UNIVERSITY OF KWAZULU-NATAL

Effects of maslinic acid and related triterpene derivatives on kidney function of male Sprague-Dawley rats

Blessing Nkazimulo Mkhwanazi

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by

Blessing Nkazimulo Mkhwanazi (206519349)

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Human Physiology in the Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences

Supervisor: **Professor C.T. Musabayane**
Discipline of Human Physiology
College of Health Science

Co-supervisor: **Professor F.R. Van Heerden**
Department of Chemistry
Faculty of Science and Agriculture

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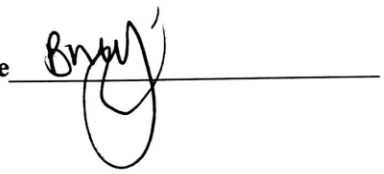
Signature 

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I, **Blessing Nkazimulo Mkhwanazi,** hereby declare that the dissertation entitled" **Effects of maslinic acid and related triterpene derivatives on kidney function of male Sprague- Dawley rats.**" is the result of my own investigation and research which has not been submitted in part or in full for any other degree or to any other university. Where use was made of the work of others, relevant acknowledgements in the text were made.

Student:	**Blessing N. Mkhwanazi**	**Signature**
Supervisor:	**Professor C.T. Musabayane**	**Signature** 10/03/2015
Co- supervisor:	**Professor F.R. Van Heerden**	**Signature**

LIST OF ABBREVIATIONS

α	Alpha
AGE	Advanced glycation end-product
BHT	Butylated hydroxytoluene
BRU	Biomedical Research Unit
BSC1	Na^+-K^+-$2Cl^-$ cotransport
Cl^-	Chloride
CPH	Chloro-Phenyl derivative
FPH	Flouro-derivative
DCM	Dichloromethane
DCMS	Dichloromethane solubles
DCT	Distal tubule
DMF	Dimethylformamide
DMSO	Dimethyl suphoxide
DN	Diabetic nephropathy
EAS	Ethyl acetate solubles
ECM	Extracellular matrix
EMEM	Eagle's Minimal Essential media
ESRD	End stage renal disease
FE_{Li}	Fractional excretion of lithium
FE_{Na}	Fractional excretion of sodium
GFR	Glomerular filtration rate
GLUTs	Glucose transporters
GP	Glycogen phosphorylase
HCl	Hydrochloric acid
HGFR	Hyper-glomerular filtration rate
LDH	Lactate dehydrogenase
Li^+	Lithium
MA	Maslinic acid

MDBK	Madin and Darby Kidney cells
Na^+	Sodium
NAD^+	Nicotinamide adenine dinucleotide
NH3	Sodium hydrogen transporter
NMR	Nuclear magnetic resonance
OA	Oleanolic acid
PH-MA	Phenly hydrazine derivative
PI-3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMSF	Phenylmethanesulfonylfluoride
PTP	Protein tyrosine phosphatase
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
SAR	Structure active site relationship
SGK1	Glucocorticoid- regulated kinase 1
SGLT	Glucose cotransporter
STZ	Streptozotocin
TAL	Thick ascending limb
TBA	Thiobarbaturic acid
TSC	Thiazide-sensitive cotransporter
UA	Ursolic acid
UKZN	University of KwaZulu-Natal

ABSTRACT

Introduction

Reports indicate that hyperglycaemia leads to development of kidney complications which result in sodium retention, decrease in glomerular filtration rate (GFR) and high blood pressure. Various biochemical processes such as polyol pathway, AGEs formation are thought to gives rise to a development and progression of these complications. Clinical trials show that there is currently no commercially available compound that lowers blood glucose concentration while alleviating diabetic nephropathy (DN). Current methods involve the use of ACE blockers which are associated with side effects. Previous reports in our laboratories indicate that triterpene constituents of *Syzygium spp.* such as oleanolic acid (OA) possess hypoglycaemic and renoprotetive effects in STZ-induced diabetic rats. The important question is whether MA, a related triterpene also possesses the same properties. MA is a hydrophobic triterpene and we therefore synthesised derivatives to improve solubility, bioavailability and efficacy. Accordingly this study was designed to investigate the effects of MA and related triterpene derivatives on renal function of STZ-induced diabetic rats.

Materials and Methods

MA was extracted from *Syzygium aromaticum* clove flower buds with dichloromethane and ethyl acetate (EA). The EA solubles rich in triterpenes were purified by silica gel column yielding a mixture of oleanolic acid (OA)/MA which was further purified and recrystallised from methanol and chloroform to obtain pure MA. The MA structure was confirmed by spectroscopic analysis using ^{1}H and ^{13}C NMR techniques. MA derivatives were then synthesized by introduction of phenylhydrazine (PH-MA), Cl-PH and F-PH at carbon 2 and 3. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) which was prepared in citrate buffer.

The cell lines (MDBK and Chang) were challenged with 19 mmol of glucose and treated with various doses (40, 80 and 160 µmol/L) of either MA or PH-MA and incubated at 37 $^{\circ}C$ in a humidified incubator with 5% CO_2. Cell viability and glucose utilisation were monitored using CellTiter-Glo Luminiscent Cell Viability assay and OneTouch glucometer after 12, 24 and 48 h incubation period. Acute effects of MA on kidney function and MAP were investigated in

anaesthetised non-diabetic and STZ-induced diabetic animals. To investigate the influence of MA and related derivatives (PH-MA, F-PH and Cl-PH) on Na^+ handling in the proximal tubule, animals received lithium 48 h prior the experiment. Following a 3½ h equilibration period, urine samples were collected at 30 min interval, over the subsequent 4 h for measurements of urine flow, Na^+, K^+, Cl^-, creatinine and Li^+ to assess proximal tubular handling of Na^+ were recorded over 4 h post-equilibration period of 1 h control, 1 h 30 min treatment as well as 1 h 30 min for recovery periods. In those animals in which the effects of either MA or derivatives were studied, MA (90 µg/min) or derivatives (22 µg/min) were added into the infusate containing creatinine for 1 ½ h treatment period.

Blood glucose concentrations were also monitored in animals treated with MA and/or metformin for 5-weeks and these were measured 6 h after treatment. Food intake, water intake, urine output and weight were measured every third day at 09h00, 24 h after treatment. Urine sample collected during the 5-week study were used for evaluation of kidney function through measurements of Na^+, K^+, Cl^-, creatinine and urea. Harvested organs after 5-weeks were used for investigation of oxidative status (MDA, SOD and GPx) in non-diabetic and STZ-induced diabetic rats. The harvested kidneys were also used to investigate the effects of MA expression on GLUT 1 and 2 and expression of GLUT 4 was investigated on muscle tissue.

Results

Acute administration of MA (40 and 80 mg/kg) significantly ($p<0.05$) reduced blood glucose concentrations in non-diabetic and STZ-induced diabetic rats by comparison with respective control animals at corresponding time periods. MA (80 mg/kg, p.o.) was more effective in lowering blood glucose concentrations and the values were comparable with metformin. The lowest dose of MA (20 mg/kg, p.o.), however reduced blood glucose to values that did not reach statistical significance by comparison with the control at corresponding time periods. The same effects were observed in STZ-induced diabetic rats treated with MA for 5-weeks; however, the reduction in non-diabetic rats did not achieve statistical significance by comparison with the control. In addition, our results indicate that MA-treatment diminished the expression of GLUT1 and GLUT2 in diabetic kidney. Interestingly, MA significantly decreased glucose utilisation by

MDBK cells line. An increase in GLUT 4 expression was observed in muscles tissue following treatment with MA. Treatment with MA (40 and 80 mg/kg, p.o.) for 5 weeks significantly ($p<0.05$) increased Na^+ output and glomerular filtration rate (GFR) without influencing urine flow, K^+, and Cl^- output. Furthermore, MA at 40 and 80 mg/kg significantly ($p<0.05$) decreased mean arterial pressure (MAP) from the 3rd week until the end of the experimental period. Acute intravenous infusion of MA significantly ($p<0.05$) increased Na^+ output, with a concomitant increase in fractional excretion of Na^+ (FE_{Na}) and lithium (FE_{Li}) without influencing GFR. Acute infusion of MA also reduced MAP significantly ($p<0.05$) in non-diabetic rats by comparison with control animals.

Discussion

Our results suggest that MA and PH-MA possess kidney ameliorative properties in part via inhibition Na^+ reabsorption in the proximal and distal tubule. We speculate that MA and PH-MA increased Na^+ excretion through inhibition of sodium hydrogen exchanger 3 (NHE3) and basolateral Na^+-K^+-ATPase which are known to increase reabsorption of Na^+ in the proximal tubule. Interestingly, treatment of MDBK cells with MA and PH-MA diminished expression of epithelial sodium channels (ENaC), indicating that Na^+ handling was not only the proximal tubule. These findings were in agreement with a decrease in plasma aldosterone following treatment with MA and PH-MA. Our results also showed that the elevated concentrations of MDA (marker of lipid peroxidation) in diabetic rat tissues of the liver, kidney and were restored within normal values after treatment with MA after 5-weeks. The reduction in MDA could be due to improved glycaemic levels and increased antioxidants such as SOD and GPx. Cell viability studies also revealed that the natriuretic effects of MA were not associated with toxicity. Although PH-MA exerted toxicity after 24 and 48 h treatment, we suspect that the doses used in this study were very high as they were the same as those of the parent compound.

Summary

In summary, the findings of this study suggest that MA and PH-MA possess kidney ameliorative properties in part via inhibition of Na^+ reabsorption in the proximal and distal tubules. This was

evidenced by an increase in FE_{Na} and FE_{Li} and decreased expression of ENaC, which suggests that these triterpenes inhibit proximal tubular and distal tubular reabsorption. This, therefore, resulted in pronounced increase in urinary Na^+ excretion without inducing any toxicity effects on the kidney and liver cell lines.

Conclusion

We provide the first report on multiple mechanisms that mediate natriuretic effects of MA and related triterpene derivatives. Additionally, this study indicates for the first time that MA reduced blood pressure while exerting no toxic effects to the kidney and liver cell lines. Taken together, the results suggest that MA and PH-MA indeed may alleviate some complications associated with diabetes mellitus.

Future recommendations

The future studies should investigate the effects of PH-MA on glucose homeostasis in STZ-induced diabetic rats. These studies should also investigate the effects of MA on Na^+ transporters such NHE in the proximal tubule to further elucidate the mechanism of action.

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administrated orally to the mice twice every third day and con the controls animals received DMSO saline

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CHAPTER 1

INTRODUCTION/LITERATURE REVIEW

1.0 Preamble

The focus of the investigations described in this thesis were to establish whether maslinic acid (MA) and related triterpene derivatives can improve impaired renal fluid and electrolyte handling of STZ-induced diabetic rats. Reports indicate that diabetes mellitus (DM) leads to impaired kidney function marked by decrease in glomerular filtration rate (GFR), sodium (Na^+) retention and increased blood pressure. Clinical trials suggest that there is no effective treatment for diabetic nephropathy (DN) and this has become a serious medical challenge. However, angiotensin II (AII) receptor antagonists have been shown to be reno-protective in patients with DM. Despite the efficacy of the aforementioned treatments, patients still remain at high risk of complication even during high intensive therapy. Primary goal to prevent the onset of diabetic nephropathy (DN) is tight glycaemic control. We have reported that *Syzygium* spp-derived oleanolic acid (OA) lowers blood glucose concentration and ameliorates kidney function of streptozotocin (STZ)-induced diabetic rats. Guided by this fundamental observation we speculated that MA, a related triterpene possesses similar effects. However, important question is whether MA can improve the impaired renal function in diabetes. To further enhance the efficacy of MA a series of derivatives were synthesised. Research is indeed showing that most synthetic drugs derived from natural products ameliorate kidney function in diabetes. Accordingly, the focus of this study was to determine whether MA and related triterpene derivatives can improve the impaired renal fluid and electrolyte handling often seen in experimental animals and humans. In addition the study investigated whether MA influences renal expression of glucose and Na^+ transporters in STZ-induced diabetic rats. High expression of these transporters has been implicated in the pathogenesis of DN. The following section describes DN and effects of triterpenes on kidney function.

1.1 Introduction/Literature review

1.1.1 Diabetic nephropathy

Hyperglycaemia leads to the development and progression of end-stage renal disease (ESRD), the principal cause of death as a result of diabetic nephropathy (DN). Multiple mechanisms that contribute to development and progression of DN include oxidative stress, protein kinase C (PKC), polyol/aldose reductase, advanced glycation end product (AGE)–receptor of AGE (RAGE) pathways and renin-angiotensin system (Arora and Singh, 2013). These pathways metabolize excess glucose to toxic metabolites which perturb intra-renal haemodynamics via glycosylation of intrarenal proteins that induce hyperfiltration and glomerular dysfunction (Whiteside and Dlugosz, 2002). An increase in glucose fluxes through epithelial pathways result in the increased expression and activity of aldose reductase, protein kinase C, and transforming growth factor-β, which have all been implicated in causing DN (Wood and Trayhurn, 2003; Abisambra *et al.*, 2013). To date there is no apparent common element linking these mechanisms. The clinical trials of inhibitors of these pathways in patients were all disappointing. Researchers have hypothesized that all of these mechanisms are linked to a common upstream event and that the failure to block all of the downstream pathways could explain the disappointing clinical trials with single-pathway inhibitors (Rodrigo and Rivera, 2002). Reports have established that all of these different mechanisms do reflect a single hyperglycaemia-induced process of the overproduction of superoxide by the mitochondrial electron transport chain which causes the oxidative stress (Evans *et al.*, 2002). This study investigated whether treatment with MA can modulate the oxidative stress in diabetic kidneys. Hyperglycaemia induced oxidative stress is thought to mediate a wide range of renal impairments ranging from acute renal failure (Shah, 2001) obstructive nephropathy (Klahr, 2001) and glomerular damage (Kitamura *et al.*, 2002). Thus, increased levels of malondialdehyde and F2-isoprostanes, two products of lipid peroxidation have been reported in various clinical settings associated with renal damage (Martín-Mateo *et al*., 1999), although most of these studies have been performed in rats or mice.

Manifestation of these toxic metabolites result in various ultrastructural changes which include basement membrane thickening, glomerular and tubular hypertrophy, mesangial expansion, glomerulosclerosis and tubulointerstitial fibrosis (Parving *et al.*, 2000). Whilst most attention has focused on glomerular changes, renal tubules are now increasingly recognized to play an important

role in the pathogenesis of DN (Magri and Fava, 2009). The proximal tubule plays a crucial role in the pathogenesis of diabetic kidney disease, being uniquely susceptible to a variety of metabolic and haemodynamic factors associated with diabetes (Bagby, 2007). The proximal tubules react to the different offences with a variety of incompletely understood changes such as tubular hypertrophy, tubular atrophy, and reduced organic ion transport (Thomas *et al.*, 2005).

1.2 Aetiology of diabetic nephropathy (DN)

Early stages of DN are associated with about 30% increase in glomerular filtration rate (GFR) (Kalk *et al.*, 2010). An increase in proximal tubular reabsorption has also been shown within the spectrum of the early changes observed in diabetic kidneys suggesting an alternative hypothesis to explain the increase in GFR. In normal and pathophysiological conditions, glomeruli and tubules are functionally linked to ensure maintenance of adequate extracellular volume and consequently systemic perfusion (Thomson and Blantz, 2008). Two mechanisms are involved in this process. Glomerulotubular balance modulates proximal tubular reabsorption according to changes in GFR in order to guarantee a constant percentage of tubular reabsorption of filtered solutes. This study focused on investigating whether MA and derivatives can decrease proximal tubular reabsorption. The mechanism mainly depends on parallel changes in peritubular capillary oncotic pressure that accompany changes in filtration fraction (Thomson *et al.*, 2001). However, glomerulotubular balance is not fully efficient in maintaining fractional reabsorption constant. Due to the limitation of glomerulotubular balance, part of any change in GFR will pass along the nephron eliciting the tubuloglomerular feedback response, which will counteract some of the original disturbance. An initial reduction in proximal tubular reabsorption results to an increase in NaCl in the thick ascending limb of Henle and the distal tubule, which in turn lead to decrease in GFR. Conversely a reduction in distal delivery driven by increased reabsorption in the proximal tubule induces an increase in single-nephron GFR. This study also focused on investigating whether treatment with either MA or derivatives influence transport proteins (ENaC) which modulate NaCl reabsorption in the distal tubule. Before dwelling too much on the pathology of DN, the following section describes how oxidative stress may lead to kidney injury in diabetes.

1.2.1 Oxidative stress

As mentioned in previous sections, evidence indicates that oxidative stress is the common denominator link for the major pathways involved in the development and progression of diabetic micro- as well as macrovascular complications of diabetes (Ha and Kim, 1999). The reactive oxygen species (ROS) are a family of molecules including molecular oxygen and derivatives, superoxide anion (O_2), hydroxyl radical, hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), nitric oxide (NO) and lipid radicals. Many ROS possess unpaired electrons and thus are regarded as free radicals. Excessive amounts of ROS, after surpassing various endogenous anti-oxidative defensive mechanisms, oxidize various tissue biomolecules, such as, DNA, protein, carbohydrates and lipids; and this devastating state has been commonly referred to as an oxidative stress (Brownlee, 2001; Papaharalambus and Griendling, 2007). In mammalian cells, potential sources of ROS include mitochondrial respiratory chain, xanthine oxidase, NADH/NADPH oxidases, NO synthase and certain other haemoproteins. These different sources of ROS are operative under hyperglycaemic state and are considered to play a significant role in pathogenesis of DN (Forbes *et al.*, 2008). The increased production ROS is thought to overwhelm the endogenous anti-oxidative defense systems in the body (Ceriello *et al.*, 2000). The oxidative stress is regarded as a common and major factor that couples hyperglycemia with vascular complications via two mechanisms first, the metabolic modifications of target tissue molecules and second, the alterations in the renal hemodynamics.

One of the most important mechanisms that leads to overproduction of ROS is the polyol-pathway. The polyol pathway is based on a family of aldo-keto reductase enzymes that can use as substrates a wide variety of carbonyl compounds and reduce these by NADPH to their respective sugar alcohols (polyols) (Brownlee, 2001). Aldose reductase (AR) converts excess glucose to sorbitol which is then oxidized into fructose by sorbitol dehydrogenase (SDH), with NAD^+ as a cofactor. Aldose reductase is found in tissues such as nerve, retina, lens, glomerulus, and vascular cells. In many of these tissues, glucose uptake is mediated by insulin-independent GLUTs intracellular glucose concentrations, therefore, rise in parallel with hyperglycemia. Several mechanisms have been proposed to explain how hyperglycaemia-induced increases in polyol pathway flux could damage the tissues involved. The most cited is an increase in redox stress caused by the

consumption of NADPH**.** Depletion of NADPH, a cofactor required to regenerate reduced glutathione (GSH) which is an important scavenger of ROS is thought to exacerbate intracellular oxidative stress. (Brownlee, 2001). Indeed, overexpression of human aldose reductase is implicated in various complications such as DN (Rodrigo and Rivera, 2002).

1.2.2 Oxidative stress in diabetic kidney

The kidney is thought to be vulnerable to early structural and functional damage caused by hyperglycaemia induced oxidative stress. The kidney may be particularly vulnerable to early structural and functional damage caused by hyperglycemia-induced oxidative stress (Singh *et al.*, 2011). Several factors might contribute directly or indirectly to this susceptibility. The glomerular mesangial cells and tubular cells do not require insulin for glucose uptake and consequently have no control over glucose movement across the cells (Heilig, 1995). Chronic hyperglycemia in the diabetic kidney stimulates the production of AGEs, the polyol pathway and activation of PKC, all of which lead to increased ROS formation and oxidative stress (Pacher *et al.*, 2005). Renal cells such as glomerulus, tubules and fibroblasts express NADPH oxidase and contribute to ROS formation in small amounts in the healthy state. In chronic hyperglycemia, dysfunctional renal cells can increase ROS generation which may enhance renal tissue injury. In addition to structural and functional derangements, presence of excessive ROS in the renal milieu can promote dysregulation of renal medullary blood flow leading to renovascular hypertension. Furthermore, hyperglycemia-induced oxidative stress plays a major role in extracellular matrix expansion, since high glucose-induced collagen production in cultured rat mesangial cells was effectively prevented by two antioxidants, taurine and vitamin E (Trachtman *et al.*, 1993). Studies have also demonstrated that hydroxyl radical scavengers at concentrations inhibits high glucose-induced lipid peroxidation, suppressed TGF-β1 and fibronectin mRNA expression and protein synthesis by mesangial cells cultured under high levels of glucose (Ha *et al.*, 1997). TGF-β is a final common mediator of the principal lesions of renal disease in diabetes mellitus such as renal/glomerular hypertrophy and extracellular matrix expansion (Ziyadeh and Han, 1997). Hydrogen peroxide increases TGF-β1 and fibronectin production in mesangial cells, further suggesting an important role of oxidative stress in the expansion of extracellular matrix seen in DN.

1.3 Proximal tubular reabsorption

We have shown that that OA inhibits reabsorption of sodium in the proximal tubule using lithium clearance studies (Madlala *et al.*, 2012). The proximal tubule is known to reabsorb about 50% of the filtered Na^+. Sodium hydrogen transporter type 3 (NH3) is the most predominant hence lithium clearance studies were carried out to investigate proximal handling of $Na^{+\cdot}$ The (NHE's) are a family of transmembrane proteins that mediates electroneutral transport of Na^+ for H^+ across cellular membranes. The reabsorption process is controlled by different regulatory factors which include glomerulotubular balance, angiotensin II, endothelin, sympathetic innervation, parathyroid hormone, dopamine and acid base status. The apical membrane Na^+/H^+ exchanger NHE3 is responsible for quantitative transcellular NaCl reabsorption, and two thirds of the transcellular $NaHC0_3$ reabsorption, as well as for 70% of H^+ secretion in the proximal tubule (Swasti *et al.*, 2007). This transport protein is regulated by a large variety of hormones, growth factors and physical parameters, including osmotic and haemodynamic factors. NHE3 is stimulated by hormones such as angiotensin II, endothelin and insulin and is inhibited by dopamine, parathyroid hormone and angiotensin. This study also investigated the effects of MA and derivatives on the plasma concentrations of renally active aldosterone and arginine vasopressin (AVP) in STZ-induced diabetic rats. Insulin has been shown increase NH3 expression by facilitating Na^+ reabsorption in the proximal tubule cells (Klisic *et al.*, 2002). In addition to NH3, cotransporter type II (NaPi-2) is responsible for reabsorption of K^+ coupled to Na^+ (Vogel *et al.*, 2000). An increase in phosphate reabsorption by NaPi-2 is concomitant with increase sodium reabsorption in the proximal tubule given no changes in other apical transporter activity. Interestingly, insulin has also been shown to increase K^+ reabsorption in the proximal tubule brush border from dog kidney (Swasti *et al.*, 2007). This therefore, indicates the importance of insulin in regulation of kidney function. Na^+ handling is not only limited to the proximal tubule, the next section explains the importance of other transport proteins in renal function. This study investigated the effects of MA and derivatives of Na^+ handling in the proximal tubule using lithium as a marker.

1.3.1 The loop of Henle

The loop of Henle is a heterogeneous nephron segment which reabsorbs about 30-40% of the filtered load. This segment consists of the straight portion of the proximal tubule (pars recta), the descending and ascending thin limbs, and the thick ascending limb (TAL). In the TAL 20% of Na^+ of the filtered load is reabsorbed but water is not. This therefore, creates a steep osmotic gradient in the medullary interstitium which permits vasopressin-dependent water reabsorption in the collecting duct. Most of sodium transport in the TAL results directly or indirectly from Na^+-K^+-$2Cl^-$ cotransport BSC1/NKCC2 in efficiently work in the presence of K^+ to recycle across the apical membrane through a K^+ channel (ROMK) and chloride to exit basolaterally through a chloride channel (Mullins *et al.*, 2006). Potassium recycling creates an electrical potential difference which drives the reabsorption of cations through the paracellular pathway. NKCC2/BSC1 increase sodium reabsorption and through counter-current multiplication, increase urinary concentrating ability (Dongun *et al.*, 2002). These findings tend to support the hypothesis that the increases NKCC2/BSC1 proteins during uncontrolled diabetes are compensatory changes that prevent a progressive decline in urinary concentrating ability despite the continuing osmotic diuresis (Dongun *et al.*, 2002). Interestingly, several studies have been conducted to demonstrate increased sodium or chloride reabsorption in response to insulin in this segment (Swasti *et al.*, 2007).

1.3.2 The distal tubule and collecting duct

Sodium reabsorption in the early distal tubule (DCT1 and DCT2) is mediated by the thiazide-sensitive NaCl cotransporter (TSC), ENaC and to a lesser extent, by sodium-hydrogen exchange (NHE-2). The remaining reabsorption is achieved in the connecting tubule and cortical collecting duct via ENaC. This is a segment where fine tuning of sodium reabsorption occurs and this is controlled by aldosterone. The actions of insulin in the DCT are not clear since this segment is short and hence not readily perfusable in most species. However, research indicates that the DCT has a fairly high abundance of insulin binding sites (Swasti *et al.*, 2007) suggesting that there is a clear potential for regulation of electrolyte transport. This suggests the importance of insulin in the systemic circulation, lack of it in case of diabetes mellitus results in several adverse effects. This

study, therefore, also investigated the effects of MA and derivatives on expression of ENaC in MDBK cells.

1.3.3 Expression of glucose/sodium transporters in diabetes mellitus

The increase in proximal tubular reabsorption and consequent reduction in distal delivery of sodium chloride in patients with types 1 and 2 DM was originally was described more than 20 years ago in studies using lithium clearance (Ditzel *et al.*, 1989). Based on these observations, a decrease in distal sodium delivery is thought to contribute to diabetic glomerular hyperfiltration through a reduction in tubuloglomerular feedback–mediated vasoconstriction. Subsequently, micropuncture studies in diabetic rats confirmed that increased proximal tubular reabsorption has a major role in the development of glomerular hyperfiltration in the early stages of DM (Thomson *et al.*, 2001). In these studies, increased proximal tubular reabsorption of chloride anions and sodium and potassium cations led to a 20%-28% reduction in the concentration of these ions in the distal tubule compared with non-diabetic rats. Indeed, clinical studies show a higher prevalence of glomerular hyperfiltration associated with enhanced proximal sodium reabsorption in Africans with impaired fasting glucose or type 2 DM (Pruijm *et al.*, 2010). Most of the filtered glucose is recovered by tubular reabsorption to protect the individual against wide variations in glucose supply and demand. Glucose is removed from the tubular lumen by active transport at the level of the proximal segments. The amount of glucose reabsorbed is proportional to the filtered load and hence depends on plasma glucose. The ability of the proximal tubule to reabsorb glucose increases with increments in the filtered load secondary to higher GFR or plasma glucose levels. When the filtered load reaches the maximal transport level, any additional increase results in glycosuria (glucose above 0.5 g/day). Experimental studies in animal models described two different glucose transporters located in the proximal tubule (Barfuss and Schafer, 1981) a “high-flux low affinity” transporter (SGLT2, located in the S1 segment) and a “low-flux high-affinity” transporter (SGLT1, located in the S3 segment). The S1 segment reabsorbs the largest proportion of the filtered glucose and sodium load, while the remaining 10% is reabsorbed by the S3 segment (Vallon *et al.*, 2011). In diabetic patients glycosuria is limited by upregulation of tubular glucose reabsorption (Quamme and Freeman, 1987). Early experiments in human kidney tubular cells harvested from urine of patients with type 2 DM demonstrate an increase in the expression, protein concentration, and a-

methylglucose transport capacity of SGLT2 compared with non-diabetic individuals (Rahmoune *et al.*, 2005). This indicates that high expression of SGLT2 is involved in pathogenesis of diabetic nephropathy. More recent insights have been provided by studies in non-diabetic mice lacking expression of SGLT1 (SGLT2/2). These studies demonstrated that SGLT1 is required for complete reabsorption of filtered glucose in the kidney (Gorboulev *et al.*, 2012). Furthermore, during SGLT2 inhibition, the glucose load to the SGLT1-expressing S2/S3 segments of the proximal tubule is enhanced and a compensatory increase in SGLT1-mediated transport occurs. The contribution of SGLT1 may explain, at least in part, why SGLT2 inhibition is associated with excretion of only 50%-60% of filtered glucose. These results are also consistent with the observation that combined SGLT1 and SGLT2 knockout is associated with higher glucose excretion and improved glycaemia control compared to inhibition of SGLT2 alone. The bulk of filtered glucose is ~90% reabsorbed by the low-affinity/high-capacity sodium glucose cotransporter (SGLT) 2 located in the S1 segments of proximal tubule (Tabatabai *et al.*, 2009). Residual glucose is then absorbed by the high-affinity/low-capacity SGLT1 in the S3 segment. Transcellular glucose transport is facilitated by two basolateral membrane glucose transporters: the low-affinity GLUT2 in the S1 segment and the high-affinity GLUT1 in the S3 segment (Tabatabai *et al.*, 2009). A study in humans with type 1 diabetes showed a significant increase in the renal transport maximum of glucose (Wood and Trayhurn, 2003). In addition, glucose transport in the diabetic kidney is upregulated and has been implicated in the pathogenesis of progressive DN. Renal tubular glucose reabsorption mediated by facilitative glucose transporter (GLUT) proteins and energy-dependent sodium glucose luminal transporters. Long-term diabetes has been reported increase GLUT1 levels in the renal proximal tubules and expression indicating that this transporter plays an important role in glucose reabsorption (Linden *et al.*, 2006). Inhibition of GLUT 1 and GLUT 2 transporters has therefore become another approach for ameliorating diabetes and kidney function. To establish the mechanism through which MA exerted hypoglycaemic effects, expression of GLUTs was investigated in STZ-induced diabetic rats. The following section will further describes other mechanisms involved in pathogenesis of DN.

1.3.4 The renin-angiotensin system (RAS)

Reports indicate that the intrarenal renin-angiotensin system (RAS) is activated by hyperglycaemia leading to synthesis of kidney Ang II (Burns, 2000). Ang II is thought to increase sodium reabsorption by influencing various transporters involved in Na^+ handling (Riquier-Brison *et al.*, 2010). Suppressing RAS activity in patients with DM using either an angiotensin-converting enzyme (ACE) inhibitor or an angiotensin receptor blocker (ARB) slows the decline in kidney function. ARBs have been found to be effective throughout the entire spectrum of DN with greater efficacy in terms of nephroprotection when therapy is started early in the course of the disease (Brenner *et al.*, 2001; Lewis *et al.*, 2001). Measurements of parameters such as GFR, electrolytes handling and fractional excretion of lithium give an idea of what MA and derivatives do on RAS related complications of the kidney. This will helps us explore other mechanisms that these triterpenes may use to ameliorate kidney function in diabetes. Various classes of agents such as oral hypoglycaemic agents, insulin, anti-RAS, and diuretics are currently available for the prevention and treatment of DN however, use of these agents can be limited by side effects. More importantly there has been no substantial improvement in the prognosis of DM despite increasing awareness and screening of DM and implementation of more intensive and personalized therapies in these patients (Rossing and de Zeeuw, 2011). Thus, new therapeutic strategies are desperately needed to limit the burden of DM-induced kidney diseases and associated morbidity and mortality. The following section describes various methods for management of DN.

1.3.5 Aldosterone

A growing interest in understanding the effects on aldosterone on kidney function and diabetes mellitus has been explored over the years. Aldosterone is a steroid hormone that is primarily produced in the zona glomerulosa, the outer layer of the adrenal cortex. Effects of aldosterone are known to promote sodium (Na^+) retention and potassium (K^+) loss in various sites of the kidney which include sites including the distal tubule, collecting duct of the nephron, salivary and sweat glands and colon. Sodium crosses the outer membrane of polarized cells via the amiloride-sensitive sodium channel (ENaC) and reaches then the plasma compartment via the Na^+, K^+ATPAse of inner membrane (Tomlinson *et al.*, 1992). The latter step thus promotes potassium excretion, and

aldosterone receptor antagonists have been mainly used in clinical practice as potassium-sparing diuretics. Reports indicate that aldosterone may contribute to tissue injury through mechanisms beyond influence on blood pressure (Tomlinson *et al.*, 1992). Studies also show that type 1 diabetes leads to elevation of plasma aldosterone levels, which fell sharply after administrations of angiotensin receptor blocker. This study therefore, also investigated the effects of MA and derivatives on plasma aldosterone levels in STZ-induced diabetic rats.

1.4 Current methods used to manage DN

The major antihypertensive drug classes that are widely used include thiazide diuretics, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), β-blockers, central sympatholytic agents and calcium channel antagonists. Other newly developed drugs include GLUT 2 and NHE3 inhibitors. The following section further describes these conventional treatments.

1.4.1 GLUT transport inhibitors

The insulin-responsive facilitative glucose transporters SGLT1 and GLUT1 found in the proximal convoluted tubule are responsible for re-absorbing filtered glucose from the tubule back into circulation. These co-transporters are up-regulated in the diabetic kidney (Marks *et al.*, 2003) and result in greater re-absorption of glucose and thus exacerbating the pre-existing hyperglycaemia (Abdul-Ghani and Defronzo, 2014). This led us into evaluating whether our triterpenes can inhibit reabsorption of glucose in renal tubules and therefore lead to hypoglycaemia. Administration of SGLT2 inhibitors in diabetic patients produces both dose-dependent glycosuria and a significant reduction in plasma glucose concentrations (Wood and Trayhurn, 2003). Inhibition of SGLT2 transport resets the system by lowering the threshold for glycosuria eventually correcting the hyperglycaemia. This decreases insulin resistance in muscle by augmenting insulin signaling, GLUT4 and glycogen synthase activity (Brosius and Heilig, 2005). In the liver correction of hyperglycaemia decreases glucose- 6-phosphatase and pyruvate carboxykinase activity, which results in decreased gluconeogenesis and total hepatic glucose production, with a resulting decrease in fasting plasma glucose levels (Rahmoune *et al.*, 2005).

Inhibiting glucose transporters (GLUT) transporters that facilitate glucose reabsorption has been promising in management of kidney diseases related to diabetes. Under normal circumstances almost all of the reabsorbed glucose is reabsorbed by the proximal tubule back of the nephron (Chao and Henry, 2010). Glucose is reabsorbed by sodium-glucose co-transporters (SGLTs) and GLUTs (Chao and Henry, 2010). SGLT2 and GLUT2 are located in the early portion of the proximal convoluted tubule of the renal nephron and are responsible for the majority of glucose reabsorption from tubule back into the circulation (Chao and Henry, 2010; Vallon *et al.*, 2011). Other glucose transporters such as SGLT1 and GLUT1 are present in the distal portion of the proximal tubule account for reabsorption of additional glucose. These co-transporters are up-regulated in the diabetic kidney (Vestri *et al.*, 2001) and result in greater re-absorption of glucose and thus contributing to the pre-existing hyperglycaemia. The up-regulation of these transporters has been implicated in the pathogenesis of diabetic nephropathy (Dominguez *et al.*, 1994). Accordingly, we investigated the effects of MA and derivatives on renal expression of glucose transporters of STZ-induced diabetic rats. This study was designed to establish whether administrations of MA and derivatives can modulate kidney dysfunction associated with diabetes mellitus as assessed by effects on renal fluid and electrolyte handling in STZ-induced diabetic rats. The SGLT2 inhibitors therefore block reabsorption of glucose into circulation facilitating excretion of glucose hence decrease plasma glucose (List and Whaley, 2011). Moreover, because the mechanism of action of SGLT2 inhibitors does not depend on the presence of insulin, the efficacy of SGLT2 inhibitors would not be primarily affected by the magnitude of insulin resistance or impairment of pancreatic β-cell function that accompanies type 2 diabetes progressions. Reports also indicate that combination of SGLT2 inhibitors with other anti-diabetic compounds such as insulin can improve glycaemic control. Some of the SGLT2 inhibitors such as dapagliflozin and canagliflozin are in clinical trials (Edward and Chao, 2014). They have been shown to decrease systolic and diastolic blood pressure (Ferrannini *et al.*, 2010; Amin *et al.*, 2011). Phlorizin inhibits SGLTs and suppresses renal glucose reabsorption, resulting in an increased urinary excretion of glucose and a reduction of plasma glucose levels in diabetic model animals. This decrease in blood pressure may be related to SGLT2 inhibitor–induced diuresis and body weight changes. Such findings indicate that inhibition of SGLTs and GLUTs is of biological importance in management of diabetes mellitus. These inhibitors have been found to induce weight loss in type 1 diabetic patients. This could work as a disadvantage as this inhibitor can exacerbate

this weight loss. Valentine, 2012 suggested that these inhibitors might work better when combined with other anti-diabetic drugs (Valentine, 2012). This could however, be of a challenge to the general population in developing countries. Therefore, there is a need to look into other therapeutic interventions that would be of benefit to everyone. This study also investigated whether MA and derivatives have effects on GLUT transporters.

1.4.2 Sodium transport inhibitors

An absence of insulin or a substantial decrease in the activity of insulin at the cell level leads to impaired glucose uptake from plasma, hyperglycaemia, and glucosuria. Studies indicate that STZ-induced diabetic rats increase protein expression of the majority of primary apical sodium transport proteins of the thick ascending limb (TAL) through the collecting duct (CD), including NKCC2, NCC, and the β-, α, γ subunits of ENaC (Figure 1) (Song *et al.*, 2003). Hyperglycaemia has also been shown increase expression of sodium hydrogen transporter 3 (NH3) which is abundant in the proximal tubule (Riazi *et al.*, 2006). High expression of these transporters lead to Na^+ reabsorption and therefore retention of Na^+. An inability to excrete sodium leads to increased blood pressure in humans and experimental animals. To date there is no treatment has been shown to lower blood glucose while inhibiting any of the above mentioned transport proteins. In our laboratory we have shown that OA-evoked natriuretic effects are due to inhibition of sodium reabsorption in the proximal tubule as shown by lithium clearance studies (Madlala *et al.*, 2012) suggesting that OA attenuating expression of NH3 transporters. This study, therefore, investigated whether treatment with MA and derivatives can down regulate expression of these transporters in diabetic rats. The question that remains unclear is whether this Na^+ handling is only limited to the proximal tubule or to other segments of the nephron particularly the distal tubule (DCT). Sodium reabsorption in the early distal tubule (DCT1 and DCT2) is mediated by the thiazide-sensitive NaCl cotransporter (TSC) and to a lesser extent by sodium-hydrogen exchange (NHE-2). The remaining reabsorption is achieved in the connecting tubule and cortical collecting duct via ENaC. This is a segment where fine tuning of sodium reabsorption occurs and this is controlled by aldosterone. To further elucidate this mechanism we investigated effects of MA and derivatives on expression of at ENaC in MDBK cells.

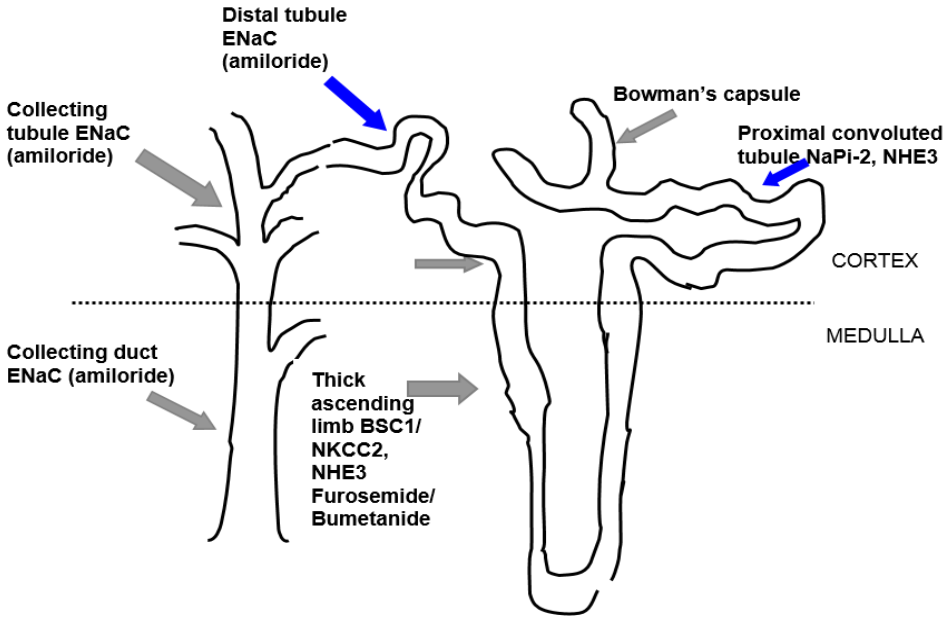

Figure 1: Expression of various transport proteins responsible for electrolyte and fluid handling. For our current research interest we focused mostly on the proximal tubule and distal tubules shown in blue. Proximal convoluted tubule: NOPi, NH3; distal tubule: ENaC and collecting duct: EnaC transporters.

