsen and Shirley, 1997). Renal clearances (C) and fractional excretions (FE) were calculated with the standard formulae $C = U \times V/P$ and $FE = C/GFR$, where U is the urinary concentration, V is the urine flow rate and P is the plasma concentration. $FE_{Na \text{ distal}}$ was calculated as C_{Na}/C_{Li} .

Glomerular filtration rate (GFR), as assessed by creatinine clearance was calculated at 30 min intervals in anaesthetized rats and in the 5th week in conscious animals using the standard formulae from measurements of the plasma and urinary concentrations of creatinine and urine flow rate.

2.7. Cell culture studies

The HEK293 cell line was obtained from University of the Witwatersrand, Faculty of Health Sciences, MDBK and HepG2 cells were purchased from Highveld Biological (Johannesburg, South Africa). Cell lines were initially propagated in 25 cm² flasks (Bibby-Sterilin, Staffordshire, England), at 37°C in Earle's minimum essential medium (EMEM) (Gibco BRL, Inchinnan, Scotland) (5 mL) containing 10% (v/v) foetal bovine serum, 20 mM HEPES, 10 mM

NaHCO₃, 100 U/ml penicillin G and 100 µg/ml streptomycin sulphate (Whittaker Bioproducts, Walkersville, Maryland, USA) at pH 7.5. Cells were divided 1:3 every 3-4 days and stored in a biofreezer (-80°C) in complete medium containing 10% dimethyl sulphoxide (DMSO).

2.7.1. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was assessed using the MTT assay originally described by Mosmann (1983). Cells were trypsinised and seeded into 48-well plates (Bibby-Sterilin, Staffordshire, England) at a seeding density of 1.8×10^4 cells/well and incubated for 24 h to permit attachment and growth of cells to semi-confluency. Thereafter, the medium (0.5 mL) was replaced and OA (0, 5, 10, 20, 40, and 80 µmol/L) was added to the wells and cells incubated at 37°C for 24, 48 and 72 h respectively. After each incubation period, the medium was removed and MTT solution (5 mg/ml in phosphate buffered saline, 200 µL) was added to wells. The cells were incubated for 4 h to allow for the formation of blue formazan crystals. The MTT solution was then replaced with DMSO (200 µl/well) and absorbance measured at 570 nm in a UV-visible spectrophotometer (Thermoscientific Biomate, Cambridge, UK). The percentage cell viability was then calculated as follows: $[A_{570} \text{ treated cells} - \text{background}] / [A_{570} \text{ control cells} - \text{background}] \times 100$.

Technique: Viable cells have active mitochondrial dehydrogenase enzymes which reduce MTT dye by cleaving the tetrazolium rings of the pale yellow MTT to form dark blue formazan crystal (Bernas and Dobrucki, 2002). These crystals are largely impermeable to cell membranes and accumulate within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals which are then solubilised also by the detergent. The number of surviving cells is directly proportional to the level of the purple formazan product (Bernas and Dobrucki, 2002). This colored solution can be quantified by measuring its absorbance at a certain wavelength (between 500 and 600 nm) using a spectrophotometer. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated cells, the effectiveness of the agent in causing death or changing metabolism of cells can be deduced through calculation of percentage cell viability using the above mentioned formula.

2.7.2. Single cell gel electrophoresis (comet) assay

DNA damage was evaluated using a previously validated protocol (Singh, McCoy, Tice and Schneider, 1988, Tice, Agurell, Anderson, Burlinson, Hartmann, Kobayashi, Miyamae, Rojas, Ryu and Sasaki, 2000). Frosted microscope slides were each covered with 400 μ l of 1% normal melting point agarose in Ca^{2+} and Mg^{2+} free Kenny's solution (0.4M NaCl, 9mM KCl, 0.7mM K_2HPO_4 , 2mM NaHCO_3), a coverslip added and the agarose allowed to solidify. The coverslips were removed and approximately 1.8×10^4 cells were added to 175 μ l of 0.5% low melting point agarose in Kenny's solution. The last layer of 200 μ l 1% normal melting point agarose was then added. The coverslips were removed, and the slides placed in ice cold, freshly prepared lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% Triton X-100 and 10% DMSO, pH 10) for 1 h. Thereafter slides were subjected to electrophoresis at 35 min at 25 V (0.83 V/cm) in a BioRad horizontal gel electrophoresis unit (BioRad, Richmond, CA, USA) containing chilled electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH 13.5). Slides were washed with three changes of neutralization buffer (0.4 M Tris, pH 7.5), each for 5 min, to remove alkali and detergents. Slides were then stained with ethidium bromide (20 μ g/ml, 50 μ l) a coverslip added and analysed. Slides were viewed using an Olympus inverted fluorescent microscope with a CC12 fluorescent camera (excitation filter of 450-490 nm and a barrier filter of 520 nm) (Wirsam Scientific & Precision Equipment LTD, Johannesburg, South Africa) which allowed for computerized image analysis for determination of DNA tails, linearly related to the frequency of DNA strand breaks (McKelvey-Martin, Ho, McKeown, Johnston, McCarthy, Rajab and Downes, 1998).

Technique: Undamaged DNA is highly organized and is closely associated with matrix proteins in the nucleus, however, if it is damaged this organization is interrupted. This causes the compact double strands of the DNA to break and single DNA strands move out of the nucleus (Ostling and Johanson, 1984; Tice *et al.*, 2000). When exposed in an electric field, undamaged DNA does not migrate while the damaged one moves from the anode towards the cathode (Ostling and Johanson, 1984; McKelvey-Martin *et al.*, 1998). Therefore, the amount of DNA that leaves the nucleus is a measure of the amount of DNA damage in the cell (Singh *et al.*, 1988; Tice *et al.*, 2000). The image analysis measures the overall intensity of the fluorescence for the migrated and unmigrated DNA and compares the two signals. The stronger the signal from the migrated DNA

the more damage there is present. The overall structure resembles a comet with a circular head corresponding to the undamaged DNA that remains in the cavity and a tail of damaged DNA (Singh *et al.*, 1988; McKelvey-Martin *et al.*, 1998; Tice *et al.*, 2000). The level of damage is determined by the intensity and length of the tail.

2.8. Analysis of data

All data were expressed as means \pm S.E.M. Statistical comparisons between treated groups and control were performed with GraphPad InStat Software (version 4.00, GraphPad Software, Inc., San Diego, California, USA) using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. A value of $p < 0.05$ was considered significant.

CHAPTER 3

Results

3.0. General

This chapter describes the following:

- i. spectroscopic data of OA;
- ii. renal function effects of OA in conscious male Sprague-Dawley rats;
- iii. effects of OA on proximal tubular Na⁺ handling in anaesthetized animals and
- iv. *in vitro* effects of OA in liver and kidney cell lines.

3.1. OA structure

The percentage yield of OA obtained varied from 0.79% to 1.72%. Figure 2 shows ¹H-NMR and ¹³C-NMR spectra of hydrogen and carbon atoms in the OA molecule. The two carbon signals at 143.6 and 122.7 ppm correspond to the carbon-carbon olefinic double bond at position 12 and 13 which is peculiar to the triterpenoids (Mahato and Kundu; 1994).

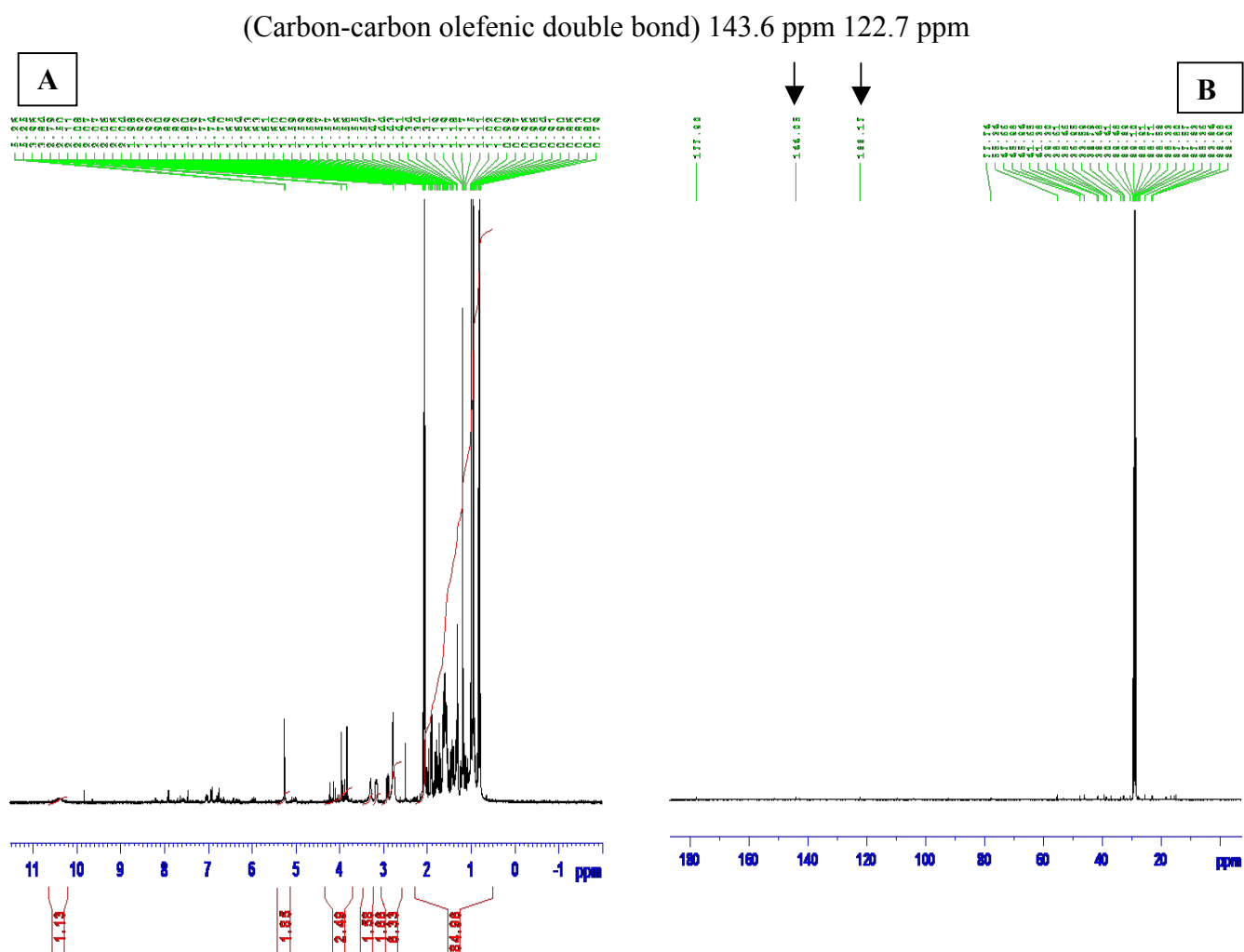


Figure 2: *Syzygium aromaticum*-derived OA ^1H (A) and ^{13}C - NMR (B) spectroscopic spectra. Pure OA was obtained following recrystallisation of EAS with methanol and elucidated using ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy.