1.4.3 Insulin and synthetic drugs

The goal in the management of diabetes mellitus is not only on maintaining optimal blood glucose control, but also alleviating associated complications. There is sparse information with regards to the effectiveness of current diabetes conventional therapy in the prevention or alleviation of renal complications associated with diabetes mellitus. However, insulin has been shown to be anti-natriuretic (Sampanis, 2008) agent increasing the activity of ENaC and NCC activity hence leading to Na^+ retention in diabetes (DeFronzo *et al.*, 1975).

Most of the synthetic drugs influence the haemodynamic factors that alter GFR and also the pathways involved in the development of the DN (Mogensen *et al*, 2000). Furthermore, drugs that interfere with the different pathways involved in the development of DN preserve the structure of the kidneys. These drugs may inhibit formation of advanced glycation end products (AGE's) (Jandeleit-Dahm, 2004) or inhibit AGE signal transduction (Hartog *et al*., 2007). Additionally,

some drugs have been shown to decrease electrolyte reabsorption and these include beta-blockers, alpha 1-blockers, alpha 2-adrenoceptor agonists and calcium channel-blockers (Heintzen and Strauer, 1994).

1.4.4 ACE inhibitors

Angiotensin II inhibitors have been developed and investigated as therapeutic intervention for management of in DN (Yu and Khraibi, 2008). ACE inhibitors such as captopril, lisinopril, imidapril, ramipril, perindopril, cilazapril, benazapril, trandolapril, enalapril and fosinopril and AT1 receptor blockers such as losartan, irbesartan, olmesartan and candisartan have been observed to have therapeutic potential in the treatment of DN in numerous experimental and clinical studies (Giacchetti *et al.*, 2005). ACE II is a vasoconstrictor that also leads to retention of sodium in experimental animals**.** Enalapril has previously been shown to inhibit sodium reabsorption in the proximal tubule suggesting that this inhibitor could have beneficial effects in diabetic animals (Simaratanamongkol *et al.*, 2014). However, synthetic ACE inhibitors produce some side effects such as coughing, rashes and taste aversion. There is therefore, a need for new natural ACE inhibitors that may be safer and also more economical to use (Simaratanamongkol *et al.*, 2014). Natural products have attracted much interest due to their lack in toxicity and affordability in developing countries. Several plant extracts, such as those from *Glycine max* (Rho *et al.*, 2009) *Cassia tora* (Hyun *et al.*, 2009) *Rosa damascene* (Kwon *et al.*, 2010) *Curcuma longa* (Bhullar *et al.*, 2013) and *Lactuca sativa* (Lagemann *et al.*, 2012) have been reported to possess ACE inhibiting properties. Interestingly *Olea lancea lam*, a plant extract that is rich in triterpenes such as MA has also been found to inhibit ACE *in vitro* improving renal injury (Hansen *et al.*, 1996). This, therefore, shows that plant derived extracts could be used as alternative methods for management of kidney dysfunction that arise due to DM.

1.4.5 Drugs with antioxidant properties

In diabetes, hypoinsulinaemia increases the activity of the fatty acyl coenzyme A oxidase, an enzyme which initiates β oxidation of fatty acids resulting in lipid peroxidation. Increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity and changing the

activity of membrane-bound enzymes and receptors. The lipid peroxidation products (lipid radical and lipid peroxide) are harmful to the cells in the body and are associated with atherosclerosis, brain and kidney damage (Zhang and Kwong-Huat Tan, 2000). Therefore, this research also investigated whether triterpenes can improve the oxidative status seen in diabetes mellitus. Research indicates that antioxidant treatment with vitamins C and E improves renal function, lessens renal injury, and decreases arterial pressure in Dahl salt-sensitive hypertension (Tian *et al.*, 2005)**.** Reports indicate that several plant extracts modulate diabetes associated oxidative stress processes (Pari and Latha, 2004). Against this background are observations that phytochemicals containing quercetin and flavonoids decrease blood glucose concentrations and oxidative stress in experimental diabetes (Azuma *et al.*, 2007). In this study, we focused on the effects of MA on oxidative stress in the liver, heart and kidney of STZ-induced diabetic rats. The decline in renal function of diabetic patients may be as a result of excess glucose in the kidneys**.** This excess glucose concentration may indirectly activate pathways within the kidney such as the polyol pathway and accumulation of advanced glycation end products (AGEs) (Cooper *et al.*, 2001). Some findings suggest that the kidneys' susceptibility to oxidative stress during early stages of diabetes mellitus may be an important factor in development of diabetic nephropathy (Jakus, 2000).

1.5 Medicinal plants

Syzygium aromaticum is traditionally used for treating burns, dental care to relieve pain and to treat gum infections when used at high concentrations (Prashar *et al.*, 2006). Pharmacological studies revealed that this is due to high concentration of euginol present in the plant extract. Reports also indicate that this extract contains a number pentacyclic triterpene including betulinic acid (BA) ursolic acid (UA), oleanolic acid (OA) and maslinic acid (MA). These triterpenes have gained considerable interest because of their multiple pharmacological effects which include anti-HIV (Fujioka *et al.*, 1994), antiviral (Pavlovaa *et al.*, 2003) and anticancer (Fulda, 2008). We have previously shown that medicinal plants and their bio-active compounds acid (OA) isolated from *Syzygium cordatum* (*S. cordatum*), *Syzygium aromaticum* (*S. aromaticum*) may have beneficial effects on some of the processes that are associated with renal derangement (Madlala *et al.*, 2012). Studies in our laboratory indicate that OA, a related triterpene does not only reduce blood glucose

concentrations, but also has beneficial effects on the kidney through the amelioration of GFR and increasing Na^+ excretion rate in animals that have been observed to retain Na^+ Hoek (Hoek *et al.*, 2008; Madlala *et al.*, 2012). The increase in Na^+ excretion also accompanied by a concomitant decrease in blood pressure (Madlala *et al.*, 2012). OA has also shown the ability to decrease plasma creatinine with a concomitant increase in GFR (Mapanga *et al.*, 2009). MA has previously been shown reduce blood glucose concentration in partially diabetic rats.

1.5.2 Other medicinal plants

Natural products have always been an ideal source of drugs and many of the currently available drugs have been derived directly or developed from plants (Narender *et al.*, 2013). Plant-derived active chemical compounds like terpenoids, flavonoids, alkaloids, guanidines, steroids, carbohydrates and amino acids exhibit activity against diabetes mellitus (Narender *et al.*, 2013). Metformin is currently used as anti-diabetic agent in the treatment of diabetes. Metformin and related analogues were synthesized on the basis of a natural product, galegine, which was isolated from the seeds of *Galega officinali* (Bailey and Day, 1989). The ethnobotanical information reports that about 800 plants may possess anti-diabetic potential (Puranik *et al.*, 2010). Our goal is not only to reduce blood glucose concentrations, but to also ameliorate complications that arise in diabetes. Studies in our laboratory have investigated whether ethno-botanic plants can improve the impaired renal function often seen in diabetes (Musabayane, 2012). Results observed showed that *Hypoxis hemerocallidea* corm aqueous extract (APE) had hypoglycaemic effects, but may have deleterious effects on kidney function (Gondwe *et al.*, 2008). However, other studies from our laboratories have shown that *Opuntia megacantha* leaf hypoglycaemic effects reversed the inability of the kidney to excrete Na^+ in STZ diabetes mellitus (Musabayane *et al.*, 2007) suggesting that this plant may be beneficial in diabetes management. Plants such as *Sclerocarya birrea* [(A. Rich) Hochst.] [Anachardiaceae] *Helichrysium ceres* (Gondwe *et al.*, 2008) and *Syzygium cordatum* (Mapanga *et al.*, 2009) have been shown to have reno-protective effects in diabetic animals. Other medicinal plants have been demonstrated to have anti-diabetic and renoprotective effects, these are further discussed in the following section.

Allium sativum

Garlic (*Allium sativum* L., Liliaceae) is a well-known as a food flavouring agent that has been used since ancient time. In the past decade some protective effects of garlic have been well established by epidemiological studies and animal experiments. Commercially available garlic preparations in the form of garlic oil, garlic powder, and pills are widely used therapeutic purposes, including lowering blood pressure and improving lipid profile (Reuter, 1995; Poonam *et al.*, 2013). Several studies have investigated diuretic effects of *A. sativum* in anaesthetized animals Pantoja (Sharafatullah *et al.*, 1986; Pantoja *et al.*, 1991). A powdered extract of *A. sativum* increased urine volume (UV) and urinary Na^+ (UNa) excretion rate in experimental animal models. Blood pressure was also decreased by this extract. The second trial a purified fraction of *A. sativum* was intravenously injected and increased UV and UNa in a dose-dependently manner with a concomitant decrease in arterial blood pressure (Pantoja *et al.*, 2000), The natriuretic effects of *A. sativum* was suggested to be via inhibition of kidney membrane N^+, K^+- ATPase. These studies demonstrated the effects of medicinal extracts on kidney function. Our study also aimed at investigating the effects of MA and derivatives on kidney function.

Zea

Zea mays L. (Gramineae) is commonly known as corn silk and has been used in folk medicine as a decoction for diuretic treatment. This plant extract has been shown to possess anti-hyperglycaemic and kidney ameliorative properties in diabetic animals (Suzuki *et al.*, 2005). Additionally, *Zea* demonstrates anti-hypertensive and anti-diabetic properties in the treatment of renal disease (Velazquez *et al.*, 2005). This plant extract has also been shown to prevent formation of kidney stones in experimental animals (Maksimovic *et al.*, 2004; Andrade-Cetto and Heinrich, 2005). Studies by Ribeiro *et al*. showed UV was significantly increased after treatment (Ribeiro *et al.*, 1988). These effects were seen after 1 day of treatment and were sustained throughout the course of treatment (Maksimovic *et al.*, 2004). No changes in serum potassium, urea and creatinine were observed (Evans *et al.*, 2002). These findings demonstrate an important relationship between treatment of diabetes and that of renal function using medicinal plants. For more information see Table 1.

Table 1: Summary of studies reporting effects of different medicinal plants on diabetes and renal function.

Plant species	**Bioactive compounds**	**Anti-diabetic properties**	**Renal function advantages**	**References**
Lepidium latifolium	Flavonoids	Anti-hyperglycaemic agent	Increase electrolyte and urinary excretion rate	(Navarro *et al.*, 1994)
Allium sativum	allicin	anti-hyperglycaemic and anti-hyperlipidaemic agent	↑ Urinary excretion rate and FENa	(Pantoja *et al.*, 1996)
Euphorbia hirta (aqueous)	Saponins, alkaloids and Flavonoids	↓ Serum cholesterol and HDLP	↑ Urinary excretion rate and Na^+	(Kumar *et al.*, 2010) (Johnson *et al.*, 1999)
Spergularia purpurea	Flavonoids	↓ Plasma glucose	↑ Urinary excretion rate and Na^+	(Jouad *et al.*, 2000)
Hypoxis hemerocallidea	Sterols and stanols	↓ [blood glucose]	Nephroprotective	(Gondwe *et al.*, 2008)
Withania somnifera	Flavonoids	Antioxidant properties Restores β-pancreatic cells	↑ Urinary volume excetions and Na^+ excretion	(Anwer *et al.*, 2012)
Syzygium cordatum	Triterpenes	Hepatic glycogen	↑ Na^+ excretion ↑ Creatinine	(Mapanga *et al.*, 2009)

1.6 Maslinic acid

MA is a natural pentacyclic triterpene found in many plant species, but abundant in olive fruit skin (Qiu *et al.*, 2009). MA (2a,3b-2,3-dihydroxy-olean-12- en-28-oic acid), which is abundant in olive has attracted much attention due to anti-tumor, anti-HIV, anti-oxidation and hypoglycemic properties. This pentacyclic triterpene has been shown to reduce high diet fat induced diabetic-induced hyperglycaemia and hyperinsulinaemia *in vivo* (Liu *et al.*, 2014).

Triterpenes such as MA are generally hydrophobic and therefore are difficult to dissolve in water before administration. According to Williams *et al*. poor water solubility is a significant risk factor in low oral absorption because drug molecules must completely dissolve in water before oral administration (Williams *et al.*, 2013). Therefore, medicinal chemistry seeks to modify physicochemical properties of different drugs to enhance their solubility. This study also focused on improving efficacy of MA by synthesizing a series of derivatives which would more soluble in water compared to the parent compound. Research indicates that synthesized or semi-synthesized drugs can have toxic effects on the cells. We also investigated the effects of synthesized derivatives on cell viability of kidney and liver cell lines**.** The following section describes synthesis of these triterpene derivatives based on their active sites.

1.7 Triterpene derivatives

Synthesis of triterpene derivatives was performed based on the currently known MA active sites. The first active site is the carboxylic group which has previously been shown to be responsible for glycogen phosphorylase (GP) inhibition (Wen *et al.*, 2006). The second active site is C2-C3 which has previously been shown to inhibit expression of protein tyrosine phosphatase 1 (PTP1) (Li *et al.*, 2004). The following section describes how inhibition of these enzymes may provide beneficial effects of on renal function.

1.7.1 Active site I: glycogen phosphorylase (GP) inhibition

Glycogen phosphorylase catalyzes the breakdown of glycogen to glucose-1-phosphate in liver and tissues leading to increased glucose in diabetics. Inhibition of hepatic GP is a promising treatment strategy for attenuating hyperglycaemia in diabetes mellitus. Glycogen phosphorylase inhibitors (GPis) have been shown to reduce liver GP activity and to dramatically attenuate hyperglycaemia without producing hypoglycaemia (Ogawa *et al.*, 2003). These inhibitors have also been shown to be more potent in reducing hepatic glucose output in the presence of high glucose concentrations. MA is a natural GPi, the rate-limiting enzyme of glycogen degradation, and has therefore, been regarded as a promising therapeutic approach for diabetes mellitus and related complications. The carboxylic group shown in structure (Figure 2) has been identified as the active site for GP (Fi) (Wen *et al.*, 2006). Several derivatives have been synthesized with an effort to improve water solubility of this triterpene, as they are well known to have poor water solubility . The results were, however, disappointing as incorporation of hydrophilic groups on carbon 28 significantly decreased potency. According to Wen *et al*. introduction of hydrophobic groups (bromobutane) on carbon 28, carboxylic groups gives rise to a 7- fold increase effects compared to lead MA. Accordingly we opted to incorporate hydrophobic groups on c-28 to increase potency of MA. Reports indicate that MA uses two currently known active sites to mediate biological effects. The following section describes the synthesis of PTP1 inhibitors which are a second active site for MA

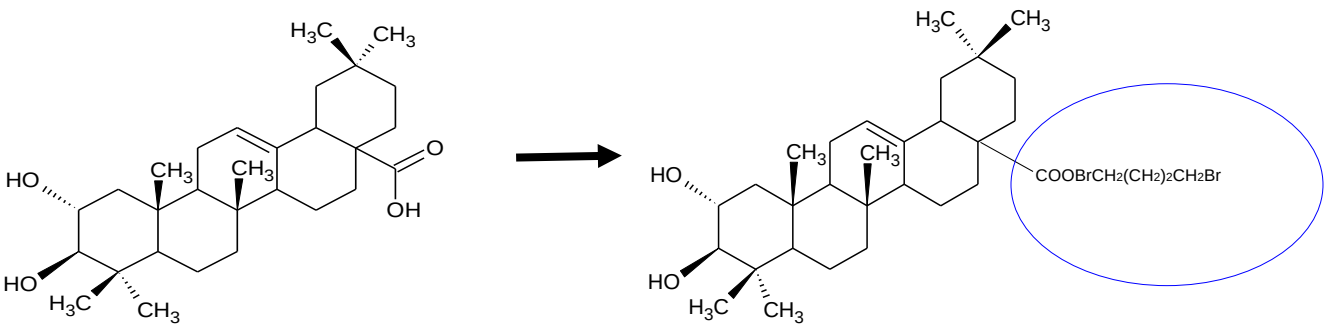

Figure 2: Synthesis of maslinic acid derivative through addition of bromobutane and bromohexane on the carboxylic group.

1.7.2 Active site II: Protein tyrosine phosphatase inhibition

Discovery of clinically available PTP1B inhibitors has been difficult as they exhibit lack in selectivity. In recent years, there has been a growing interest in anti-diabetic agents from natural products. They represent an alternative mode for therapy as most of the anti-diabetic drugs have some side effects and fail to significantly alter the course of the disease. However, natural PTP1B inhibitors such as UA, OA and MA have been identified and demonstrated to possess blood glucose lowering effects (Na *et al.*, 2006; Qiu *et al.*, 2009). Indeed, we have observed that triterpenes such as OA do not only lower blood glucose but also improve kidney function in STZ-induced diabetic rats a long term complication for diabetes mellitus. In searching for novel types of PTP1B agonists, researchers have found that pentacyclic triterpenoids such as OA to be moderate PTP1B inhibition (Zhang *et al.*, 2006). Based on these results, some potent inhibitors with good cell permeability were obtained by modifying long hydrophobic chains at C-3 and C-28 positions. However, these synthetic compounds had poor water solubility and had no obvious selectivity on PTP1B (Zhang *et al.*, 2006; Zhang *et al.*, 2008). MA has, however, been screened for PTP1B inhibition along with isomers such as 3-epi-maslinic acid and augustic acid on PTP1B. Among the MA isomers, MA demonstrated good PTP1B inhibition (IC_{50} = 5.93 µM). Hence our current research focuses on synthesizing MA derivatives with an aim to improve solubility; efficacy and bioavailability. To achieve this, a series of heterocyclic rings were incorporated in C-2 and C-3 position based on the evidence that heterocyclic incorporating heterocyclic rings

improved efficacy of MA (Figure 2). The synthesis was based from previous studies which demonstrated the efficacy of MA derivatives after incorporating heterocyclic rings. To date no studies have been performed on these derivatives to investigate their effects on kidney function. Our goal was not only to manage blood glucose concentration, but to also evaluate whether MA and derivatives ameliorate kidney function in STZ-induced diabetic rats.

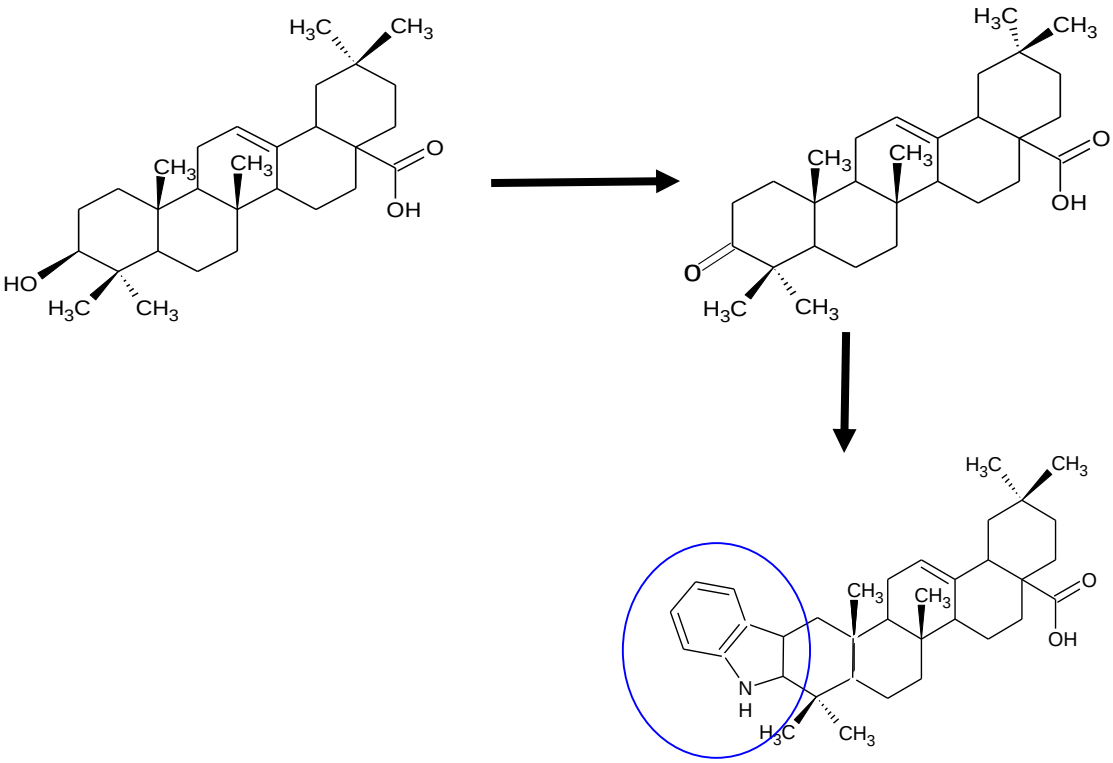

Figure 3: Synthesis of tyrosine phosphatase inhibitor by insertion of phenyl hydrazine in carbon 2 and 3.

1.8 Methods to assess kidney function

1.8.1 Lithium clearance

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 has been used for determination of Li^+ concentrations in plasma and urine samples. This is a controversial technique for proximal Na^+

handling as there is evidence that lithium can be reabsorbed by the distal nephron under certain conditions (e.g. Na^+ and K^+ depletion) (Shirley and Walter, 1997). However, since the animals used in the study were Na^+ and K^+ replete Li^+ was assumed as a valid marker of proximal tubule function. Lithium (Li^+) is, therefore, an ideal marker for proximal tubule function as this element is absorbed in the proximal tubule and does not induce any tubular secretions (Boer *et al.*, 2005). In addition, Li^+ is not actively transported in the proximal tubules, but is also transported at the same rate as Na^+ and water (Holstey *et al.*, 1985; Weder, 1986). Research indicates that most of the Li^+ is reabsorbed in the proximal tubule at a range of 73 to 82% of the whole kidney. Fractional excretion of lithium (FE_{Li}) in these rats has been reported to range from 25 to 35% (Shirley *et al.*, 2002).The proximal tubular reabsorption would, therefore, contain of 65-75 % of the filtrate. This evidence indicates that Li^+ can, indeed be used to asses functioning of the proximal tubule in the kidney. Li^+ clearance should, therefore, be used to assess the effects of anti-diabetic drugs, in this way it would be clear if they have any effects on renal electrolytes handling electrolytes handling.

1.8.2 Cell culture studies

Cell culture studies are important as they serve as a 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). Understanding the toxicity of drugs and chemicals is essential for progress in the pharmaceutical industry, medical science and academic research (Farkas and Tannenbaum, 2005). A variety of cell viability study techniques are available and are used to screen drugs in pharmaceutical industries. However, current methods are relatively inconvenient, insensitive. These include direct cell counting by dye invasion, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays (Fernandez and Sanchez, 2001) and metabolism-based methods such as oxidation of MTT (Boncler *et al.*, 2014). The LDH-release method involves either direct measurement of LDH enzymatic activity by observing the reduction of NAD^+ to NADH at 340 nm or indirect measurements of pyruvate depletion (Stewart *et al.*, 1961). Neither method is highly sensitive both suffer from the possible presence of interfering substances in biological or cell-culture mixtures, and both are open to the objection that

many substances of biological interest contain moderate or large amounts of LDH. We, therefore opted to use CellTiter-Gloluminescent cell viability (ATP) assay due to its sensitivity and ability to give reproducible results. The ATP assay is considered the fastest cell viability assay to use, the most sensitive, and is less prone to artifacts than other viability assay methods. The luminescent signal reaches a steady state and stabilizes within 10 minutes after addition of reagent and glows with a half-life greater than 5 hours. The ATP assay has the advantage step with a population of viable cells to convert a substrate (such a tetrazolium or resazurin) into a colored compound. This also eliminates a plate handling step because you do not have to return cells to the incubator to generate signal (Riss *et al.*, 2013).

1.9 Basis of the study

Despite the currently available commercial drugs for alleviating DN, morbidity of patients with DN is increasing. Alternative methods to ameliorate kidney function are needed. Clinical trials indicate that tight glycaemic control may prevent or delay the onset of DN. This study, therefore, focused on using MA and related derivatives on kidney function of STZ-diabetic rats.

1.9.1 Objectives

The objectives were to investigate the effects of MA and MA triterpene derivatives on renal Na^+ handling and to further elucidate the mechanism through which these compounds exert their effects.

CHAPTER 2

MATERIALS AND METHODS

2. Drugs and chemicals

Drugs were obtained from different sources as indicted: Sodium chloride (NaCl), metformin (1, 1-dimethylbiguanide hydrochloride), streptozotocin, dimethyl suphoxide (DMSO), sodium citrate ($Na_3C_6H_5O_7$), lithium choride (LiCl), inactin (5-ethyl-5(1-methylpropyl)-2-thiobarbiturate), halothane (2-Bromo-2-Chloro-1, 1, 1-trifluoroethane) (Sigma St Louis, MO, USA);

Ethyl acetate (EtOAc), silica gel, dichloromethane (DCM), ethanol (C_2H_5OH), methanol (CH_3OH), chloroform ($CHCl_3$) (Merck, Waderville, South Africa);

Phosphate buffered saline (PBS), Eagle's Minimal Essential Medium (EMEM), L-glutamine, fungizone/penicillin/streptomycin and trypsin (Highveld Biological, Johannesburg, South Africa) and

Isofor inhalation anaesthetic (Safeline Pharmaceuticals (PTY) LTD, Weltevreden Park, Rooderport, South Africa).

All chemicals were of analytical grade.

2.1. Methods

2.1.1 Extraction of OA and MA

MA and OA (Figure 4) were isolated from *Syzygium aromaticum* [(Linnaeus) Merrill & Perry] [Myrtaceae] (cloves) flower buds using a standard protocol that has been validated in our laboratory with minor changes (Mapanga *et al.*, 2009; Madlala *et al.*, 2012) . Briefly, air-dried *S.*

aromaticum flower buds (500 g) were sequentially extracted twice at 24 h intervals at room temperature with 1 L dichloromethane (DCM), and ethyl acetate (720 ml) on each occasion. Removal of the solvent from the extract under reduced pressure at 55±1°C using a rotary evaporator yielded dichloromethane solubles (DCMS, 63 g) and ethyl acetate solubles (EAS, 85 g). The EAS containing mixtures of oleanolic/ursolic acid and methyl maslinate/methyl corosolate were purified by silica gel 60 column chromatography with a hexane: ethyl acetate 9:1, 8:2 solvent system increasing polarity. This yielded OA. The polarity was then increased to 6:4 to obtain MA**.** The resulting white powder was recrystallized from chloroform-methanol (1:1, v/v). The structure of MA was confirmed by spectroscopic analysis using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) techniques. The total of 10.5 g MA was obtained.

MASLINIC ACID **OLEANOLIC ACID**

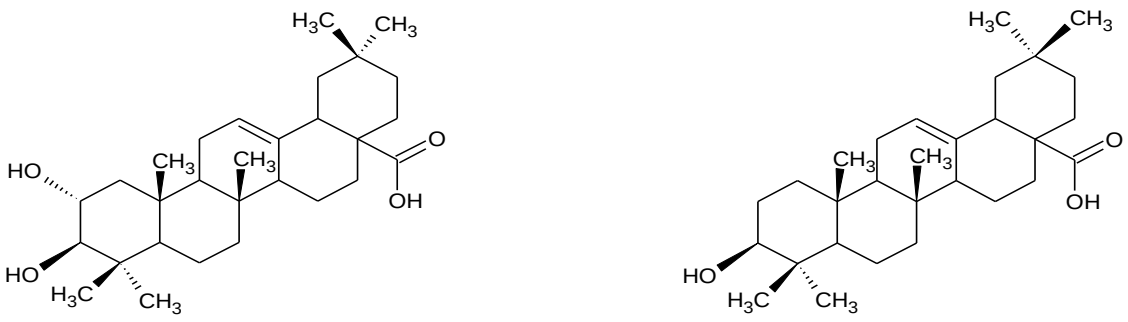

Figure 4: Chemical structure of maslinic acid (MA) and oleanolic acid (OA).

2.2 Synthesis of MA derivatives

2.2.1 Glycogen phosphorylase inhibition

2.2.1.1 Substitution with bromobutane

Reaction of MA (1.0 g, 2.1 mmol) with 1,4 bromobutane (1.1 g, 6.3 mmol) in the presence of potassium carbonate (0.029g, 2.1 mmol) in dimethylformamide [DMF (10 mL)] at room temperature resulted in formation of bromobutane derivative (Figure 4) . After 12 hours, the

reaction was diluted with H_2O and bromobutane derivative was extracted with ethyl acetate. Column chromatography over silica gel hexane ethyl acetate and elution with 7:3 afforded pure bromobutane derivative which was recrystallized from methanol and chloroform affording 72% yield.

BROMOBUTANE DERIVATIVE

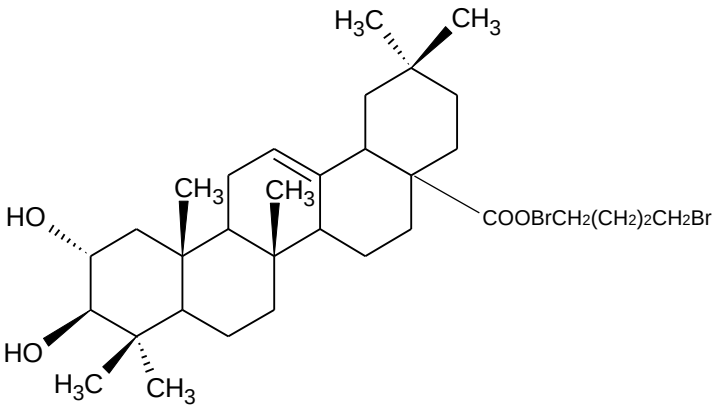

Figure 5: Chemical structure of bromobutane substituted derivative.

2.2.1.2 Substitution with bromohexane

Reaction of MA (1.0 g, 2.1 mmol) with 1,6 bromohexane (1.5 g, 6.3 mmol) in the presence of potassium carbonate (0.029 g, 2.1 mmol) in dimethylformamide [DMF (10 ml)] at room temperature resulted in formation of bromohexane derivative (Figure 5). After 12 hours, the reaction was diluted with H_2O (25 mL) and bromohexane derivative was extracted with ethyl acetate (60 x 3). Column chromatography over silica gel hexane ethyl acetate and elution with 7:3 afforded compound a final product which was recrystallized from methanol and chloroform.

BROMOHEXANE DERIVATIVE

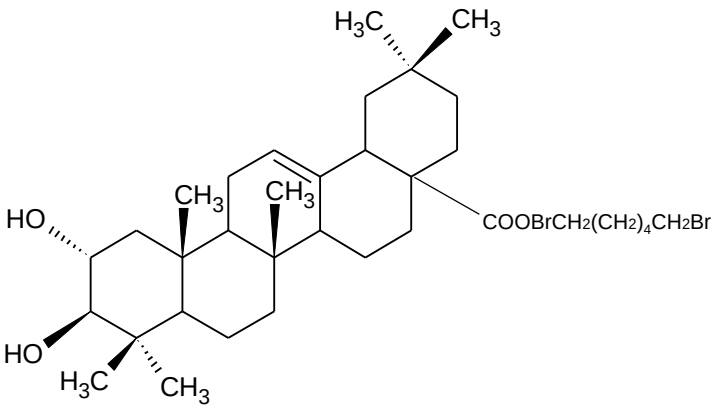

Figure 6: Chemical structure of bromohexane substituted derivative

2.2.2 Tyrosine phoshorylase (PTP1B) inhibition

2.2.2.1 Oxidation of oleanolic acid

Oxidation of OA was performed according a methods described by Zhang *et al*.(Zhang *et al.*, 2006). A suspension of oleanolic acid (1.0 g, 2.2 mmol) in 10 mL CH_2CL_2/C_3H_6O (1/1) was cooled at 5 ˚C and a solution of Jones reagent (1.2 mL, 5 equiv) was added dropwise over 30 min and the reaction was allowed to run for 1 hour until the colour changed dark brown**.** Isopropanol and H_2O (mL) were added to the reaction mixture. The reaction mixture was then stirred at room temperature for 15 minutes. H_2O and CH_2CL_2 were added to the mixture and the layers were separated. The organic phase was washed with brine and concentrated to give 0.90 g of oxidized oleanolic acid (Figure 6). Pure product of oxidized OA was obtained by silica gel chromatography (7:3 hexane: ethyl acetate) and recrystallized from chloroform/methanol (1/1).

OXIDISED OA

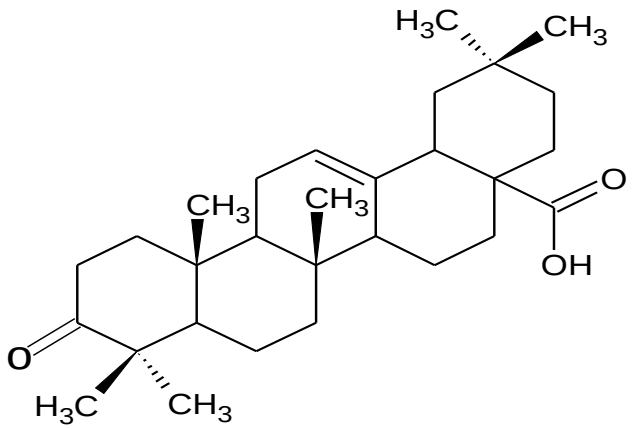

Figure 7: Chemical structure of oxidised oleanolic acid.

2.2.2.2 Phenyl hydrazine

Fischer indole synthesis was done according to a method described by Alonso *et al.* (Alonso *et al.*, 2004). Briefly, a mixture of ketone (1 g, 2.2 mmol), phenylhydrazine (0.8 mL 0.9 mmol) and glacial acetic acid (5 mL) was heated at reflux under N_2 for 1 h. During this period the colour changed from colourless to bright yellow. The reaction mixture was pipetted into distilled water (50 mL) and extracted with ether (4 x 20 mL). The combined ether extracts were washed with 5% aqueous NaOH (2 x 20 mL) and brine (2 x 20 mL), dried (Na_2SO_4), and concentrated in vacuo to give a yellow solid product. Chromatography over silica gel and elution with hexane: ethyl (7:3) acetate afforded indole 11 (Figure 7) (86%) as a yellow solid.

PHENYLHYDRAZINE (PH) DERIVATIVE

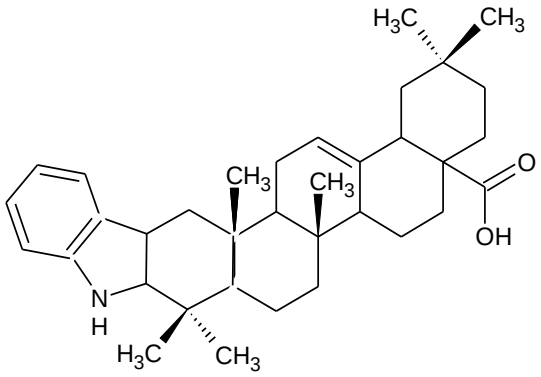

Figure 8: Chemical structure of phenylhydrazine (PH) substituted derivative

2.2.2.3 3-chloro phenyl hydrazine

In similar manner as section 2.2.2.2 a ketone (1.0 g, 2.2 mmol) was mixed with 3-chloro phenyl-hydrazine hydrochloric acid (1.6 g 8.8 mmol) in acetic acid (5 mL) heated at reflux under N_2 for 1 h to produce 3-chloro phenyl hydrazine (Figure 9).

CHLORO PHENYL HYDRAZINE (CPH) DERIVATIVE

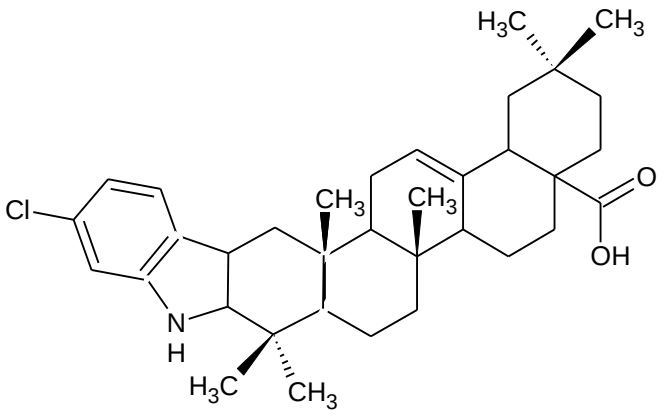

Figure 9: Chemical structure of chloro phenyl hydrazine (CPH) substituted derivative

2.2.2.4 3-Flouro phenyl hydrazine

Fischer indole synthesis was done according to a method described by Alonso at al. (Alonso *et al.*, 2004). Briefly, a mixture of oxidised OA (1 g, 2.2 mmol), 3-fluorophenylhydrazine hydrochloride (0.8 mL, 0.9 mmoL) and glacial acetic acid (5 mL) was heated at reflux under N_2 for 1 h. During this period the colour changed from colourless to bright yellow. The reaction mixture was pipetted into distilled water (50 mL) and extracted with ether (4 x 20 mL). The combined ether extracts were washed with 5% aqueous NaOH (2 x 20 mL) and brine (2 x 20 mL). These extracts were then dried in (Na_2SO_4), and concentrated *in vacuo* to give a yellow solid product (Figure 10). Chromatography over silica gel and elution with hexane: ethyl (7:3) acetate afforded an indole (86%) as a yellow solid (3-flouro phenyl hydrazine derivative).

FLUOROPHENYLHYDRAZINE DERIVATIVE

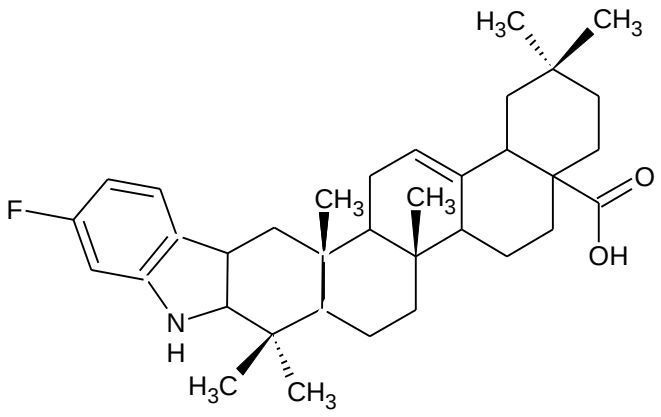

Figure 10: Chemical structure of 3-fluorophenylhydrazine hydrochloride derivative

2.3 Animal experiments

2.3.1 Animals

Male Sprague-Dawley rats weighing 250-300 g were obtained from Biomedical Research Unit (BRU), University of KwaZulu-Natal Westville campus. The animals were kept under maintained laboratory conditions of constant temperature (22±1 ˚C); $C0_2$ (<5000 p.p.m,), humidity of 55±5% and illumination (12 h light/dark cycles). The animals had full exposure to food standard rat chow (Meadows Feeds, Pietermaritzburg, South Africa) and water *ad libitum*.

2.3.2 Ethical consideration

The experiments and treatments of animals were performed according to University of KwaZulu-Natal ethics committee guidelines for experimental animals. The ethical clearance was obtained from the animal ethics committee of the University of KwaZulu-Natal (UKZN) (002/13/Animal and 029/14/Animal).

2.3.3 Induction of experimental diabetes

Diabetes was induced with a single intraperitoneal injection of STZ (60 mg/kg) dissolved in 0.1 M citrate buffer pH 6.3 (Musabayane *et al.*, 2005; Hadebe *et al.*, 2014). Control animals were injected with a vehicle, citrate buffer. Animals showing glucosuria after 24 h, tested by urine strips (Rapidmed Diagnostics, Sandton, South Africa) were considered diabetic. Blood glucose concentration of 20 mmol/L measured after one week was considered as stable diabetic state and these animals were used for carrying out the study.

2.4 Experimental design

Non-diabetic and STZ-induced diabetic rats were divided into separate groups to study the following: (1) acute effects of MA and derivatives on proximal tubule function (2) sub-chronic (5 weeks) effects of MA on blood glucose, kidney function and oxidative stress in livers, hearts and kidneys, (n=6 in each group). MDBK and Chang cells were divided into control and treated groups to study the effects of MA and PH on viability and glucose utilisation by these cells. (Figure 11).

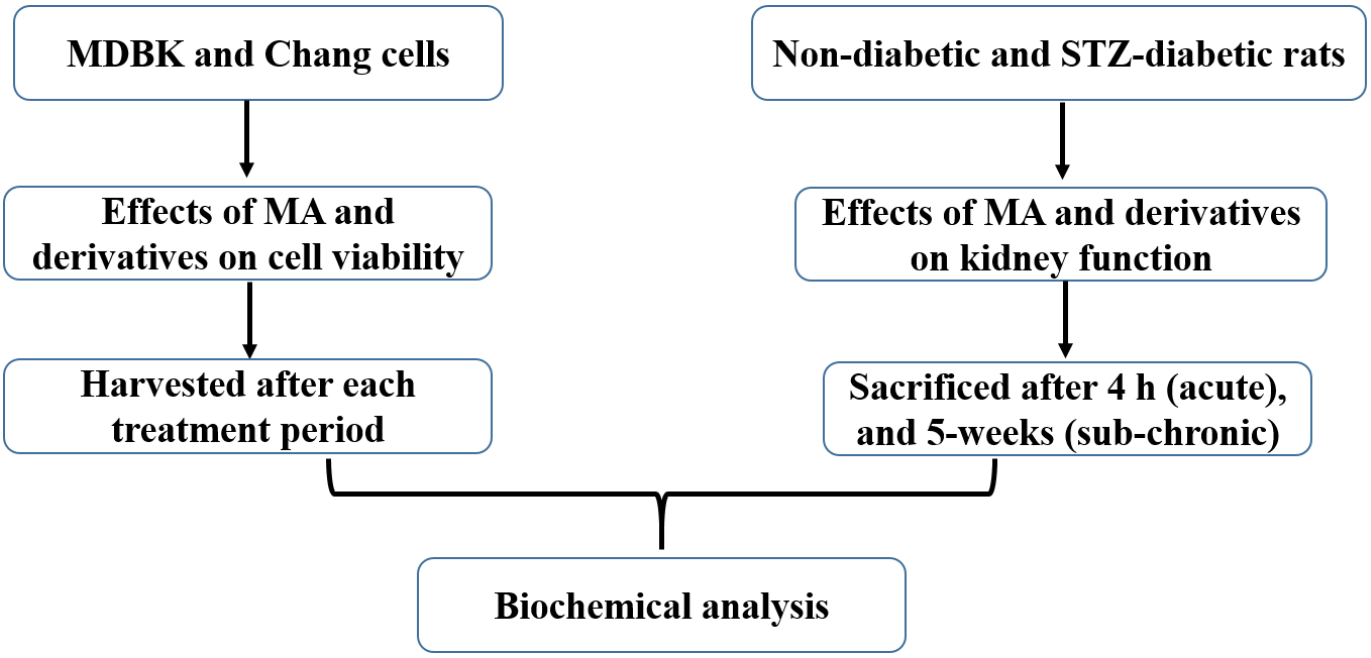

Figure 11: Experimental design for proximal tubular function, animals were treated with 3 MA derivatives for 1.5 h treatment period for acute studies. Subchronic, rats were treated with various doses of MA for a period of five weeks, organs, kidney, liver and heart were harvested and further used for biochemical analysis. Cell culture studies, where cells were treated with various doses of MA for 12, 24 and 48 h period.

2.5 Cell viability

2.5.1 Cell culture

The Chang cell lines were kindly donated by Dr Christo J.F. Muller from the Diabetes Discovery Platform at the South African Medical Research Council (MRC), Cape Town, South Africa. Cell culture was conducted using a well-established cell culture protocol (Czifra *et al.*, 2006) . Briefly, the reconstituted muscle and liver cell lines were plated in 25 cm^3 flasks followed by addition of 10 mL fully supplemented EMEM media, respectively. Thereafter, the flasks were incubated at 37 ºC in a humidified incubator (Shel Lab, Cornelius, Oregon, USA) with 5% CO_2. The cells were allowed to grow, attach and become confluent. The attached confluent cells were trypsinized with trypsin (1 mL) after washing three times with PBS. The trypsinized cells were sub-cultured into new flasks and some were stored in a nalgene cooler (Sigma-Aldrich, St Louis, Missouri, USA) at -80ºC for subsequent studies.

2.5.2 Protocol

Chang and MDBK cell lines were divided into control and treated groups. Cell viability effects of MA and phenylhydrazine derivative were then evaluated on these cell lines. The effects of MA and PH derivative were investigated on cell viability and glucose utilization. Kidney (MDBK) and liver (Chang) cells were seeded in a 24 well plate at a density of (1.5×10^5 cells/mL). Both cell lines were allowed to attach and reach 80% confluent monolayer over 2 days. Each cell line was divided as follows: group 1 remained untreated and served as the control, group 2 was treated with MA and phenyl hydrazine derivatives at various doses (40, 80 and 160 µmol/L) and group 3 was treated with metformin (50 µmol/L).

2.5.3 Effects of MA

Cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, Wisconsin, USA). The assay reagent produces luminescence in the presence of adenosine triphosphate (ATP) from viable cells. The luminescence produced is proportional to the amount of ATP produced by viable cells. Both kidney and liver cells (10, 000 cells/mL) were seeded in 96- well plates and incubated in their respective media (200 µL) overnight. Thereafter, the cells were treated with MA or derivative (40, 80, and 160 µmol/L) some wells remained untreated, serving as a control. After every 12, 24 and 48 hours, plates were equilibrated at room temperature for 30 minutes. The assay reagent (100 µL) was added to each well, followed by shaking the plates for 2 minutes to induce cell lysis. After shaking, plates were incubated at room temperature for 10 minutes to stabilize the luminescence signal; thereafter the luminescence was read on the Promega Microplate Luminometer (Promega, Madison, Wisconsin**,** USA). All plates had wells media without cells, serving as a blank for background correction. Data were expressed as a percentage of viable cells (i.e., treatment value-blank/ control value-blank)

2.5.4 Glucose utilization

Glucose utilization was performed to investigate whether our triterpenes exhibited glucose lowering effects in Chang and MDBK cells. The glucose utilization experiments were performed as previously described Favaro *et al.* (Favaro *et al.*, 2012) with slight modifications. MA or phenylhydrazine derivative (40, 80 and 160 µmol/L) and metformin (50 µmol/L) were introduced on separate wells in the 24 well plates containing cells. The 24 well plates were then incubated at 37 ºC in a humidified incubator with 5% CO_2. Glucose concentrations were measured after 12, 24, and 48 h hours using the OneTouch select glucometer (Lifescan, Mosta, Malta, and United Kingdom). After 48 h experimental period, cells were harvested for further biochemical analysis. At the end of the experiment, cells and media were stored at -70 for further analysis.

2.5.6 ENaC

To investigate the effects of PH and derivative on expression of ENaC, harvested MDBK cells (section 2.5.4) were used. Cells (1 ml) were sonicated on ice in isolation buffer (0.5mM Na2EDTA, 0.1 M KH_2PO_4, 0.1mM dithiothreitol, 0.25M sucrose) and then centrifuged at 400xg for 10 minutes (4^oC). The protein content was quantified using the Lowry method and all the samples were standardized to one concentration (1mg/mL). SDS-PAGE gel electrophoresis was performed as described in section 2.7.4, except that the PDVD membrane was immuno-probed with monoclonal anti-ENaC (1:1 000 in 1% BSA, Neogen, USA) for 1 h at room temperature (RT).

2.6 Acute studies

2.6.1 Proximal tubular function

Sodium (Na^+) reabsorption in the proximal tubule and, by implication, in the distal nephron was assessed through measurement of lithium clearance (C_{Li}) (Thomsen and Shirley, 1997). The effects of MA and related triterpenes on MAP and renal function were investigated in separate groups anaesthetized of non-diabetic and STZ-induced diabetic male rats (Tecniplats, Labotec, South Africa) (n = 6 in each group). 48 h prior to experimentation the rats were fed standard rodent chow

supplemented with lithium chloride (12 mmol/kg dry weight) in order to raise plasma lithium to measurable concentrations without affecting renal sodium or water excretion (Shalmi and Thomsen, 1989) . Subsequently, renal clearance studies were conducted in inactin-anaesthetized (0.11 g/ kg) hypotonic saline infused non-diabetic and STZ-induced rats, a model which has been extensively used our laboratory (Madlala *et al.*, 2012). Briefly, the right jugular vein was cannulated to allow a continuous intravenous infusion of hypotonic saline (0.077M NaCl) at 9 mL/ h (Harvard syringe infusion Pump 22, Harvard Apparatus, Holliston, Massachusetts, USA). A catheter was inserted into the left carotid artery for withdrawal of blood samples and to record mean 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). The urinary bladder was also cannulated via an incision in the lower abdomen for the collection of urine samples. Animals were given a priming dose of creatinine (3 µg in 0.3 mL 0.077 M NaCl) and then placed on a continuous infusion of 0.077 M NaCl containing creatinine (0.15 µg/ml) of at 9 mL/h to allow calculation of creatinine clearance as a measure of GFR. After a 3.5 h equilibration period, blood samples (200 µL) were drawn at 1 h intervals and urine collections were made every 30 min over 4 h of 1 h control, 1.5 h treatment and 1.5 h recovery periods for measurement of electrolyte and clearance marker concentrations. In those animals in which the effects of MA and 3 MA derivatives were examined, the infusate was changed during the 1.5 h treatment period to one identical in ionic composition, but containing MA (90 µg/ h) and 22.5 µg/h of 3 MA derivatives.

2.7. Sub-chronic effects on kidney function

Non-diabetic and STZ-induced diabetic male Sprague-Dawley rats were divided into treated and untreated groups. MA (20, 40 and 80 mg/kg) was administered to non-diabetic and STZ-induced diabetic rats twice every third day at 9h00 and 15h00 for 5 weeks by means of ball-tipped, 18 gauge gavage needle (Kyron Laboratories (Pty) LTD, Benrose, South Africa). The control and positive control animals received deionised water (0.3 mL/kg, p.o.) and metformin (500 mg/kg, p.o.), respectively. These were kept individually in Makrolonpolycarbonate metabolic cages (Techniplats, Labotec, South Africa) at the Biomedical Research Unit, University of KwaZulu-Natal). Blood glucose concentration was measured after 6 hours of treatment using Bayer's glucometer Elite (Elite Pty Ltd. Health care division, South Africa). Bodyweight changes, urine

output, food and water intake were measured after 24 h of MA administration ever third day. Urine samples were collected for further analysis of renal function.

2.7.1 Sub-chronic effects of MA on mean arterial pressure (MAP)

MAP was monitored every third day at 09h00 by in conscious male Sprague Dawley rats for five weeks using non-invasive tail cuff method (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, CA) previously described by Musabayane *et al*. (Musabayane *et al.*, 2005) . The system uses a sensitive photoelectric sensor measurement of blood pressure pulses at a 37 ˚C. The results obtained from the sensors are displayed on the computer screen.

2.7.2 Sub-chronic effects of MA on glucose homeostasis

Non -diabetic and STZ-induced diabetic male Sprague-Dawley rats were divided into treated and untreated groups, standard rat chow (Meadows, Pietermaritzburg, South Africa) and water *ad libitum* was provided to the rats. MA (20, 40 and 80 mg/kg, p.o.) was administered to non-diabetic and STZ-induced diabetic rats twice every third day, at 9h00 and 15h00 for 5 weeks by means of bulbled steel tubes. The control and positive control animals received deionised water (0.3 ml/kg b.wt, p.o.) and metformin (500 mg/kg b.wt, p.o.), respectively. These were kept individually in Makrolonpolycarbonate metabolic cages (Techniplats, Labotec, South Africa) at the Biomedical Research Unit, University of KwaZulu-Natal). Blood glucose concentration was measured after 6 hours of treatment using Bayer's glucometer Elite® (Elite Pty Ltd. Health care division, South Africa) and parameters such as weight change, urine output, food and water intake were measured after 24 h of MA administration. Urine samples were collected for further analysis of renal function.

2.7.3 Analytical methods

Urine flow was determined gravimetrically. Na^+, K^+, urea and creatinine were analysed using the Beckman Coulter Counter (Synchron CX3 Clinical Systems, Fullerton, California, USA) with commercial diagnostic kits from Beckman Coulter, Dublin Ireland. Lithium was determined flame emission spectroscopy at 670.8 nm (Optima 2100 DV, Perkin Elmer, Shelton, Connecticut, USA) using a modified procedure that has been previously described by Madlala *et al*. (Madlala *et al.*, 2012). Fractional excretions (FE) rates of Na^+ (Fe_{Na}) and Li (Fe_{Li}) were determined simultaneously. Lithium clearance (C_{Li}) was used as a marker for the output of Na^+ from the proximal tubules (Thomsen, 1990) . Renal clearances (C) and fractional excretions (FE) were calculated with the standard formulae C = U *x* V/P and FE = C/GFR, where U is the urinary concentration, V is the urine flow rate and P is the plasma concentration. Glomerular filtration rate (GFR), as assessed by creatinine clearance was calculated at 1 h intervals in anaesthetized rats and in the 5^{th} week in conscious animals using the standard formulae from measurements of the plasma and urinary concentrations of creatinine and urine flow rate.

2.7.4 Terminal studies

At the end of the 5 week experimental period, all animals were sacrificed by exposing to halothane for 3 min via a gas anaesthetic chamber (100 mg/kg). Thereafter, livers, hearts, kidneys and gastrocnemius muscles were removed, snap frozen in liquid nitrogen and stored in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at -70 °C until use. Blood was collected from all rats by cardiac puncture into individual pre-cooled heparinized containers at the end of the 5-week experimental period and centrifuged using an eppendorf centrifuge 5403, Mazian Med Equip, Germany for 15 min at 3500 rpm G x 100 at 4 °C to separate the plasma. Plasma was frozen in a Bio Ultra freezer (Snijers Scientific, Holland, -80 °C) until used for different assays. Assays include measurements of electrolytes ($Na^{+,}$ K^+, Cl^- and Li^+), creatinine and urea.

2.7.5. Biochemical measurements

2.7.5.1 Evaluation of oxidative stress

To establish the effects of MA on oxidative stress in the liver, heart and kidney of STZ-induced diabetic rats, we compared levels MDA, a commonly known marker of oxidative stress and of antioxidant defense enzymes SOD and GPx between non-diabetic control rats and MA-treated diabetic animals. All organs were analyzed for protein content in addition to other biochemical parameters. The protein content was quantified using the Lowry method (Lowry *et al.*, 1951) with 2mg/mL BSA as standard (0-1mg/mL). Kidney, liver and heart samples (0.5 g) homogenized using isolation buffer (1 mL). The homogenate (0.2 mL) was made up to 0.5 mL using distilled water and 5 ml alkaline reagent was then added and the mixture incubated for 15 min at 40˚C. After 15 min 0.5 mL of Folin Ciocalteu reagent (diluted 0.5 with deionized water) was added. The absorbances were read at 600 nm after standing for 30 min. An alkaline reagent consisted of 100 volumes of 4% sodium carbonate, 1 volume of 4% coppers sulphate and 4% of sodium potassium tartrate. A calibration curve was set up using BSA standards.

2.7.5.2 MDA

Tissues (50 mg) were homogenised in 500µl of 0.2% phosphoric acid. The homogenate was centrifuged at 400 x G for 10 minutes. Thereafter, 400 µL of the homogenate was supplemented with 400µL 2% phosphoric acid and then separated into two glass tubes, each receiving equal volumes of the solution. Subsequently, 200 µL of 7% phosphoric acid was added into both glass tubes followed by the addition of 400 µL of thiobarbaturic acid (TBA)/butylated hydroxytoluene (BHT) into one glass tube (sample test) and 400µL of 3mM hydrochloric acid (HCl) into the second glass tube (blank). To ensure an acidic pH of 1.5, 200 µL of 1M HCl was added to sample and blank test tubes. Both solutions were heated at 100^{o}C for 15 minutes, and allowed to cool to room temperature. Butanol (1.5 mL) was added to the cooled solution; the sample was vortexed for 1 minute to ensure rigorous mixing and allowed to settle until 2 phases could be distinguished. The butanol phase (top layer) was transferred to eppendorf tubes and centrifuged at 13200xg for 6 minutes. The samples were aliquoted into a 96-well microtitre plate in triplicate and the absorbance was read at 532nm (reference λ 600nm) on a BioTek µQuant spectrophotometer (Biotek

Johannesburg, South Africa). The absorbances from these wavelengths were used to calculate the concentration of MDA using Beer's Law.

$$[MDA]\ (nmol\ g^{-1}) = \frac{Average\ absorbance}{Absorption\ coefficient\ (156\ mM^{-1})}$$

2.7.5.3 SOD

SOD activity was assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine in a convenient 96 well format. SOD activity was measured using the Biovision SOD Assay Kit according to manufacturers' instructions (BioVision Research Products, Mountain View, California, USA). Rat liver, kidney and heart tissues (50mg) were homogenized in ice cold 0.1M Tris /HCl (pH 7.4) containing 0.5% Triton X-100, 5mM β-mercaptoethanol (ME) and 0.1mg mL^{-1} phenylmethanesulfonylfluoride (PMSF). The tissue homogenate was centrifuged at 14000 x G for 5 minutes at 4ºC. The supernatant obtained was added to each sample (20µL) and blank 2 (20 µL) well, while blank 1 and blank 3 wells received 20 µl of H_2O. Thereafter, 200 µL of working solution was added to each well. Subsequently, dilution buffer (20 µL) was added to each blank 2 and blank 3 well, while each sample and blank 1 well received enzyme working solution (20 µL). The solutions were mixed thoroughly before reading the plate. Inhibition activity of SOD was measured on Anthos Venytch-200 Spectrophotometer (Biochrom limited, Cambridge, United Kingdom) after a reaction period of 20 minutes at 37 ºC.

$$SOD\ activity\ (nmol\ min^{-1}\ mL^{-1}) = \frac{(A\ blank\ 1 - A\ blank\ 3)\ -\ (A\ sample\ 1 -\ A\ blank\ 2)}{(A\ blank\ 1 - A\ blank\ 3)} \times 100$$

2.7.5.4 GPx

Glutathione peroxidase (GPx) is an antioxidant enzyme which reduces liquid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water through the oxidation of reduced glutatione (GSH) to oxidized glutathione (GSSG). GPx activity was measured in rat liver, kidney and heart tissues using the Biovision GPx Assay Kit according to manufacturers' instructions

(BioVision Research Products, Mountain View, USA). The tissues (50 mg) were homogenized on ice in cold assay buffer (0.2 mL) and subsequently centrifuged at 10000 x G for 15minutes at 4°C. The resultant supernatant (100 µL) was loaded into a 96-well plate in duplicate. The NADPH standard curve was prepared by diluting the 1mM NADPH standard through a series of concentrations (0, 20,40,60,80, 100 nmol per well). The optical density of the standards (OD) was measured at 340nm using Anthos Venytch-200 Spectrophotometer (Biochrom limited, Cambridge, United Kingdom) and the standard curve was constructed from the values obtained. A reaction mix (90 µL) containing assay buffer, NADPH, glutathione reductase and GSH was added into each sample well, mixed thoroughly and incubated for 15 minutes at room temperature. The OD was then measured at 340 nm followed by the addition of cumene hydroperoxide (10 µL) and measurement of OD (T1) and another reading following a 5 minute incubation in the dark (25°C). GPx activity was calculated using following equation: GPx activity:

$$Px\ activity\ (nmol\ min^{-1}mL^{-1}) = \frac{(B\ -\ B^{0})}{(T2\ -\ T1)\ \times\ V}\ \times sample\ dilution$$

Where: B - NADPH amount that was decreased between T1 and T2

B° - background change without cumene hydroperoxide between T1 and T2

T1 - time of first reading

T2 - time of second reading

V- pretreated sample volume added into the reaction well

2.7.6 GLUT1, GLUT2 and GLUT4

To evaluate the mechanism through which MA exerts its effects on blood glucose concentrations, expression of GLUT 1, 2 and 4 was investigated in kidney samples. Harvested organs from five week study were used to further elucidate mechanism through which MA exert blood glucose lowering effects**.** Kidney tissues were analyzed for GLUT1 and GLUT2 content while GLUT 4 was analysed in gastrocnemius muscles. The tissue (0.1g) was homogenized on ice in isolation buffer (0.5mM Na_2EDTA, 0.1m KH_2PO_4, 0.1mM dithiothreitol, 0.25M sucrose) and then centrifuged at 400xG for 10 minutes (4°C). The protein content was quantified using the Lowry

method described above and all the samples were standardized to one concentration (1 mg/mL) for consistency. The proteins were then denatured by boiling in laemmli sample buffer (0.5M Tris-HCl, glycerol, 10% sodium dodecyl sulphate (SDS), 2-mercaptoethanol, 1% bromophenol blue) for 5min. The denatured proteins were loaded (25µL) on prepared resolving (7%) and stacking (4%) polyacrylamide gels along with molecular weight marker (5µL). The gel was electrophoresed for 1 h in electrophorese at 150V for 1 h in electrode (running) buffer (tris base, glycine, SDS), pH 8.3. Following electrophoresis, the resolved proteins were electro-transferred to an equilibrated polyvinylidene difluoride (PVDF) / nitrocellulose membrane for one hour in transfer buffer (192mM glycine, 25mM tris, 10% methanol). After transfer, the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS) (20mM tris, 150mM NaCl, KCl, 0.05% Tween-20). The membrane was then immuno-probed with monoclonal anti-GLUT-1/2 (1:1 000 in 1% BSA, Neogen, USA) for 1 h at room temperature (RT). The PVDF membrane was then subjected to 5 washes (10 minutes each with gentle agitation) with TTBS. Following which, the membrane was incubated in horse radish peroxidase (HRP)-conjugated secondary antibody (rabbit anti-mouse 1:10 000; Bio-Rad) for 1 hour at RT. After further washing, antigen-antibody complexes were detected by chemiluminescence using the Immune-star™ HRP substrate kit (Bio-Rad). Chemiluminescent signals were detected with the Chemi-doc XRS gel documentation system and analysed using the quantity one software (Bio-Rad, Johannesburg, South Africa). Band intensity analysis was conducted on the resultant bands.

2.7.7 AVP assay

A standard enzymatic method was used to determine plasma AVP concentrations. The assays were performed on an Arg^8-Vasopressin ELISA Kit, using reagents purchased from the manufacturer (Abcam, Cambridge, Massachusetts, USA). The lower and upper limits of detection were 4 pmol/L - 923 pmol/L respectively. The intra assay analytical coefficient of variation ranged from 5.9 - 10.6 % and the inter-assay coefficient variation from 6.0 - 8.5 %. The kit components included a 96 well plate coated goat antibodies, vasopressin conjugate, vasopressin antibody, assay buffer, wash buffer concentrate, vasopressin standards, p-nitrophenyl phosphatesubstrate (pNpp) and a stop solution. The Arg^8-Vasopressin ELISA Kit is a competitive immunoassay for the quantitative determination of vasopressin in samples. The assay uses a polyclonal antibody-vasopressin

conjugate to bind covalently in a competitive manner with vasopressin in unknown samples. During the incubation period AVP in the sample reacts with phosphatase-conjugate anti-vasopressin antibodies and anti-vasopressin antibodies bound to the micro-titration well. The washing step removes unbound enzyme labelled antibody, leaving the bound conjugate which reacts with pNpp. This reaction is stopped by adding an acid to give a colorimetric endpoint that is read spectrophotometrically on a microplate reader (BMG Labtech GmbH, Ortenberg, Germany) at 405 nm.

A volume of 100 µL of vasopressin standards (0.29, 0.58, 1.15, 2.30 and 4.6 pmol/L) was added into anti-vasopressin wells. Samples (100 µL) were then added to the remaining wells followed by 50 µL vasopressin conjugate into all standard and sample wells. The plates were incubated at 4 ºC for 24 hrs. Following incubation, the reaction volume was emptied. 400 µL of wash buffer was added to all wells and aspirated. The process was repeated 3 times. After the final wash, the plates were inverted firmly against absorbent paper to remove all the liquid. 200 µL of substrate pNpp was added to all wells and incubated at 37 ºC for 1 h. The reaction was stopped by adding 50 µL of stop solution to all wells and mixing on the shaker for 5 minutes. The absorbance was read at 405 nm using a Spectrostar Nano microplate reader (BMG Labtech GmbH, Ortenberg, Germany). A standard curve was constructed from the absorbance values of the known concentrations of the standards using the spline regression curve in GraphPad Prism Instat Software (version 5.00). The respective AVP concentrations of the unknown samples were then extrapolated from the standard curve.

2.7.8 Aldosterone assay

The kit components included a 96 well plate coated with a polyclonal rabbit antibody, aldosterone conjugate, wash buffer concentrate, aldosterone standards, and a stop solution. The aldosterone ELISA kit is a solid phase enzyme-linked ELISA based on the principle of competitive binding. The microtiter wells are coated with a polyclonal rabbit antibody directed towards an antigenic site of the aldosterone molecule. Aldosterone in the sample competes with an aldosterone-horseradish peroxide conjugate for binding to the coated antibody. After incubation the unbound

conjugate is washed off. After addition of the substrate solution, the intensity of the colour is inversely proportional to the concentration of aldosterone in the sample.

The assay procedure was as follows, 50 µL of each standard (0.11, 0.22, 0.43, 0.86, 1.73, 3.46 and 6.92 pmol/L), control and samples were dispensed into appropriate wells, 150 µL of the aldosterone conjugate was then added into each well followed by mixing for 10 minutes and then incubation for 1 h at room temperature. The contents of the wells were shaken out and rinsed with 300 µL of wash buffer was added to all wells and aspirated. The process was repeated 3 times. After the final wash, the plates were inverted firmly against absorbent paper to remove all the liquid followed by incubation of 30 minutes at room temperature. The reaction was stopped by adding 100 µL of the stop solution to all well. The absorbance was read at 405 nm using a Spectrostar Nano microplate reader (BMG Labtech GmbH, Ortenberg, Germany). A standard curve was constructed from the absorbance values of the known concentrations of the standards using the spline regression curve in GraphPad Prism Instat Software (version 5.00). The respective AVP concentrations of the unknown samples were then extrapolated from the standard curve.

2.8 Statistical analysis

All data were presented as means or standard deviation (SEM). Statistical comparisons in different groups were performed by using one way analysis of variances (ANOVA), followed by Tukey-Kramer multiple comparison test. All statistical analysis were performed with Graph-pad InStat Software (version 5, GraphPad Software, San Diego, California USA). A value of $p<0.05$ was considered statistically significant.

CHAPTER 3

RESULTS

3.1 Structure elucidation

3.2 MA and OA

The percentage yield of MA obtained from EAS (10 g) varied from 0.02 to 0.03%. The carbon signals at 68.8 ppm, 121.9 ppm and 143.7 ppm correspond to carbon-OH (C-2) and carbon-carbon double bond (C-12 and C-13), respectively.

3.2.1 ^{13}C NMR of MA

The data below depicts different carbon positioning for MA dissolved in deuterated methanol.^{13}C NMR (400 MHz,CD_3OD): 46.2 (C-1), 68.3 (C-2), 83.3 (C-3), 39.1 (C-4), 55.0 (C-5), 18.1 (C-6), 32.7 (C-7), 39.0 (C-8), C-9 (47.4), C-10 (38.0), C-11 (23.2), C-12 (121.9), C-13 (143.7), C-14 (41.6), C-15 (27.4), C-16 (23.0), C-17 (46.2), C-18 (41.0), C-19 (45.7), C-20 (30.4), C-21 (33.6), C-22 (32.3), C-23 (28.3), C-24 (16.6), C-25 (16.5), C-26 (16.4), C-27 (23.2), C-28 (178.5), (C-29) 32.2, C-30 (23.2). For supporting information (see appendices). The purity of the plant-derived MA was approximately 98%.

3.2.2 ^{13}C NMR of OA

The data below depicts different carbon positioning for OA dissolved in deuterated methanol **^{13}C NMR (400 MHz, $CDCl_3$):** δ 183.5, 143.8, 122.8, 79.2, 55.4, 47.8, 46.7, 46.1, 41.8, 41.2, 39.4, 38.9, 38.6, 37.3, 34.0, 33.2, 32.8, 32.6, 31.8, 28.3, 27.9, 27.3, 26.1, 23.6, 23.1, 22.9, 18.5, 17.3, 15.7, 15.5

3.3 MA derivatives

3.3.1 ^{1}H NMR $CDCl_3$ of Bromobutane derivative

^{1}H NMR data below depicts different proton positioning for MA dissolved in deuterated chloroform. δ, 0.71, 0.80, 0.88, 0.91, 0.96, 1.01, 1.1 (each 3H, s), 2.86 (1h, dd, *J* = 4.5), 2.97 (1H,

d, J = 9.7), 3.40 (2H, t, J = 6.7), 3.70 (1H, m), 4.05 (2H, t, *J* = 6.3), 5.27 (1H, t, *J* = 4.0). The percentage yield of this derivative was 50% after recrystallization from methanol and chloroform (1:1 v/v).

3.3.2 ^{1}H NMR $CDCl_3$ of Bromohexane derivative

^{1}H NMR data below depicts different proton positioning for bromohexane derivative dissolved in deuterated chloroform. δ, 0.71, 0.80, 0.88, 0.90, 0.96, 1.04, 1.11 (each 3H, s), 2.85 (1H, d, J = 9.51), 3.39 (2H, t, J = 6.7), 3.67 (1H, m), 4.00 (2H, dd, J = 6.6), 5.26 (1H, t, J = 3.5). Data indicated that (2H, t, *J* = 6.7), 3.67 (1H, m), 4.00 (2H, dd, *J* = 6.6), 5.26 (1H, t, *J* = 3.5) indicating that two more hydrogens were introduced hence verifying that this was indeed a bromohexane derivative. A yield of 50% was obtained after recrytallisation from methanol and chloroform (1:1 v/v) and pure compound was obtained as white needles

3.3.3 Oxidised OA

^{1}H NMR data below depicts different proton positioning of oxidised OA dissolved in deuterated chloroform. ^{1}HNMR $CDCl_3$, δ 0.80, 0.88, 0.91, 1.01, 1.02, 1.06, 1.13, (each 3H, s). 1.2-2.0 (m, 21), 2.30-2.38 (m, 21), 2.48-2.57 (m, 2), 5.28 (br, s 1). White needles were obtained after recrystallisation affording 100% yield. ^{1}H NMR data was comparable to previously oxidized OA.

3.3.4 ^{1}HNMR $CDCl_3$ of Phenylhydrazine (PH-MA) derivative

^{1}H NMR data below depicts different proton positioning for phenylhydrazine (PH-MA) derivative dissolved in deuterated chloroform. ^{1}HNMR $CDCl_3$, δ 0.85. 0.90, 0.94, 1.16, 1.17, 1.24, 1.27 (each 3H, s), 2.21 (IH, d, *J* = 14.5), 2.77 (1H,d, *J* = 14.7), 2.87 (1H, m,), 5.28 (1H, s,), 7.05 (1H, m), 7.10 (1H,m), 7.29 (1H, d, *J* = 7.8), 7.40 (1H, d, *J* = 7.5), 7.69 (1H, s, NH). The total of 80% was obtained for phenyl-hydrazine derivative, and brown particles were obtained.

3.3.5 ^{1}HNMR $CDCl_3$ of chloro-phenyl hydrazine derivative

^{1}H NMR data below depicts different carbon positioning of choloro-phenylhydrazine (PH-MA) derivative dissolved in deuterated chloroform**.** ^{1}HNMR $CDCl_3$, δ 0.84, 0.92, 0.94, 1.16, 1.17, 1.24, 1.27 (each 3H, s), 2.17 (1H, d, *J* = 1.44) 2.73 (1H, d, *J* = 14.79), 2.87 (1H, m), 5.37 (1H, m), 6.99 (1H, m), 7.01 (IH, m), 7.29 (1H, d, *J* =8.45), 7.7 (1H, s), 8.0 (1H, s). The total of 75% yield was obtained for 3-phenyl-hydrazine derivative in a form of brown particles.

3.3.6 ^{1}HNMR $CDCl_3$ of 3- flouro-phenyl hydrazine

^{1}H NMR data below depicts different proton positioning for 3-flouro-phenylhydrazine (F-PH) derivative dissolved in deuterated chloroform. δ 0.85. 0.90, 0.94, 1.16, 1.17, 1.24, 1.27 (each 3H, s), 2.21 (IH, d, *J* = 14.5), 2.77 (1H,d, *J* = 14.7), 2.87 (1H, m,), 5.28 (1H, s,), 7.05 (1H, m), 7.10 (1H,m), 7.29 (1H, d, *J* = 7.8), 7.40 (1H, d, *J* = 7.5), 7.69 (1H, s, NH). Yield (86%) as a yellow solid (3-flouro phenyl hydrazine derivative) was obtained from recystallisation.

3.4 Cell viability studies

3.4.1 Effect of MA on MDBK and Chang cells

Figure 12 shows percentage viability of MDBK and Chang cell lines after treatment with either MA or PH-MA. The viability of all controls remained stable at 100 % from 12-48 h experimental period. All MA doses exerted no significant effects on cell viability of MDBK cells after 12 and 24 h treatment (Figure 12 A). However, MA (80 µmol/L) significantly ($p < 0.05$) increased cell viability of MDBK cells after 48 h treatment by comparison with the control. Administration of MA (40 and 80 µmol/L) also exerted no significant effects on viability of Chang cells after 12 and 24 h treatment period. However, the highest dose (160 µmol/L) significantly ($p<0.05$) increased cell viability by comparison with the control (Figure 13 B). Administration of PH-MA (40 and 80 µmol/L) significantly increased viability of MBK cells after 12 h treatment, however, doses 80 and 160 µmol/L significantly ($p<0.05$) decreased cell viability by MDBK cells by comparison with respective controls (Figure 13 A). All PH-MA doses significantly ($p< 0.05$) decreased cell viability of MDBK cells after 48 h treatment. However, PH-MA showed no significant changes in cell viability of Chang cells at all-time intervals (Figure 13 B).

3.4.2 Effects of MA on glucose utilisation

Glucose utilisation by MDBK and Chang was investigated following exposure of these cell lines to different concentrations of MA or PH-MA. Results indicate that MA exerted no effects on glucose utilisation from 12 to 24 h treatment of MDBK cells. However, a significant ($p<0.05$) decrease in glucose utilisation at 48 h MA treatment (80 and 160 µmol/L) on MDBK cells (Figure 14 A). In contrary, Chang cells showed no significant change on glucose utilisation following treatment with various doses of MA (Figure 14 B). PH administration showed no significant effects on glucose utilisation by MDBK cells (Figure 15 A). However, all PH-MA doses significantly increased glucose utilisation from 24-48 h treatment period. These results were comparable to that of metformin (Figure 15 B).

3.4.3 Effects of MA and PH on ENaC expression

Effects of MA and PH-MA on ENaC expression were evaluated using Western blot analysis. Cells were harvested after 48 h of treatment with MA. Cells treated with PH were harvested after 12 h since this triterpene showed deleterious effects on viability after 24 and 48 h. Harvested cells were then used to evaluate expression of these transporters. Results indicate that treatment with various doses of MA significantly ($p<0.05$) decreased expression of ENaC by comparison with the control (Figure 16). Administration of PH-MA significantly ($p<0.05$) decreased expression of ENaC by comparison with control. However, no significant change was observed in cells treated with metformin.

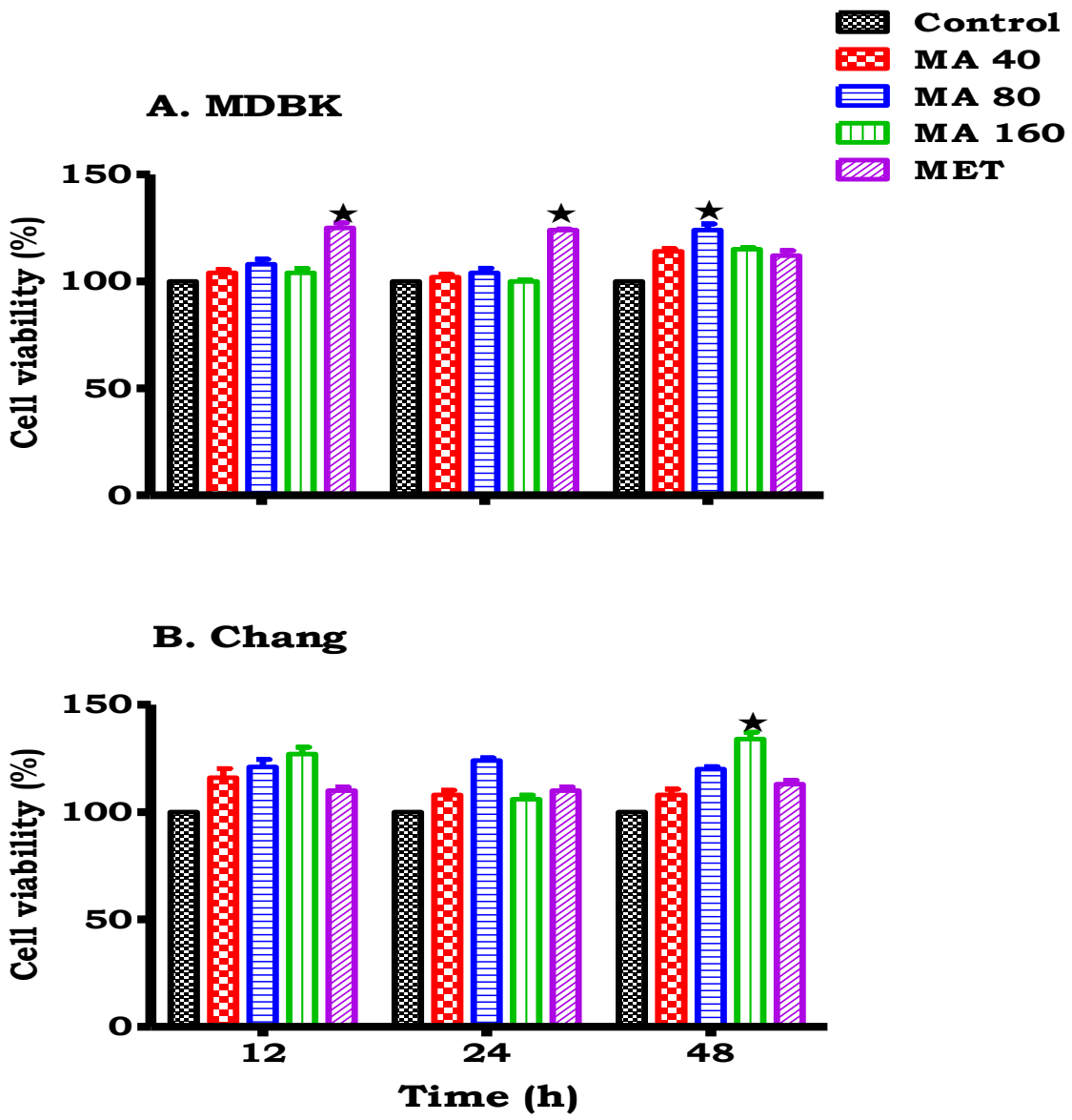

Figure 12: Viability of MDBK (A), and Chang (B) cell lines exposed to different doses of MA at 12, 24 and 48 h time periods. Values are presented as means, and vertical bars indicate SEM (n=6 in each group.★ $p<0.05$ by comparison with controls at each time corresponding time.