3.2. OA effects on body weight, food and water intake

Table 2 compares the effects of various doses of OA (30, 60, 120 mg/kg, p.o.) on food and water intake, and body weight in control and experimental animals over the 5-week period. OA administration had no significant influence on food and water intake, and body weight changes throughout the experimental period when compared with control animals.

Table 2: Comparison of the effects of OA administration on food and water intake, and body weight with control animals. Values are presented as means \pm SEM (n=6 in each group).

Experimental protocol	Parameter	Time (weeks)				
		1	2	3	4	5
Control	Food intake (g/day)	30 \pm 1	31 \pm 1	30 \pm 1	29 \pm 1	28 \pm 1
OA 30		28 \pm 1	27 \pm 1	31 \pm 1	30 \pm 1	31 \pm 1
OA 60		29 \pm 1	30 \pm 1	29 \pm 1	26 \pm 3	28 \pm 1
OA 120		27 \pm 2	28 \pm 1	28 \pm 1	28 \pm 1	27 \pm 1
Control	Water intake (ml/day)	33 \pm 1	31 \pm 1	33 \pm 1	30 \pm 1	28 \pm 1
OA 30		30 \pm 1	32 \pm 1	32 \pm 1	29 \pm 1	28 \pm 1
OA 60		32 \pm 1	33 \pm 1	30 \pm 1	29 \pm 3	32 \pm 1
OA 120		33 \pm 2	28 \pm 1	29 \pm 1	30 \pm 1	29 \pm 1
Control	% b.wt change/week	7 \pm 2	13 \pm 2	18 \pm 2	22 \pm 2	24 \pm 1
OA 30		8 \pm 1	13 \pm 1	19 \pm 3	21 \pm 3	24 \pm 2
OA 60		6 \pm 2	12 \pm 2	18 \pm 2	20 \pm 2	23 \pm 1
OA 120		8 \pm 3	14 \pm 1	17 \pm 2	22 \pm 1	25 \pm 1

3.3. Renal fluid and electrolyte handling

3.3.1. Anaesthetized animals

Figure 3 shows urinary Na⁺ excretion rate of control and OA-administered animals during the 4 h experimental period. Na⁺ excretion rate of control animals which ranged from 711 to 756 μmol/h throughout the experiment compared with the infusion rate of 693 μmol/h. However, OA infusion at 90 μg/h for 1½ h significantly ($p < 0.05$) increased urinary Na⁺ outputs from the pre-treatment value of 713 to 847 μmol/h at the end of the treatment period. OA infusion, however, had no influence on urine flow and urinary K⁺ and Cl⁻ outputs in comparison with control animals (Figure 4).

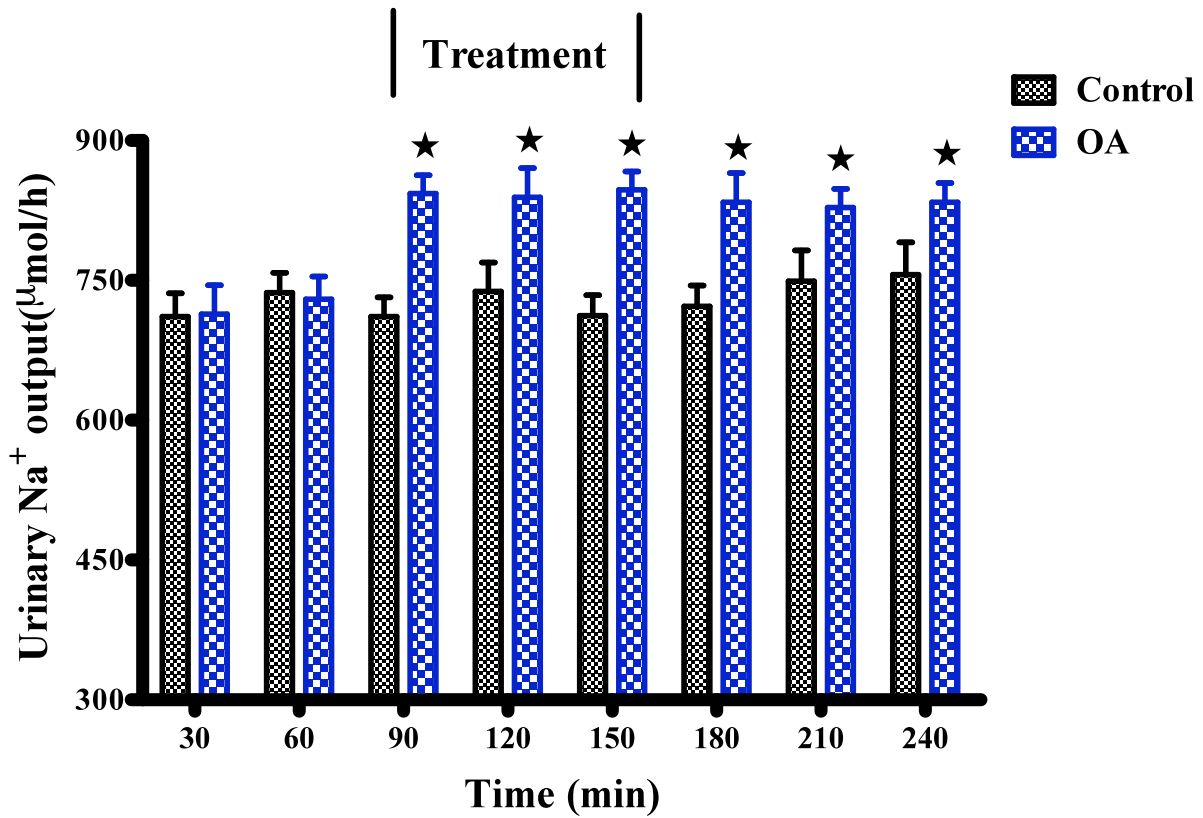


Figure 3: Comparison of urinary Na⁺ excretion (A) rate of control rats with animals administered OA. OA was administered for 1½ h during the treatment period. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★p< 0.05 by comparison with control animals at each corresponding time.

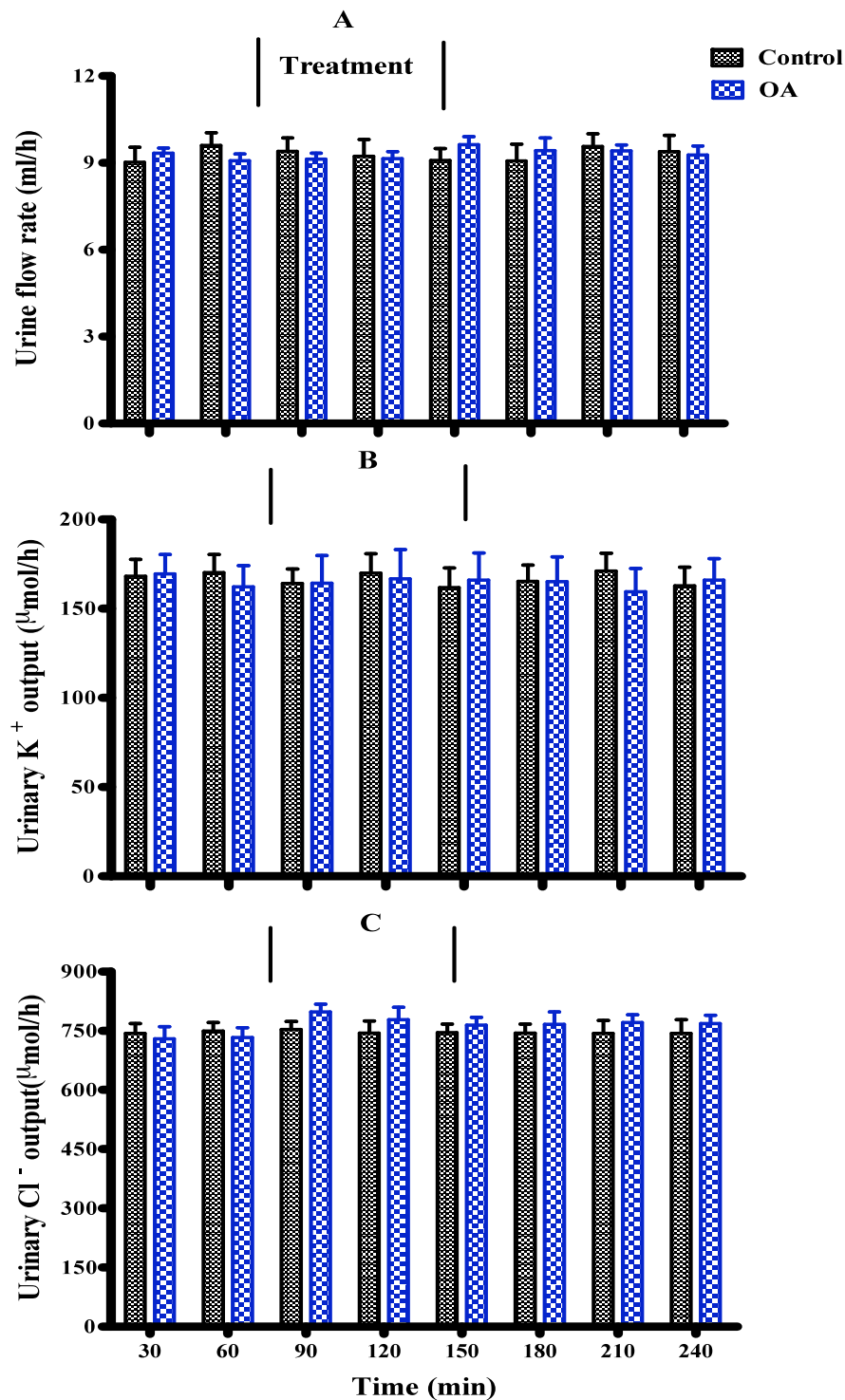


Figure 4: Comparison of urine flow (A), K⁺ (B) and Cl⁻ excretion (C) rates of control rats with animals administered OA. OA was administered for 1½ h during the treatment period. Values are presented as means, and vertical bars indicate SEM (n=6 in each group).