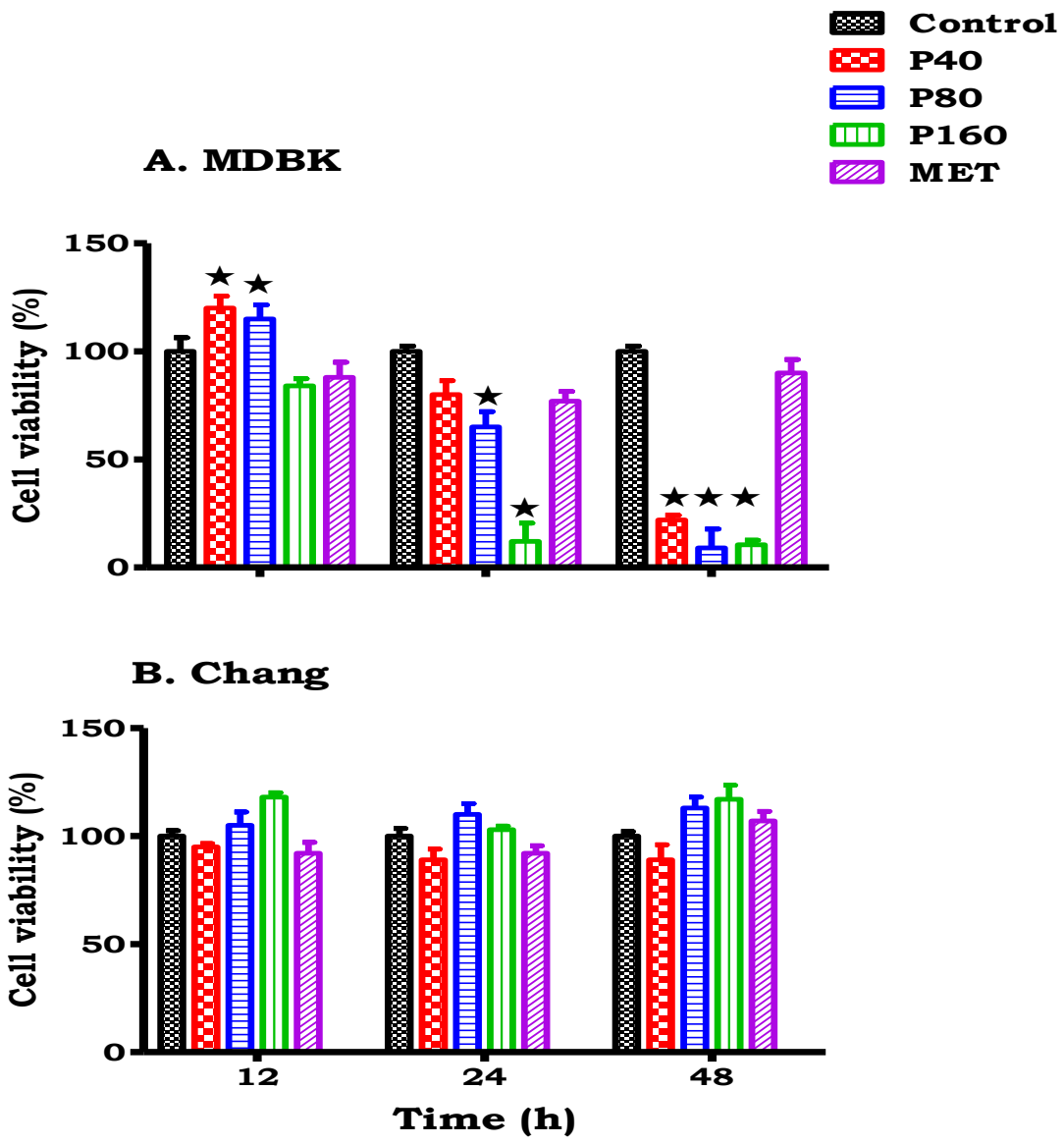

Figure 13: Viability of MDBK (A) and Chang (B) cell lines exposed to different doses of PH-MA at 12, 24 and 48 h time periods. Values are presented as means, and vertical bars indicate SEM (n=6 in each group.★ $p<0.05$ by comparison with controls at each time corresponding time.

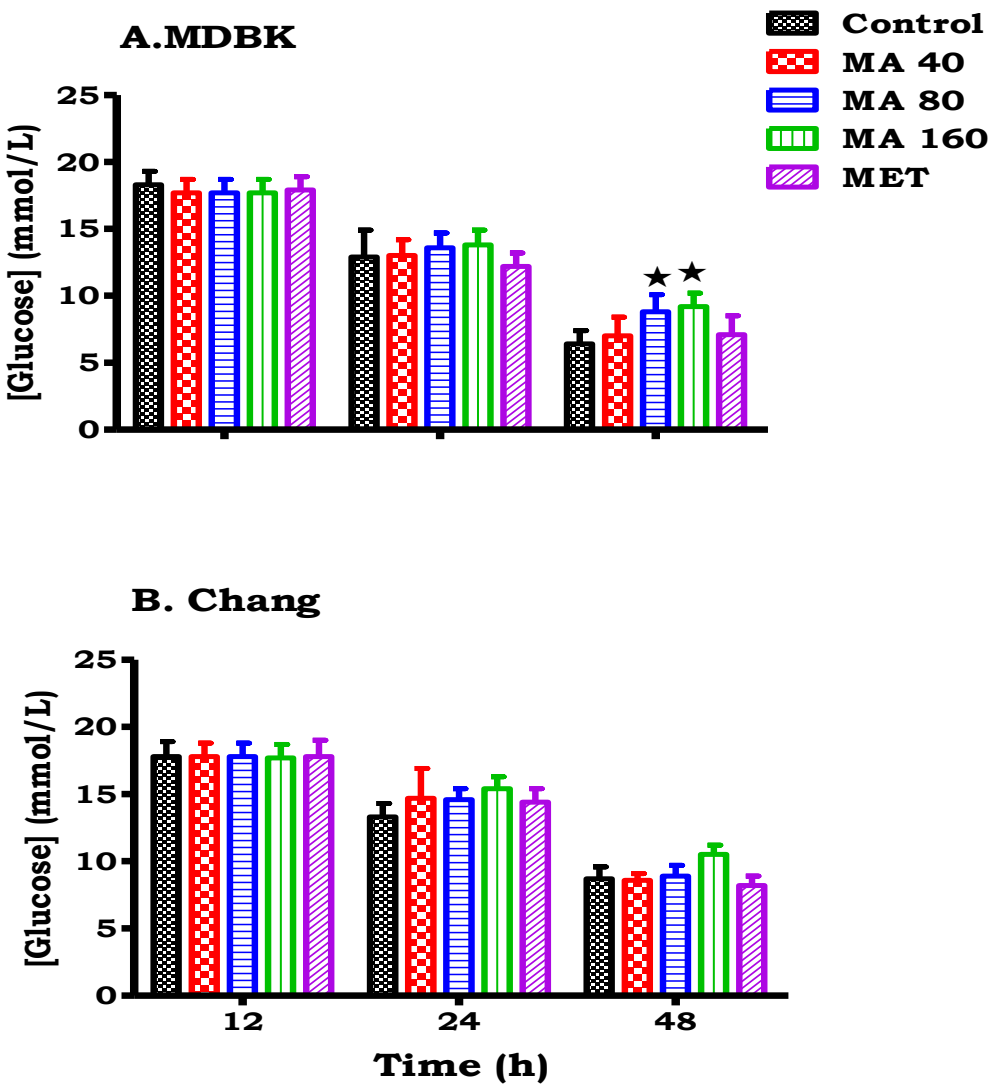

Figure 14: Effects of MA on glucose utilisation on MDBK (A) and Chang cells (B) following treatment at 12, 24 and 48 h. Cell lines were exposed to 19 mmol of glucose prior the experiment Values are presented as means, and vertical bars indicate SEM (n=6 in each group. ★ $p<0.05$ by comparison with controls at each corresponding time.

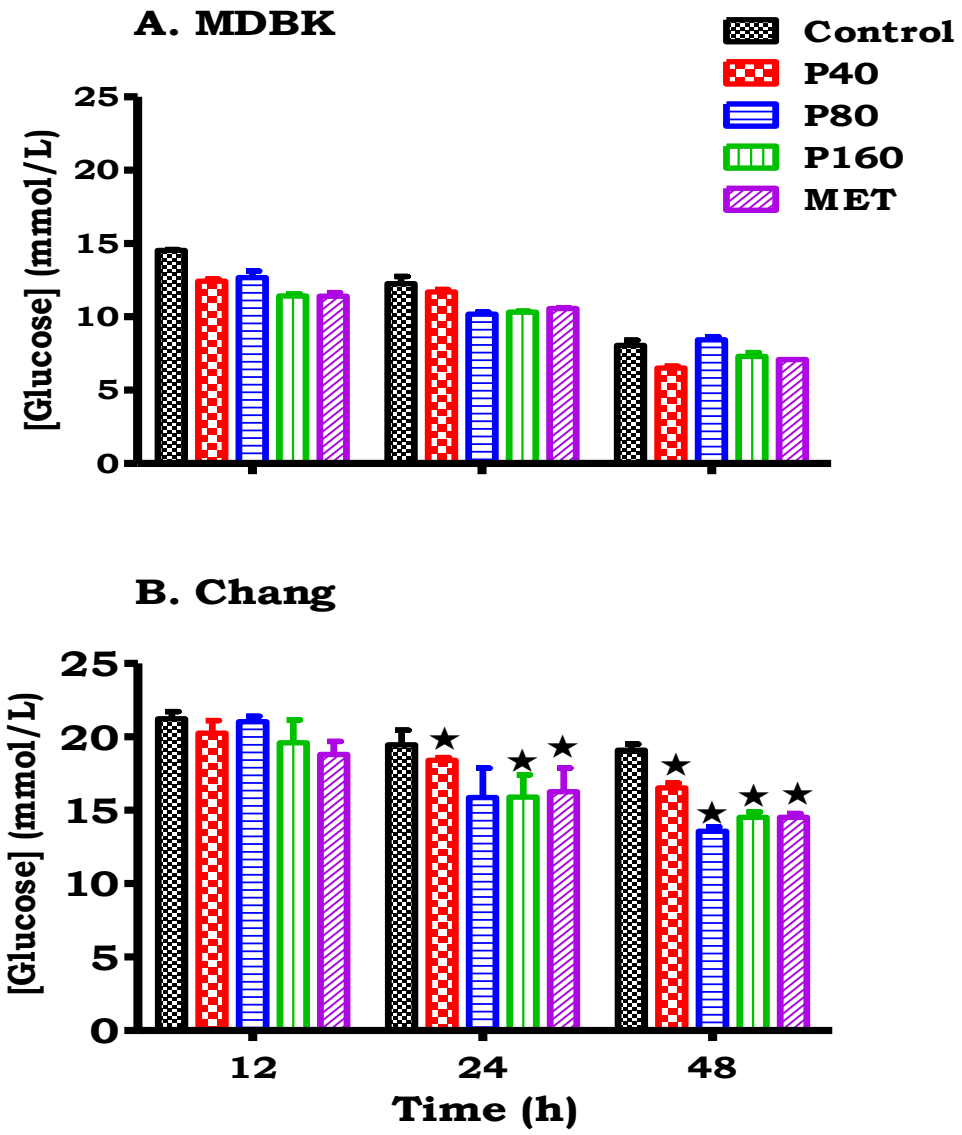

Figure 15: Effects of PH-MA on glucose utilisation on MDBK (A) and Chang (B) cells following treatment at 12, 24 and 48. Cell lines were exposed to 19 mmol of glucose prior the experiment. Values are presented as means, and vertical bars indicate SEM (n=6 in each group. ★ $p<0.05$ by comparison with controls at each corresponding time.

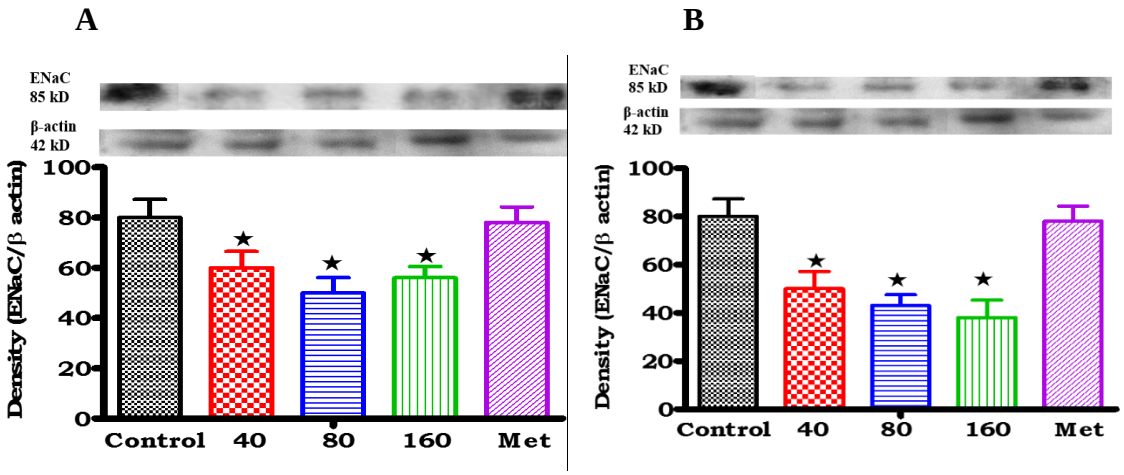

Figure 16: Effects of MA (A) and PH-MA (B) on expression of epithelial sodium channel γ-ENaC transport protein as determined by Western blotting. Groups were divided into control, MA, PH and metformin-treated MDBK cells**.** ★ $p<0.05$ by comparison with the control (n=6).

3.5 Acute effects on kidney function

3.5.1 Effects of MA on proximal tubular function

The effects of MA and derivatives on proximal tubular function were investigated in anaesthetized non-diabetic and STZ-induced diabetic rats. The animals were challenged with hypotonic saline after 3½ h equilibration for 4 h of 1 h control, 1½ h treatment and 1 ½ h recovery periods. Figure 17 shows urine flow and urinary excretion rate of Na^+, K^+ and Cl^- of control and MA treated animals. STZ-induced diabetic control rats exhibited lower urine flow and electrolyte excretion rates by comparison with non-diabetic rats. The urinary excretion Na^+ of control non-diabetic animals ranged from 711 to 756 µmol/h throughout the 4 h post experimental period compared to the infusion rate of 693 µmol/h. Infusion of MA (90 µg/h) during 1½ h treatment significantly ($p<0.05$) increased Na^+ excretion from the pre-treatment mean value of 650 to 818 µmol/h (Figure 17, A). However, treatment with MA did not change urinary excretion of K^+ and Cl^- (Figure 17, B-C). Figure 17 E shows that the urinary excretion Na^+ of untreated STZ-induced diabetic animals ranged from 290 to 313 µmol/h throughout the 4 h post experimental period. Infusion of MA (90 µg/h) during 1½ h treatment significantly ($p<0.05$) increased Na^+ excretion from the pre-treatment mean value of 304 to 431 µmol/h. MA, however, did not change urinary K^+ and Cl^- excretion rates by comparison with control animals (Figure 17, F-G).

3.5.2 Effects of MA derivatives (PH-MA, F-PH and Cl-PH) on proximal tubular function

The infusion of PH-MA at 22 µg/h significantly ($p<0.05$) increased Na^+ excretion of non-diabetic and STZ-induced diabetic rats (696 to 1154 and 312 to 477 µmol/h, respectively) (Figure 18, A and E respectively). Infusion of F-PH and Cl-PH also significantly ($p<0.05$) increased Na^+ excretion in non-diabetic rats, however, Cl-PH resulted in significant decrease in Na^+ during recovery period (Figure 19, A and D). The Effects of Cl-PH and F-PH were only investigated in non-diabetic animals due to limited sample size. PH-MA, Cl-PH and F-PH did not change urine flow, urinary K^+ excretion.

3.5.3 Cumulative data

The mean total volume of urine and amounts of Na^+ K^+ and Cl^- excreted in 1 h 50 min of the treatment period are presented in Table 2. STZ- diabetic rats treated with MA or PH showed a significantly low urinary Na^+, K^+ and Cl^- by comparison with non-diabetic animals. However, PH (22 µg) was more effective in excreting urinary Na^+ when compared to MA (90 µg) in non-diabetic animals. In all MA or PH -treated groups, the total urine, K^+ and Cl^- excretion outputs were not significantly altered by any of the treatments.

3.5.4 Renal clearance

The effects of MA on proximal tubular Na^+ clearance were estimated by comparing renal lithium clearance (FE_{Li^+}) between anaesthetized non-diabetic, STZ-induced diabetic controls and MA-treated rats. These effects were only investigated on animals treated with MA and PH since they were more effective than the other triterpenes. Infusion of MA (90 µg/h) for 1½ h significantly ($p<0.05$) increased fractional excretion rates of sodium (FE_{Na+}) and lithium (FE_{Li+}) in non-diabetic and STZ-induced diabetic rats by comparison with respective controls (Figure 20). Like MA, infusion of PH (22 µg/h) for 1½ h significantly ($p<0.05$) increased fractional excretion rates of sodium (FE_{Na+}) and lithium (FE_{Li+}) in non-diabetic and STZ-induced diabetic rats by comparison with respective controls (Figure 21).

3.5.5 MAP and GFR

The MAP of control non-diabetic and untreated STZ-induced diabetic animals did not show any significant variations throughout the 4 h post equilibration period. For purpose of this study acute effects on MAP were investigated on MA treated animals only. The MAP values in untreated diabetic rats (119 to 120 mmHg) were higher than that of control non-diabetic rats (104 to 105 mmHg). However, MA administration significantly ($p<0.05$) decreased blood pressure in both groups of animals which persisted during 4 h post-treatment. Blood pressure decreased from 104 to 91 mmHg and 119 to 98 mmHg for non-diabetic and STZ-induced diabetic rats respectively (Figure 22). Figure 23 shows that the GFR values of untreated diabetic rats (range 0.39-0.45

mL/100 g) were lower than that control non-diabetic rats (range 0.76-0.80 mL min/100 g). MA and PH-MA administration for 1.5 h significantly increased (0.39 to 0.60 and 0.39 to 0.65 mL/min/100g respectively) GFR in STZ-induced diabetic rats. However, administration of PH-MA or MA did not alter GFR in non-diabetic animals (Figure 23).

3.6 Effects of MA on plasma hormones

3.6.1 AVP and aldosterone

Table 4 shows the concentration of plasma aldosterone and AVP measured in non-diabetic and STZ-diabetic rats following 1.5 h treatment period. Plasma AVP levels of untreated STZ-diabetic rats were significantly elevated (2.82 ± 0.47 to 4.45 ± 0.24 pmol/L) when compared to non-diabetic rats. Administration of MA or PH-MA during 1.5 h of treatment showed no significant difference in AVP levels for both non-diabetic and STZ-diabetic rats. Like AVP, aldosterone levels of untreated STZ-induced diabetic rats were significantly elevated (0.78 ± 0.04 to 1.45 ± 0.03 nm/L) when compared to non-diabetic animals. Interestingly, MA and PH-MA significantly ($p<0.05$) decreased aldosterone levels of STZ-induced diabetic rats when compared to the control group. However, MA or PH did not alter aldosterone levels on non-diabetic rats.

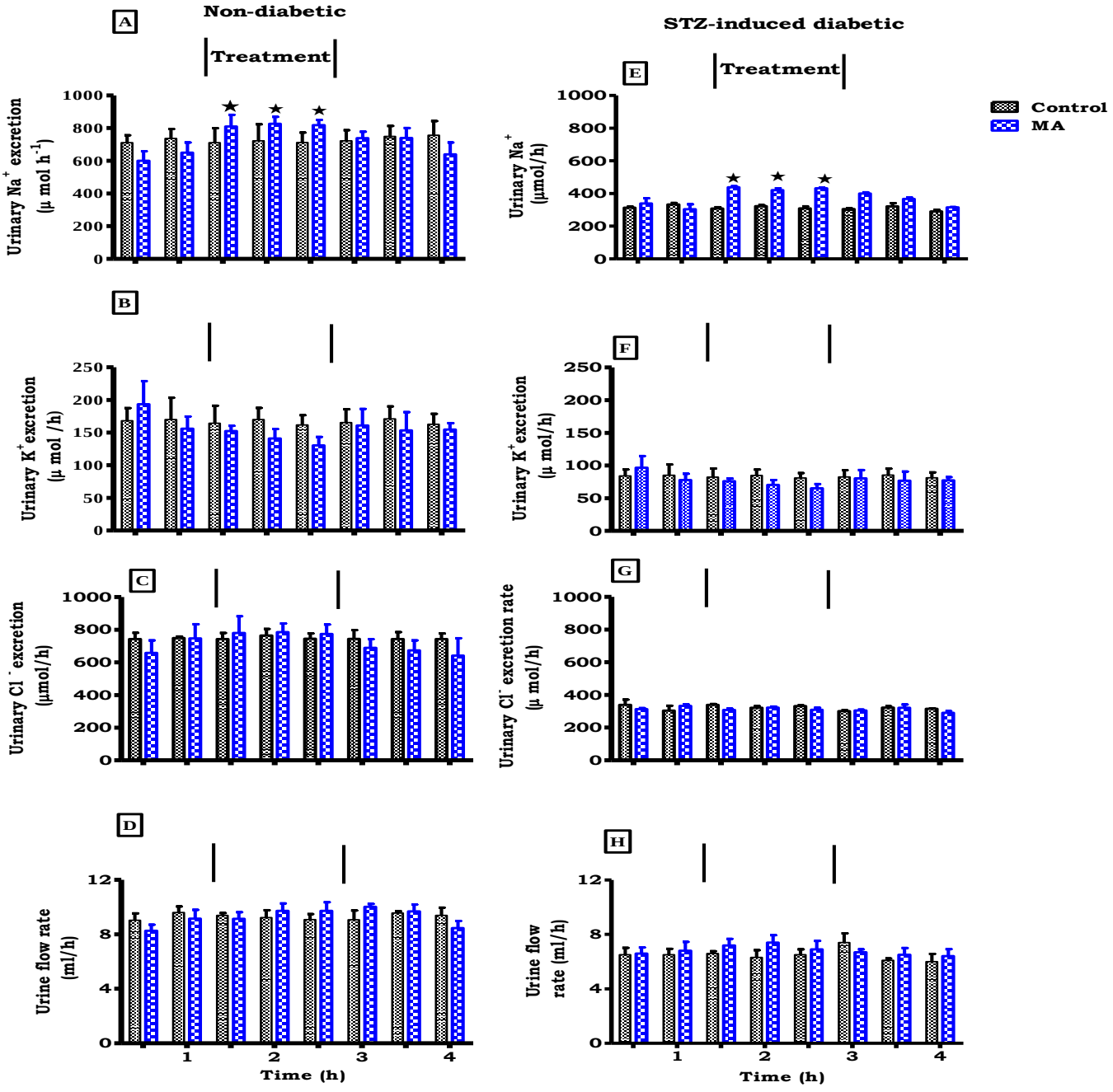

Figure 17: Comparison of the acute effects of MA administration of on urinary excretion rates of Na^+, K^+ and Cl^- and urinary output of non-diabetic rats (A–D) and STZ-induced diabetic rats (E–H) with respective control animals. MA was infused at 90 µg/h for 1.5 h during the treatment period. Values are presented as means for each 30 min collection; vertical bars indicate SEM of means (n=6 rats in each group).★ $p<0.05$ by comparison with control animals.

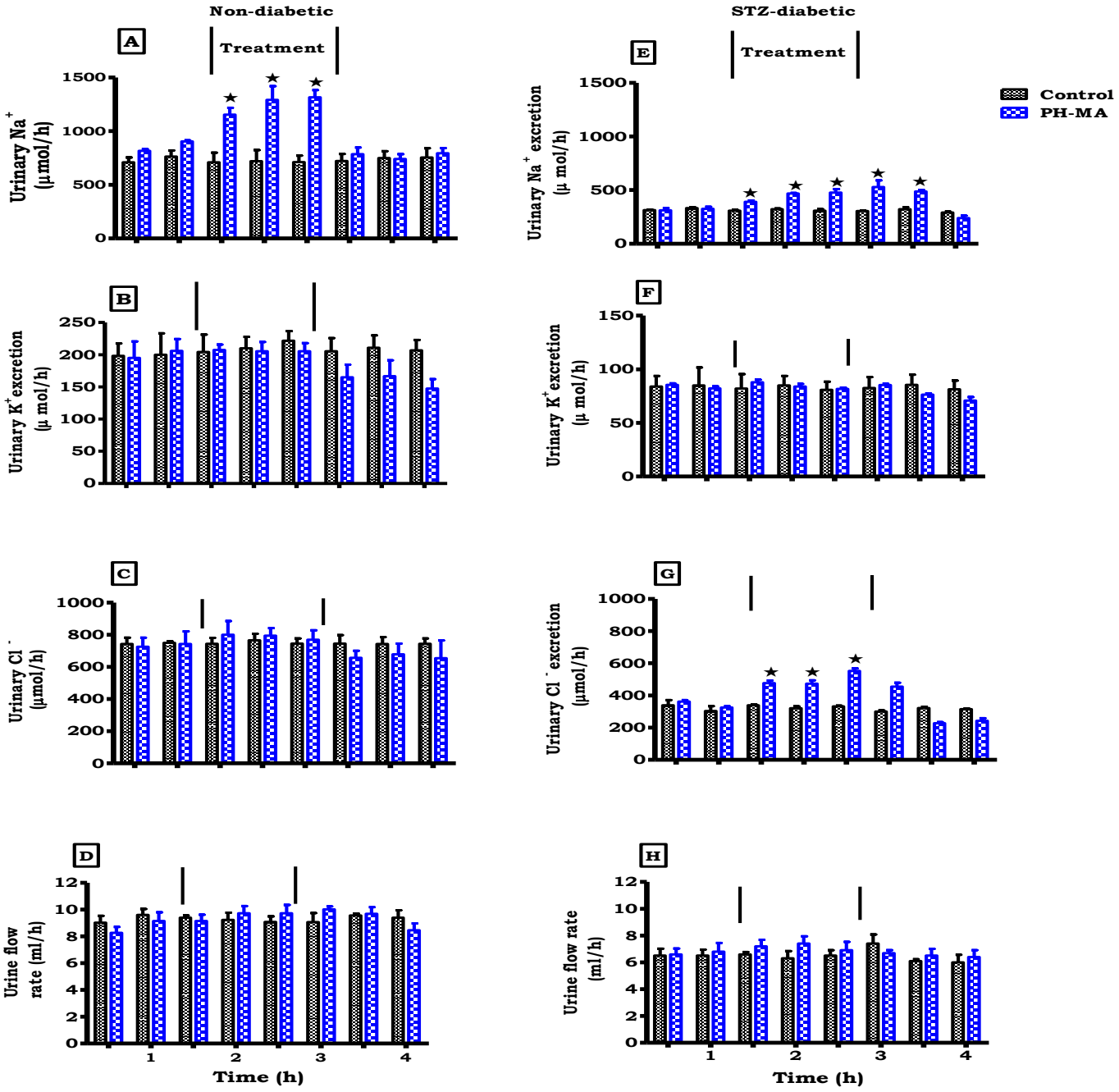

Figure 18: Comparison of the acute effects of PH-MA administration of on urinary excretion rate of Na^+, K^+ and Cl^- and urinary output of non-diabetic rats (A–D) and STZ-induced diabetic rats (E–H). PH was infused at 90 µg/h for 1.5 h during the treatment period. Values are presented as means for each 30 min collection; vertical bars indicate SEM of means (n=6 rats in each group). ★$p<0.05$ by comparison with control animals.

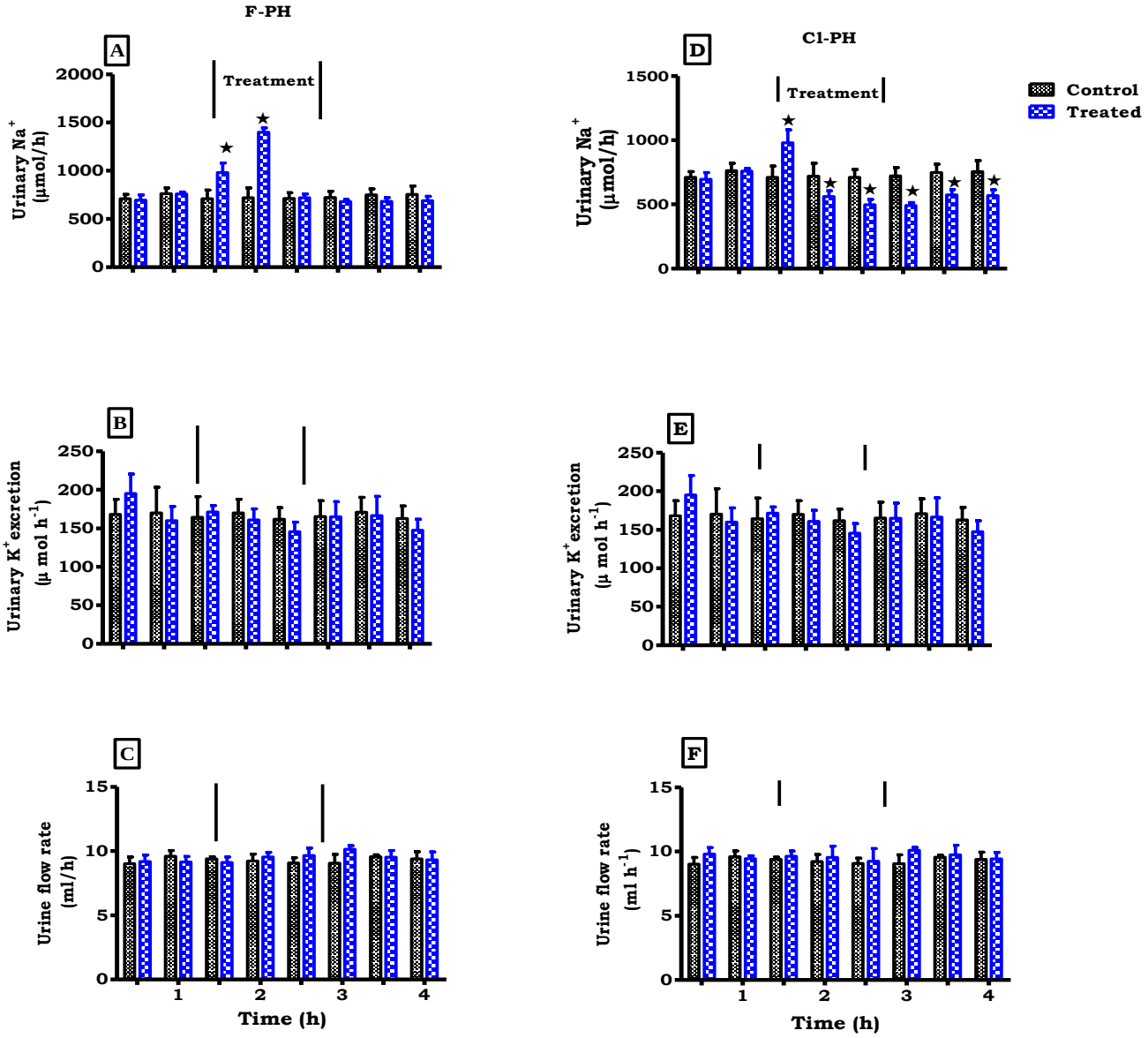

Figure 19: Comparison of the acute effects of F-PH (A-C) and Cl-PH (D-F) administration on urine flow, Na^+ and K^+ excretion rate of non-diabetic rats. F-PH and Cl-PH were infused at 22 µg/h for 1.5 h during the treatment period. Values are presented as means for each 30 min collection; vertical bars indicate SEM of means (n=6 rats in each group). ★ $p<0.05$ by comparison with control animals

Table 2: Comparison of the effects MA and PH-MA in 1.5 h on the total amounts of urine voided and Na^+, K^+ and Cl^- excreted during 1.5 h treatment of non-diabetic and STZ-induced diabetic rats with respective control groups. Data are expressed as mean ± SEM, n = 6 in each group.

Group		**UV (mL)**	**Na^+ (mmol)**	**K^+ (mmol)**	**Cl^- (mmol)**
Non-Diabetic	MA	14.10±0.06	1.22±0.02	1.22±0.02	1.18±0.02
	PH-MA	14.55±0.06	1.94* ±0.02	1.08±0.02	1.20±0.02
STZ-Diabetic	MA	13.50±0.06	0.24±0.08#	0.32±0.02 #	0.46±0.02#
	PH-MA	13.07±0.03	0.86* ±0.02#	0.31±0.01#	0.74±0.02#

* $p<0.05$ by comparison with respective individual groups (e.g. non-diabetic MA and non-diabetic PH-MA)

# $p<0.05$ by comparison between non-diabetic and STZ-diabetic groups

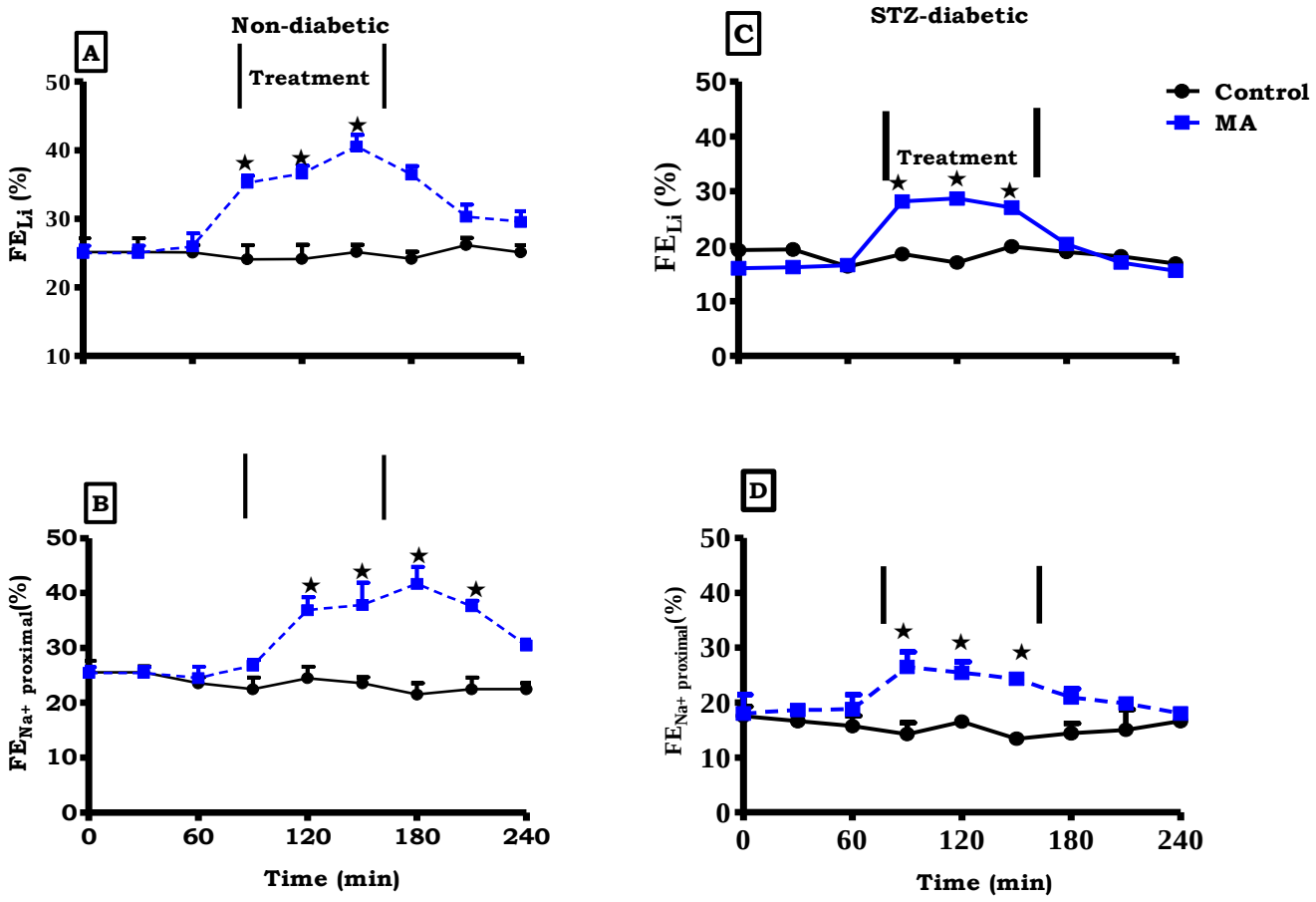

Figure 20: Effects of intravenously infused MA on FE_{Li+} and FE_{Na+} in non-diabetic (A-B) and STZ-induced diabetic (C-D) rats (n=6 in each group). Values are presented as means, and vertical bars indicate SEM (n=6 in each group).★$p<0.05$ by comparison with respective control.

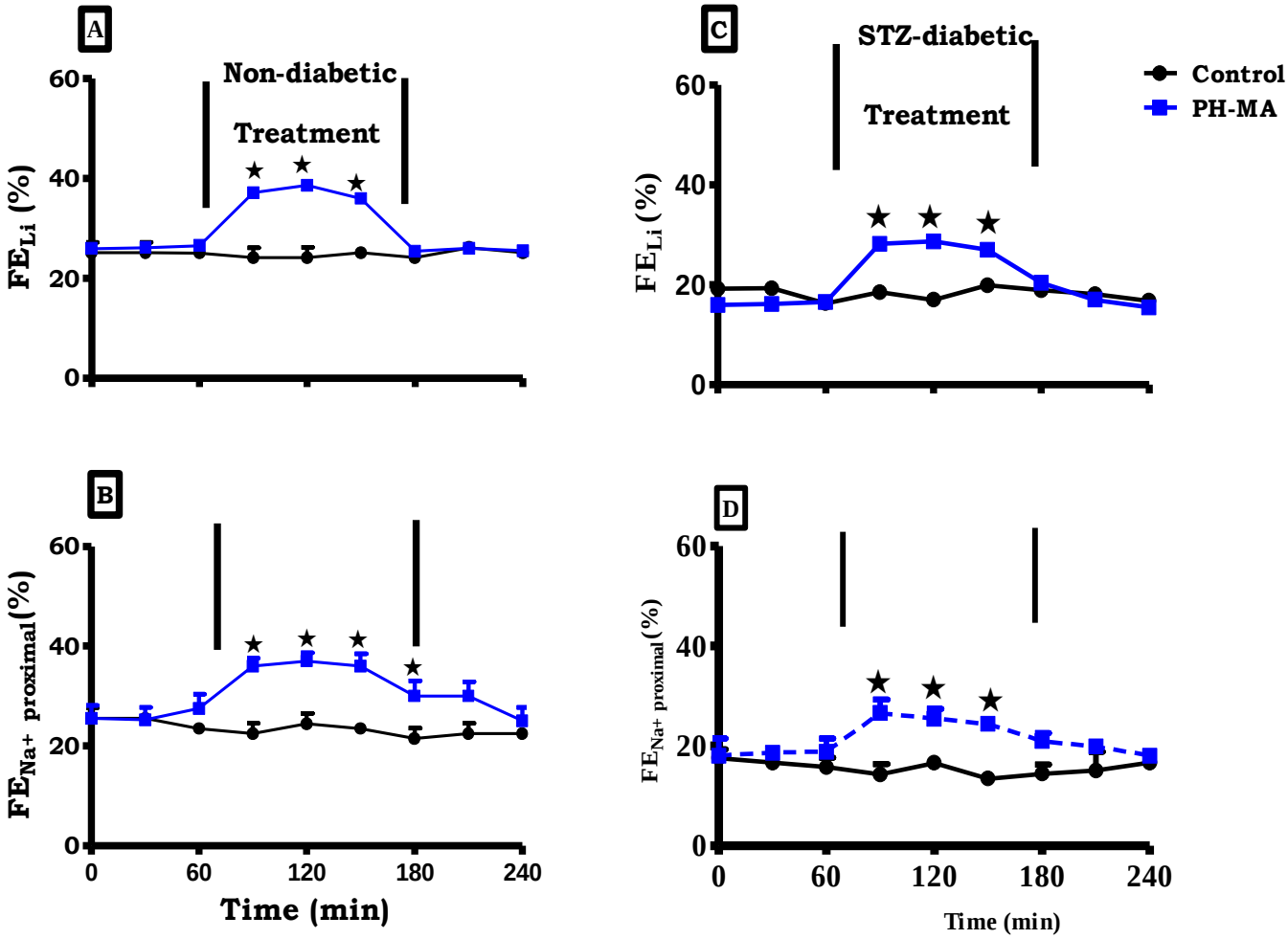

Figure 21: Effects of intravenously infused PH-MA on FE_{Na+} and FE_{Li+} in non-diabetic rats (A-B) and STZ-induced diabetic (C-D) (n=6 in each group). Values are presented as means, and vertical bars indicate SEM (n=6 in each group).★ $p<0.05$ by comparison with respective control.

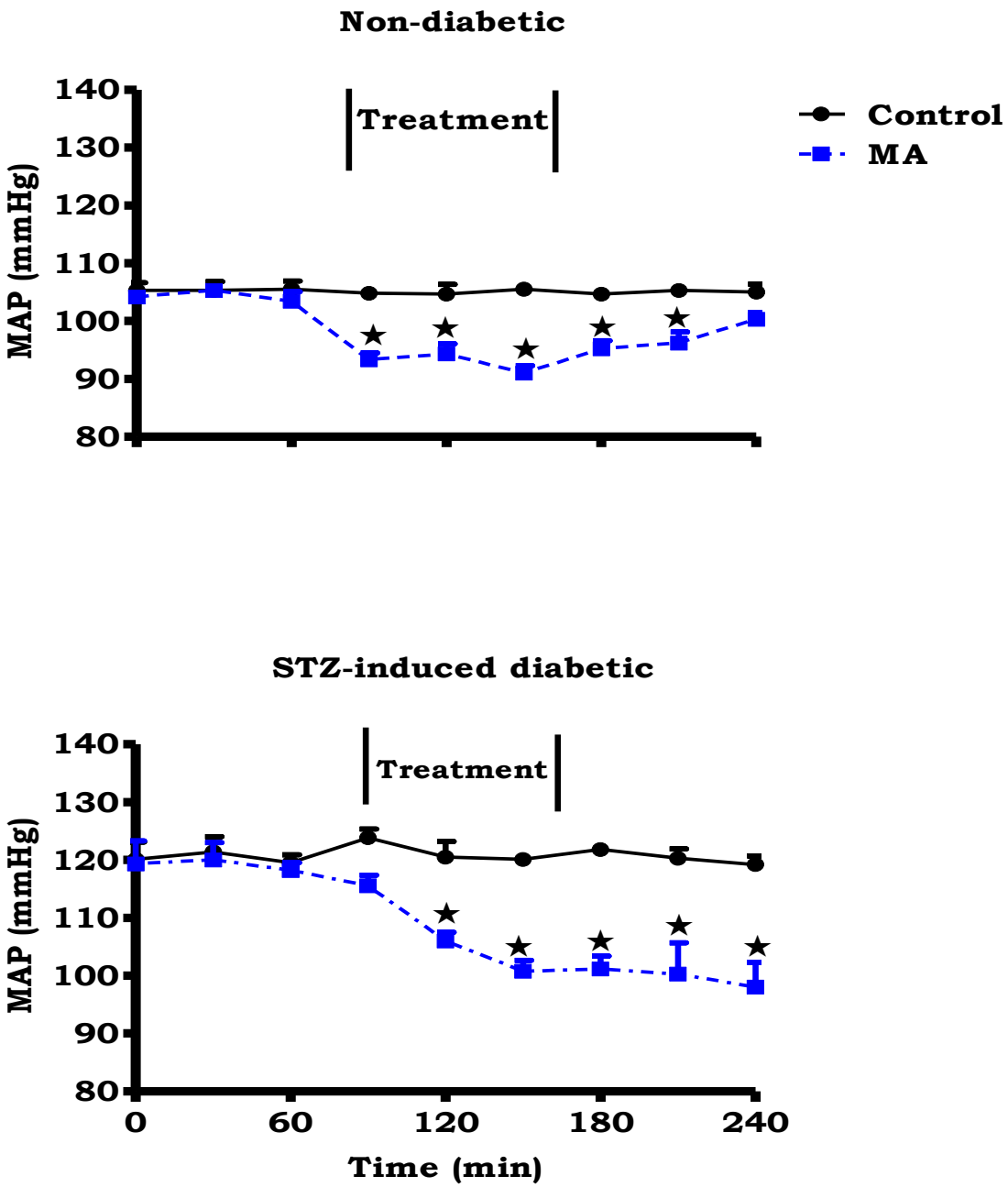

Figure 22: Effects of intravenously infused MA (1½ h) on MAP in non-diabetic rats. Values are presented as means, and vertical bars indicate SEM (n=6 in each group).★$p<0.05$ by comparison with the control animals.

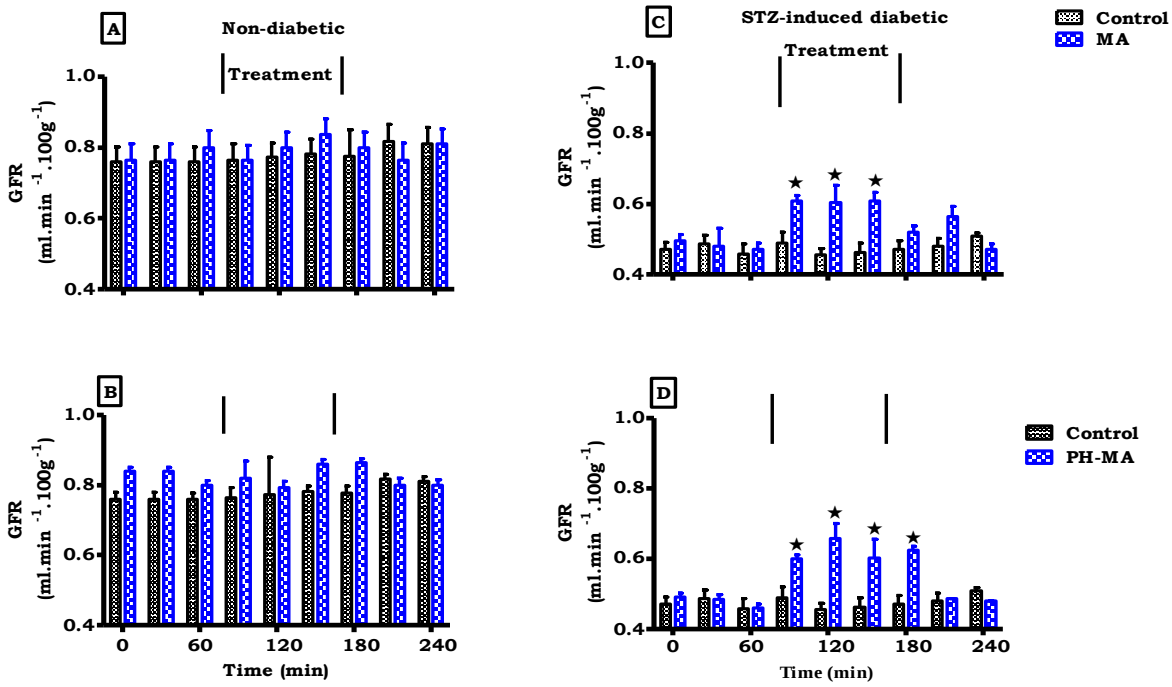

Figure 23: Comparison of the acute effects of MA (A and C) and PH-MA (B and D) administration on GFR rate of non-diabetic and STZ-induced diabetic. MA or PH-MA was infused at 90 µg/h for 1.5 h during the treatment period. Values are presented as means for each 30 min collection; vertical bars indicate SEM of means (n=6 rats in each group).★ $p<0.05$ by comparison with control animals

Table 3: Plasma hormone concentration in rats after MA or PH-MA administration for 90 min of treatment.

Groups	**AVP (pmol/L)**	**Aldosterone (nm/L)**
Non-diabetic control	2.82 ± 0.47	0.78 ± 0.04
Non-diabetic MA treated	2.38 ± 0.21	0.70 ± 0.01
Non-diabetic PH treated	2.44 ± 0.10	0.75 ± 0.07
STZ-diabetic control	4.45 ± 0.24#	1.45 ± 0.03#
STZ-diabetic MA treated	4.00 ± 0.26	0.79 ± 0.05*
STZ-diabetic PH treated	4.42 ± 0.33	0.80 ± 0.07*

* $p<0.05$ by comparison with respective control animals;

# $p<0.05$ by comparison with non-diabetic animals

3.7 Sub-chronic

3.7.1 MA effects on body weight, food and water intake

Synthesis of derivatives afforded lower yields of derivatives, hence only MA was used to carry out the short-term studies. Table 4 shows the effects of MA on mean weekly food intake, water intake and body weight change in non-diabetic and STZ-induced diabetic rats measured 24 h after treatment over a 5-week study period. Untreated diabetic rats exhibited severe wasting despite the significantly higher weekly intake of food and water when compared with non-diabetic control rats. In contrast, non-diabetic control animals progressively gained weight from the 2^{nd} week until the end of the experimental period. Administration of various doses of MA (20, 40 and 80 mg/kg, p.o.) to STZ-induced diabetic rats, however, stabilised body weight from the 4^{th} to the 5^{th} week of the experimental period. Like-wise, reduction in body weight of STZ-induced diabetic rats was stabilised by metformin from the 3^{rd} to the 4^{th} week of the experimental period. Kidney weight was significantly increased in STZ-diabetic rats by comparison with non-diabetic animals. Administration of MA or metformin did not affect the kidney mass in non-diabetic and STZ-diabetic rats (Table 5).

3.7.2 Effect of MA on renal fluid and electrolyte handling

The mean weekly urine volume was elevated in untreated STZ-induced diabetic by comparison with non-diabetic control animals throughout the 5-week experimental period. Administration of MA did not have significant effects on urine volume for both non-diabetic and STZ-induced diabetic animals (Figure 24). Urinary Na^+, K^+ and Cl^- outputs of untreated STZ-induced diabetic rats were significantly lower than those of control non-diabetic animals throughout the 5–week period (Figure 24). However, administration of MA (40 mg/kg, p.o.) to non-diabetic rats significantly ($p < 0.05$) increased mean weekly urinary Na^+ output from the 1^{st} week value of 171 to 277 mmol/L and the 80 mg/kg, p.o. dose increased urinary Na^+ output from the 1^{st} week value of 171 to 281 mmol/L without affecting weekly urinary K^+ and Cl^- outputs. Like in non-diabetic animals, MA (40 mg/kg, p.o.) significantly ($p<0.05$) increased urinary Na^+ output of STZ-diabetic rats from the 1^{st} week value of 22 to 27 mmol/L and MA (80 mg/kg, p.o.) increased weekly Na^+ excretion from the 1^{st} week value of 22 to 38 mmol/L at the end of the experiment without influencing the urine flow rate, urinary K^+ and Cl- excretion rate. An increase in Na^+ output by

MA was not reflected in plasma collected at the end of the experiment as there was no significant difference in non-diabetic control and treated animals (Table 5).

At the end of 5-week experimental period, plasma creatinine concentrations were significantly ($p<0.05$) elevated in control STZ-induced diabetic rats by comparison with untreated non-diabetic rats (Table 5). Administration of MA (40 and 80 mg/kg, p.o.) significantly decreased plasma creatinine concentration of STZ-diabetic rats with a concomitant increase in GFR with no influence on GFR on non-diabetic rats.

3.7.3 Effects of MA on MAP

Figure 25 shows the effects of MA on MAP of non-diabetic and STZ-induced diabetic conscious rats during the 5 week study period. MAP of non-diabetic control animals was stable varying from 99 to 100 mmHg throughout the 5-week experimental period. MAP of the untreated STZ-induced diabetic rats was significantly ($p<0.05$) elevated compared to non-diabetic control animals. Administration of MA (20 mg/kg, p.o.) did not have significant effects on MAP. However, administration of various doses of MA (40 and 80 mg/kg, p.o.) significantly ($p<0.05$) reduced MAP from the 3rd week until the end of the experiment by comparison with the control animals at corresponding time periods (Figure 25 A). Treatment of the STZ with MA (40 and 80 mg/kg, p.o.) significantly ($p<0.05$) reduced MAP from the 1st week value of 119 to 100 mm Hg (MA 40 mg/kg, p.o.) and from the 1st week value of 120 to 96 mmHg (MA 80 mg/kg, p.o.) at the end of the experimental period (Figure 25 B).

3.7.4 GLUT 1 and 2 expression

The effects of MA on the expression of glucose transporters was evaluated in glucose transporters expression, renal GLUT 1 and 2 were analysed using Western blotting in tissues harvested from non-diabetic and STZ-induced diabetic rats at the end of 5-week MA treatment. The GLUT1 and GLUT2 expression were significantly elevated in kidney tissues of untreated STZ-induced diabetic rats compared with control non-diabetic animals (Figure 26). In comparison with respective control groups, MA treatment not only abolished the diabetes- induced increase in GLUT1 and GLUT2 but also decreased the expression of these transporters in non-diabetic animals.

3.7.5 GLUT 4 expression

Figure 27 shows the effects of MA on expression of GLUT4 in gastrocnemius of non-diabetic and STZ-induced diabetic rats. GLUT4 content in gastrocnemius muscle of diabetic rats was markedly lower as compared with non-diabetic control animals. MA did not have any significant effects on the expression of GLUT4 in the muscle of non-diabetic rats. Surprisingly, MA increased GLUT4 content in diabetic rat skeletal muscle, but no effect of metformin was observed. Moreover, administration of MA (40 and 80 mg/kg p.o.) significantly ($p<0.05$) reduced glucose concentrations from the second week of treatment in STZ-induced diabetic rats by comparison with untreated STZ-induced diabetic rats. However, MA exerted no significant changes on blood glucose concentrations of non-diabetic animals (Figure 28).

3.7.6 Oxidative stress

The concentrations of MDA and antioxidants enzymes (SOD and GPx) in non-diabetic control animals represent baseline levels found in the tissues used (Table 6). Significant ($p<0.05$) increases of MDA and decreases of enzymatic oxidants, SOD and GPx were found in the liver heart and kidney tissues of diabetic group by comparison with controls to animals. Treatment of diabetic rats with MA significantly ($p<0.05$) reduced MDA levels in all tissues and decreased SOD activities of SOD and GPx in the liver and kidney compared to untreated diabetic group. On the other hand, GPx activity was undetectable (<0.01 nm/min/mL g protein) in the heart for all groups except for metformin treated STZ-induced diabetic group.

Table 4: Effects of MA on food and water intake, and body weight change with control animals during the 5-week study (n=6 in each group). Values are expressed as mean ± SEM. * $p<0.05$ by comparison with control animals.

			Time (weeks)				
Parameter	**Treatment**		**1**	**2**	**3**	**4**	**5**
Food intake (g/100 g)	Control	ND	10.0± 0.4	9.2 ± 0.4	10.0 ± 0.4	10.1 ± 0.4	11.0 ± 0.3
		STZ	50.0±0.4	50.0±1.0	49.1±1.1	50.0±1.0	48.2±1.2
	MA 20	ND	10.0±0.4	11.1±0.2	10.3±0.4	11.4±0.4	10.2±0.4
		STZ	10.2±0.4	11.2±0.2	10.1±0.4	10.1±0.4	10.0±0.4
	MA 40	ND	10.0±0.2	9.1±0.4	10.2±0.4	10.1±0.4	9.0±0.2
		STZ	11.1±0.4	11.0±0.4	12.0±0.2	12.0±0.4	13.0±0.6
	MA 80	ND	10.1±0.2	11.1±0.4	11.1±0.2	10.0±0.4	11.1±0.4
		STZ	10.1±0.2	10.0±0.4	10.2±0.4	9.0±0.4	8.1±0.2
	Metformin	ND	10.0±0.4	12.2±0.4	12.3±0.4	12.2±0.3	10.1±0.4
		STZ	11.0±0.5	11.0±0.4	11.4±0.4	12.3±0.4	12.2±0.4
Water intake (mL /100 g)	Control	ND	11.1±0.4	12.2±0.4	12.2±0.4	12.1±0.4	11.2±0.4
		STZ	48.1±0.4	49.2±2.2	48.2±1.1	50.1±1.4	50.0±1.6
	MA 20	ND	11.5±0.4	10.3±0.2	13.2±0.2	12.2±0.4	11.1±0.4
		STZ	50.4±1.1	49.2±1.0	49.3±1.3	48.2±1.2	50.0±1.0
	MA 40	ND	10.2±0.4	10.1±0.1	10.0±0.4	11.2±0.4	10.3±0.4
		STZ	47.1±0.4	48.2±1.0	50.1±1.0	50.2±1.0	50.1±1.4
	MA 80	ND	10.2±0.2	11.3±0.2	11.4±0.4	11.±0.4	9.2±0.4
		STZ	49.1±1.8	48.2±2.6	48.2±2.2	46.1±3.5	45.7±2.0
	Metformin	ND	9.1±0.2	10.0±0.4	11.0±0.4	10.0±0.2	11.0±0.4
		STZ	50.0±1.6	46.0±0.1	45.2±1.0	40.2±1.3	38.2±1.2
% b.wt change/week	Control	ND	7.0±1.1	6.1±2.0	8.2±1.3	13.1+1.2	22.0±1.0
		STZ	8.2±1.1	-1.0±1.1	-10.1±1.0	-2.5±1.1	-5.0±1.1
	MA 20	ND	4.1±1.0	2.1±1.0	5.2±2.3	9.2±1.2	14.1±1.1
		STZ	-1.3±0.1	-1.2±1.0	2.1±1.0	3.2±1.1	3.2±1.2
	MA 40	ND	6.0±0.8	8.0±1.2	12.0±1.0	15.1±1.0	23.0±1.1
		STZ	-1.0±0.2	-2.1±1.2	2.1±1.0	*8.2±1.0	*7.0±1.0
	MA 80	ND	7.2±1.1	9.2±2.0	10.1±1.2	14.0±2.1	25.0±1.1
		STZ	-1±2.2	-2.0±1.1	1.2±1.2	3.2±2.3	*4.2±1.3
	Metformin	ND	8.0±2.0	7.2±1.0	12.1±2.1	16.±1.2	22.1±1.1
		STZ	-2.1±0.2	-1.0±1.0	2.0±2.1	*6.0±1.01	*8.2±1.1

Table 5: The effects of various MA doses in plasma biochemical parameters in non-diabetic and STZ-induced diabetic rats treated every third day for 5-weeks. Values are presented as mean ± SEM.

Parameters								
Treatment		**Na^+ (mmol/L)**	**K^+ (mmol/L)**	**Cl^- (mmol/L)**	**Urea (mmol/L)**	**Creatinine (µmol)**	**GFR (ml/min/ 100 g)**	**Kidney mass (g/100g)**
Control	ND	139±1	4.8±0.3	105±1	6.7±1.3	26.0±1.1	1.0±0.1	0.5±0.1
	STZ	138±3	3.8±0.1	106±3	9.2±1.4	33.2±1.0♦	0.3±0.1♦	0.9±0.2♦
MA 20	ND	136±1	4.1±0.2	102±4	7.3±0.8	24.1±1.1	0.9±0.1	0.6±0.1
	STZ	137±2	4.2±0.2	108±2	8.9±1.1	26.2±1.0	0.2±0.1♦	0.8±0.1♦
MA 40	ND	138±3	4.2±0.3	100±3	7.0±1.5	20.1±1.3*	0.8±0.1	0.6±0.1
	STZ	132±1	3.9±0.2	104±1	9.5±0.7	22.0±1.0*	0.6±0.1*	0.8±0.1♦
MA 80	ND	138±2	4.5±0.1	103±1	7.6±1.1	18.0±1.0*	0.9±0.1*	0.5±0.1
	STZ	135±4	4.3±0.3	97±1	9.0±0.4	22±1. 0*	1.0±0.3*	0.8±0.1♦

* $p<0.05$ by comparison with respective control animals
♦$p<0.05$ by comparison with respective non-diabetic animals

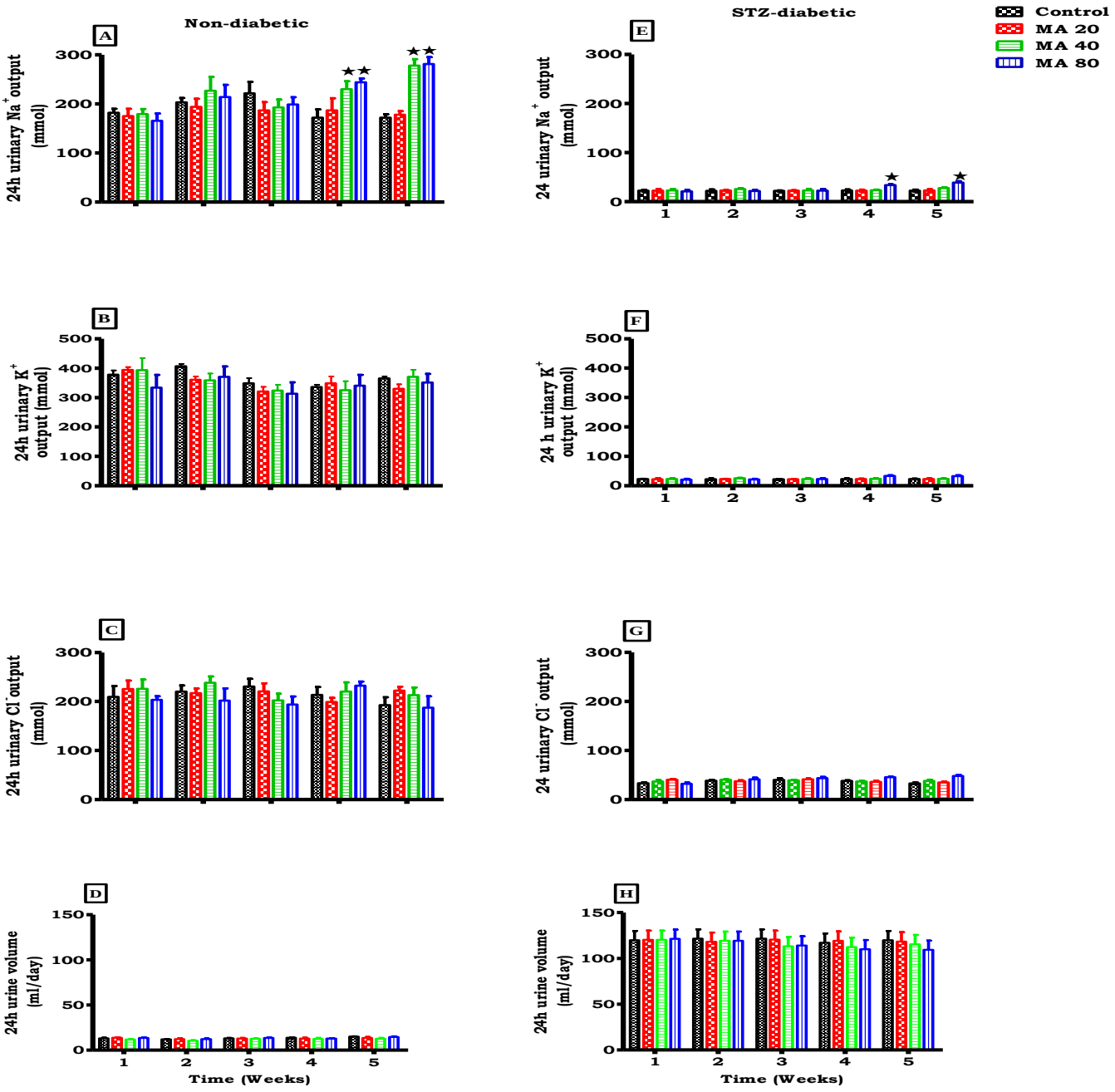

Figure 24: Comparison of the effects of the administration of MA (80 mg kg, p.o.) twice daily on urinary excretion rate of Na^+, K^+ Cl^- and urinary outputs in non-diabetic rats (A–D) and STZ-induced diabetic rats (E–H) with respective control animals. Values are presented as means for each week; vertical bars indicate SEM of means (n= 6 rats in each group). ★ $p<0.05$ by comparison with control animals.

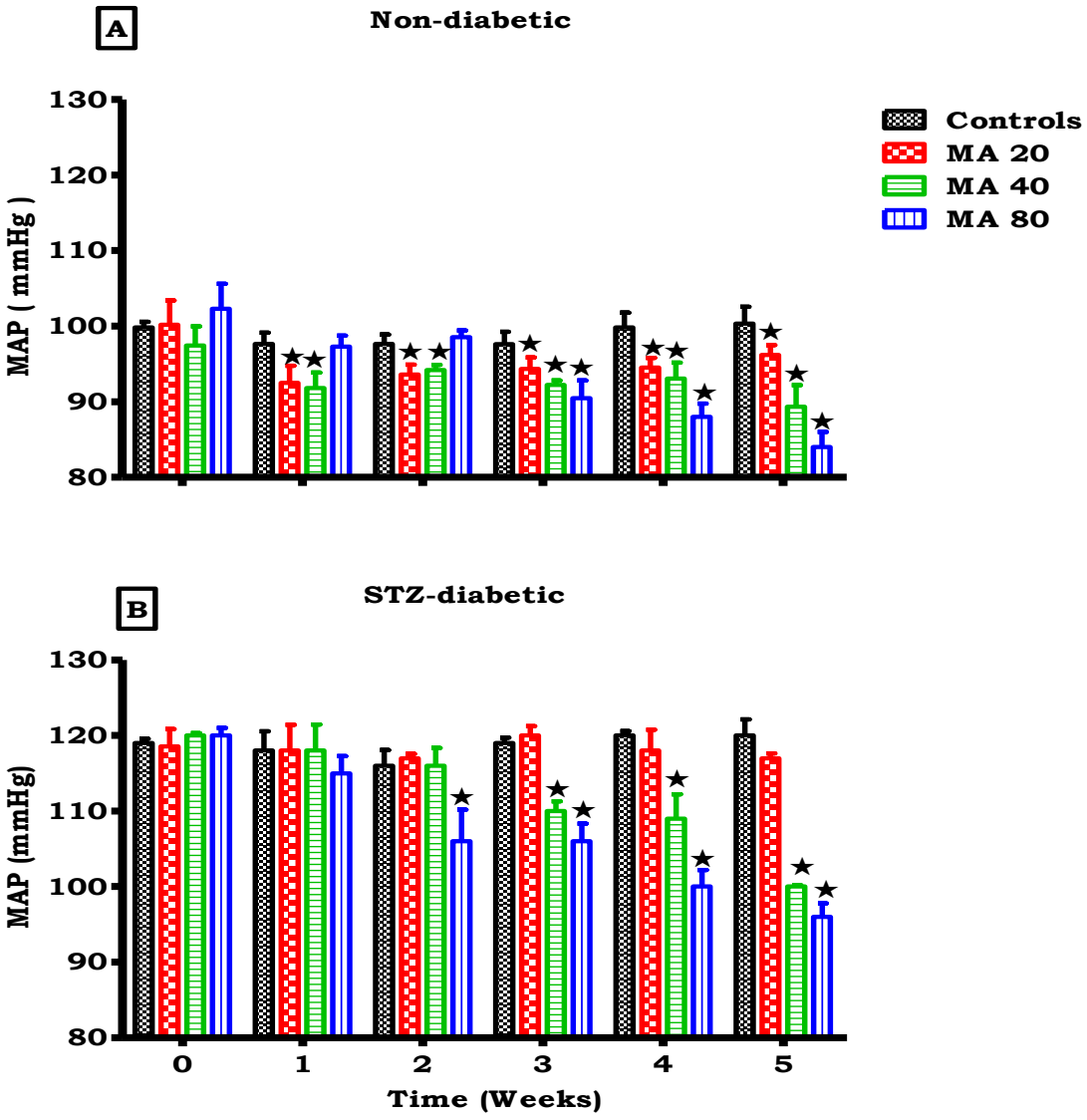

Figure 25: The effects of MA on 24h MAP of non-diabetic (**A**) and STZ-induced diabetic rats (**B**). Values are presented as means, and vertical bars indicate SEM (n=6 in each group).★$p<0.05$ by comparison with control animals at each corresponding time.

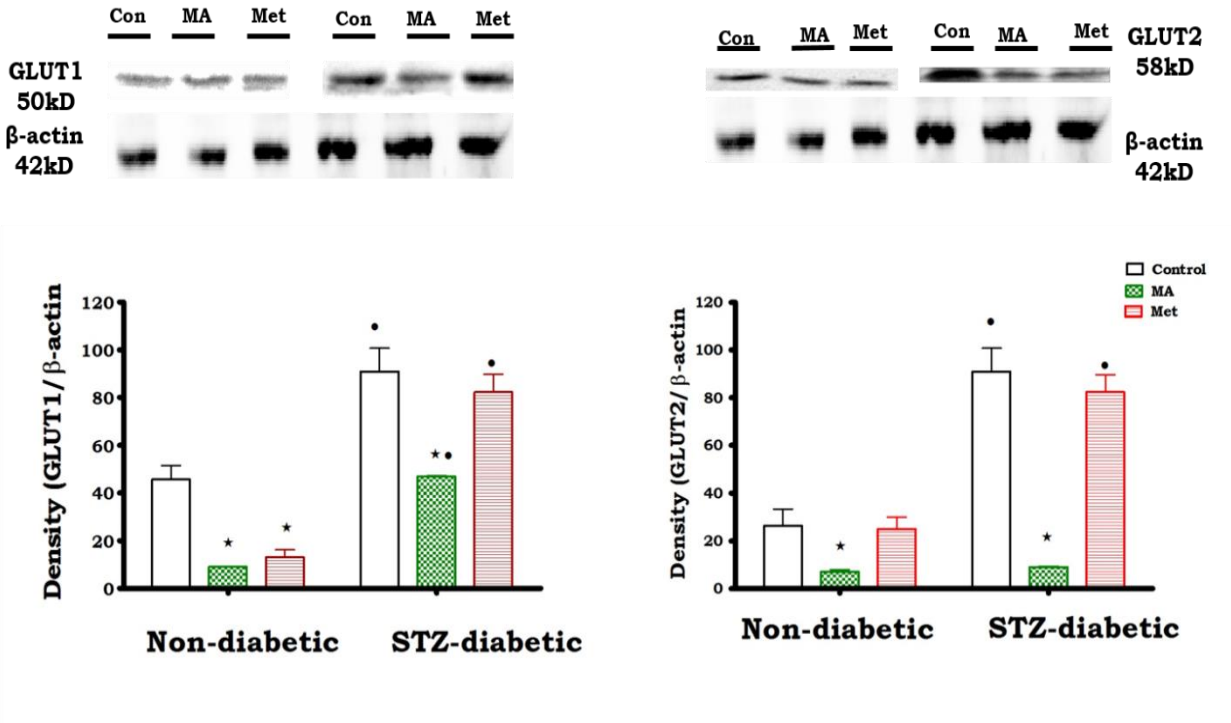

Figure 26: Effects of MA on expression of GLUT1and GLUT2 as determined by Western blotting of control, MA-treated and metformin-treated kidney tissues of non-diabetic and STZ-induced diabetic rats. Values are expressed as mean ±SEM, ★ $p<0.05$ by comparison with control animals at each corresponding time. Values were obtained from Western blots for six preparations.

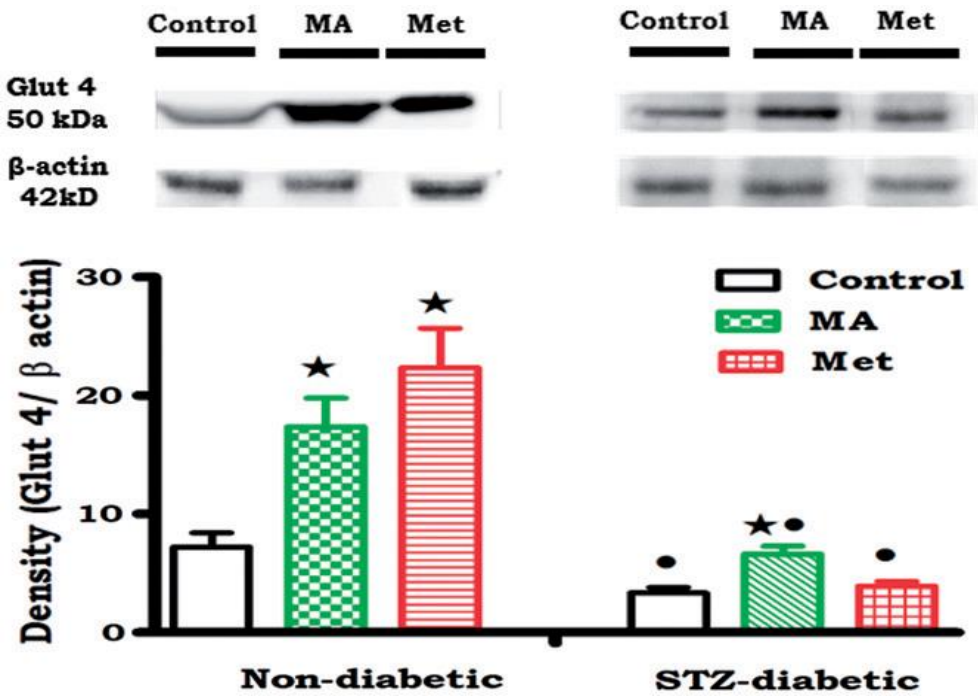

Figure 27: Effects of MA on GLUT4 expression as determined by Western blotting of control, MA-treated and metformin-treated skeletal muscle tissues of non-diabetic and STZ-induced diabetic rats. Values are expressed as mean ± SEM. Values were obtained from Western blots for six preparation. ★$p<0.05$ by comparison with control animals at each corresponding time, •$p<0.05$ by comparison of non-diabetic with STZ-induced diabetic.

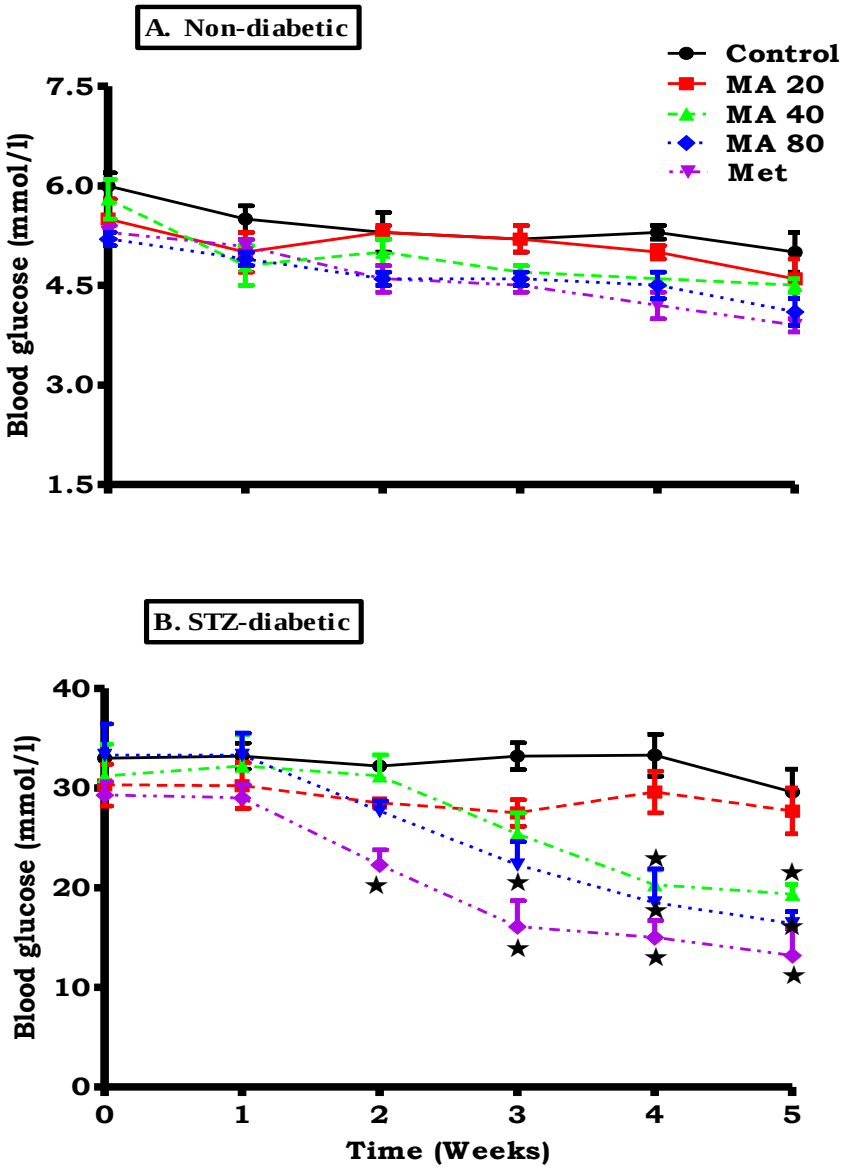

Figure 28: Effects of various doses of MA on blood glucose concentration in non-diabetic (A) and STZ-induced diabetic (B) rats. MA (20, 40 and 80 mg/kg) doses were administrated orally to the rats twice every third day and the controls animals received DMSO saline. Glucose concentrations were measured 6 h following treatment. Values are presented as means, and vertical bars indicate SEM (n=6 in each group).★$p<0.05$ by comparison with respective control animals

Table 6: Comparison of MDA concentration, activities of SOD and GPx in the liver, kidney and heart of STZ-induced diabetic rats treated twice every third day for 5 weeks with control non-diabetic and diabetic animals. Values are expressed as mean ± SEM.

		Organ		
Parameter measured	**Treatment**	**Liver**	**Kidney**	**Heart**
MDA µmol (nmol g^{-1} protein)	Non-diabetic control	38.40± 4.30	20.42± 6.50	28.40± 4.80
	STZ-diabetic untreated	46.50±5.20#	41.70±1.60	47.20 ±1.20#
	STZ-diabetic MA	41.60±1.60	25.60±2.60*	45.90±6.40#
	STZ-diabetic Met	41.70±3.22	11.10±2.80*#	56.00±12.80#
SOD Activity (nmol/min/mL/g)	Non-diabetic control	8.11±0.93	18.34±3.60	3.00±0.14
	STZ-diabetic untreated	1.22±0.02#	5.50±0.10	3.83±0.15
	STZ-diabetic MA	8.16±1.28*#	16.04±0.46*	2.39±0.33
	STZ-diabetic Met	6.53±1.00*#	7.40±1.09*	5.62±1.10*#
GPx Activity (pmol/min/mL/g)	Non-diabetic control	26±1	49±6	UNDETECTABLE
	STZ-diabetic untreated	0.34 ±0.04#	7±1*#	UNDETECTABLE
	STZ-diabetic MA	25±4*#	44±6*#	UNDETECTABLE
	STZ-diabetic Met	22±7*#	98±3*#	55±8*#

* $p<0.05$ by comparison with respective control animals;
# $p<0.05$ by comparison with non-diabetic animals

CHAPTER 4

DISCUSSION

This study determined the effects of *S. aromaticum* derived-MA and related triterpenes derivatives on renal function and blood pressure of STZ-induced non-diabetic and diabetic rats with the hope of providing evidence for possible use of the compounds in the management of diabetes mellitus. The results indicate that MA and the relative derivate PH-MA were effective in ameliorating renal fluid and electrolyte handling and expression of renal tubule glucose transporters. The results also indicate that MA reduces mean arterial blood pressure of non-diabetic and STZ-induced diabetic rats. The findings contextualize the renal, hypoglycaemic and hypotensive effects of *S. aromaticum* derived-MA and related triterpenes derivatives in the management of diabetes.

The results were obtained using plant derived MA and derivatives whose structures were confirmed by NMR spectroscopy. Previous studies indicate that MA possess hypoglycaemic effects in partially diabetic rats (Liu *et al*., 2007). MA was used in this study in an effort to advance current knowledge about this triterpene on kidney function. Triterpenes such as MA are generally hydrophobic and therefore are difficult to dissolve in water before administration. According to Williams *et al*. poor water solubility is a significant risk factor in low oral absorption because drug molecules must completely dissolve in water before oral administration (Williams *et al.*, 2013). Therefore, MA derivatives were synthesised in order to improve efficacy of this compound. Derivatives were synthesised based on the currently known MA active sites. The first active site is the carboxylic group which has previously been shown to be responsible for glycogen phosphorylase (GP) inhibition and the second site is which is responsible for PTP1 inhibitor. Several derivatives have been synthesized with an effort to improve water solubility of this triterpene, as they are well known to have poor water solubility. The results were, however, disappointing as incorporation of hydrophilic groups on carbon 28 significantly decreased potency. According to Wen *et al*. introduction of hydrophobic groups (bromobutane) on carbon 28, carboxylic groups gives rise to a 7- fold increase effects compared to lead MA (Wen *et al.*, 2006). Accordingly, we opted to incorporate hydrophobic groups on c-28 to increase potency of MA. MA has, however, been screened for PTP1B inhibition along with isomers such as 3-epi-maslinic acid and augustic acid on PTP1B. Among the MA isomers, MA demonstrated good PTP1B inhibition

(IC_{50} = 5.93 µM). Hence our current research also focused on synthesizing MA derivatives with an aim to improve solubility, efficacy and bioavailability. To achieve this, a series of heterocyclic rings were incorporated in C-2 and C-3 position based on the evidence that heterocyclic incorporating heterocyclic rings improved efficacy of MA (Qiu *et al.*, 2009). The synthesis was based from previous studies which demonstrated the efficacy of MA derivatives after incorporating heterocyclic rings (Qiu *et al.*, 2009) .

MA was extracted from the ethyl acetate solubles rich in triterpenes (Mapanga *et al.*, 2009). The structure of MA and derivatives was elucidated using ^{1}HNMR and ^{13}C NMR spectra compared to the previously reported data (Júlio *et al.*, 2003) . The C-2 represents a carbon-OH bond that is absent in other triterpenes like oleanolic acid (OA) and ursolic acid (UA) had the resonance shift that was identical to previously extracted MA (Júlio *et al.*, 2003) . Additionally, C-12 and C-13 representing the olefenic bond peculiar to triterpenes served as a confirmation that the compound was indeed a triterpene and the carbon signal was identical to previously extracted MA (Júlio *et al.*, 2003). This information confirms our previous findings that *Syzygium* species are rich in triterpenes mixtures. Fischer indole synthesis was used for synthesis of MA derivatives from OA. Fused heterocyclic derivatives of triterpenes and alkaloids are well documented (Heather *et al.*, 2000) and biologically active indole-fused examples are of particular interest. Our syntheses of the target compounds PH-MA, Cl-PH and F-PH are based on the Fischer indole synthesis (Claas *et al.*, 2007; Qiu *et al.*, 2009). The ^{1}HNMR for PH derivative indicates that there were four singlets, three duplets and three multiplets which were comparable to that of (Finlay *et al.*, 2002). Indeed, Fl-PH and CL-PH had the similar number of singlets and dublets with an addition of corresponding resonance peaks for flouro and chlouro. These results, therefore, indicate the authenticity of our fused-heterocyclic triterpene derivatives. The GP inhibitors were all insoluble in water even after preparing in DMSO, indicating that introduction of hydrophobic rings does indeed influence solubility of compounds.