3.3.2. Conscious animals

Figure 5 shows urine flow and urinary Na⁺ excretion rates of control and OA-administered animals during the 5-weeks experimental period. Daily Na⁺ excretion rate of control animals was stable ranging from 210 to 223 mmol/day throughout the experiment. However, administration of various doses of OA (30, 60, 120 mg/kg p.o.) significantly ($p < 0.05$) increased daily Na⁺ output from the 2nd week until the end of the study period in a dose independent manner. OA administration, however, had no influence on urine flow and urinary K⁺ and Cl⁻ outputs (Figure 6). The OA-evoked increase in Na⁺ excretion was not reflected in plasma collected at the end of the experiment, however, there was a significant ($p < 0.05$) decrease in plasma creatinine at the end of the experiment (Table 3). OA administration had no influence on plasma K⁺, Cl⁻, urea concentrations and kidney mass compared to control animals (Table 3).

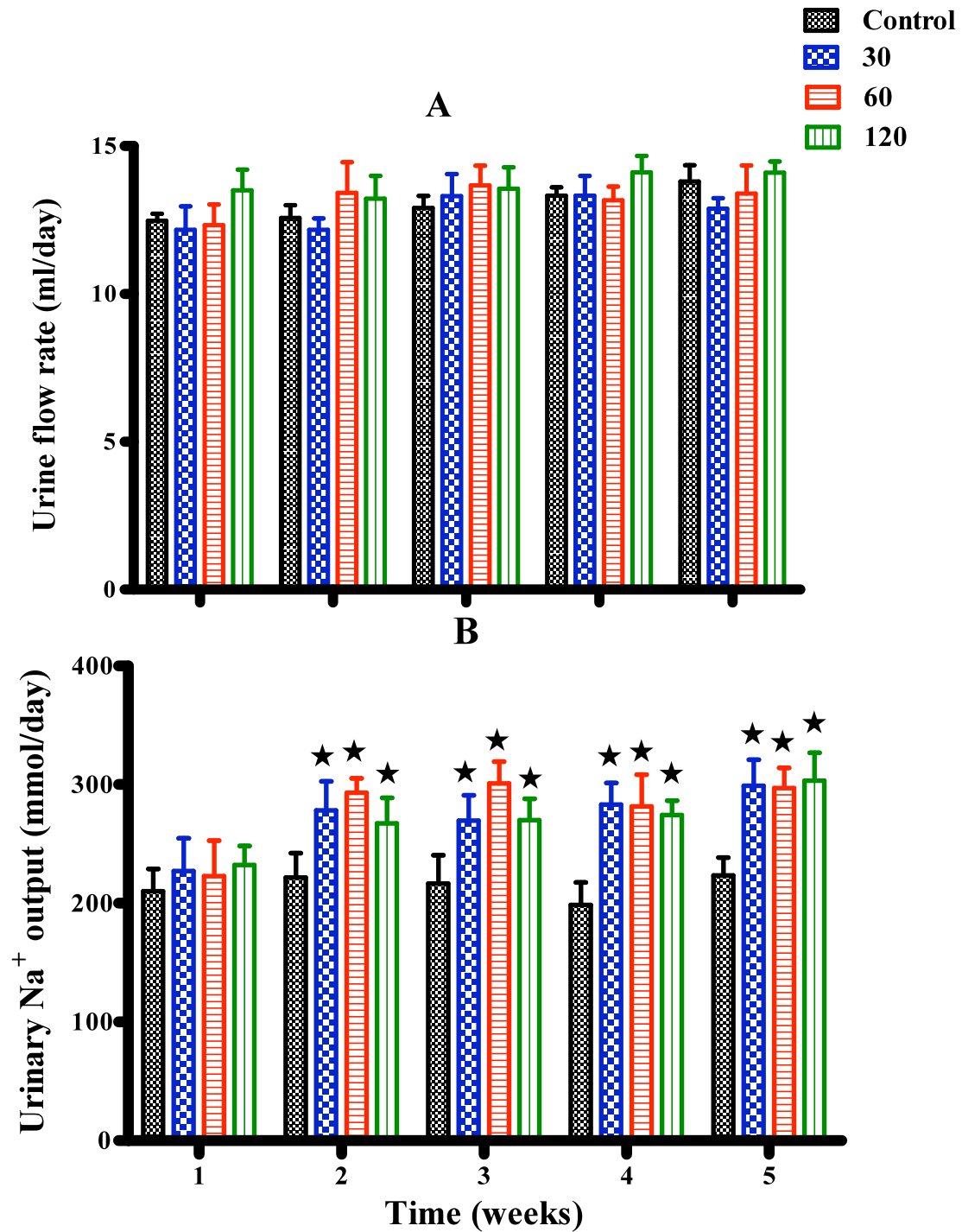


Figure 5: Comparison of the effects of various doses of OA administration on 24 h urine flow (A) and Na⁺ excretion (B) rates with control animals. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★p < 0.05 by comparison with control animals at each corresponding time.

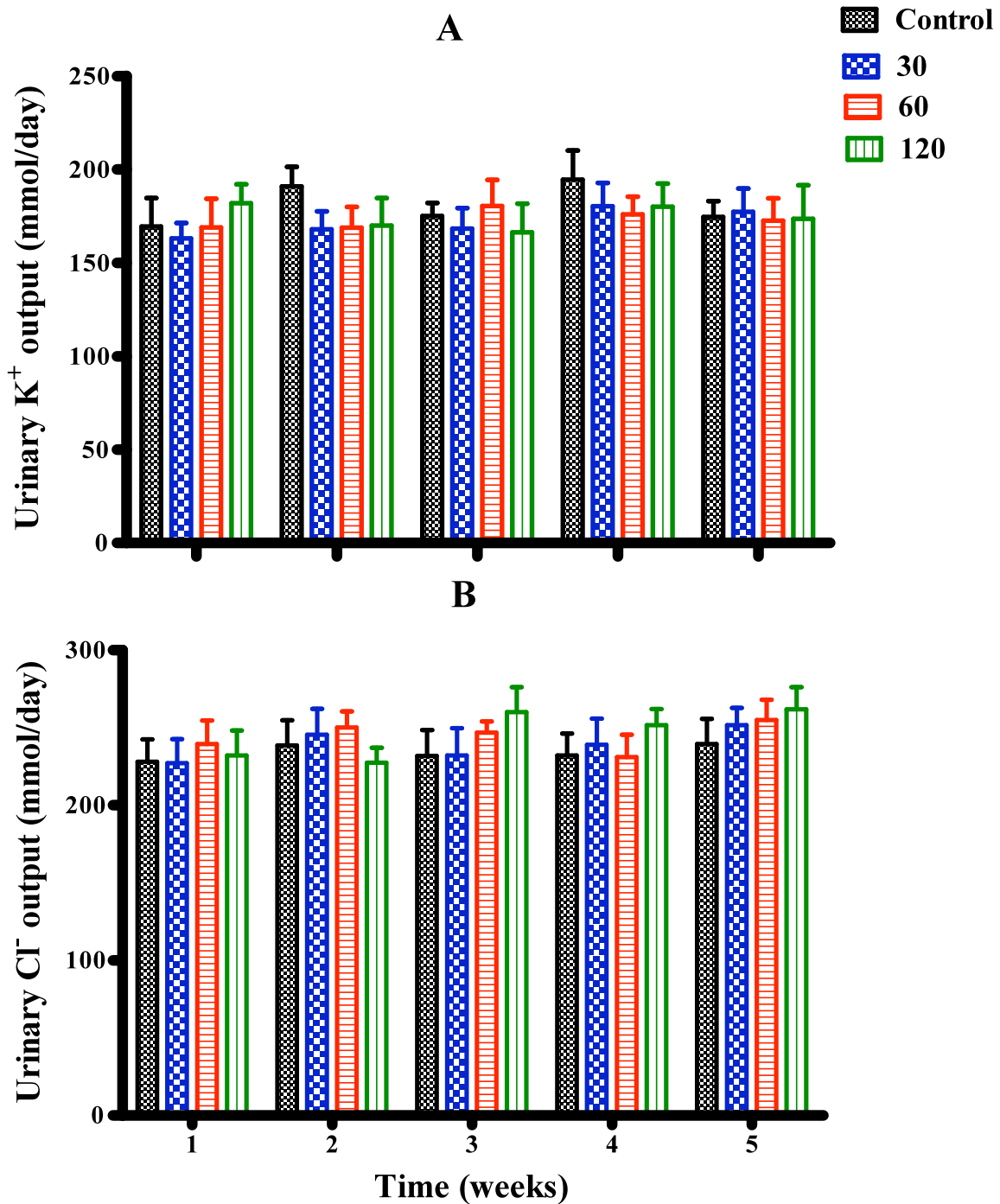


Figure 6: Comparison of the effects of various doses of OA administration on urinary K^+ (A) and Cl^- excretion (B) rates with control animals. Values are presented as means, and vertical bars indicate SEM (n=6 in each group).

3.4. Effects of OA on MAP

3.4.1. Anaesthetized animals

MAP of untreated and OA-treated rats over the 4 h experimental period is shown in figure 7. Following infusion of hypotonic saline to control rats, no significant variations were seen in the MAP throughout the 4-h post-equilibration period. However, intravenous infusion of OA for 1 ½ h in the experimental group significantly ($p < 0.05$) reduced MAP from a pre-treatment value of 105 to 92 mmHg at the end of the treatment period ($n=6$). The hypotensive effect of OA did not revert back (94 mmHg) to normal during the post-treatment period.