Experimental diabetes was induced with a single intraperitoneal STZ (60 mg/kg) injection which selectively destroys or impairs secretion of insulin from pancreatic β- cells and the systemic changes which occur following this are related to the induced diabetic state (Li *et al.*, 2005; Lenzen, 2008). Indeed, untreated STZ-diabetic rats exhibited significantly low plasma insulin

concentration and increased plasma glucose concentrations compared to non-diabetic rats perhaps due to destruction of pancreatic β-cells (Pinent *et al.*, 2004; Lenzen, 2008). This was also in agreement with previous immunohistological studies which showed complete obliteration pancreatic β- cells by STZ injection (Coskun *et al.*, 2005). STZ is translocated in cells through the GLUT2 transporter to crosslink the vital structure (Beckman *et al.*, 1990; Kroncke *et al.*, 1991). Oxygen free radicals and superoxide dismutase have also been shown induce toxicity of pancreatic β cells (Wohaieb and Godin, 1987) . We were, therefore able to investigate the effects of the extract without an influence of insulin secretion, mimicking type 1 diabetes.

STZ-induced diabetic rats exhibited weekly decreases in urinary Na^+ excretion and elevated plasma creatinine concentration at the end of five weeks with concomitant reduction in GFR. We suggest that the impaired renal function in diabetic animals was not due to the STZ nephrotoxicity since the dose of STZ (60 mg/kg) used to induce diabetes has been reported to have minimal kidney toxicity in experimental animals (Kuramochi and Homma, 1993; Koulmanda *et al.*, 2003; Lenzen, 2008). Chronic hyperglycaemia due to diabetes mellitus leads to several kidney complications which include Na^+ retention, reduction in glomerular filtration rate (GFR) and elevation of blood pressure presumably due to elevated oxidative stress (MDA levels) (Stengel *et al.*, 2003; Magri and Fava, 2009; Raju *et al.*, 2013). These effects eventually lead to renal failure and hence death of diabetic patients (Nangaku, 2006). Indeed, impaired renal dysfunction has long been associated with experimental diabetes (Musabayane *et al.*, 1995). Laboratory evidence indicates that MA and PH do not only reduce blood glucose concentration, but also exhibit beneficial effects in the kidney through amelioration of GFR and increased Na^+ excretion rate in those animals that have been observed to retain Na^+. GFR is the important marker for kidney function and correlation between urinary Na^+ excretion and GFR has been reported by other authors (Satirapoj *et al.*, 2006 ; Hoek *et al.*, 2008). GFR was assessed by creatinine clearance (Ccr) which has been extensively used in our laboratory and other researchers to evaluate renal tubular function (Rebsomen *et al.*, 2005; Kumar *et al.*, 2008; Mapanga *et al.*, 2009; Madlala *et al.*, 2012). An increase in GFR by triterpenes, particularly OA has indeed been reported in our laboratory (Madlala *et al.*, 2012). MA is clinically used as an anti-tumour drug (Li *et al.*, 2010) which means this triterpene may increase cell viability in the kidney. This was confirmed by the ability of MA to increase cell viability in MDBK cells, indicating that the natriuretic effects were not associated with nephrotoxicity. Although PH-MA

exerted toxicity after 24 and 48 h treatment, we suspect that the doses used in this study were very high. Hence for acute studies, relatively low doses of PH-MA were used compared to that of MA. A decrease in GFR is also associated with increased reabsorption of Na^+ mainly in proximal tubule's sodium hydrogen transporter (NHE3) (Vallon *et al.*, 1999). The data obtained in this suggest that the beneficial effects of MA in diabetes mellitus are complimentary. Our results showed that the elevated concentrations of MDA (marker of lipid peroxidation) in diabetic rat tissues of the liver, kidney and were restored within normal values after treatment with MA after 5-weeks. The reduction in MDA levels could be due to the improvement in glycaemic control and also increased antioxidant status, since MA significantly stimulated increased activities of SOD and GPx. Studies indeed show that tight reduction of blood glucose may prevent oxidative stress and therefore delay the onset of diabetic complications (Brosius and Heilig, 2005). The increase in lipid peroxidation products due to hyperglycaemia has been previously reported in tissues of diabetic rats and suggested to partly contribute to long term kidney damage (Raju *et al.*, 2013). MA has previously been shown to decrease nitric oxide levels, another potent tissue damage oxidant, therefore supporting our current findings (Montilla *et al.*, 2003). MA attenuated these changes mediated possibly in part via enhancement of the antioxidant status thus supporting previous reports that extracts containing MA reduce the severity of kidney dysfunction in diabetes (Xu *et al.*, 2009). Furthermore, Yamabe *et al*. reported that antioxidants and good control of diabetes led to improved renal function. Thus, oxidative stress could, at least partially, play a central role in cardio-renal dysfunction in diabetes (Yamabe *et al.*, 2006). Against these observations are reports of the absence of improvement and even worsening of diabetic nephropathy with antioxidant treatment (Agardh *et al.*, 1992). Diabetic nephropathy has indeed been observed unregulated diabetes characterized by increased expression of GLUT1, and 2 in proximal (Marks *et al.*, 2003) and distal tubule (Linden *et al.*, 2006). Interestingly, treatment with MA for 5-weeks decreased GLUT1 and GLUT 2 expressions in the kidneys of diabetic rats to perhaps reduce urinary glucose leak. In the absence of urinary glucose measurements, further studies are clearly needed to support this speculation. The renal reabsorption of glucose which occurs mostly in the proximal tubule involves luminal Na^+ glucose transporters (SGLTs) and facilitative diffusion through GLUT1 and GLUT2 in the brush border membrane (Thorens *et al.*, 1990). In diabetic animals insertion of GLUT2 into the brush border membrane of the proximal tubule is stimulated thereby increasing facilitative glucose uptake (Marks *et al.*, 2003) .

Interestingly, cell culture studies indicate that MA inhibited glucose uptake by MDBK cells which correlates with results obtained chronically whereby MA abolished expression of GLUT1 and 2 in diabetic kidneys. MA has indeed been shown to reduce glucose absorption by decreasing the levels of SGLT and GLUT2 in the small intestines of STZ-induced (Khathi *et al.*, 2013). Interestingly both MA (Liu *et al.*, 2007) and metformin (Stumvoll *et al.*, 1995) have been shown to reduce plasma insulin in diabetic animals. Moreover, among other triterpenes such as OA and UA, MA and PH have previously been shown to selectively inhibit PTP1B expression which leads to increased insulin sensitivity. This could be another mechanism through which these triterpenes (MA and PH) improve kidney function. Indeed, the tight glycaemic control and kidney ameliorative properties of triterpenes has previously been shown in previous studies (Madlala *et al.*, 2012) . Additionally, MA treatment also increased GLUT4 expression in the skeletal muscle of diabetic rats suggesting reduced blood glucose concentration by this triterpene is indeed linked to renal function. Studies indicate that hyperglycaemia is the main cause of decline in renal function in diabetic patients (Gnudi *et al.*, 2007).

Our aim to improve efficacy solubility and efficacy of MA had proven to be successful as PH was more effective in improving glucose homeostasis in liver cell lines. This was indeed in agreement with previous researcher whereby PH derivatives exhibited 7-fold effects against protein tyrosine phosphatase when compared to MA (parent compound) (Qiu *et al.*, 2009). Treatment with MA stabilized body weight of STZ-induced diabetic rats, this could be biological importance as diabetic patients are known to lose weight over time. The decrease in weight could be due to hypoglycaemic properties of MA.

This study also attempted to clarify the effects of MA and PH-MA on proximal tubule Na^+ handling and using lithium clearance as a marker, a technique that is widely used in animal studies to assess proximal tubular function (Boer *et al.*, 1995). Acute administration of MA, PH-MA, F-PH and Cl-PH increased urinary Na^+ output in STZ-induced diabetic animals. This increase in Na^+ was in concomitant with FE_{Na}^+ and FE_{Li}^+ in anaesthetised non-diabetic and STZ-induced diabetic animals. C_{Li}^+ was used to estimate the amount of Na^+ in the proximal tubules. Renal C_{Li}^+ studies have been widely used to estimate proximal tubular function in both animals and mammals (Koomans *et al.*, 1989; Shirley and Walter, 1990; Walter and Shirley, 1991). Renal C_{Li+} technique is based on the fact that Na^+ is reabsorbed at the similar rate as $Li^{+\cdot}$. Studies conducted by five

different researchers, have however, indicated that most of the Li^+ is reabsorbed in the proximal tubule at a range of 73 to 82% of the whole kidney (Gutman *et al.*, 1973; Hayslett and Kashgarian, 1979; Thomsen *et al.*, 1981; Thomsen *et al.*, 1981; Haas *et al.*, 1986; Kirchner, 1987). Oliveria *et al*. showed that insulin increased urinary Na^+ output with a concomitant increase in FE_{Li+} and FE_{Na+} that on STZ-diabetic rats (Oliveira *et al.*, 2010) . This evidence also suggests that Li^+ is indeed an ideal marker for Na^+ handling in the proximal tubule. Our results indicate that MA and PH-MA evoked increase in FE_{Na}^+ without GFR resulted in increased proximal tubular Na^+ excretion of non-diabetic rats. However, a significant increase in GFR was observed in STZ-induced diabetic rats. These results, therefore, suggest that MA and PH-MA influences Na^+ handling in this segment of the nephrons. An increase in FE_{Na}^+ and FE_{Li}^+ suggests that the triterpenes inhibit proximal tubular reabsorption of Na^+ hence resulting in a pronounced increase in urinary Na^+ excretion. The exact mechanism (s) and transporters that are involved in impaired proximal tubular Na^+ reabsorption could not be elucidated in this study. The proximal tubules reabsorbs about 70% of total filtered Na^+. The reabsorption involves coupling of Na^+ entry via sodium-proton exchanger type 3 (NHE3) transporters where by the Na^+ extrusion primarily occurs through Na^+-K^+-ATPase (Cai *et al.*, 2008). Another important transporter found in the proximal tubule is sodium-bicarbonate co-transporter (NBCe1) which mediates Na^+ exit from the tubular cells (Horita *et al.*, 2011). We, therefore, speculate that MA may be involved in inhibiting apical NHE3 and basolateral Na^+-K^+-ATPase stimulated basolateral NBCe1 transported which accounts for the exit of Na^+ from the proximal tubular. MA and PH-MA possible inhibit these transporters in a way that favours increase Na^+ output seen in experimental animals. Research conducted in hypertensive rats showed an increase in expression of apical NHE3 also increased absorption of Na^+ in the proximal tubule (Anne *et al.*, 2009). However, drugs like a-adrenoceptor agonists and inhibitory parathyroid hormone (PTH) regulate NHE3 and therefore, Na^+ reabsorption (Gesek *et al.*, 1989) . To further elucidate the mechanism of action the effects of MA and PH-MA on expression of ENaC transporter were investigated in the distal tubule cells. Our results suggest that MA and PH-MA also increase Na^+ excretion by attenuation ENaC expression in distal tubule cells with PH showing more efficacy than the parent compound. In the kidney distal nephron and collecting duct sodium entry from the lumen into the cells is mediated by the amiloride-sensitive sodium channel, ENaC at the apical membrane (Duc *et al.*, 1994) and the Na, K-ATPase at the basolateral membrane, which provides the driving force for apical sodium reabsorption. Studies by O'Neill *et al.* showed

that STZ-induced diabetic rats exhibit a marked increase in ENaC expression which in concomitant with increased plasma AVP (O'Neill *et al.*, 2008). This was indeed in agreement with the observed increases in plasma for our STZ-induced diabetic animals, although MA and PH-MA reduced AVP to values that did not reach significance. However, this could be of biological importance. Na^+ reabsorption by ENaC is also known to be regulated by aldosterone (Masilamani *et al.*, 1999). The results of this study also show that aldosterone levels were elevated in STZ-induced diabetic rats. In contrast MA and PH-MA decreased plasma aldosterone with a concomitant decrease in ENaC expression. This outcome of MA and PH-MA was similar to that of amiodipine which was shown by other researchers to decreased plasma aldosterone, attenuate oxidative stress and thus ameliorates diabetes induced renal injury via (Matavelli and Siragy, 2013). Our cumulative data revealed that PH was more effective in ameliorating acute kidney function as shown by significant increased Na^+ excretion. A large number of heterocyclic indole derivatives have been shown to have improved pharmacological effects when compared to parent compounds (Wagaw *et al.*, 1998). Indeed, PH has previously been shown to possess 7 times fold efficacy on PTPB1 inhibition by comparison to lead MA, therefore, correlating to our current findings (Qiu *et al.*, 2009).

In addition to Na^+ renal function studies, the effects of MA were also investigated on MAP of non-diabetic and STZ-induced diabetic rats. MA significantly reduced MAP, this effect has previously been reported in triterpenes like UA (Somova *et al.*, 2003) and OA (Mapanga *et al.*, 2009) in Dahl sensitive and STZ-induced diabetic rats, respectively. The results are of significant importance as it is well know that diabetes leads to high blood pressure that eventually results in cardiovascular complications (Cooper *et al.*, 2006; Kanetsuna *et al.*, 2006). The mechanism by which MA lowered MAP was not elucidated in this study.

In addition, this study shows for the first the efficacy of MA in reducing the mean arterial blood pressure of STZ-induced diabetic rats. The findings contextualize the renal, hypoglycaemic and hypotensive effects of *S. aromaticum* derived-MA and related triterpenes derivatives in the management of diabetes.

CHAPTER 5

CONCLUSIONS

5.0 Conclusions

The results described in this study demonstrate that the *S. aromaticum*-derived MA and related triterpene derivatives improve kidney function associated with diabetes mellitus. Additionally, this study indicates for the first time that MA reduced blood pressure while exerting no toxic effects to the kidney and liver cell lines. Taken together, the results suggest that MA and PH-MA indeed may alleviate some complications associated with diabetes mellitus.

5.1 Limitations of the study

This study only investigated the effects of MA and PH-MA on proximal and distal tubular Na^+ handling. Other parameters such as blood pressure were not determined for PH and higher doses of PH-MA were used for cell viability studies. The effects of PH-MA were only investigated acutely for Na^+ handling studies, the effects of PH chronically remain unknown.

5.2 Future recommendations

The future studies should, therefore, investigate the effects of MA and PH-MA on Na^+ handling in other segments of the kidney. Future studies should also investigate the chronic effects of PH-MA on Na^+ handling and blood pressure. In addition, for toxicity studies lower doses of PH-MA should be used to evaluate the efficacy of PH-MA.

CHAPTER 6

REFERENCES

1. Abdul-Ghani M and Defronzo R (2014). Lowering plasma glucose concentration by inhibiting renal sodium-glucose co-transport. *Journal of internal medicine*; **10:** 1244-1248.

2. Abisambra J, Jinwal UK, Miyata Y, Rogers J, Blair L, Li X *et al.*, (2013). Allosteric heat shock protein 70 Inhibitors rapidly rescue synaptic plasticity deficits by reducing aberrant tau. *Biological Psychiatry*; **74:** 367-374.

3. Agardh C, Eckert B and Agardh E (1992). Irreversible progression of severe retinopathy in young type I insulin-dependent diabetes mellitus patients after improved metabolic control. *Journal of Diabetes and its Complications*; **6:** 96-100.

4. Alonso A, Sasin J, Bottini N, Friedberg I, Friedberg I, Osterman A *et al.*, (2004). Protein tyrosine phosphatases in the human genome. *Cell*; **117:** 699-711.

5. Amin N, Wang X, Nucci G and Rusnak J (2011). The sodium glucose co-transporter-2 (SGLT2) inhibitor, PF04971729, yielded BP lowering in hypertensive patients with type 2 diabetes mellitus (T2DM. *Diabetes*; **60:** 6-14.

6. Andrade-Cetto A and Heinrich M (2005). Mexican plants with hypoglycaemic effect used in the treatment of diabetes. *Journal of Ethnopharmacology*; **99:** 325-348.

7. Anne DMR, Donna HL and Alicia AM (2009). Renal NHE3 and NaPi2 partition into distinct membrane domains. *American Journal of Physiology*; **296:** C900-C910.

8. Anwer T, Sharma M, Pillai KK and Khan G (2012). Protective effect of *Withania somnifera* against oxidative stress and pancreatic beta-cell damage in type 2 diabetic rats. *Acta Poloniae Pharmaceutica*; **69:** 1095-1101.

9. Arora MK and Singh UK (2013). Molecular mechanisms in the pathogenesis of diabetic nephropathy: An update. *Vascular Pharmacology*; **58:** 259-271.

10. Azuma K, Minami Y, Ippoush I and Terao J (2007). Lowering effects of onion intake on oxidative stress biomarkers in streptozotocininduced diabetic rats. *Journal of Clinical Biochemistry and Nutrition*; **40:** 131-140.

11. Bagby SP (2007). Diabetic nephropathy and proximal tubule ROS: challenging our glomerulocentricity. *Kidney International*; **71:** 1199-1202.

12. Bailey CJ and Day C (1989). Traditional plant medicines as treatments for diabetes. *Diabetes Care*; **12:** 553-564.

13. Barfuss D and Schafer J (1981). Differences in active and passive glucose transport along the proximal nephron. *American Journal of Hypertension*; **241:** F322-F332.

14. Beckman J, Beckman T, Chen J, Marshall P and Freeman B (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proceedings of the National Academy of Sciences*; **87:** 1620-1624.

15. Bhullar K, Jha A, Youssef D and Rupasinghe H (2013). Curcumin and its carbocyclic analogs: Structure–activity in relation to antioxidant and selected biological properties. *Molecules*; **18:** 5389–5404.

16. Boer PA, Morelli JM, Figueiredo JF and Gontijo JAR (2005). Early altered renal sodium handling determined by lithium clearance in spontaneously hypertensive rats (SHR): Role of renal nerves. *Life Sciences*; **76:** 1805-1815.

17. Boer W, Fransen R, Shirley D, Walter S, Boer P and Koomans H (1995). Evaluation of the lithium clearance method: direct analysis of tubular lithium handling by micropuncture. *Kidney International*; **47:** 1023–1030.

18. Boncler M, Różalski M, Krajewska U, Podsędek A and Watala C (2014). Comparison of PrestoBlue and MTT assays of cellular viability in the assessment of anti-proliferative effects of plant extracts on human endothelial cells. *Journal of Pharmacological and Toxicological Methods*; **69:** 9-16.

19. Brenner B, Cooper M and de Zeeuw D (2001). Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *The New England Journal of Medicine*; **861-869**.

20. Brosius FC and Heilig CW (2005). Glucose transporters in diabetic nephropathy. *Pediatric Nephrology*; **20:** 447-451.

21. Brownlee M (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature*; **414:** 1813-1820.

22. Burns K (2000). Angiotensin II and its receptors in the diabetic kidney. *American Journal of Kidney Diseases*; **36:** 449-467.

23. Cai H, Wu L, Qu W, Malhotra D, Xie Z and Shapiro J (2008). Regulation of apical NHE3 trafficking by ouabain-induced activation of the basolateral Na^+-K^+-ATPase receptor complex. *American Journal of Cell Physiology*; **294:** 555–563.

24. Ceriello A, Morocutti A, Mercur iF, Quagliaro L, Moro M, Damante G *et al.*, (2000). Defective intracellular antioxidative enzyme production in type 1 diabetic patients with nephropathy. *Diabetes*; **49:** 2170–2177.

25. Chao E and Henry R (2010). SGLT2 inhibition: a novel strategy for diabetes treatment. *Review of Drug Discovery*; **9:** 551–559.

26. Claas LD, Wolfgang F and Jens C (2007). Linear versus angular Fischer indole annulation: relative configuration determines regioselectivity. *European Journal of Organic Chemistry*; **2007:** 4731-4737.

27. Cooper M, Bonnet F, Oldfield M and Jandeleit-Dahm K (2001). Mechanisms of diabetic vasculopathy: an overview. *American Journal of Hypertension*; **14:** 475–486.

28. Cooper W, Hernandez-diaz S, Arbogast P, Dudley J, Dyer S and Gideon P (2006). Major congenital malformations after first-trimester exposure to ACE inhibitors. *The New England Journal of Medicine*; **354:** 2443–2451.

29. Coskun O, Kanter M, Korkmaz A and Oter S (2005). Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and β-cell damage in rat pancreas. *Pharmacological Research*; **51:** 117-123.

30. Czifra G, Tóth I, Juhász R, Kovács I and Ács P (2006). Insulin-like growth factor-I-coupled mitogenic signaling in primary cultured human skeletal muscle cells and in C2C12 myoblasts. A central role of protein kinase Cδ,. *Cellular signalling*; **10:** 1461–1472.

31. DeFronzo RA, Cooke CR, Andres R, Faloona GR and Davis PJ (1975). The effect of insulin on renal handling of sodium, potassium, calcium, and phosphate in man. *Journal of Clinical Investigations*; **55:** 845-855.

32. Ditzel J, Lervang H and Brochner-Mortensen J (1989). Renal sodium metabolism in relation to hypertension in diabetes. *Diabetes Metabolism*; **15:** 292-295.

33. Dominguez J, Camp K, Maianu L, Feister H and Garvey T (1994). Molecular adaptations of GLUT1 and GLUT2 in renal proximal tubules of diabetic rats. *American Journal of Physiology*; **266:** F238-290.

34. Dongun K, Jeff MS and Janet DK (2002). Changes in renal medullary transport proteins during uncontrolled diabetes mellitus in rats. *American Journal of Physiology*; **285:** F303-F309.

35. Duc C, Farman N and Canessa C (1994). Cell-specific expression of epithelial sodium channel alpha, beta, and gamma subunits in aldosterone-responsive epithelia from the rat: localization by in situ hybridization and immunocytochemistry. *The Journal of Cell Biology*; **127:** 1907-1921.

36. Dunham J and Guthmiller P (2008). (Doing good science: Authenticating cell line identity. *Cell Notes*; **22:** 16-17.

37. Edward C and Chao D (2014). SGLT-2 Inhibitors: a new mechanism for glycemic control. *Clinical Diabetes*; **32:** 4-11.

38. Evans LJ, Goldfine ID, Maddux BA and Grodsky GM (2002). Oxidative stress and stress-activated signaling pathways: A unifying hypothesis of type 2 diabetes. *Endocrine Reviews*; **23:** 559-662.

39. Farkas D and Tannenbaum S (2005). *In-vitro* methods to study chemically-induced hepatotoxicity: a literature. *Current Drug Metabolism*; **6:** 111-125.

40. Favaro E, Bensaad K, Chong MG, Tennant DA, Ferguson DJP, Snell C *et al.,* (2012). Glucose utilization via glycogen phosphorylase sustains proliferation and prevents premature senescence in cancer cells. *Cell Metabolism*; **16:** 751–764.

41. Fernandez M and Sanchez J (2001). Viability staining and terminal deoxyribonucleotide transferase-mediated dUTP nick end labelling of the mycelium in submerged cultures of Streptomyces antibioticus ETH7451. *Journal of Microbiological Methods*; **47:** 293-298.

42. Ferrannini E, Ramos S, Salsali A, Tang W and List J (2010). Dapagliflozin monotherapy in type 2 diabetic patients with inadequate glycemic control by diet and exercise: a randomized, double-blind, placebo-controlled, phase 3 trial. *Diabetes Care*; **33:** 2217–2224.

43. Finlay HJ, Honda T and Gribble GW (2002). Synthesis of novel [3,2-b]indole fused oleanolic acids as potential inhibitors of cell proliferation. *Archive for Organic Chemistry*; **12:** 38-46.

44. Forbes J, Coughlan M and Cooper M (2008). Oxidative stress as a major culprit in kidney disease in diabetes. *Diabetes*; **57:** 1446–1454.

45. Fujioka T, Kashiwada Y, Kilkuskie R, Cosentino L, Ballas L, Jiang J *et al.*, (1994). Anti-AIDS agents, 11. Betulinic acid and platanic acid as anti-HIV principles from *Syzygium claviflorum*, and the anti-HIV activity of structurally related triterpenoids. *Journal of Natural Products*; **57:** 243-247.

46. Fulda S (2008). Betulinic acid for cancer treatment and prevention. *International Journal of Molecular Sciences*; **9:** 1096-1107.

47. Gesek F, Cragoe J and Strandhoy J (1989). Synergistic cyl and cy2 adrenergic stimulation of rat proximal synergistic cyl and cy2 adrenergic stimulation of rat proximal nephron Na/H exchange. *Journal of Pharmacology and Experimental Therapeutics*; **249:** 694-700.

48. Giacchetti G, Sechi L, Rilli S and Carey R (2005). The renin–angiotensin–aldosterone system, glucose metabolism and diabetes. *Trends in Endocrinology and Metabolism*; **16:** 120-126.

49. Gnudi L, Thomas S and Viberti G (2007). Mechanical forces in diabetic kidney disease: A trigger for impaired glucose metabolism. *Journal of the American Society of Nephrology*; **18:** 2226–2232.

50. Gondwe M, Kamadyaapa D, Tufts M, Chuturgoon A and Musabayane C (2008). *Sclerocarya birrea* [(A. Rich.) Hochst.] [*Anacardiaceae*] stem-bark ethanolic extract

(SBE) modulates blood glucose, glomerular filtration rate (GFR) and mean arterial blood pressure (MAP) of STZ-induced diabetic rats. *Phytomedicine*; **15:** 699-709.

51. Gorboulev V, Schurmann A and Vallon V (2012). D-glucose cotransporter SGLT1 is pivotal for intestinal glucose absorption and glucose-dependent incretin secretion. *Diabetes*; **61:** 187-196.

52. Gutman Y, Hochman S and Wald H (1973). The differential effect of Li^+ on microsomal ATPase in cortex, medulla, and papilla of the rat kidney. *Biochema et Biophysica Acta*; **298:** 284-290.

53. Ha H and Kim KH (1999). Pathogenesis of diabetic nephropathy: the role of oxidative stress and protein kinase C. *Diabetes Research and Clinical Practice*; **45:** 147-151.

54. Ha J, Lee SH and Kim KH (1997). Effects of rebamipide in a model of experimental diabetes and on the synthesis of transforming growth factor-beta and fibronectin, and lipid peroxidation induced by high glucose in cultured mesangial cells. *Journal of Pharmacology and Experimental Therapeutics*; **281:** 1457-1462.

55. Haas J, Granger J and Knox F (1986). Effect of renal perfusion pressure on sodium reabsorption from proximal tubules of superficial and deep nephrons. *American Journal of Physiology*; **250:** 425-429.

56. Hadebe SI, Ngubane PS, Serumula MR and Musabayane CT (2014). Transdermal delivery of insulin by amidated pectin hydrogel matrix patch in streptozotocin-induced diabetic rats: effects on some selected metabolic parameters. *Plos One*; **9:** 1-10

57. Hansen K, Adsersen A, Christensen SB, Jensen SR, Nyman U and Smitt UW (1996). Isolation of an angiotensin converting enzyme (ACE) inhibitor from *Olea europaea* and *Olea lancea*. *Phytomedicine*; **2:** 319-325.

58. Hayslett JP and Kashgarian M (1979). A micropuncture study of the renal handling of lithium. *Pflugers Arch*; **380:** 159—163.

59. Heather JF, Tadashi H and Gordon WG (2000). Synthesis of novel [3,2-b]indole fused oleanolic acids as potential inhibitors of cell proliferation. *Archive for Organic Chemistry*; **12:** 38-46.

60. Heilig CW (1995). Overexpression of glucose transporters in rat mesangial cells cultured in a normal glucose milieu mimics the diabetic phenotype. *Journal of Clinical Investigation*; **96:** 1802–1814.

61. Hoek I, Lefebvre H, Kooistra H, Croubels S, Binst D and Peremans K (2008). Plasma clearance of exogenous creatinine, exo-iohexol, and endo-iohexol in hyperthyroid cats before and after treatment with radioiodine. *Journal of Veterinary Internal Medicine*; **22:** 879-885.

62. Holstey R, Svenden U and Leysaac P (1985). Effects of acute volume loading on kidney function in patients with essential hypertension, as estimated by the lithium clearance method. *Journal of Hypertension*; **3:** 511-516.

63. Horita S, Seki G, Yamada H, Suzuki M, Koike K and Fujita T (2011). Insulin resistance, obesity, hypertension, and renal sodium transport. *International Journal of Hypertension*; **2011:** 391762.

64. Hyun SK, Lee H, Kang SS, Chung HY and Choi JS (2009). Inhibitory activities of *Cassia tora* and its anthraquinone constituents on angiotensin-converting enzyme. *Phytotherapy Research*; **23:** 178–184.

65. Jakus V (2000). The role of free radicals, oxidative stress and antioxidant systems in diabetic vascular disease. *Bratisl Lek Listy*; **101:** 541-551.

66. Johnson PB, Abdurahman EM, Tiam EA, Abdu-Aguye I and Hussaini IM (1999). *Euphorbia hirta* leaf extracts increase urine output and electrolytes in rats. *Journal of Ethnopharmacology*; **65:** 63-69.

67. Jouad H, Eddouks M, Lacaille-Dubois MA and Lyoussi B (2000). Hypoglycaemic effect of *Spergularia purpurea* in normal and streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology*; **71:** 169-177.

68. Júlio C, Gentil J and Cleuza C (2003). A new tormentic acid Derivative from *Luehea divaricata* Mart. (Tiliaceae). *Journal of the Brazilian Chemical Society*; **14:** 475-478.

69. Kalk WJ, Raal FJ and Joffe BI (2010). The prevalence and incidence of and risk factors for, micro-albuminuria among urban Africans with type 1 diabetes in South Africa: An inter-ethnic study. *International Journal of Diabetes Mellitus*; **2:** 148-153.

70. Kanetsuna Y, Hirano K, Nagata M, Gannon MA, Takahashi K, Harris RC *et al.*, (2006). Characterization of diabetic nephropathy in a transgenic model of hypoinsulinemic diabetes. *American Journal of Physiology*; **291:** 1315-1322.

71. Khathi A, Serumula MR, Myburg RB, Van Heerden FR and Musabayane CT (2013). Effects of *Syzygium aromaticum*-derived triterpenes on postprandial blood glucose in streptozotocin-induced diabetic rats following carbohydrate challenge. *Plos One*; **8:** e81632.

72. Kirchner K (1987). Lithium as a marker for proximal tubular delivery during low salt intake and diuretic infusion. *Am J Physiol*; **253:** l88-96.

73. Kitamura M, Ishikawa Y, Moreno-Manzano V, Xu Q, Konta T, Lucio-Cazana J *et al.*, (2002). Intervention by retinoic acid in oxidative stress-induced apoptosis. *Nephrology Dialysis Transplantation*; **17:** 84–87.

74. Klahr S (2001). Urinary tract obstruction. *Seminars in Nephrology*; **21:** 133–145.

75. Klisic J, Hu MC, Nief V, Reyes L, Fuster D, Moe OW *et al.*, (2002). Insulin activates Na(+)/H(+) exchanger 3: biphasic response and glucocorticoid dependence. *American Journal of Physiology*, *Renal Physiology*; **283:** F532-539.

76. Koomans HA, Boer WH and Mess EJD (1989). Evaluation of lithium clearance as a marker of proximal tubule sodium handling. *Kidney International*; **36:** 2-12.

77. Koulmanda M, Qipo A and Chebrolu S (2003). The effect of low versus high dose of streptozotocin in cynomolgus monkeys (*Macaca fascilularis*). *American Journal of Transplantation*; **3:** 267–272.

78. Kroncke K, Kolb-Bachofen V, Berschick B, Burkart V and Kolb H (1991). Activated macrophages kill pancreatic syngeneic islet cells via arginine-dependent nitric oxide generation. *Biochemical and Biophysical Research Communications*; **175:** 752-758.

79. Kumar GS, Shetty AK and Salimath PV (2008). Modulatory effect of bitter gourd (Momordica charantia LINN.) on alterations in kidney heparan sulfate in streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology*; **115:** 276-283.

80. Kumar S, Malhotra R and Kumar D (2010). Antidiabetic and free radicals scavenging potential of *Euphorbia hirta* flower Extract. *Indian Journal of Pharmaceutical Sciences*; **72:** 533-537.

81. Kuramochi G and Homma S (1993). Effects of furosemide on renal oxygen consumption after ischemia in normal and streptozotocin diabetic rats. *Nephron Physiology*; **64:** 436–442.

82. Kwon EK, Lee DY, Hyungjae L, Kim DOK, Baek NIN and Kim YE (2010). Flavonoids from the buds of *Rosa damascena* inhibit the activity of 3-hydroxy-3-methylglutaryl-coenzyme a reductase and angiotensin I-converting enzyme. *Journal of Agricultural and Food Chemistry*; **58:** 882–886.

83. Lagemann A, Dunkel A and Hofmann T (2012). Activity-guided discovery of (S)-malic acid 1′-O-β-gentiobioside as an angiotensin I-converting enzyme inhibitor in lettuce (Lactuca sativa). *Journal of Agricultural and Food Chemistry*; **60:** 7211–7217.

84. Lenzen S (2008). The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia*; **15:** 216–226.

85. Lewis E, Hunsicker L and Clarke W (2001). Collaborative Study Group. Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. *The New England Journal of Medicine*; **345:** 851-860.

86. Li A, Bode C and Sakai Y (2004). A novel in vitro system, the integrated discrete multiple organ cell culture (IdMOC) system, for the evaluation of human drug toxicity: comparative cytotoxicity of tamoxifen towards normal human cells from five major organs and MCF-7adenocarcinoma breast cancer cells. *Chem Biol Interact*; **150:** 129-136.

87. Li C, Yang Z, Zhai C, Qiu W, Li D, Yi Z *et al.,* (2010). Maslinic acid potentiates the anti-tumor activity of tumor necrosis factor alpha by inhibiting NF-kappaB signaling pathway. *Mol Cancer*; **9:** 73.

88. Li Y, Wen S, Kota B, Peng G and Li G (2005). *Punica granatum* flower extract, a potent a-glucosidase inhibitor, improves postprandial hyperglycemia in Zucker diabetic fatty rats. *Journal of Ethnopharmacology*; **99:** 239–244.

89. Linden KC, DeHaan CL, Zhang Y, Glowacka S, Cox AJ, Kelly DJ *et al.,* (2006). Renal expression and localization of the facilitative glucose transporters GLUT1 and GLUT12 in

animal models of hypertension and diabetic nephropathy. *American Journal of Physiology, Renal Physiology*; **290:** F205–F213.

90. List J and Whaley J (2011). Glucose dynamics and mechanistic implications of SGLT2 inhibitors in animals and human. *Kidney International*; **79:** S20–S27.

91. Liu J, Sun H, Duan W, Mu D and Zhang L (2007). Maslinic acid reduces blood glucose in KK-A(y) mice. *Biological & Pharmaceutical Bulletin*; **30:** 2075-2078.

92. Liu J, Sun H, Duan W, Mu D and Zhang L (2007). Maslinic acid reduces blood glucose in KK-Ay mice. *Biological & Pharmaceutical Bulletin*; **30:** 2075-2078.

93. Liu J, Wang X, Chen Y-P, Mao L-F, Shang J, Sun H-B *et al.*, (2014). Maslinic acid modulates glycogen metabolism by enhancing the insulin signaling pathway and inhibiting glycogen phosphorylase. *Chinese Journal of Natural Medicines*; **12:** 259-265.

94. Lowry O, Rosebrough N, Farr A and Randall R (1951). Protein measurement with the folin phenol reagent. *The Journal of Biological Chemistry*; **193:** 265-275.

95. Madlala HP, Masola B, Singh M and Musabayane CT (2012). The effects of *Syzygium aromaticum*-derived oleanolic acid on kidney function of male Sprague-Dawley rats and on kidney and liver cell lines. *Renal failure*; **34:** 767-776.

96. Magri CJ and Fava S (2009). The role of tubular injury in diabetic nephropathy. *European Journal of Internal Medicine*; **20:** 551-555.

97. Maksimovic Z, Dobric S, Kovacevic N and Milovanovic Z (2004). Diuretic activity of *Maydis stigma* extract in rats. *Pharmazie*; **59:** 967-971.

98. Mapanga RF, Tufts MA, Shode FO and Musabayane CT (2009). Renal effects of plant-derived oleanolic acid in streptozotocin-induced diabetic rats. *Renal failure*; **31:** 481-491.

99. Marks J, Carvou N, Debnam E, Srai S and Unwin R (2003). Diabetes increases facilitative glucose uptake and GLUT2 expression at the rat proximal tubule brush border membrane. *Journal of Physiology*; **553:** 137–145.

100. Masilamani S, Kim GH, Mitchell C, Wade JB and Knepper MA (1999). Aldosterone-mediated regulation of ENaC alpha,beta, and gamma subunit proteins in rat kidney. *Journal of Clinical Investigation*; **104:** R19-R23.

101. Matavelli LC and Siragy HM (2013). Reduction of aldosterone production improves renal oxidative stress and fibrosis in diabetic rats. *J Cardiovasc Pharmacol*; **61:** 17-22.

102. Montilla M, Agil A and Navarro M (2003). Antioxidant activity of maslinic acid, a triterpene derivative obtained from *Olea europaea*. *Journal of Natural Products*; **69:** 472–474.

103. Mullins LJ, Bailey MA and Mullins JJ (2006). Hypertension, kidney, and transgenics: a fresh perspective. *Physiological Reviews*; **86:** 709-746.

104. Musabayane C, Gondwe M, Kamadyaapa D, Chuturgoon A and Ojewole J (2007). Effects of *Ficus thonningii* (Blume) [Morarceae] stem-bark ethanolic extract on blood glucose, cardiovascular and kidney functions of rats, and on kidney cell lines of the proximal (LLC-PK1) and distal tubules (MDBK. *Renal failure*; **29:** 389-397.

105. Musabayane C, Ndhlovu C and Balment R (1995). Renal fluid and electrolyte handling in streptozotocin induced diabetic rats. *Renal failure*; **17:** 107-116.

106. Musabayane CT (2012). The effects of medicinal plants on renal function and blood pressure in diabetes mellitus. *Cardiovascular Journal of Africa*; **23:** 462–468.

107. Musabayane CT, Mahlalela N, Shode FO and Ojewole JA (2005). Effects of *Syzygium cordatum* (Hochst.) [Myrtaceae] leaf extract on plasma glucose and hepatic glycogen in streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology*; **97:** 485-490.

108. Na M, Yang S, He L, Oh H, Kim BS, Oh WK *et al.*, (2006). Inhibition of protein tyrosine phosphatase 1B by ursane-type triterpenes isolated from *Symplocos paniculata*. *Planta Med*; **72:** 261-263.

109. Nangaku M (2006). Chronic hypoxia and tubulointerstitial injury: a final common pathway to end-stage renal failure. *Journal of the American Society of Nephrology*; **17:** 17-25.

110. Narender T, Madhur G, Jaiswal N, Agrawal M, Maurya CK, Rahuja N *et al.*, (2013). Synthesis of novel triterpene and N-allylated/N-alkylated niacin hybrids as α-glucosidase inhibitors. *European Journal of Medicinal Chemistry*; **63:** 162-169.

111. Navarro E, Alonso J, Rodriguez R, Trujillo J and Boada J (1994). Diuretic action of an aqueous extract of *Lepidium latifolium L*. *Journal of Ethnopharmacology*; **41:** 65-69.

112. O'Neill H, Lebeck J, Collins PB, Kwon TH, Frokiaer J and Nielsen S (2008). Aldosterone-mediated apical targeting of ENaC subunits is blunted in rats with streptozotocin-induced diabetes mellitus. *Nephrology Dialysis Transplantation*; **23:** 1546-1555.

113. Ogawa A, Willoughby C, Bergeron R, Ellsworth K, Geissler W, Myers R *et al.*, (2003). Glucose-lowering in a db/db mouse model by dihydropyridine diacid glycogen phosphorylase inhibitors. *Bioorganic and Medicinal Chemistry Letters*; **13:** 3405 –3340.

114. Oliveira P, Michelotto J, Zapparoli A and Gontijo J (2010). Intracerebroventricular insulin injection on renal sodium handling: role of centally nitric oxide synthase inhibition in rats. *Advanced Studies in Biology*; **2:** 1-14.

115. Pacher P, Obrosova IG, Mabley JG and Szabó C (2005). Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complication. *Current Medicinal Chemistry*; **12:** 267–275.

116. Pantoja CV, Chiang LC, Norris BC and Concha JB (1991). Diuretic, natriuretic and hypotensive effects produced by *Allium-sativum* (Garlic) in anesthetized dogs. *Journal of Ethnopharmacology*; **31:** 325-331.

117. Pantoja CV, Martin NT, Norris BC and Contreras CM (2000). Purification and bioassays of a diuretic and natriuretic fraction from garlic (*Allium sativum*). *Journal of Ethnopharmacology*; **70:** 35-40.

118. Pantoja CV, Norris BC and Contreras CM (1996). Diuretic and natriuretic effects of chromatographically purified fraction of garlic (*Allium sativum*). *Journal of Ethnopharmacology*; **52:** 101–105.

119. Papaharalambus C and Griendling K (2007). Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. *Trends in Cardiovascular Medicine*; **17:** 84–54.

120. Pari L and Latha MP (2004). Protective role of *Scoparia dulcis* plant extract on brain antioxidant status and lipidperoxidation in STZ diabetic male Wistar rats. *BMC Complementry and Alternative Medicine*; **4:** 16–24.

121. Parving HH, Osterby R and Ritz E (2000). Diabetic nephropathy. *Kidney international*; **14:** 1731–1773.

122. Pavlovaa N, Savinovaa O, Nikolaevaa S, Boreko E and Flekhterb O (2003). Antiviral activity of betulin, betulinic and betulonic acids against some enveloped and non-enveloped viruses. *Fitoterapia*; **74:** 489-492.

123. Pinent M, Blay M, Blade M, Salvado M and Arola L (2004). Grape seedderived procyanidins have an anti-hyperglycaemic effect in streptozotocin-induced diabetic rats and insulino-mimetic activity in insulin-sensitive cell lines. *Endocrinology and Metabolism Clinics of North America*; **145:** 4985–4990.

124. Poonam T, Prakash GP and Kumar LV (2013). Influence of *Allium sativum* extract on the hypoglycemic activity of glibenclamide: an approach to possible herb-drug interaction. *Drug Metabolism and Drug Interaction*; **28:** 225-230.

125. Prashar A, Locke I and Evans C (2006). Cytotoxicity of clove (*Syzygium aromaticum)* oil and its major components to human skin cells. *Cell Proliferation*; **39:** 241-248

126. Pruijm M, Wuerzner G and Maillard M (2010). Glomerular hyperfiltration and increased proximal sodium reabsorption in subjectswith type 2 diabetes orimpaired fastingglucose in a population of the African region. *Nephrology and Dialysis Transplantation*; **25:** 2225-2231.

127. Puranik N, Kammar KF and Devi S (2010). Anti-diabetic activity of *Tinospora cordifolia (Willd.)* in streptozotocin diabetic rats; does it act like sulfonylurea? *Turkish Journal of Medical Sciences*; **40:** 265–270.

128. Qiu WW, Shen Q, Yang F, Wang B, Zou H, Li J-Y *et al.,* (2009). Synthesis and biological evaluation of heterocyclic ring-substituted maslinic acid derivatives as novel inhibitors of protein tyrosine phosphatase 1B. *Bioorganic & Medicinal Chemistry Letters*; **19** 6618–6622.

129. Quamme G and Freeman H (1987). Evidence for a high-affinity sodium-dependent D-glucose transport system in the kidney. *American Journal of Physiology*; **253:** F151-F157.

130. Rahmoune H, Thompson P, Ward J, Smith C, Hong G and Brown J (2005). Glucose transporters in human renal proximal tubular cells isolated from the urine of patients with non-insulindependent diabetes. *Diabetes*; **54:** 3427-3434.

131. Raju DSSK, Lalitha DL and Kiranmayi P (2013). A Study of lipid profile and lipid peroxidation in chronic kidney disease with special reference to hemodialysis. *Clinical Research & Bioethics*; **4:** 1-5.

132. Rebsomen L, Pitel S, Boubred F, Buffat C, Feuerstein JM, Raccah D *et al.*, (2005). C-peptide replacement improves weight gain and renal function in diabetic rats. *Diabetes Metabolism*; **32:** 223-228.

133. Reuter HD (1995). *Allium sativum* and *Allium ursinum*: Part 2 pharmacology and medicinal application. *Phytomedicine*; **2:** 73-91.

134. Rho SJ, Lee JS, Chung YI, Kim YW and Lee HG (2009). Purification and identification of an angiotensin I-converting enzyme inhibitory peptide from fermented soybean extract. *Process Biochemistry*; **44:** 490–493.

135. Riazi S, Maric C and Ecelbarger C (2006). 17-beta Estradiol attenuates streptozotocin-induced diabetes and regulates the expression of renal sodium transporters. *Kidney International*; **69:** 471– 480.

136. Ribeiro RA, de Barros F, de Melo MM, Muniz C, Chieia S, Wanderley M das G *et al.*, (1988). Acute diuretic effects in conscious rats produced by some medicinal plants used in the state of Sao Paulo, Brasil. *Journal of Ethnopharmacology*; **24:** 19-29.

137. Riquier-Brison AD, Leong PK, Pihakaski-Maunsbach K and McDonough AA (2010). Angiotensin II stimulates trafficking of NHE3, NaPi2, and associated proteins into the proximal tubule microvilli. *American Journal of Physiology, Renal Physiology*; **298:** F177-118.

138. Riss TL, A M, Niles AL, Benink HA, Worzella TJ and Minor L (2013). Cell viability Assays *Assay guidance manual*; **9:** 1-23.

139. Rodrigo R and Rivera G (2002). Renal damage mediated by oxidative stress: a hypothesis of protective effects of red wine. *Free Radical Biology and Medicine*; **33:** 409-422.

140. Rossing P and de Zeeuw D (2011). Need for better diabetes treatment for improved renal outcome. *Kidney International Supplements*; **120:** S28-S32.

141. Satirapoj B, Supasyndh O, Patumanond J and Choovichian P (2006). Estimating glomerular filtration rate in Asian patients with chronic kidney diseases from bioelectrical impedance analysis. *Journal of the Medical Associaion of Thailand*; **89:** 1584-1591.

142. Shah SV (2001). Role of iron in progressive renal disease. *American Journal of Kidney Diseases*; **37:** S30-S33.

143. Shalmi M and Thomsen K (1989). Alterations of lithium clearance in rats by different modes of lithium administration. *Renal Physiology and Biochemistry*; **12:** 273-280.

144. Sharafatullah T, Khan MI and Ahmad SI (1986). Diuretic action of garlic extract in anaesthetised normotensive dogs. *JPMA. The Journal of the Pakistan Medical Association*; **36:** 280-282.

145. Shirley D and Walter S (1997). Renal tubular lithium reabsorption in potassium-depleted rats. *The Journal of Physiology*; **501:** 663–670.

146. Shirley DG, Walters J and Noormohamed FH (2002). Natriuretic effect of caffeine: assessment of segmental sodium reabsorption in humans. *Clinica Science*; **103:** 461-466.

147. Shirley G and Walter SJ (1990). The lithiuretic effect of fritsemide: a micropuncture study in anaesthetized rats. *Journal of Physiology*; **430:** 27.

148. Simaratanamongkol A, Umehara K, Noguchi H and Panichayupakaranant P (2014). Identification of a new angiotensin-converting enzyme (ACE) inhibitor from Thai edible plants. *Food Chemistry*; **165:** 92-97.

149. Singh DK, Winocour P and Farrington K (2011). Oxidative stress in early diabetic nephropathy: fueling the fire. *Nature Reviews Endocrinology*; **7:** 176-184.

150. Somova LO, Nada A, Rammana P and Shode FO (2003). Cardiovascular, antihyperlipidemic and antioxidant effects of oleanolic and ursolic acids in experimental hypertension. *Phytomedicine*; **10:** 115-121.

151. Song J, Knepper M, Verbalis J and Ecelbarger C (2003). Increased renal NaC subunit and sodium transporter abundances in streptozotocininduced ype 1 diabetes. *American Journal of Renal Physiology*; **285:** F1125–F1137.

152. Stengel B, Billon S, Dijk PCWv, Jager KJ, Dekker FW, Simpson K *et al.*, (2003). Trends in the incidence of renal replacement therapy for endstage renal disease in Europe,. *Nephrology Dialysis Transplantation*; **18:** 1824-1183.

153. Stewart W, Stewart TW and Warburton FG (1961). Serum LD estimations in myocardial infarction. *British Heart Journal*; **23:** 236.

154. Stumvoll M, Nujahn N, Perriello G, Dailey G and ERICH G (1995). Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. *The New England Journal of Medicine*; **10:** 550-554.

155. Suzuki R, Okada Y and Okuyama T (2005). The favorable effect of style of *Zea mays* L. on streptozotocin induced diabetic nephropathy. *Biological and Pharmaceutical Bulletin*; **28:** 919-920.

156. Swasti T, Shahla R and Carolyn AE (2007). Insulin's impact on renal sodium transport and blood pressure in health,obesity, and diabetes. *American Journal of Physiology*; **293:** F974–F984.

157. Tabatabai NM, Sharma M, Blumenthal SS and Petering DH (2009). Enhanced expressions of sodium–glucose cotransporters in the kidneys of diabetic Zucker rats. *Diabetes Research and Clinical Practice*; **83:** e27-e30.

158. Thomas MC, Burns WC and Cooper ME (2005). Tubular changes in early diabetic nephropathy. *Advances in Chronic Kidney Disease*; **12:** 177-186.

159. Thomsen K (1990). Lithium clearance as a measure of sodium and water delivery from the proximal tubules. *Kidney International*; **37:** 10-16.

160. Thomsen K, Holstein-Rathlou N and Leyssac P (1981). Comparison of three measures of proximal tubular reabsorption: Lithium clearance, occlusion time, and micropuncture. *American Journal of Physiology*; **241:** 348-355.

161. Thomsen K, Holstein-Rathlou NH and Leyssac PP (1981). Comparison of three measures of proximal tubular reabsorption: Lithium clearance, occlusion time, and micropuncture. *Am J Physiol*; **241:** 348-355.

162. Thomsen K and Shirley DG (1997). The validity of lithium clearance as an index of sodium and water delivery from the proximal tubules. *Nephron*; **77:** 125-138.

163. Thomson S, Deng A, Bao D, Satriano J, Blantz R C and Vallon V (2001). Ornithine decarboxylase, kidney size, and the tubular hypothesis of glomerular hyperfiltration in experimental diabetes. *Journal of Clinical Investigation*; **107:** 217-224.

164. Thomson SC and Blantz RC (2008). Glomerulotubular Balance, Tubuloglomerular Feedback, and Salt Homeostasis. *Journal of the American Society of Nephrology*; **19:** 2272–2275.

165. Thorens B, Lodish H and Brown D (1990). Differential localization of two glucose transporter isoforms in rat kidney. *American Journal of Physiology*; **259:** C286–C294.

166. Tian N, Kristina D. Thrasher, Paul D. Gundy, Michael D. Hughson and Manning RD (2005). Antioxidant treatment prevents renal damage and dysfunction and reduces arterial pressure in salt-sensitive hypertension. *Jornal of american heart association*; **45:** 934-939.

167. Tomlinson K, Gardiner S, Hebden R and Bennett T (1992). Functional consequences of streptozotocin-induced diabetes mellitus, with particular reference to the cardiovascular system. *Pharmacological Reviews*; **44:** 103–131.

168. Trachtman H, Futterweit S and Bienkowski RS (1993). Taurine prevents glucose-induced lipid peroxidation and increased collagen production in cultured rat mesangial Cells. *Biochemical and Biophysical Research Communications*; **191:** 759-765.

169. Valentine V (2012). The role of the kidney and sodium-glucose cotransporter-2 inhibition in diabetes Management. *Clinical Diabetes*; **30** 151-155.

170. Vallon V, Platt K, Cunard R, Schroth J, Whaley J, Thomson S *et al.*, (2011). SGLT2 mediates glucose reabsorption in the early proximal tubule *Journal of the American Society of Nephrology*; **22**.

171. Vallon V, Richter K, Blantz RC, Thomson S and Osswald H (1999). Glomerular hyperfiltration in experimental diabetes mellitus : potential role of tubular reabsorption *Journal of American Society of Nephropathy*; **11:** 71-79.

172. Velazquez DVO, Xavier HS, Batista JEM and de Castro-Chaves C (2005). *Zea mays L*. extracts modify glomerular function and potassium urinary excretion in conscious rats. *Phytomedicine*; **12:** 363-369.

173. Vestri S, Okamoto M, Freitas H, Dos S and Nunes M (2001). Changes in sodium or glucose filtration rate modulate expression of glucose transporters in renal proximal tubular cells of rat. *Journal of Membrane Biology*; **182:** 105-112.

174. Vogel M, Kranzlin B, Biber J, Murer H, Gretz N and Bachmann S (2000). Altered expression of type II sodium/phosphate cotransporter in polycystic kidney disease. *Journal of the American Society of Nephrology*; **11:** 1926-1932.

175. Wagaw S, YangBryant H and Buchwald SL (1998). A Palladium-catalyzed strategy for the preparation of indoles: a novel entry into the fischer indole synthesis. *Journal of American Chemical Society*; **120:** 6621-6622.

176. Walter SJ and Shirley DG (1991). Effect of frusemide on lithium clearance and proximal tubular reabsorption in anaesthetized rats. *Journal of Physiology*; **437:** 85-93.

177. Weder A (1986). Red cell lithium sodium countertransport and renal lithium clearance in hypertension. *New England Journal of Medicine*; **201:** 198-314.

178. Wen X, Zhang P, Liu J, Zhang L, Wu X, Ni P *et al.*, (2006). Pentacyclic triterpenes. Part 2: Synthesis and biological evaluation of maslinic acid derivatives as glycogen phosphorylase inhibitors. *Bioorganic and Medicinal Chemistry Letters*; **16:** 722-726.

179. Whiteside CI and Dlugosz JA (2002). Mesangial cell protein kinase C isozyme activation in the diabetic milieu. *American Journal of Physiology, Renal Physiology*; **282:** 975–980.

180. Williams HD, Trevaskis NL, Charman SA, Shanker RM, Charman WN, Pouton CW *et al.*, (2013). Strategies to address low drug solubility in discovery and development. *Pharmacological Reviews*; **65:** 315-499.

181. Wohaieb SA and Godin DV (1987). Alterations in free radical tissue-defense mechanisms in streptozocin-induced diabetes in rat. Effects of insulin treatment. *Diabetes*; **36:** 1014-1018.

182. Wood IS and Trayhurn P (2003). Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. *British Journal of Nutrition*; **89:** 3 –9.

183. Xu Q, Chen G and Fan J (2009). Chemical constituents of roots of *Boehmeria nivea*. *China Journal of Chinese Materia Medica*; **34:** 2610–2612.

184. Yamabe N, Yokozawa T, Oya T and Kim M (2006). Therapeutic potential of (-)-epigallocatechin 3-O-gallate on renal damage in diabetic nephropathy model rats. *Journal of Pharmacology and Experimental Therapeutics*; **319:** 228-236.

185. Yu T and Khraibi AA (2008). Enalapril treatment restores the decreased proximal tubule reabsorption in response to acute volume expansion in diabetic rats. *Life Sciences*; **83:** 364-368.

186. Zhang W, Hong D, Zhou Y, Zhang Y, Shen Q, Li JY *et al.,* (2006). Ursolic acid and its derivative inhibit protein tyrosine phosphatase 1B, enhancing insulin receptor phosphorylation and stimulating glucose uptake. *Biochim Biophys Acta*; **1760:** 1505-1512.

187. Zhang YN, Zhang W, Hong D, Shi L, Shen Q, Li JY *et al.,* (2008). Oleanolic acid and its derivatives: new inhibitor of protein tyrosine phosphatase 1B with cellular activities. *Bioorg Med Chem*; **16:** 8697-8705.

188. Ziyadeh F and Han DC (1997). Involvement of transforming growth factor-β and its receptors in the pathogenesis of diabetic nephropathy. *Kidney International*; **52:** S7–S11.

APPENDIX I

UNIVERSITY OF
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INYUVESI
YAKWAZULU-NATALI
Research Office
Animal Ethics Research Committee
Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
Email: animalethics@ukzn.ac.za

02 October 2012

Reference: 002/13/Animal

Mr BN Mkhwanazi
School of Laboratory Medicine
and Medical Sciences
University of KwaZulu-Natal
WESTVILLE

Dear Mr Mkhwanazi

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 **2012/2013** on the following project:

"The effects of plant derived maslinic acid (MA) and related triterpenes on kidney function of Streptozotocin-induced diabetic rats."

Yours sincerely

HT Coetzer

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar – Prof. J Meyerowitz
Research Office – ~~Mr N Moodley~~ Dr N. Singh
Supervisor, Prof. C Musabayane
Head of School – Prof. W Daniels
BRU, Dr S Singh

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APPENDIX II

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

20 December 2013

Reference: 029/14/Animal

Mr B Mkhwanazi
Discipline of Physiology
School of Laboratory Medicine &
Medical Sciences
WESTVILLE Campus

Dear Mr Mkhwanazi

RENEWAL: Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for **2014** on the following project:

"Effects of maslinic acid (MA) and related triterpene derivatives on kidney function of streptozotocin-induced diabetic rats."

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

Cc Registrar – Prof. J Meyerowitz
Research Office – Dr N Singh
Supervisor – Prof. C Musabayane
Head of School – Prof. W Daniels
BRU – Dr S Singh

Animal Ethics Committee
Professor Theresa HT Coetzer (Chair)
Postal Address: Room 105, John Bews Building, Private Bag X01, Pietermaritzburg, 3201, South Africa
elephone: +27 (0)33 260 5463/35 **Facsimile:** +27 (0)33 260 5105 **Email:** animalethics@ukzn.ac.za **Website:** www.ukzn.ac.za
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

APPENDIX III: Conference presentations

Endocrine Abstracts (2012) **28** P207

Effects of *Syzygium aromaticum*-derived maslinic acid on blood glucose of streptozotocin induced-diabetic rats

Blessing Mkhwanazi[1], Andile Khathi[1], Masola Bubuya[2], Fanie van Heerden[3] & Cephas Musabayane[1]

Available evidence suggests that *Syzygium* spp ethyl acetate solubles (EAS) contain triterpene mixtures (oleanolic/ursolic acid and methyl maslinate/methyl corosolate) with hypoglycaemic properties in streptozotocin (STZ)-induced diabetic rats. Accordingly, maslinic acid (MA) was isolated from S. aromaticum. Air-dried powdered flower buds of S. aromaticum were sequentially extracted thrice at 24 h intervals with 3 litres on each occasion of hexane, dichloromethane, ethyl acetate and methanol. The stereostructure of MA obtained following recrystallization with chloroform and methanol was elucidated by 1 h- and 13C-NMR spectroscopy (1D and 2D) on chemical and physicochemical evidence. Oral glucose tolerance (OGT) responses to various doses of MA (20, 40 and 80 mg.kg-1, p.o.) were monitored in non-diabetic and STZ-induced diabetic rats after an 18-h fast. Rats treated with deionized water or metformin acted as untreated and treated positive controls, respectively. Blood glucose concentrations were measured at 15-min intervals for the first hour, and hourly thereafter for 3 h. Short-term effects were monitored after 6 h in animals treated with MA twice daily by means of a bulbed steel tube for a period of 5 weeks while, food and water intake as well as body weight were monitored 24 hours after treatment every third day. All results are presented as mean± standard error means where $P<0.05$ denotes statistical significance. All doses of MA significantly decreased blood glucose of non-diabetic and STZ-induced diabetic rats throughout the experimental period. Comparisons of the blood glucose lowering effects of the MA doses at each time appeared to be dose-dependent in STZ-induced diabetic rats. MA administration reduced the body weight loss of STZ-induced diabetic rats without altering food intake. The results suggest that MA, like metformin, contains blood glucose lowering properties suggesting that it is a potential drug for the management of type 1 diabetes mellitus.

Declaration of interest: There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding: No specific grant from any funding agency in the public, commercial or not-for-profit sector.

ANTIOXIDANT EFFECTS OF *SYZYGIUM AROMATICUM*-DERIVED MASLINIC ACID IN LIVERS, HEARTS AND KIDNEYS OF STREPTOZOTOCIN-INDUCED DIABETIC RATS: EFFECTS ON KIDNEY FUNCTION

Mkhwanazi, BN; [1] Serumula, M; [1] Myburg, R; [2]Van Heerden F R & Musabayane, CT

Discipline of [1] Human Physiology and [2] Chemistry

Previous studies indicate that hyperglycaemia-induced oxidative stress triggers the development and progression of microvascular and macrovascular complications in diabetes. Medicinal plants contain active ingredients such as phenolic substances which have been previously shown to possess some antioxidants effects in animals. Accordingly, we hypothesized that *Syzygium aromaticum* derived-maslinic acid (MA) may alleviate these complications due to its antioxidant properties.

To verify this, we investigated the effects of 5-week MA treatment on anti-oxidative status of cardiac, hepatic and renal tissues of streptozotocin (STZ)-induced diabetic rats as well as on kidney function and blood glucose concentration. Proximal tubular effects of MA were studied in anaesthetized rats challenged with hypotonic saline after a 3.5-h equilibration for 4h of 1 h control, 1.5 h treatment, and 1.5 h recovery periods using lithium clearance. MA was added to the infusate during the treatment period.

Oral glucose tolerance responses to MA were monitored in rats given a glucose load after an 18-h fast. By comparison with STZ-induced diabetic control, MA significantly reduced malondialdehyde (MDA, a marker of lipid peroxidation) levels and increased the activity of antioxidant enzymes; superoxide dismutase and glutathione peroxidase in hepatic, cardiac and renal tissues. MA administration increased urinary Na^+ outputs and additionally the FE_{Na} and FE_{Li} indicating that at least part of the overall reduction in Na^+ reabsorption occurred in the proximal tubules.

These results suggest that antioxidant effects of MA can in part be involved in improving kidney function in diabetes mellitus

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The effects of *Syzygium aromaticum*-derived oleanolic acid on reactive oxygen species in the heart, liver and kidney of STZ-induced diabetic rats

Blessing Mkhwanazi, Ntethelelo Sibiya, Metse Serumala, Rene Myburg & Cephas T Musabayane

The onset of diabetic complications is attributed to sustained hyperglycemia which triggers the generation of free radicals and oxidative-related damage in the retina, renal glomerulus and peripheral nerves. Recent studies report that intense glycaemic control by the subcutaneous administration of insulin cannot completely restore the balance between reactive oxygen species and antioxidants. Preliminary studies in our laboratory indicate that transdermally delivered *Syzygium aromaticum*-derived oleanolic acid (OA) has the ability to lower blood glucose in experimental diabetes mellitus due to the sustained release of the triterpene. However, no work has been done to determine the effects of OA on reactive oxygen species (ROS). Research has indicated that some bioactive compounds such as flavonoids and tannins have antioxidant properties. Accordingly, this study was designed to investigate and evaluate the effects of *S. aromaticum*-derived OA on ROS levels. The acute effects of OA were evaluated on malondialdehyde (MDA) and glutathione (GSH) concentrations in STZ-induced diabetic rats following a glucose load after an 18-h fast. Rats administered pectin-free OA or transdermally delivered insulin acted as untreated and treated positive controls, respectively, while non-diabetic rats served as absolute controls. The transdermal patches were applied for 6 h, thereafter the animals were sacrificed. The heart, liver and kidney were collected for ROS biomarkers (MDA) and antioxidants (GSH) analysis. MDA levels were significantly reduced in the heart (233.0±0.1 vs 47.0±0.1 nmol/l) and liver (233.0±0.1 vs 130.0±0.1 nmol/l). Interestingly, GSH levels were also significantly increased in the heart (196.0±0.4 vs 313.0±0.5 nmol/l). These results suggest that *S. aromaticum*-derived OA is potentially effective in ameliorating the oxidative stress observed in diabetes mellitus in the heart. It can therefore, be concluded that OA is a potential drug for diabetes mellitus that would not only lower blood glucose but also can avert complications that arise due to oxidative stress.

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ORAL PRESENTATION 22

EFFECTS OF MASLINIC ACID (MA) DERIVATIVES ON KIDNEY FUNCTION OF MALE SPRAGUE-DAWLEY RATS

AUTHOR/S	INSTITUTE	EMAIL
[1]B N Mkhwanazi [2]F R Van Heerden [1]C T Musabayane	Schools of [1]Laboratory Medicine & Medical Sciences; [2]Chemistry and Physics University of KwaZulu-Natal	206519349@stu.ukzn.ac.za

ABSTRACT

Studies indicate that *Syzygium* spp-derived maslinic acid (MA) enhances renal function of streptozotocin (STZ)-induced diabetic rats as evidenced by its reversal of the previously reported inability of the kidney to excrete Na^+ in these animals. Further studies indicated that may partly mediate natriuresis via the proximal tubule as shown by lithium clearance. MA is, however, hydrophobic and therefore difficult to dissolve. Derivatives were synthesizes with an aim to improve solubility and efficacy of lead MA. This study, therefore, investigated the effects of MA derivatives on renal function. MA derivatives were synthesized by introduction of phenylhydrazine (PH), floro phenyl hydrazine (FPH) and choloro-phenyl hydrazine (CPH) on the carbon 2 and 3 of the triterpene. Renal effects of MA derivatives were studied in anaesthetized rats challenged with hypotonic saline after a 3.5 h equilibration for 4 h of 1 h control, 1.5 h treatment, and 1.5 h recovery periods. Derivatives were added to the infusate during the treatment period. Administration of these derivatives significantly increased urinary Na^+ outputs with PH-derivative exerting more potency when compared to lead MA. These results indicate that PH-derivative is a promising derivative for ameliorating kidney dysfunction.

APPDENDIX IV: PUBLICATIONS

RENAL FAILURE

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LABORATORY STUDY

Antioxidant effects of maslinic acid in livers, hearts and kidneys of streptozotocin-induced diabetic rats: effects on kidney function

Blessing N. Mkhwanazi[1], Metse R. Serumula[1], Rene B. Myburg[1], Fanie R. Van Heerden[2], and Cephas T. Musabayane[1]

[1]School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa and [2]School of Chemistry and Physics, University of KwaZulu-Natal, Durban, South Africa

Abstract

Studies indicate that hyperglycemia-induced oxidative stress triggers the development of microvascular and macrovascular complications in diabetes. Accordingly, we hypothesized that maslinic acid (MA) prevents these complications due to its antioxidant properties. We, therefore, investigated the effects of 5-week MA treatment of streptozotocin (STZ)-induced diabetic rats on anti-oxidative status of cardiac, hepatic and renal tissues as well as on kidney function. Proximal tubular effects of MA were studied in anesthetized rats challenged with hypotonic saline after a 3.5 h equilibration for 4 h of 1 h control, 1.5 h treatment and 1.5 h recovery periods using lithium clearance. MA was added to the infusate during the treatment period. Oral glucose tolerance responses to MA were monitored in rats given a glucose load after an 18 h fast. Compared with untreated diabetic rats, MA-treated diabetic animals exhibited significantly low malondialdehyde (MDA, a marker of lipid peroxidation) and increased the activity of antioxidant enzymes; superoxide dismutase and glutathione peroxidase in hepatic, cardiac and renal tissues. The expressions of gastrocnemius muscle GLUT4 and kidney GLUT1 and GLUT2 were assessed to elucidate the mechanism of the hypoglycemic effects of MA. MA-treatment diminished the expression of GLUT1 and GLUT2 in diabetic kidney and reduced glycemia values of diabetic rats. MA administration increased urinary Na^+ outputs and additionally the FE_{Na} indicating that at least part of the overall reduction in Na^+ reabsorption occurred in the proximal tubules. These results suggest antioxidant effects of MA can ameliorate oxidative stress and improve kidney function in diabetes mellitus.

Keywords

Antioxidant, diabetes, glutathione peroxidase, kidney, maslinic acid, malondialdehyde and superoxide dismutase

History

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Introduction

The pentacyclic triterpenes ursolic acid (UA), oleanolic acid (OA) and maslinic acid (MA) with wide-spread occurrence throughout the plant kingdom possess a broad spectrum of pharmacological properties which include anti-inflammatory, anti-humoral, hepatoprotective, hypoglycemic, antibacterial and anti-HIV effects.[1,2] MA (2α,3β-2,3-dihydroxy-olean-12-en-28-oic acid), which is abundant in olive fruit skin, has attracted much attention due to its anti-tumor,[3] anti-HIV,[4] anti-oxidation[5] and hypoglycemic[6–8] activities. However, important questions whether MA can prevent oxidative stress to consequently alleviate or ameliorate microvascular and macrovascular complications in diabetes remain unanswered. Indeed, experimental evidence suggests that oxidative stress plays a dominant role in the pathogenesis of diabetic complications.[5,9] Reports indicate that several plant extracts modulate diabetes associated oxidative stress processes.[10] Against this background are observations that phytochemicals containing quercetin and flavonoids decrease blood glucose concentrations and oxidative stress in experimental diabetes.[11] In this study, we focus on the effects of plant-derived MA on oxidative stress in the liver, heart and kidney of STZ-induced diabetic rats. We were also interested in determining whether MA can improve renal function often impaired in experimental diabetes[12,13] and in end stage renal disease (ESRD).[14] ESRD characterized by functional and structural abnormalities of the kidney are a common and often severe complication attributed in part to decreases in antioxidants.[15,16] Therefore, we also evaluated the effects of MA on renal proximal tubular Na^+ handling, the site for the reabsorption of approximately two thirds of the Na^+ that enters the tubular fluid by glomerular filtration.[17] In addition, this study investigated whether MA influences renal expression of the facilitative glucose transporters in STZ-induced diabetic rats. The up-regulation of these transporters has been implicated in the pathogenesis of progressive diabetic nephropathy.[18]

Address correspondence to Professor C.T. Musabayane, Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, University of KwaZulu Natal, Private Bag X54001, Durban 4000, South Africa. Tel: +27 31 260 7975; Fax: +27 31 260 7132; E mail: musabayanec@ukzn.ac.za

Materials and methods

Drugs and chemicals

Drugs were sourced from standard pharmaceutical suppliers. All other chemicals which were of analytical grade quality were purchased from standard commercial suppliers.

Isolation of MA

MA was isolated from *Syzygium aromaticum* [(Linnaeus) Merrill & Perry] [Myrtaceae] (cloves) flower buds using a standard protocol that has been validated in our laboratory,[19,20] with minor changes. Air-dried *S. aromaticum* flower buds (500 g) were sequentially extracted twice at 24 h intervals at room temperature with 1 L dichloromethane (DCM), and ethyl acetate (720 mL) on each occasion. Removal of the solvent from the extract under reduced pressure at 55 ± 1 °C using a rotary evaporator yielded dichloromethane solubles (DCMS, 63 g) and ethyl acetate solubles (EAS, 85 g). The EAS containing mixtures of oleanolic/ursolic acid and methyl maslinate/methyl corosolate were purified by silica gel 60 column chromatography with a hexane: ethyl acetate (7:3 solvent system. This yielded MA which was further purified by recrystallization from chloroform–methanol (1:1, v/v). The structure of MA was confirmed by spectroscopic analysis using 1D and 2D, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopic techniques.

Animals

Male Sprague-Dawley rats (250–300 g) bred at the Biomedical Research Unit, University of KwaZulu-Natal were used in this study. The animals were kept and maintained under standard laboratory conditions of temperature, humidity, 12 h day:12 h night cycle and allowed access to standard rat chow (Meadow Feeds, Pietermaritzburg, South Africa) and water *ad libitum*. All animal experimentation was reviewed and approved by the Animal Ethics Committee of the University of KwaZulu-Natal (102/11/Animal).

Induction of diabetes mellitus

Type 1 diabetes mellitus was induced by a single intraperitoneal injection of 60 mg kg^{-1} STZ in freshly prepared 0.1 M citrate buffer (pH 6.3). Control group received the vehicle, citrate buffer through the same route. Animals that exhibited glucosuria after 24 h, tested by urine strips (Rapidmed Diagnostics, Sandton, South Africa) were considered diabetic. Seven days later, the blood glucose concentration of STZ-induced diabetic rats greater than 20 mmol L^{-1} was considered as stable diabetes.

Experimental design

Non-diabetic and STZ-induced diabetic rats were divided into separate groups to study the following: (1) short-term (5 weeks) effects of MA on blood glucose, kidney function and oxidative stress in livers, hearts and kidneys, (2) effects of MA on proximal tubule function and (3) oral glucose tolerance (OGT) responses to MA ($n = 6$ in each group).

Short-term studies

The short-term effects of MA on blood glucose, mean arterial pressure (MAP) and renal function were monitored for 5 weeks in separate groups of non-diabetic and STZ-induced diabetic male rats housed individually in Makrolon polycarbonate metabolic cages (Tecniplats, Labotec, South Africa). Various doses of MA (20, 40 and 80 mg kg^{-1}, p.o.) were administered twice every third day at 09h00 and 15h00 in separate groups of rats by means of a bulbed steel tube. Animals which received DMSO/saline (3 mL kg^{-1}, p.o.) and metformin (500 mg kg^{-1}, p.o.) acted as control and positive control, respectively.

Renal function

Urine volume and urinary concentrations of creatinine, urea, Na^+, K^+ and Cl^- were determined daily while mean arterial blood pressure (MAP) was monitored every third consecutive day using non-invasive tail cuff method with photoelectric sensors (ITC Model 31 Computerized Blood Pressure Monitor, Life Sciences, Woodland Hills, CA). The unit works with ITC hardware system to measure blood pressure and heart rate in conscious rats. The animals were warmed at ±30 °C in an enclosed chamber (ITC Model 303sc Animal Test Chamber ITC Life Sciences, Woodland Hills, CA) for 30 min before taking blood pressure readings. All measurements were conducted at 09h00. Blood samples were collected by cardiac puncture into individual pre-cooled heparinized containers at the end of the 5-week experimental period for biochemical analysis. Glomerular filtration rate (GFR), as assessed by creatinine clearance (C_{Cr}) was calculated using the standard formulae from measurements of the plasma and urinary concentrations of creatinine and urine flow rate in the fifth week.

Proximal tubular function

The reabsorption of Na^+ in the proximal tubule and, by implication, in the distal nephron was assessed through measurement of lithium clearance (C_{Li}).[21] The rats were fed standard rodent chow supplemented with lithium chloride (12 mmol kg^{-1} dry weight) for 48 h prior to experimentation in order to raise plasma lithium to measurable concentrations without affecting renal sodium or water excretion.[22] Subsequently, renal clearance studies were conducted in inactin-anesthetized (0.11 g kg^{-1}) hypotonic saline infused rats, a model which has been extensively used our laboratory.[12,19,23] Briefly, the right jugular vein was cannulated to allow a continuous intravenous infusion of hypotonic saline (0.077 M NaCl) at 9 mL h^{-1} (Harvard syringe infusion Pump 22, Harvard Apparatus, Holliston, MA). A catheter was inserted into the left carotid artery for withdrawal of blood samples and to record mean 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). The urinary bladder was also cannulated via an incision in the lower abdomen for the collection of urine samples. Animals were given a priming dose of creatinine (3 μg in 0.3 mL 0.077 M NaCl) and then placed on a continuous infusion of 0.077 M

NaCl containing creatinine (0.15 μg mL^{-1}) of at 9 mL h^{-1} to allow calculation of creatinine clearance as a measure of GFR. After a 3.5 h equilibration period, blood samples (200 μL) were drawn at 1 h intervals and urine collections were made every 30 min over 4 h of 1 h control, 1.5 h treatment and 1.5 h recovery periods for measurement of electrolyte and clearance marker concentrations. In those animals in which the effects of MA were examined, the infusate was changed during the 1.5 h treatment period to one identical in ionic composition, but containing MA (90 μg h^{-1}).

Urinalysis

Urine flow was determined gravimetrically. Na^+, K^+, urea and creatinine were analyzed using the Beckman Coulter Counter (Synchron CX3 Clinical Systems, Fullerton, CA) with commercial diagnostic kits from Beckman Coulter, Dublin Ireland. Lithium was determined flame emission spectroscopy at 670.8 nm (Optima 2100 DV, Perkin Elmer, Shelton, CT) using a modified procedure that has been previously described by Madlala et al.[23] Fractional excretions (FE) rates of Na^+ (Fe_{Na}) and Li^+(Fe_{Li}) were determined simultaneously.

Calculations

Renal clearances (C) and fractional excretions (FE) were calculated using the following standard formulae:

$$C = U \times V/P$$

$$FE_{Li} = 100 \times C/GFR; \quad (FE_{Li} = FE_{Na})$$

$$FE_{Na\ distal} = C_{Na}/C_{Li}$$

where U is the urinary concentration, V is the urine flow rate and P is the plasma concentration.

GFR values were calculated using creatinine clearance (C_{Cr}).

Tissue sample harvesting

At the end of the 5 week, all animals were sacrificed by exposing to halothane via a gas anesthetic chamber (100 mg kg^{-1}, for 3 min). Livers, hearts, kidneys and gastrocnemius muscles were removed, snap frozen in liquid nitrogen and stored in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at −70 °C for subsequent biochemical analysis. All organs were analyzed for protein content in addition to other biochemical parameters. The protein content was quantified using the Lowry method.[24] All the samples were standardized to one concentration (1 mg mL^{-1}).

Biochemical measurements

Evaluation of oxidative stress

To establish the effects of MA on oxidative stress in the liver, heart and kidney of STZ-induced diabetic rats, we compared levels of MDA, a commonly known marker of oxidative stress and of antioxidant defense enzymes SOD and GPx between untreated diabetic and MA-treated diabetic rats.

MDA

Tissues (50 mg) were homogenized in 500 μL of 0.2% phosphoric acid. The homogenate was centrifuged at 400 × *g* for 10 min. Thereafter, 400 μL of the homogenate was supplemented with 400 μL 2% phosphoric acid and then separated into two glass tubes, each receiving equal volumes of the solution. Subsequently, 200 μL of 7% phosphoric acid was added into both glass tubes followed by the addition of 400 μL of thiobarbituric acid (TBA)/butylated hydroxytoluene (BHT) into one glass tube (sample test) and 400 μL of 3 mM hydrochloric acid (HCl) into the second glass tube (blank). To ensure an acidic pH of 1.5, 200 μL of 1 M HCl was added to sample and blank test tubes. Both solutions were heated at 100 °C for 15 min, and allowed to cool to room temperature. Butanol (1.5 mL) was added to the cooled solution; the sample was vortexed for 1 min to ensure rigorous mixing and allowed to settle until two phases could be distinguished. The butanol phase (top layer) was transferred to Eppendorf tubes and centrifuged at 13,200 × *g* for 6 min. The samples were aliquoted into a 96-well microtiter plate in triplicate and the absorbance was read at 532 nm (reference λ 600 nm) on a BioTek μQuant spectrophotometer (Biotek, Johannesburg, South Africa). The absorbance from these wavelengths was used to calculate the concentration of MDA using Beer's Law.

$$\text{Concentration of MDA (mM)} = \frac{\text{Average Absorbance}}{\text{Absorption coefficient}(156\ \text{mmol}^{-1})}$$

SOD

SOD activity was assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine in a convenient 96 well format. SOD activity was measured using the Biovision SOD Assay Kit according to manufacturers' instructions (BioVision Research Products, Mountain View, CA). Rat liver, kidney and heart tissues (50 mg) were homogenized in ice cold 0.1 M Tris/HCl (pH 7.4) containing 0.5% Triton X-100, 5 mM β-mercaptoethanol (ME) and 0.1 mg mL^{-1} phenylmethanesulfonylfluoride (PMSF). The tissue homogenate was centrifuged at 14,000 × *g* for 5 min at 4 °C. The supernatant obtained was added to each sample (20 μL) and blank 2 (20 μL) well, while blank 1 and blank 3 wells received 20 μL of H_2O. Thereafter, 200 μL of working solution was added to each well. Subsequently, dilution buffer (20 μL) was added to each blank 2 and blank 3 well, while each sample and blank 1 well received enzyme working solution (20 μL). The solutions were mixed thoroughly before reading the plate. Inhibition activity of SOD was colorimetrically measured on an Anthos Venytch-200 Spectrophotometer (Biochrom Limited, Cambridge, United Kingdom) after a reaction period of 20 min at 37 °C. SOD activity was calculated as percentage inhibition using the equation:

$$\text{SOD activity (\%inhibition rate)} = \frac{(A_{blank1} - A_{blank3}) - A_{sample} - A_{blank2})}{(A_{blank1} - A_{blank3})}$$

GPx

Glutathione peroxidase (GPx) is an antioxidant enzyme which reduces liquid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water through the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG). GPx activity was measured in rat liver, kidney and heart tissues using the Biovision GPx Assay Kit according to manufacturers' instructions (BioVision Research Products, Mountain View, CA). The tissues (50 mg) were homogenized on ice in cold assay buffer (0.2 mL) and subsequently centrifuged at 10,000 × *g* for 15 min at 4 °C. The resultant supernatant (100 µL) was loaded into a 96-well plate in duplicate. The NADPH standard curve was prepared by diluting the 1 mM NADPH standard through a series of concentrations (0, 20, 40, 60, 80, 100 nmol per well). The optical density of the standards (OD) was measured at 340 nm using an Anthos Venytch-200 Spectrophotometer (Biochrom Limited, Cambridge, United Kingdom) and the standard curve was constructed from the values obtained. A reaction mix (90 µL) containing assay buffer, NADPH, glutathione reductase and GSH was added into each sample well, mixed thoroughly and incubated for 15 min at room temperature. The OD was then measured (340 nm) followed by the addition of cumene hydroperoxide (10 µL) and measurement of OD (T1) and another reading following a 5 min incubation in the dark (25 °C). GPx activity was calculated using following equation:

$$\text{GPx activity} = \frac{(B - B^0) \times \text{Sample dilution}}{(T_2 - T_1) \times V}$$

where ΔA_{340nm} was used to extrapolate the values of B and B^0 from the NADPH standard curve.