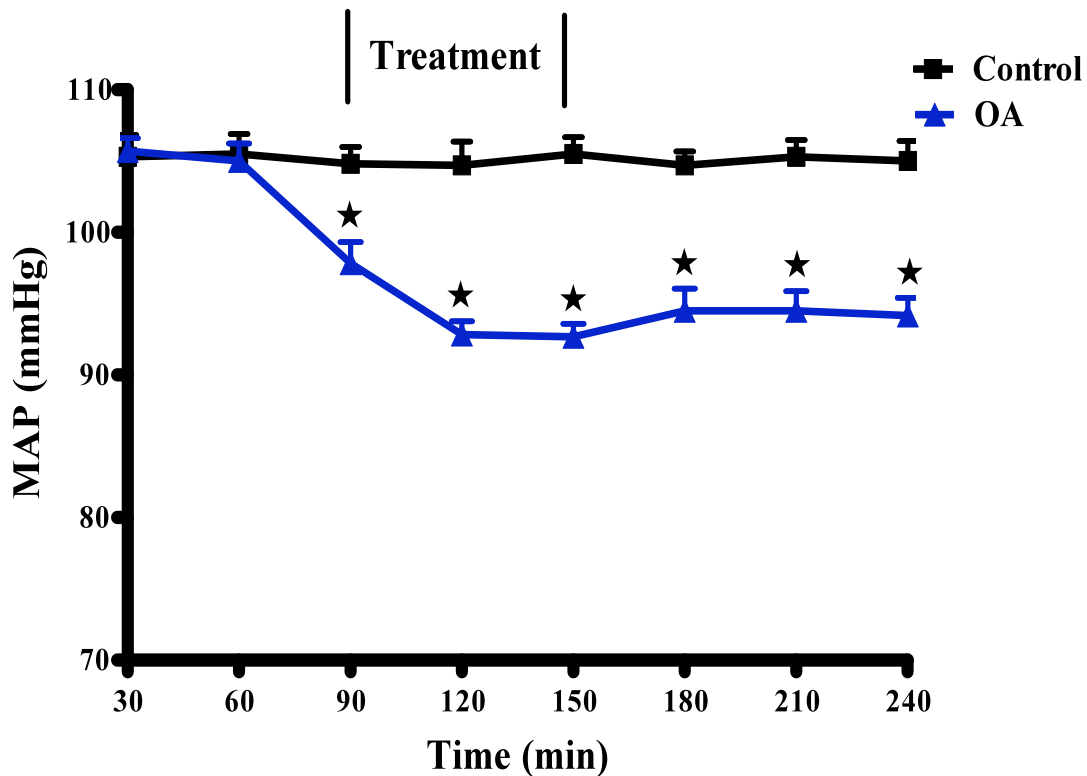


Figure 7: Comparison of MAP of control rats with animals administered OA. OA was administered for 1½ h during the treatment period. Values are presented as means, and vertical bars indicate SEM ($n=6$ in each group). ★ $p < 0.05$ by comparison with control animals at each corresponding time.

3.4.2. Conscious animals

Weekly MAP of control animals ranged from 102 to 104 mmHg throughout the experiment (Figure 8). However, administration of various doses of OA (30, 60, 120 mg/kg p.o.) significantly ($p < 0.05$) decreased MAP from the 2nd week until the end of the study period. The highest dose of OA (120 mg/kg p.o.) was the most effective in reducing the MAP (from a mean value of 103 to 86 mmHg) in weeks 3 and 4 by comparison with lower doses at corresponding time periods.

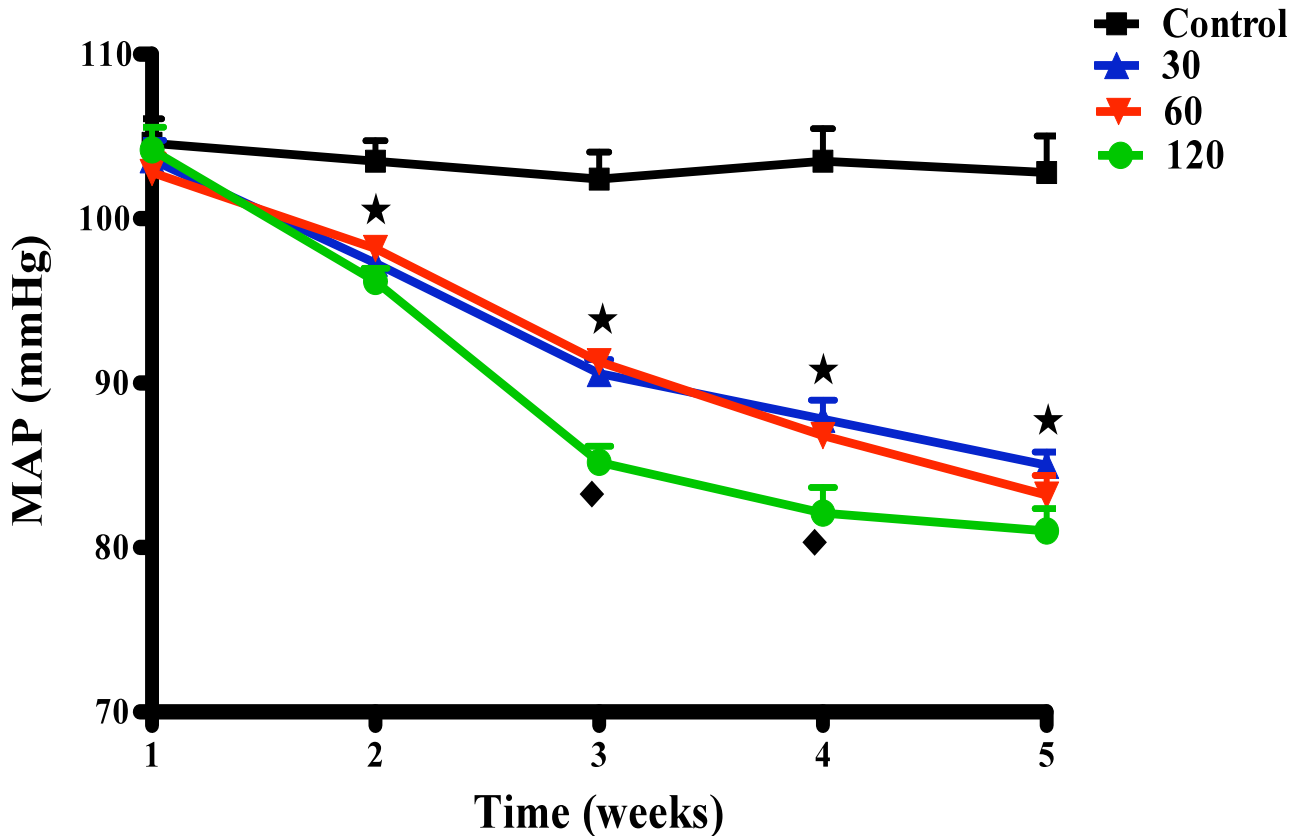


Figure 8: Comparison of the effects of various doses of OA administration on MAP with control animals. Values are presented as means, and vertical bars indicate SEM (n=6 in each group).★ $p < 0.05$ by comparison with control animals at each corresponding time. ◆ $p < 0.05$ by comparison with 30 and 60 mg/kg b.wt at each corresponding time.

3.5. Effects of OA on GFR

3.5.1. Anaesthetized animals

Following infusion of hypotonic saline to control rats, no significant variations were seen in the GFR throughout the 4 h post-equilibration period (Figure 9). However, intravenous infusion of OA slightly increased GFR to values that did not achieve statistical significance.

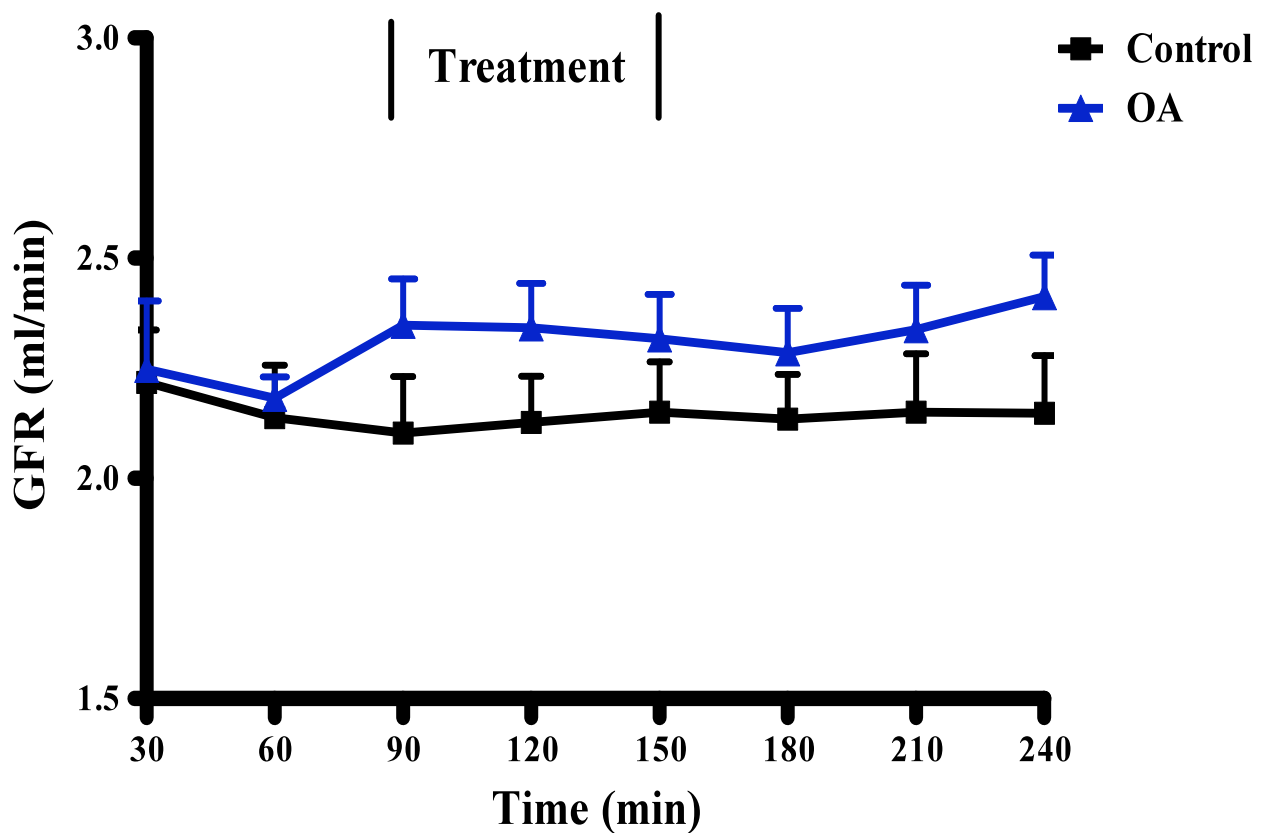


Figure 9: Comparison of GFR of control rats with animals administered OA. OA was administered for 1½ h during the treatment period. Values are presented as means, and vertical bars indicate SEM (n=6 in each group).

3.5.2. Conscious animals

Table 3 shows GFR at the end of the 5-week study period as assessed by creatinine clearance (Ccr). Administration of various doses of OA significantly ($p < 0.05$) reduced plasma creatinine concentration of experimental animals with a concomitant increase in GFR.

Table 3: The effects of OA on plasma biochemical parameters in male Sprague-Dawley rats which were administered OA twice every third day for five weeks. Values are presented as means \pm SEM (n=6 in each group).

Parameter	Experimental protocol			
	Control	OA 30	OA 60	OA 120
Na ⁺ (mmol/l)	142 \pm 1	141 \pm 2	140 \pm 2	142 \pm 1
K ⁺ (mmol/l)	4.25 \pm 0.25	4.14 \pm 0.21	4.03 \pm 0.22	4.17 \pm 0.20
Cl ⁻ (mmol/l)	104 \pm 1	102 \pm 1	103 \pm 1	101 \pm 2
Urea (mmol/l)	7.5 \pm 0.6	8.1 \pm 0.3	8.5 \pm 0.5	8.4 \pm 0.2
Creatinine (μ mol/l)	27 \pm 1	20 \pm 3*	21 \pm 2*	20 \pm 3*
GFR (ml/min)	2.58 \pm 0.16	3.04 \pm 0.16*	3.51 \pm 0.17*	3.73 \pm 0.17* [#]
Kidney weights (g/100g b.wt)	0.43 \pm 0.20	0.47 \pm 0.06	0.48 \pm 0.05	0.47 \pm 0.01

* $p < 0.05$ by comparison with control animals.

$p < 0.05$ by comparison with OA 30 mg/kg b.wt dose.

3.6. Renal clearance measurements

Lithium clearance (C_{Li}) was used as a marker for proximal tubular sodium clearance (Thomsen and Shirley, 1997). The effect of OA on proximal and distal tubular Na^+ clearance was estimated by comparing renal lithium clearance (FE_{Li}) between anaesthetized control and OA-treated rats. The lithium doses used resulted in plasma lithium concentrations between 0.2 and 0.3 mmol/l with no difference between the groups. All measured variables during the 4-h experimental period were in a steady state and did not differ between the control and the experimental groups prior to the infusion of OA. The infusion of OA at 90 $\mu g/h$ for 1½ h significantly ($p < 0.05$) increased FE_{Li} and $FE_{Na\ proximal/distal}$ by comparison with control animals at the corresponding time (Figure 10). However, the $FE_{Na\ distal}$ was slightly reduced by OA treatment to levels that did not achieve statistical significance by comparison to $FE_{Na\ proximal}$ at the corresponding time period. In all cases, the FE_{Na} was not accompanied by any changes in FE_K , and FE_{Cl} (data not shown).

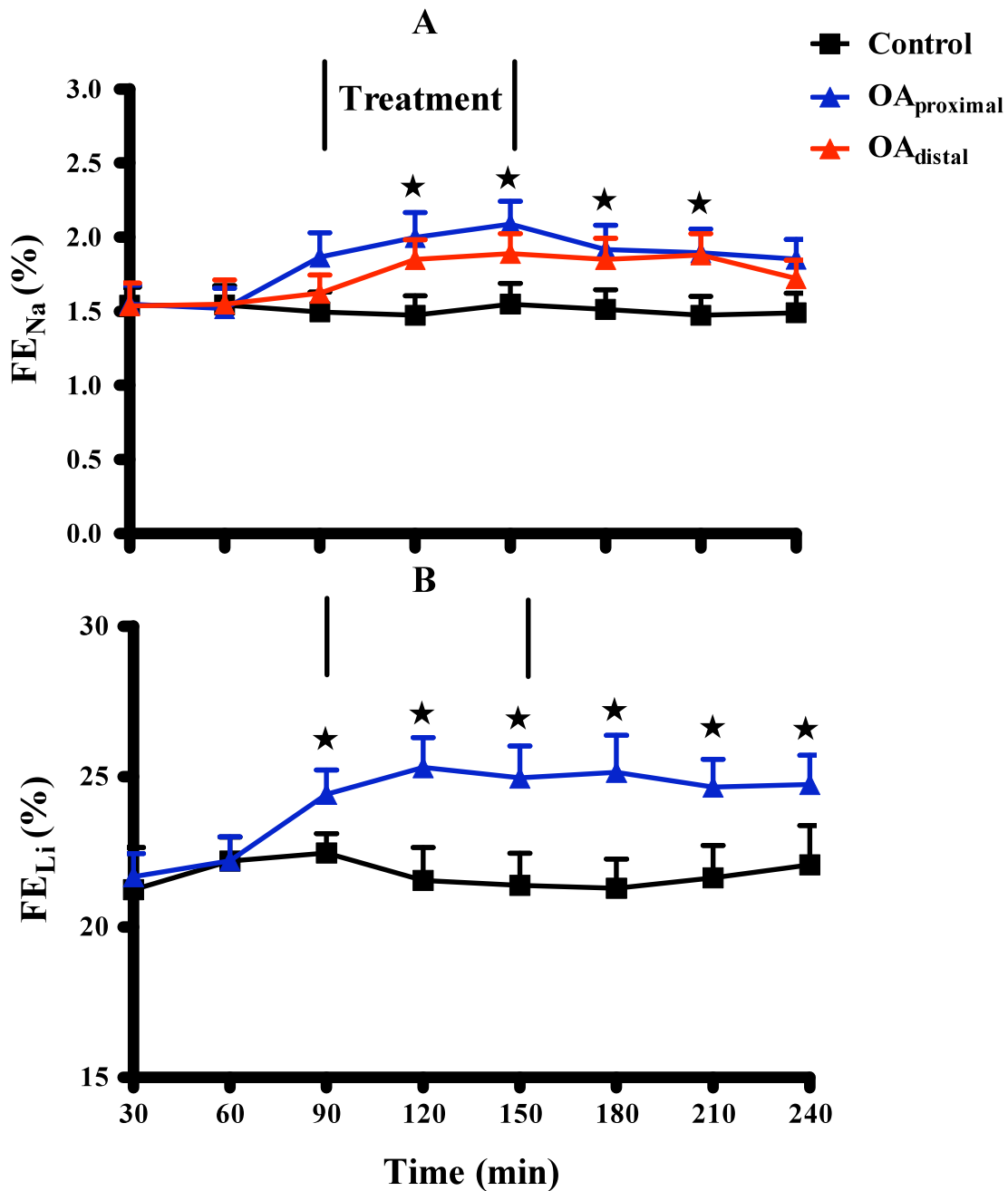


Figure 10: Comparison of FE_{Na proximal} and FE_{Na distal} (A) and FE_{Li} (B) of control rats with animals administered OA. OA was administered for 1½ h during the treatment period. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ p < 0.05 by comparison with control animals at each corresponding time.

3.7. Cell culture studies

In vitro effects of various doses of OA (5, 10, 20, 40, 80 $\mu\text{mol/l}$) were evaluated in isolated hepatocyte-derived human transformed (HepG2), human embryonic kidney (HEK293) and Madin-Darby bovine kidney cell lines (MDBK) which were exposed to OA for 24, 48 and 72 h.

3.7.1. Cell viability

Figure 11 shows the percentage cell viability of HepG2, HEK293 and MDBK cell lines. The viability and/or metabolic activity of HepG2 cells increased significantly ($p < 0.05$) after 24 and 48 h exposure periods when compared to the control. However, the cell viability and/or metabolic activity decreased significantly ($p < 0.05$) after 72 exposure period in comparison to 24 and 48 h exposure periods but it remained above that of the control. OA treatment significantly increased ($p < 0.05$) the viability and/or metabolic activity of HEK293 and MDBK in a dose dependant manner after 24 h exposure in comparison to both the control and the longer exposure periods. However, there was a slight decrease in the viability and/or metabolic activity of both kidney cell lines after 48 and 72 h exposure which did not reach statistical significance when compared to the control.

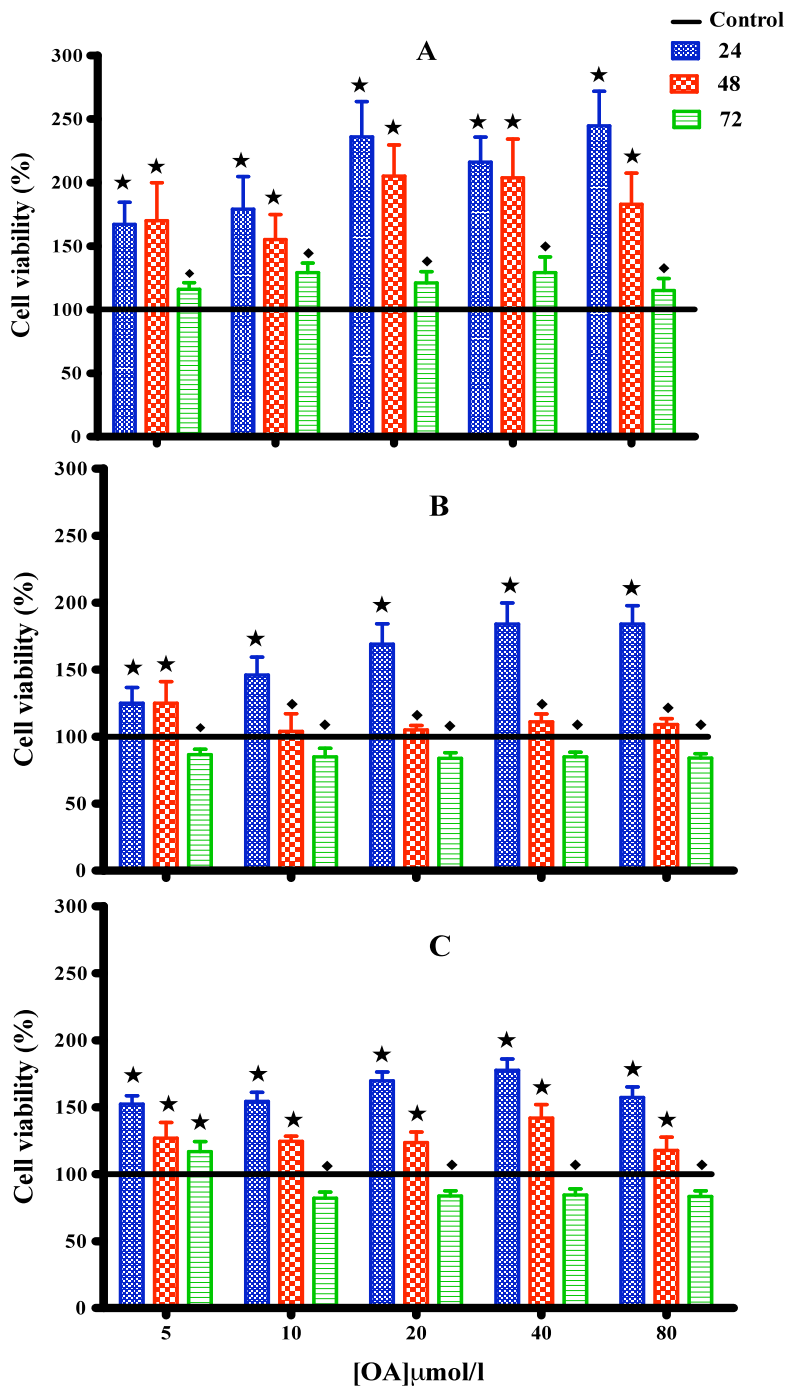


Figure 11: The effects of OA on the viability and/or metabolic activity of HepG2 (A), HEK293 (B) and MDBK (C) cells *in vitro* after exposure to various concentrations of OA for 24, 48 and 72 h. Values are presented as means, and vertical bars indicate SEM (n = 6). ★ p < 0.05 by comparison with control. ♦ p < 0.05 by comparison with 24 h (A,B & C) and 48 h (A & C).

3.7.2. DNA integrity

Figure 12 shows the effects of OA (20 and 80 $\mu\text{mol/l}$) on the DNA of liver cell lines. Image A (24 h) and B (48 h) are control cells which were only suspended in the growth medium and their DNA is not damaged. Image C is a positive control treated with hydrogen peroxide (H_2O_2) which caused DNA damage as indicated by the comet tails. Similar to the untreated control, all OA-treated cells [image D - 24 h, E - 48h, F - 72 h (20 $\mu\text{mol/l}$) and image G - 24 h, H - 48 h, I - 72 h (80 $\mu\text{mol/l}$)] had intact DNA. Figure 13 and Figure 14 show the effects of OA on DNA integrity of HEK293 and MDBK cell lines, respectively, which were also not influenced by OA exposure since all OA-treated cells had intact DNA after all the three exposure periods.

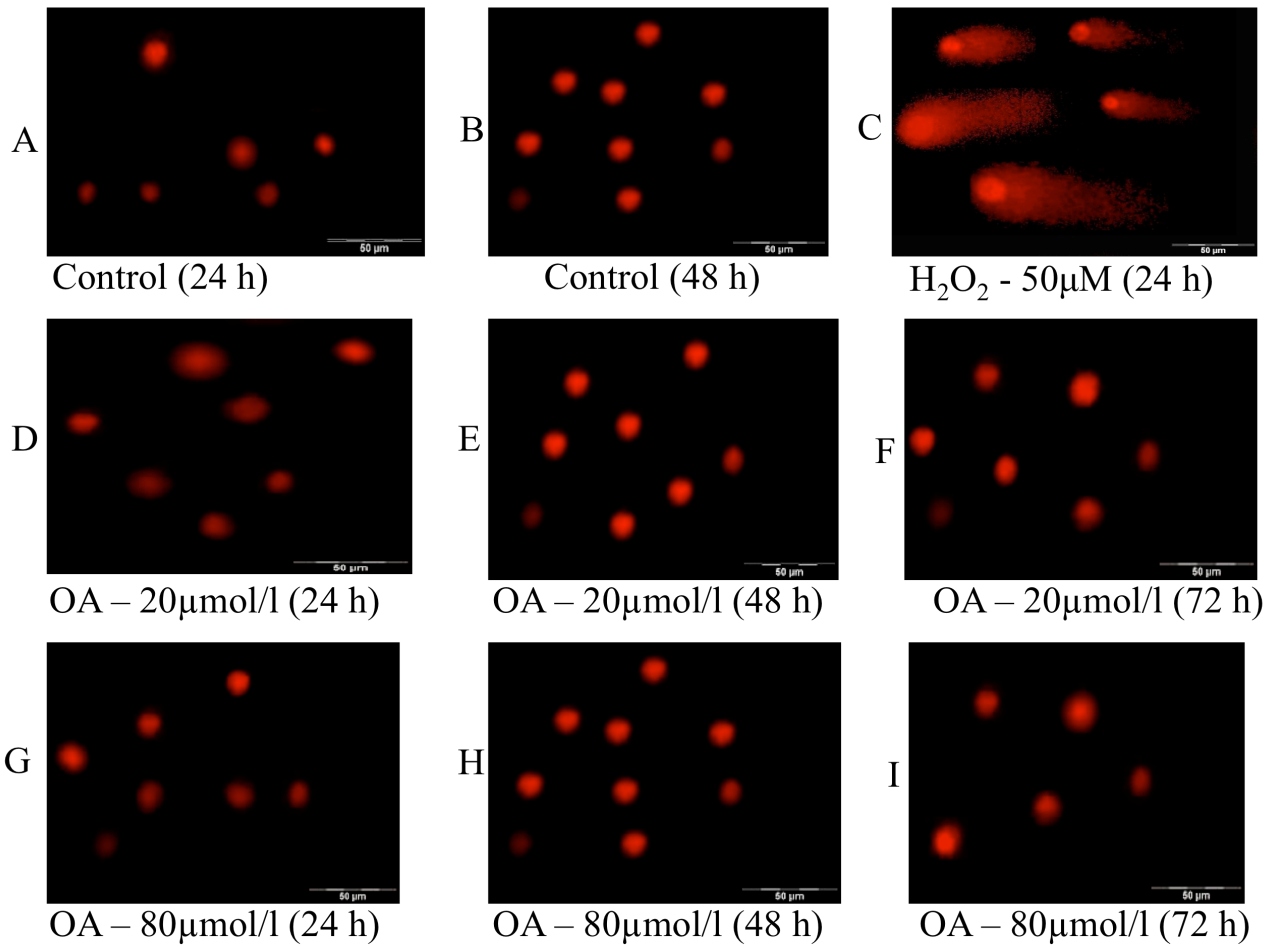


Figure 12: The effects of OA on DNA integrity of HepG2 cells *in vitro* after exposure to various concentrations of OA for 24, 48 and 72 h. Control for 24 h (A), 48 h (B), H₂O₂ - 50 μmol/l for 24h (C), OA - 20 μmol/l for 24 (D), 48h (E) & 72h (F), and 80 μmol/l for 24h (G) 48h (H) & 72h (I).

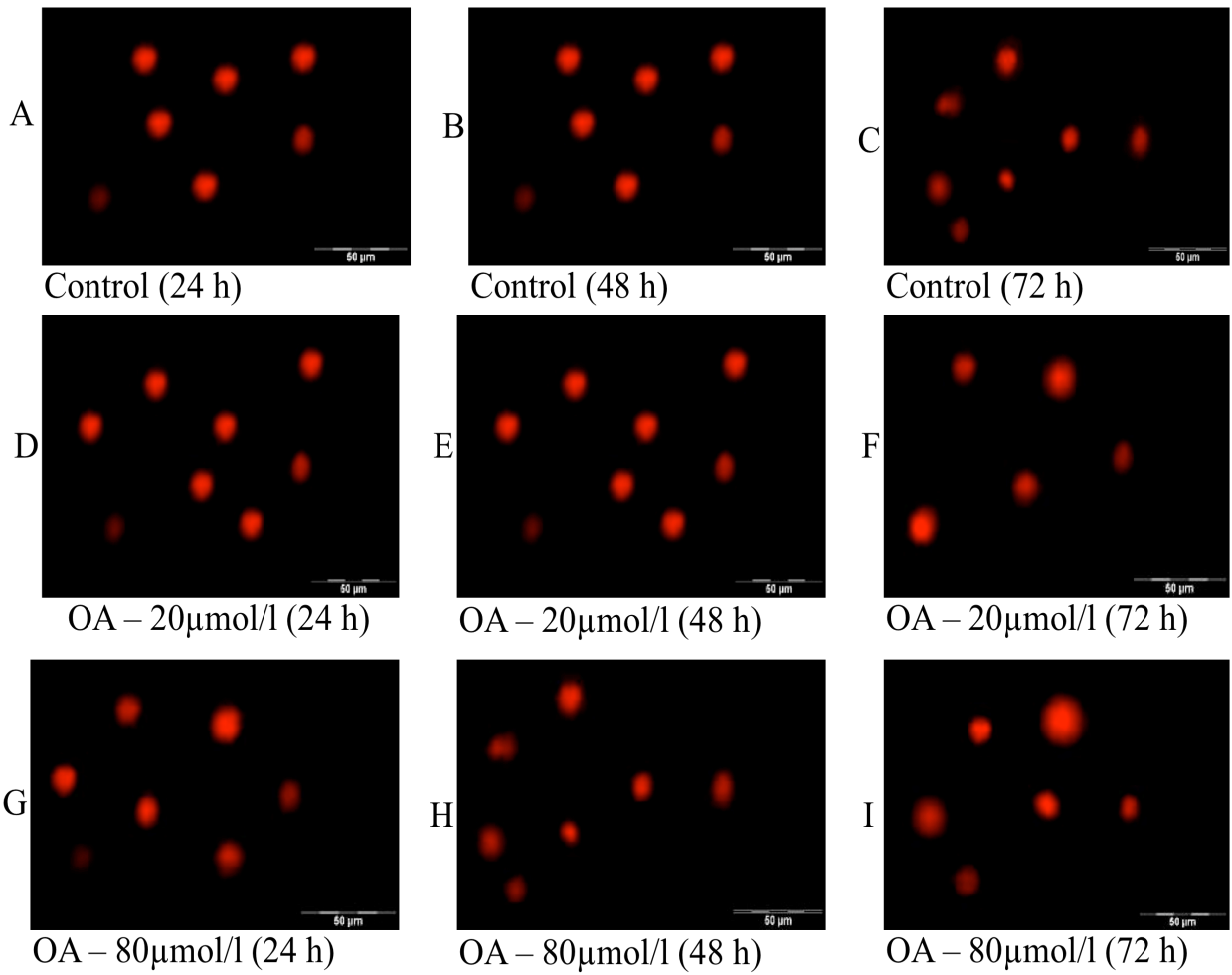


Figure 13: The effects of OA on DNA integrity of HEK293 cells *in vitro* after exposure to various concentrations of OA for 24, 48 and 72 h. Control for 24 h (**A**), 48 h (**B**) & 72 h (**C**), OA - 20 μmol/l for 24 (**D**), 48h (**E**) & 72h (**F**), and 80 μmol/l for 24h (**G**) 48h (**H**) & 72h (**I**).

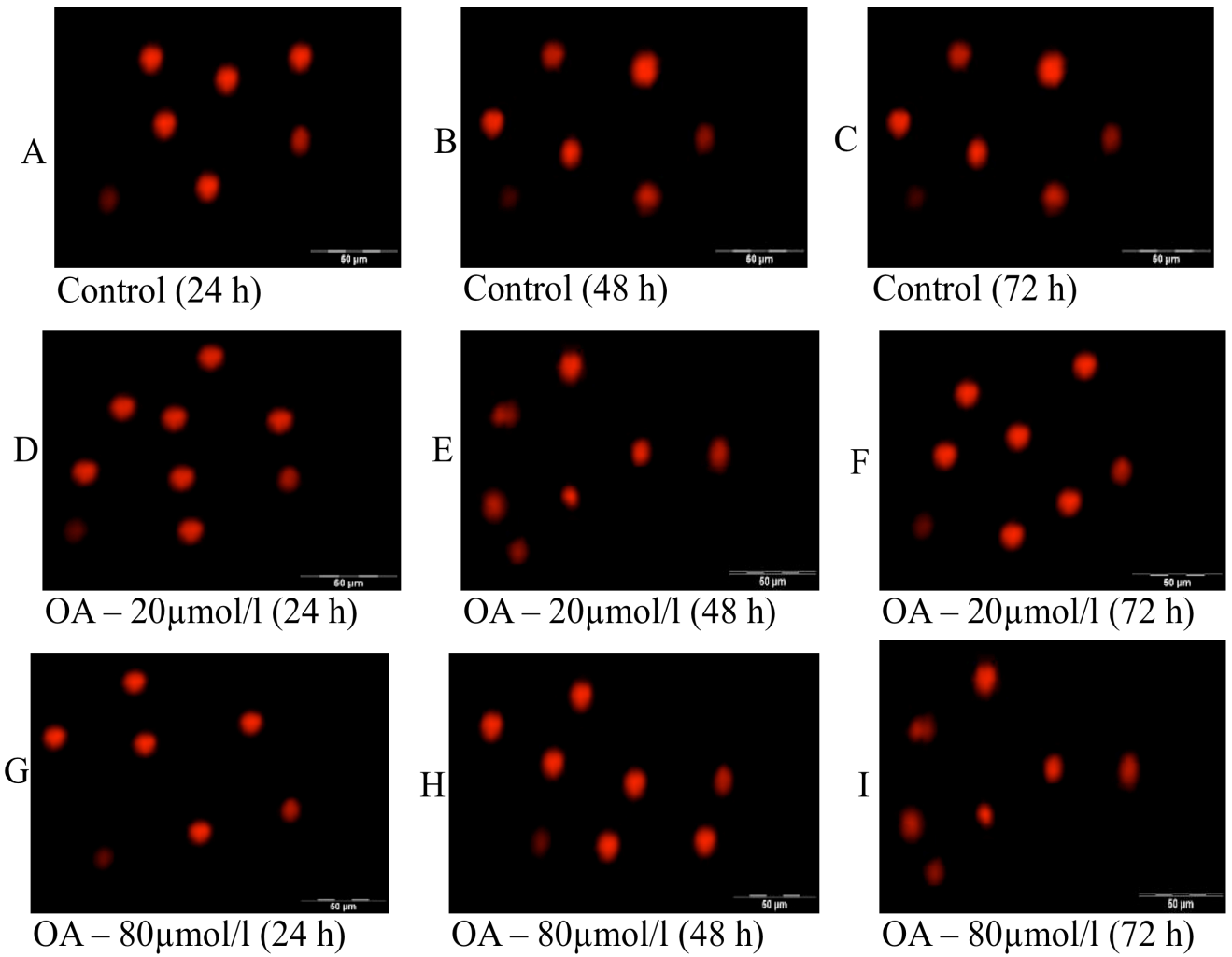


Figure 14: The effects of OA on DNA integrity of MDBK cells *in vitro* after exposure to various concentrations of OA for 24, 48 and 72 h. Control for 24 h (A), 48 h (B) & 72 h (C), OA - 20 μ mol/l for 24 (D), 48h (E) & 72h (F), and 80 μ mol/l for 24h (G) 48h (H) & 72h (I).

CHAPTER 4

Discussion

4.0. General

The aim of the present study was to investigate the effects of plant-derived OA on renal function and proximal tubular Na^+ handling in an effort to identify the site of action for the previously reported OA-evoked increases in urinary Na^+ output in male Sprague Dawley rats. This study also assessed the effects of OA in kidney and liver cell lines to establish whether this triterpene exhibits any toxicity in these organs. The results of the present study, apart from confirming our previous observations of the natriuretic effects of OA in rats (Mapanga *et al.*, 2009), indicate that this effect is in part mediated via the inhibition of proximal tubular Na^+ reabsorption by this triterpene. In addition, the results also suggest that OA may have no toxic effects in kidney and liver cell lines as indicated by the MTT and comet assays. Taken together with the previous observation of the hypoglycaemic effects of OA in streptozotocin-induced diabetic rats, these results indicate that OA may be able to prevent or alleviate kidney complications that are associated with diabetes. This is clinically relevant considering that the majority of morbidity and mortality in diabetes is caused by its microvascular complications including diabetic nephropathy (Taskinen, 2000).

The absolute stereostructure of *S. aromaticum*-derived OA elucidated from the spectra using ^1H - and ^{13}C -NMR was comparable to the previously reported data (Mahato and Kundu, 1994; Mapanga *et al.*, 2009) hence isolated OA was used to carry out all the experiments as it is less costly. Renal experimental techniques are well validated as they have been used extensively in our laboratory (Musabayane *et al.*, 2007; Mapanga *et al.*, 2009). Renal lithium clearance has been used widely in animal studies and clinical investigations as a means of assessing proximal tubular function in the mammalian kidney (Thomsen, 1984; Koomans *et al.*, 1989; Thomsen, 1990; Walter and Shirley, 1991; Shirley and Walter, 1993; Boer *et al.*, 1995; Whiting, 1999). OA significantly increased FE_{Na} and FE_{Li} in anaesthetized animals. Endogenous lithium clearance (C_{Li}) was used to estimate changes in proximal tubule Na^+ reabsorption. C_{Li} technique for measurement of proximal tubular output is based on the assumptions that Li^+ is reabsorbed in the

proximal tubules to the same degree as Na^+ . Controversially, recent studies have shown that lithium reabsorption also takes place in the distal nephron, however, this was only observed in Na^+/K^+ deprived rats (Shalmi *et al.*, 1998; Thomsen and Shalmi, 1997; Emamifar *et al.*, 2000). However, clearance and micropuncture studies in rats have confirmed that distal Li^+ reabsorption does not occur if it is used in low concentrations (Koomans *et al.*, 1989; Shirley *et al.*, 1992; Thomsen, 1990; Walter and Shirley, 1991; Shirley and Walter, 1993). In addition, Leyssac and Christensen (1994) have shown that Li^+ clearance is a valid, quantitative index of Na^+ delivery from the proximal tubules if GFR is unchanged. Indeed, Mudaliar *et al.*, (2010) used Fe_{Li} to assess the mechanisms through which thiazolidinediones (TZDs) treatment for diabetes causes fluid retention. They found that TZD-treated individuals had decreased Fe_{Li} consistent with increased Na^+ reabsorption (Skott, Vaag and Bruun, 1991; Mudaliar, Chang, Aroda, Chao, Burke, Baxi1, Griver, O'Connor and Henry, 2010). Furthermore, Boer *et al.*, (2005) used lithium clearance to investigate the long-term changes in renal Na^+ handling associated with genetic hypertension in spontaneously hypertensive rats (SHR). They observed that SHR had reduced Fe_{Li} and Fe_{Na} which suggested an increase in Na^+ reabsorption with a concomitant increase in blood pressure. However, renal denervation decreased blood pressure and significantly increased Fe_{Li} which indicated an increase in urinary Na^+ excretion and a decrease in proximal Na^+ reabsorption (Boer, Morelli, Figueiredo and Gontijo, 2005). Increased urinary Na^+ excretion was probably as a result of low aldosterone due to the blunting of the renin-angiotensin-aldosterone system (RAAS) in the absence of sympathetic stimulation (Lonn, Yusuf, Jha, Montague, Teo, Benedict and Pitt, 1994; Katz, Opsahl, Lunzer, Forbis and Hirsch, 1997).

We suggest that the OA-evoked increases in Fe_{Na} without any apparent changes in the GFR resulted in increase proximal tubular Na^+ secretion thereby decreasing reabsorption. Accordingly, these results suggest that OA influences transport processes in this portion of the nephron. In addition, OA increased Fe_{Na} in the distal tubule, however to a lesser extent than the proximal tubule. These results suggest that OA also influences transport processes in the distal tubule and that there is no compensatory increased Na^+ reabsorption due to increased Na^+ delivery to this portion of the nephron. The pronounced increase in urinary Na^+ excretion rate evoked by OA infusion during the 1½ h in the absence of significant changes in GFR suggests that OA inhibits both proximal and distal tubular epithelial Na^+ reabsorption. The precise tubular

Na⁺ transporters at which OA exerts this Na⁺ inhibitory action in the proximal and distal tubule could not be established by the current observations. Na⁺ is the major cation that controls osmolality in the extracellular fluid (ECF) (DeFronzo *et al.*, 1975). Thus Na⁺ reabsorption is vital for maintenance of ECF volume (DeFronzo *et al.*, 2001; Edwards, 2010). The proximal tubule reabsorbs approximately two-thirds of the NaCl that enters the tubular fluid by glomerular filtration (Aronson, 1996) mediated by transport proteins (Girardi *et al.*, 2008; Yingst *et al.*, 2009). The proximal tubular Na⁺ transporters found in the epithelial cells separate the plasma membrane into apical and basolateral membranes (Lorenz *et al.*, 1999). Active Na⁺ reabsorption in the apical membrane is primarily mediated via Na⁺/H⁺ exchanger (NHE3) followed by uphill active extrusion via basolateral Na⁺/K⁺ ATPase (Lorenz *et al.*, 1999; Magyar and McDonough, 2000). The literature on natriuretic hormones' actions in the proximal tubule, however, shows that transport inhibition is effected via parallel independent inhibition of apical NHE3 and basolateral Na⁺/K⁺ ATPase (Zhang, Mircheff, Hensley, Magyar, Warnock, Chambrey, Yip, Marsh, Holstein-Rathlou and McDonough, 1996). We, therefore, speculate that OA inhibited apical NHE3 and basolateral Na⁺/K⁺ ATPase in the kidney tubules accounting for increased Na⁺ excretion since NHE3 is a major Na⁺ transport pathway in the renal proximal tubule (Aronson, 1996). The results of the current study provide the first *in vivo* evidence that *S. aromaticum*-derived OA possibly increased Na⁺ secretion in the proximal tubule. However, the precise mechanism(s) by which OA leads to impaired proximal tubular Na⁺ reabsorption remains to be elucidated.

In addition to OA increasing urinary Na⁺ excretion, this triterpene also increased the GFR at the end of the 5-week study. GFR is an important marker of kidney function and a correlation between increased urinary Na⁺ excretion and elevation of GFR has been reported in experimental animals (Marin-Grez, Fleming and Steinhausen, 1986; Mapanga *et al.*, 2009). GFR was assessed by creatinine clearance (Ccr) which has been used in our laboratory and by other authors as a fundamental parameter of evaluating renal tubular function (Rebsomen *et al.*, 2006; Kumar *et al.*, 2008, Mapanga *et al.*, 2009). A progressive decline in GFR has been reported in diabetes, acute renal failure and nephritic syndrome (Kim, Jeon, Lee, Kang, Kook, Ahn, Kim, Cho, Kim, Han and Choi, 2000; Gnudi *et al.*, 2003; Mason and Wahab, 2003; Kumar *et al.*, 2008).

Treatment related increases in GFR observed in this study suggest an improvement of kidney function in experimental animals.

The results obtained in this study demonstrated that OA-evoked natriuresis is associated with a reduction in blood pressure. The hypotensive properties of OA are in line with previous observations in Dahl salt sensitive (Somova *et al.*, 2003) and normotensive male Sprague-Dawley rats (Mapanga *et al.*, 2009). The results are significant considering that long term diabetes complications often include elevated blood pressure (Stengel, Billon, van Dijk, Jager, Dekker, Simpson and Briggs, 2003).

The MTT and comet assay results showed that OA increase cell viability and does not cause DNA damage in kidney cells, respectively. Therefore, the findings of the study suggest that OA-elicited increase in proximal tubular Na⁺ secretion was not due to toxicity of this compound but may in part be mediated via modulation of cellular activity. Stimulation of cell viability and/or metabolism by OA is advantageous to this compound as some plant extracts have been shown to decrease the viability of tubular epithelial cells *in vitro* (Gondwe *et al.*, 2008). Furthermore, extract of *Aristolochia fangchi* was shown to induce cellular injury and apoptosis in proximal tubular (LLC-PK1) epithelial cell lines and hence was not a good therapeutic agent (Vanhaelen, Vanhaelen- fastre, But and Vanherweghem, 1994; Lebeau, Arlt, Schmeiser, Boom, Verroust, Devuyt and Beauwens, 2001; Balachandran, Wei, Lin, Khan and Pasco, 2005; Debelle, Vanherweghem and Nortier, 2008).

In addition, this study demonstrated that OA does not exhibit toxicity in liver cells. Drug-induced liver injury is particularly regarded as an alarming public health problem as this organ is responsible for biotransformation of drugs (Xu *et al.*, 2008). Some drugs like troglitazone have been withdrawn from the market mainly due to hepatotoxicity (The Diabetes Prevention Program Research Group, 2005). Our results suggest that OA has beneficial effects on kidney function and is not toxic to kidney and liver cell lines.

CHAPTER 5

Conclusions

5.0. Conclusions

The results described in this study introduce the first *in vivo* evidence that the previously reported OA-evoked increases in urinary Na⁺ output is in part mediated via increased proximal tubular Na⁺ secretion. In addition, the study shows that OA does not exhibit toxicity in kidney and liver cell lines.

5.1. Limitations and directions for future studies.

The limitations of the present study are that the effects of OA on renal active hormones such as aldosterone and arginine vasopressin were not established. Therefore, the results for OA effects on renal parameters could not be related to the function of these hormones. Future studies should therefore investigate the effects of OA on renin-angiotensin ratios, aldosterone and arginine vasopressin as these hormones are related to sodium homeostasis. In addition, only one marker was used for identification of the site of action of OA. Therefore, future studies should use various markers such as diuretics which target other segments of the nephron in order to fully understand the mechanisms elicited by OA in amelioration of kidney function. Furthermore, previous studies have indicated increased Na⁺ reabsorption in the proximal tubule in diabetes mellitus, with implications for the Na⁺-glucose co-transporters, therefore effects of OA on sodium transporters may explain the mechanisms for the natriuretic effects observed in this study. Toxicity studies were only performed *in vitro* and therefore future studies will look at the histology of renal tissues as well as measurements of blood markers of hepatic function like Alkaline phosphatase (ALP), Alanine Transaminase (ALT) and Gamma-glutamyl Transferase (GGT).

CHAPTER 6

References

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23 June 2010

Reference: 077/10/Animal

Miss Hlengiwe P Mudlala
School of Human Physiology
University of KwaZulu-Natal
WESTVILLE

Dear Miss Madlala

Ethical Approval of Field Research Project on Animals

I have pleasure in informing you that on recommendation of the review panel, the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2010 on the following project:

"The effects of Plant-Derived Oleanolic Acid on Kidney Function, Blood Pressure in male Sprague-Dawley Rats and on Kidney Cell Lines of the Proximal (LLC-PK1) and Distal Tubules (MDBK)"

Yours sincerely

A handwritten signature in black ink, appearing to read 'Th Coetzer'.

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar
Supervisor (Prof. C. T. Musabayane)
Research Office
Head of School



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**Research office
Animal Ethics Research Committee**

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21 December 2010

Reference: 20/11/Animal

Miss HP Madlala
School of Medical Sciences
University of KwaZulu-Natal
WESTVILLE CAMPUS

Dear Miss Madlala

Renewal: Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2011 on the following project:

"The effects of Plant-Derived Oleanolic Acid on Kidney Function, Blood Pressure in Male Sprague-Dawley Rats and on Kidney and Liver Cell Lines"

Yours sincerely

**Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee**

Cc Registrar
Research Office
Head of School (Prof. WMU Daniels)



Founding Campuses:

-  Edgewood
-  Howard College
-  Medical School
-  Pietermaritzburg
-  Westville

15 August 2011

Ref: 81

Miss Hlengiwe Madlala
University Of KwaZulu Natal
204517136@ukzn.ac.za

39th Conference of the Physiology Society of Southern Africa

University of the Western Cape, Cape Town, South Africa

Monday 29th – Wednesday 31st August 2011

Session Notification - Final Details

Please find below the final details of your presentation.

SESSION DETAILS

Title:	The effects of <i>Syzygium aromaticum</i>-derived oleanolic acid in kidney and liver cell lines, and on kidney function of male Sprague-Dawley rats
Paper Status:	Accepted as Oral
Presentation Type:	Oral Presentation
Presentation Time:	10h00 – 10h15 Note: Your presentation time within the session is subject to change, please check these details carefully.
Session Details:	Wyndham Competition Presentation Tuesday, August 30 2011
Authors:	Hlengiwe P. Madlala Bubuya Masola Moganavelli Singh Cephas T. Musabayane

Kind regards,

Fazlin Anthony

PSSA 2011 Secretariat



UNIVERSITY of the
WESTERN CAPE

PSSA 2011

Theme: Molecules to Function Forging
Physiological Frontiers through Research



This is to certify that

Hlengiwe Madlala

Attended the 39th Conference of the Physiology
Society of Southern Africa from
29 - 31 August 2011


Prof Kennedy Urlwanger
President PSSA


Prof Daneel Dietrich
Chair, Organising Committee
for the Congress