Glycogen

Samples of known weights (1–2 g) of liver and gastrocnemius muscle from untreated and treated rats were used to measure glycogen concentrations using a previously described protocol.[25] Briefly, liver and gastrocnemius muscle tissue samples (1–1.5 g) were homogenized in 2 mL of 30% potassium hydroxide solution and digested at 100 °C for 30 min and then cooled in ice-saturated sodium sulphate. The glycogen was precipitated with ethanol, pelleted and resolubilized in deionized water. Glycogen content was determined by the treatment with anthrone reagent and measured at 620 nm using a Novaspec II spectrophotometer (Biochrom Ltd., Cambridge, UK).

Western blot analysis

GLUT4, GLUT1 and GLUT2

Kidney tissues were analyzed for GLUT1 and GLUT2 content while GLUT 4 was analyzed in gastrocnemius muscles. The tissue (0.1 g) was homogenized on ice in isolation buffer (0.5 mM Na_2EDTA, 0.1 M KH_2PO_4, 0.1 mM dithiothreitol, 0.25 M sucrose) and then centrifuged at 400 × *g* for 10 min (4 °C). The protein content was quantified using the Lowry method and all the samples were standardized to one concentration (1 mg mL^{-1}). The proteins were then denatured by boiling in Laemmli sample buffer (0.5 M Tris–HCl, glycerol, 10% sodium dodecyl sulfate (SDS), 2-mercaptoethanol, 1% bromophenol blue) for 5 min. The denatured proteins were loaded (25 µL) on prepared resolving (10%) and stacking (4%) polyacrylamide gels along with molecular weight marker (5 µL). The gel was electrophoresed for 1 h at 150 V in electrode (running) buffer (Trisbase, glycine, SDS, pH 8.3). Following electrophoresis, the resolved proteins were electrotransferred to an equilibrated polyvinylidene difluoride (PVDF)/membrane for 1 h in transfer buffer (192 mM glycine, 25 mM Tris, 10% methanol). After transfer, the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS; 20 mM Tris, 150 mM NaCl, KCl, 0.05% Tween-20). The membrane was then immunoprobed with antibodies-GLUT-1 and GLUT2 (1:1 000 in 1% BSA, Neogen, USA) for 1 h at room temperature (RT). The PVDF membrane was then subjected to 5 washes (10 min each with gentle agitation) with TTBS. Following which, the membrane was incubated in horse radish peroxidase (HRP)-conjugated secondary antibody (rabbit anti-mouse 1:10,000; Bio-Rad) for 1 h at RT. After further washing, antigen-antibody complexes were detected by chemiluminescence using the Immune-star™ HRP substrate kit (Bio-Rad, Johannesburg, South Africa). Chemiluminescent signals were detected with the Chemi-doc XRS gel documentation system and analyzed using the quantity one software (Bio-Rad, Johannesburg, South Africa). Band intensity analysis was conducted on the resultant bands.

OGT response protocol

Various doses of MA (20, 40 and 80 mg kg^{-1}, p.o.) were used to evaluate OGT responses in separate groups of non-diabetic and STZ-induced diabetic rats according to the method described previously by Musabayane et al.[26] Briefly, rats were given a glucose load (0.86 g kg^{-1}, p.o.) after an 18 h fast followed by MA. The selection of these doses was based on the posology of other triterpenes used in our laboratory.[25,26] MA was freshly dissolved in dimethyl sulphoxide (DMSO, 2 mL) and normal saline (19 mL) before use in each case.[27] Rats treated with DMSO/saline (3 mL kg^{-1}, p.o.) and metformin (1,1-dimethylbiguanide hydrochloride, Sigma Aldrich, St. Louis, MO, 500 mg kg^{-1}, p.o.) served as control and positive control animals, respectively. Blood samples were collected from the tail veins at 15 min intervals for the first hour, and hourly thereafter for the subsequent 3 h for glucose measurements, using Bayer's Glucometer Elite® [Elite (Pty) Ltd, Health Care Division, Isando, South Africa].

Effects of MA on plasma insulin

Blood was collected from separate parallel groups of non-diabetic and STZ-induced diabetic rats prepared as for OGT studies into pre-cooled heparinized containers after 4 h treatment with MA (80 mg kg^{-1}, p.o.) or metformin, (500 mg kg^{-1}, p.o.) for insulin determination. The plasma insulin concentrations were measured by ultrasensitive rat insulin ELISA kit (DRG Instruments GmBH, Marburg, Germany). The immunoassay is a quantitative method utilizing two monoclonal antibodies which together are specific for insulin. The lower limit of detection was

1.74 pmol L^{-1}. The intra- and inter-assay analytical coefficients of variation ranged from 4.4% to 5.5% and from 4.7% to 8.9%, respectively.

Animal and tissue disposal

Animal carcasses and tissues were packaged in boxes with biohazard symbol approved by the Biological Safety Office for the Safety's Disposal Service, and transported to the Biomedical Research Animal Unit for disposal.

Statistical analysis

Data are presented as the means ± standard error of mean (SEM). Overall statistical comparisons between the control means and experimental groups were performed with GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, CA), using one-way analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparison test. A value of $p < 0.05$ was considered significant.

Results

Structure elucidation of MA

The MA isolated from *S. aromaticum* was identified by ^{1}H NMR and ^{13}C NMR (1D and 2D). It had ^{13}C NMR: δ_H (^{13}C NMR (400 MHz,CD3OD): 46.2 (C-1), 68.3 (C-2), 83.3 (C-3), 39.1 (C-4), 55.0 (C-5), 18.1 (C-6), 32.7 (C-7), 39.0 (C-8), C-9 (47.4), C-10 (38.0), C-11 (23.2), C-12 (121.9), C-13 (143.7), C-14 (41.6), C-15 (27.4), C-16 (23.0), C-17 (46.2), C-18 (41.0), C-19 (45.7), C-20 (30.4), C-21 (33.6), C-22 (32.3), C-23 (28.3), C-24 (16.6), C-25 (16.5), C-26 (16.4), C-27 (23.2), C-28 (178.5), (C-29) 32.2, C-30 (23.2). These data compared with those reported in literature.[28] Spectra in Figure 1 are given in the supporting information. The purity of the plant-derived MA was approximately 98% and the percentage yield varied from 0.02% to 0.03%.

Short-term effects of MA

Weight, blood glucose, food and water intake

Table 1 shows the outcome of the administration of MA to non-diabetic and diabetic rats on physical and biochemical parameters at the end of the 5 weeks. Untreated diabetic rats exhibited severe wasting, despite significantly higher weekly intake of food and water when compared with non-diabetic control rats. MA or metformin attenuated these diabetes-induced abnormalities. Compared to untreated STZ-induced diabetic rats, MA and metformin treatments significantly lowered the blood glucose concentrations. Kidney weight was significantly increased in STZ-diabetic rats by comparison with non-diabetic animals. Administration of MA or metformin did not affect the kidney mass in non-diabetic and STZ-diabetic rats (Table 1).

Oxidative stress

The concentrations of MDA and antioxidant enzymes (SOD and GPx) in non-diabetic control animals represent baseline/normal activity levels found in the tissues used (Table 2). Significant increase of MDA and decreases of SOD and GPx were found in liver, heart and kidney tissues of diabetic group as compared to control animals. Treatment of diabetic rats with MA significantly reduced the MDA in all tissues and increased the activities of SOD and GPx in the liver and kidney compared to untreated diabetic group. On the other hand, GPx activity was undetectable (<0.01 nmol min^{-1} mL^{-1}g protein) in the heart for all groups except for metformin treated STZ-induced diabetic rats group.

In summary, the liver and kidney were a lot more responsive to MA treatment than the heart which only responded to metformin treatments. MA caused a significant increase in antioxidant activity with a corresponding decrease in MDA levels.

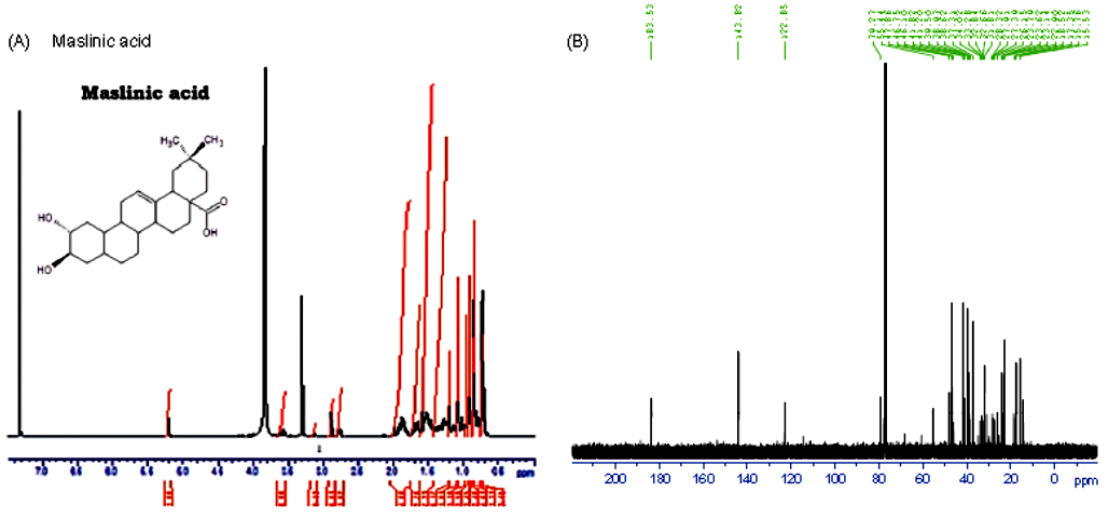

Figure 1. ^{1}H (A) and ^{13}C NMR (B) spectroscopic analysis of MA dissolved in deuterated methanol.

Table 1. Comparison of the effects of 5 week MA and metformin treatment on physical and biochemical parameters of non diabetic and STZ induced diabetic rats with respective control groups.

		Treatment		
Measure	Group	Control	MA	Metformin
Glucose (mmol)	Non diabetic	5.03 ± 0.33	3.90 ± 0.01*	4.10 ± 0.02*
	STZ diabetic	29.60 ± 2.60♦	16.40 ± 1.20*♦	13.20 ± 2.70*♦
Na^+ (mmol)	Non diabetic	139 ± 1	138 ± 3	136 ± 1
	STZ diabetic	137 ± 3	138 ± 3	137 ± 1
K^+ (mmol)	Non diabetic	3.6 ± 0.3	3.8 ± 0.2	3.6 ± 0.1
	STZ diabetic	3.8 ± 0.1	3.9 ± 0.2	3.4 ± 0.6
Urea (mmol)	Non diabetic	7.0 ± 1.3	7.6 ± 1.1	7.2 ± 1.5
	STZ diabetic	9.1 ± 1.4	9.0 ± 0.4	9.1 ± 1.6
Creatinine (μmol)	Non diabetic	26 ± 1	18 ± 1*♦	20 ± 1*
	STZ diabetic	33 ± 1♦	22 ± 1*♦	24 ± 1*
GFR (mL min 100 g $^{-1}$)	Non diabetic	1.03 ± 0.04	1.10 ± 0.08	0.96 ± 0.05
	STZ diabetic	0.63 ± 0.08♦	1.26 ± 0.01*	1.15 ± 0.01*
% body weight change	Non diabetic	+22 ± 1	+25 ± 1	22 ± 1
	STZ diabetic	−5 ± 1♦	+4 ± 1*♦	+8 ± 1*♦
Kidney mass (g 100 g $^{-1}$)	Non diabetic	0.21 ± 0.08	0.23 ± 0.12	0.22 ± 0.08
	STZ diabetic	0.37 ± 0.01♦	0.33 ± 0.01♦	0.32 ± 0.01♦
Hepatic glycogen (g 100 g $^{-1}$)	Non diabetic	26.3 ± 0.2	33.84 ± 0.80*	36.0 ± 0.5*
	STZ diabetic	15.7 ± 1.0♦	22.7 ± 0.6*♦	28.2 ± 1.2*♦
Muscle glycogen (g 100 g $^{-1}$)	Non diabetic	2.1 ± 0.4	3.6 ± 0.3*	3.4 ± 0.1*
	STZ diabetic	0.9 ± 0.1♦	2.5 ± 0.1*	2.2 ± 0.2*
Water intake (mL 100 g $^{-1}$)	Non diabetic	11 ± 1	10 ± 1	11 ± 1
	STZ diabetic	56 ± 1♦	46 ± 1*♦	38 ± 1*♦
Food intake (g 100 g $^{-1}$)	Non diabetic	9.0 ± 0.4	9.2 ± 0.2	9.1 ± 0.4
	STZ diabetic	12.0 ± 0.4*♦	8.4 ± 0.4*	8.2 ± 0.4*

Notes: Animals were treated every third day with either MA (80 mg kg $^{-1}$, p.o.) or metformin (500 mg kg $^{-1}$, p.o.) for 5 weeks. Data are expressed as mean ± SEM, $n = 6$ in each group.
*$p < 0.05$ by comparison with respective control animals.
♦$p < 0.05$ by comparison with respective non diabetic animals.

Table 2. Comparison of MDA concentration, activities of SOD and GPx in hepatic, renal and cardiac tissues of STZ induced diabetic rats treated twice every third day for 5 weeks with control non diabetic and diabetic animals.

		Organ		
Parameter measured	Treatment	Liver	Kidney	Heart
MDA (nmol g $^{-1}$ protein)	Non diabetic control	1.92 ± 0. 30	1.25 ± 0.08	0.65 ± 0.02
	STZ diabetic control	4.16 ± 0.20#	2.80 ± 0.10#	2.06 ± 0.80#
	STZ diabetic MA	1.56 ± 0.10*	1.45 ± 0.10*	1.51 ± 0.40*#
	STZ diabetic Met	1.25 ± 0.09*	1.20 ± 0.10*	1.60 ± 0.20#
SOD activity (nmol min $^{-1}$mL g $^{-1}$ protein)	Non diabetic control	8.11 ± 0.90	18.34 ± 3.60	3.00 ± 0.14
	STZ diabetic control	1.22 ± 0.20#	5.50 ± 0.10#	2.39 ± 0.33#
	STZ diabetic MA	8.16 ± 1.28*#	16.04 ± 4.60*#	3.83 ± 0.15*#
	STZ diabetic Met	6.53 ± 0.10*#	10.11 ± 0.56*#	5.62 ± 0.11*#
GPx activity (nmol min $^{-1}$mL g $^{-1}$ protein)	Non diabetic control	2.60 ± 0.10	4.90 ± 0.60	UNDECTABLE
	STZ diabetic control	0.04 ± 0.01#	0.70 ± 0.01*#	UNDECTABLE
	STZ diabetic MA	2.50 ± 0.40*#	4.40 ± 0.60*#	UNDECTABLE
	STZ diabetic Met	2.00 ± 0.30*#	5.20 ± 0.40*	5.50 ± 0.80*#

Notes: Values are expressed as mean ± SEM. UNDETECTABLE ≤0.01 nmol min $^{-1}$mL $^{-1}$g protein.
*$p < 0.05$ by comparison with respective control animals.
#$p < 0.05$ by comparison with non diabetic animals.

Renal fluid and electrolyte excretion

The mean weekly urinary Na^+, K^+ and Cl^- outputs of untreated STZ-induced diabetic rats were significantly lower than those of control non-diabetic animals throughout the 5-week period (Figure 2). Administration of MA (80 mg kg^{-1}, p.o.) increased weekly urinary Na^+ outputs of both non-diabetic and STZ-induced diabetic rats in weeks 4 and 5 without affecting urinary K^+ and Cl^- outputs (Figure 2). The loss of Na^+ was not reflected in the plasma by the end of the experimental period (Table 1). MA administration did not significantly alter the weekly volume of urine voided of both non-diabetic and STZ-induced diabetic group's rats over the 5-week study period (Figure 3).

At the end of the 5-week experimental period, plasma creatinine concentration was significantly ($p < 0.01$) elevated

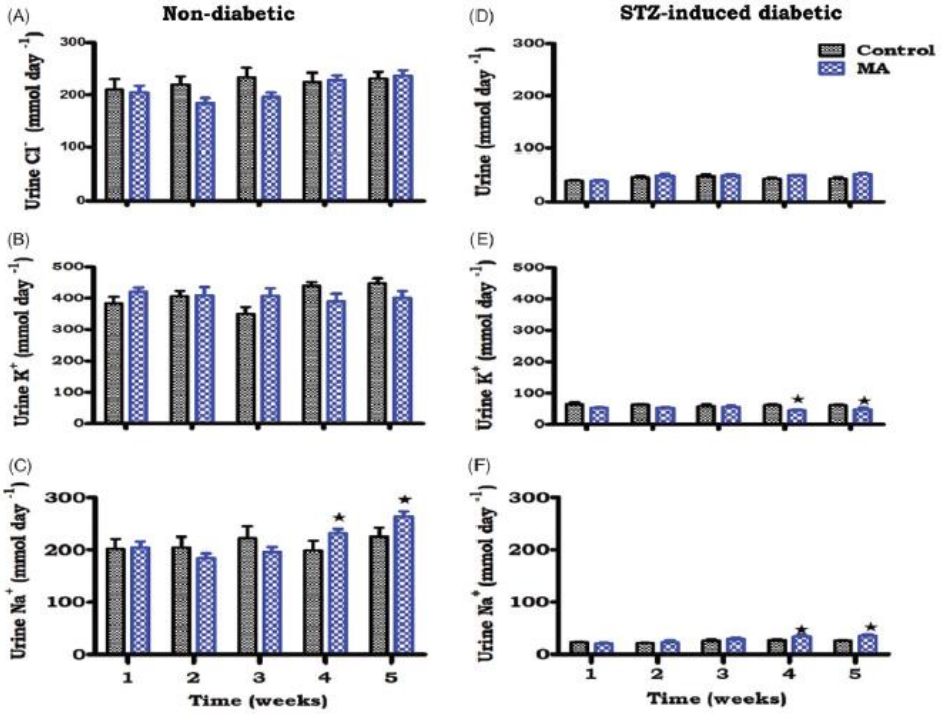

Figure 2. Comparison of the effects of the administration of MA (80 mg kg ^{1}, p.o.) twice daily on urinary Na^+, K^+ and Cl outputs of non diabetic rats (A–C) and STZ induced diabetic rats (D–F) with respective control animals. Values are presented as means for each week; vertical bars indicate SEM of means ($n = 6$ rats in each group). ★$p<0.05$ by comparison with control animals.

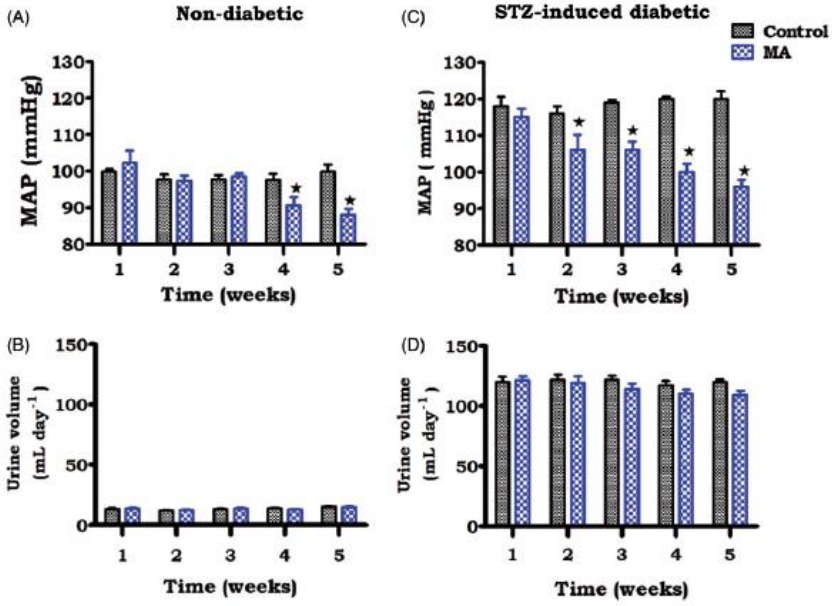

Figure 3. Comparison of the effects of the administration of MA (80 mg kg ^{1}, p.o.) twice daily on MAP and urine volume of non diabetic rats (A,B) and STZ induced diabetic rats (C,D) with respective control animals. Values are presented as means for each week; vertical bars indicate SEM of means ($n = 6$ rats in each group). ★$p<0.05$ by comparison with control animals.

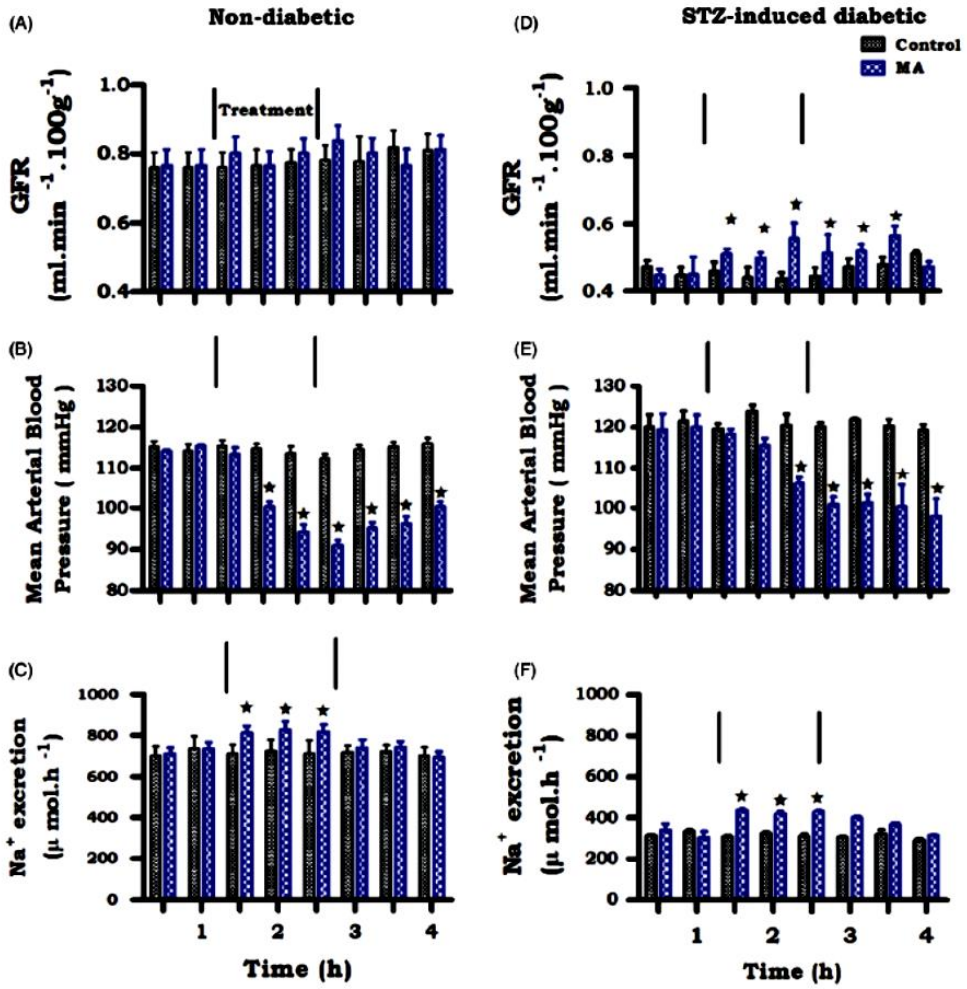

Figure 4. Comparison of the acute effects of MA administration of on GFR, MAP and Na^+ excretion rate of non diabetic rats (A–C) and STZ induced diabetic rats (D–F) with respective control animals. MA was infused at 90 μg h^{-1} for 1.5 h during the treatment period. Values are presented as means for each 30 min collection; vertical bars indicate SEM of means ($n = 6$ rats in each group). ★$p < 0.05$ by comparison with control animals.

in control STZ-induced diabetic rats by comparison with untreated non-diabetic rats while plasma urea concentration that was slightly increased to levels that did not achieve statistical significance (Table 1). MA decreased significantly ($p < 0.05$) the plasma creatinine concentration with a concomitant increase in GFR by the end of 5 weeks, but plasma urea concentration was not altered (Table 2). However, MA did not have any significant influence on GFR of the non-diabetic rats. Kidney mass was not altered by MA treatment (Table 1). The MAP of non-diabetic rats and diabetic animals was significantly ($p < 0.05$) decreased by MA from weeks 4 and 2, respectively, until the end of the 5-week experimental period (Figure 3).

Effects of MA proximal tubule function

Na^+ excretion rates, MAP and GFR in anaesthetized saline infused non-diabetic and STZ-induced diabetic rats are compared in Figure 4. The mean Na^+ excretion rates of untreated STZ-induced diabetic rats ranged from 290 to 332 μmol h^{-1} over the 4 h experimental period, values which were significantly ($p < 0.01$) lower than those of control non-diabetic animals (range 700–736 μmol h^{-1}) and the infusion rate (693 μmol h^{-1}). The infusion of MA at 90 μg h^{-1} increased Na^+ excretion rates of non-diabetic and diabetic rats within 30 min. However, MA treatment did not change the urine flow, K^+ and Cl^- excretion rates of anaesthetized animals (data not shown). The MAP of control non-diabetic and untreated STZ-induced diabetic animals did not show any significant variations throughout the 4 h post-equilibration period (Figure 4). MA administration, however, decreased blood pressure in both groups of animals which persisted during the post-treatment period. The GFR values in untreated diabetic rats (range 0.39–0.45 mL min 100 g^{-1}) were lower than that of control non-diabetic rats (range 0.76–0.80 mL min 100 g^{-1}). MA administration for 1 h

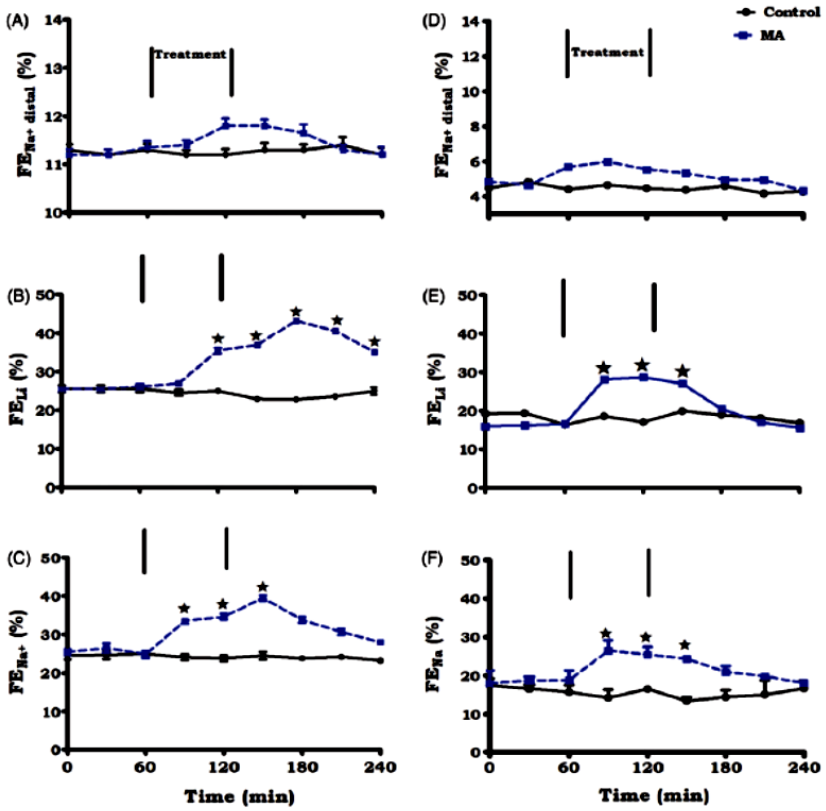

Figure 5. Comparison of the effects of MA infusion of FE_{Na}, $FE_{Na\ dist}$ and FE_{Li} of non diabetic rats (A–C) and STZ induced diabetic rats (D–F) with respective control animals. MA was infused at 90 μg h^{-1} for 1.5 h during the treatment period. Values are presented as means, and vertical bars indicate SEM ($n = 6$ rats in each group). ★$p < 0.05$ by comparison with control animals.

30 min only increased the GFR in STZ-induced diabetic rats (Figure 4).

The plasma Li^+ concentrations of non-diabetic and STZ-induced diabetic rats supplemented with lithium 48 h prior to experimentation which varied between 0.2 and 0.3 mmol L^{-1} showed no statistical difference between the groups. Prior to the infusion MA, the FE_{Li} and FE_{Na} of anesthetized saline infused non-diabetic and STZ-induced diabetic rats and experimental groups did not differ between groups (Figure 5). Infusion of MA over a 90 min period caused substantial increases in FE_{Na} and FE_{Li} (an index of end-proximal $FE_{Na\ prox}$ delivery) by comparison with respective control animals at the corresponding time (Figure 5). Finally, $FE_{Na\ dist}$ (C_{Na}/C_{Li}), used as an index of the fraction of sodium delivered to the distal nephron that escapes reabsorption therein, was also significantly elevated. In all cases, the FE_{Na} was not accompanied by any changes in FE_K, and FE_{Cl} (data not shown).

OGT responses

Blood glucose concentrations were significantly higher in untreated STZ-induced rats at all time-points during the OGT responses test, and the area under the curve (AUC) also increased significantly compared to that in non-diabetic control rats (Figure 6). Treating non-diabetic or STZ-induced rats with high MA doses (40 and 80 mg kg^{-1}) significantly reduced blood glucose levels. In addition, the blood glucose AUC was smaller in treated animals compared with respective control diabetic rats. MA at a low concentration (20 mg kg^{-1}) had no significant effect on these parameters. The standard anti-diabetic drug, metformin not only demonstrated blood glucose-lowering effects in non-diabetic and STZ-induced diabetic rats but also reduced the AUC. The AUC for increase in glucose over baseline were calculated during OGT responses by incremental method.

MA effects on insulin secretion

Plasma insulin concentrations in untreated STZ-induced diabetic rats were significantly low ($p < 0.05$) in comparison with control non-diabetic rats (3.32 ± 0.62 vs. 11.15 ± 2.24 ng mL^{-1}). Acute MA administration did not alter plasma insulin concentrations of STZ-induced diabetic animals (3.16 ± 0.53 ng mL^{-1}) and non-diabetic rats (10.80 ± 1.30 ng mL^{-1}) in comparison with respective control animals.

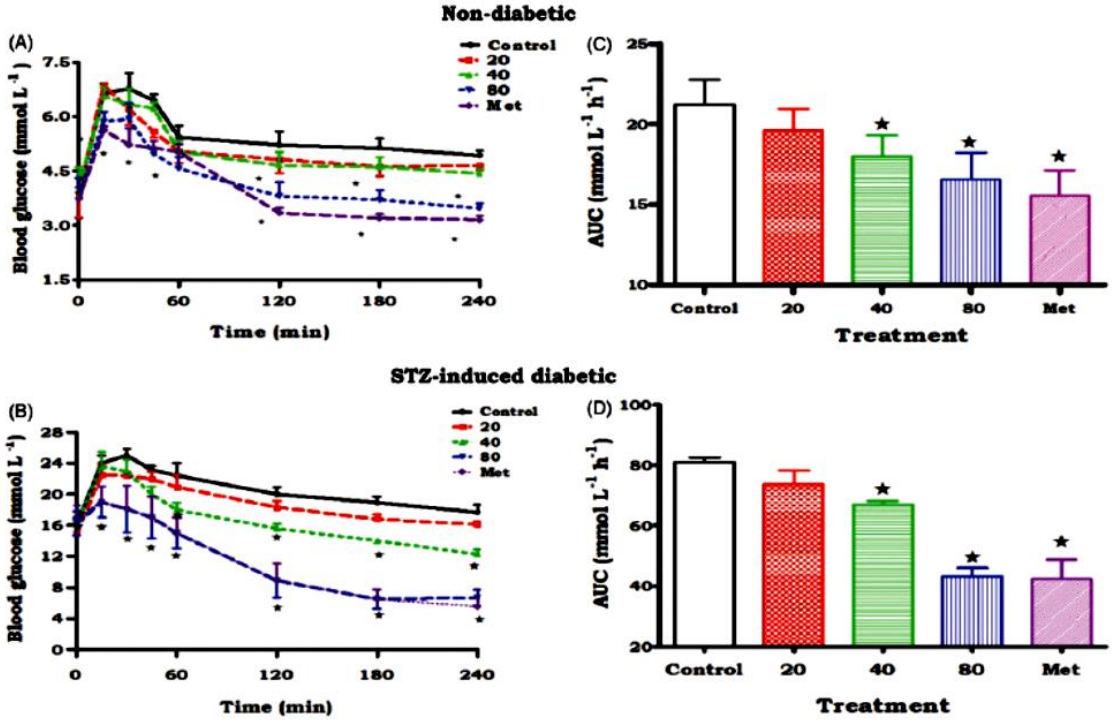

Figure 6. Comparison of OGT responses and the AUC to various doses of MA in non diabetic (A) and STZ induced diabetic (B) rats. Values are presented as means, and vertical bars indicate SEM ($n = 6$ rats in each group). ★$p < 0.05$ by comparison with control animals.

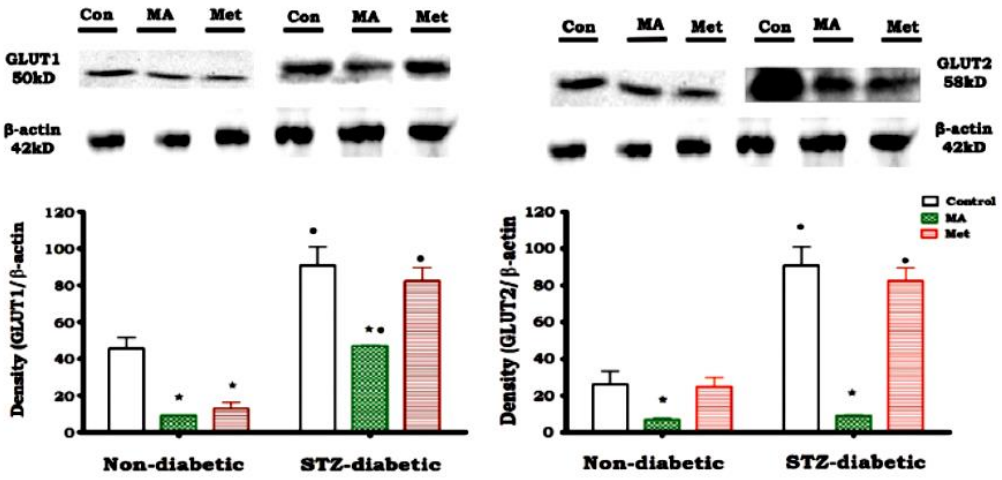

Figure 7. Effects of MA on expression of GLUT1 and GLUT2 as determined by Western blotting of control, MA treated and metformin treated kidney tissues of non diabetic and STZ induced diabetic rats. Values are expressed as mean ± SEM. Values were obtained from Western blots for six preparations. ★$p < 0.05$ by comparison with respective control animals; •$p < 0.05$ by comparison with respective non diabetic animals.

Glycogen

STZ-induced diabetic rats were characterized by the depletion of liver and muscle glycogen concentrations at the end of the 5-week experimental period (Table 1). Treatment with MA (80 mg kg^{-1}, p.o.) or metformin (500 mg kg^{-1}, p.o.) decreased blood glucose concentrations of STZ-induced diabetic rats with concomitant restoration of hepatic and muscle glycogen content to levels comparable with non-diabetic rats.

Effects of MA on glucose transporters

To evaluate the effect of MA on glucose transporters expression, renal GLUT1 and GLUT2 and skeletal muscle GLUT4 analyzed using Western blotting in tissues harvested

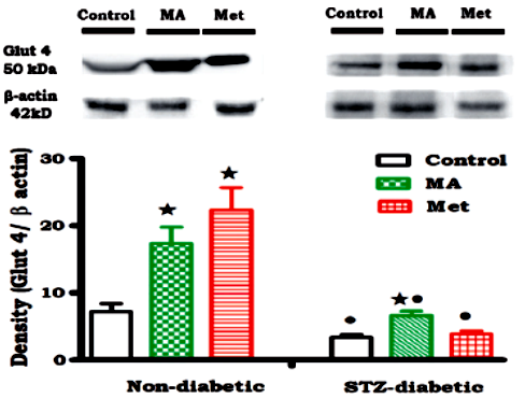

Figure 8. Effects of MA on GLUT4 expression as determined by Western blotting of control, MA treated and metformin treated skeletal muscle tissues of non diabetic and STZ induced diabetic rats. Values are expressed as mean ± SEM. Values were obtained from Western blots for six preparations. ★$p<0.05$ by comparison with respective control animals; •$p<0.05$ by comparison with respective non diabetic animals.

from non-diabetic and STZ-induced diabetic rats at the end of 5-week MA treatment. The GLUT1 and GLUT2 expression were significantly elevated in kidney tissues of untreated STZ-induced diabetic rats compared with control non-diabetic animals (Figure 7). In comparison with respective control groups, MA treatment not only abolished the diabetes-induced increase in GLUT1 and GLUT2 but also decreased the expression of these transporters in non-diabetic animals. GLUT4 content in gastrocnemius muscle of diabetic rats was markedly lower as compared with non-diabetic control animals. Both metformin and MA did not have any significant effect on the expression of GLUT 4 in the muscle of non-diabetic rats (Figure 8). Surprisingly, MA increased GLUT4 content in diabetic rat skeletal muscle, but no effect of metformin was observed.

Discussion

The study examined the effects of *S. aromaticum*-extracted MA on some metabolic disturbances in diabetes using the STZ-induced diabetic rat model which has been extensively used in our laboratory to evaluate complications associated with type 1 diabetes mellitus.[12,25,26] The spectra of the plant derived MA elucidated using ^{1}H- and ^{13}C-NMR compared with previously reported data of maslinic acid.[28,29] Streptozotocin used to induce diabetes mellitus selectively destroys pancreatic β-cells[30] and the systemic changes which occur after its injection are related to the induced diabetic state.[31] The major findings were that MA improves oxidative stress and renal function which is often impaired in diabetes. The data suggest that the beneficial effects of MA in diabetes mellitus are complementary.

Our results showed that the elevated concentrations of MDA (marker of lipid peroxidation) in diabetic rat tissues of the liver, kidney and heart were restored to within normal values after the treatment with MA. The reduction in MDA levels could be due to the improvement in glycemic control and also increased antioxidant status, since MA significantly stimulated increased activities of SOD and GPx (Table 2 and Figure 6). The increase in markers of lipid peroxidation products due to hyperglycemia has been previously reported in tissues of diabetic rats and suggested to partly contribute to long-term tissue damage.[32–35] MA has previously been shown to decrease nitric oxide levels, another potent tissue oxidant, therefore supporting our current findings.[36] This outcome of *S. aromaticum*-derived MA is similar to that of rutin, a polyphenolic flavonoid in diabetic rat.[37] The amelioration of oxidative stress by MA suggests that MA improves the disturbed metabolism associated with diabetes.

STZ-induced rats exhibited marked weekly decreases in urinary Na^+ excretion, elevated plasma creatinine concentration and reduced GFR at the end of 5 weeks suggestive of the deterioration of kidney function. Impaired renal function has long been associated with experimental diabetes.[12,13] MA attenuated these changes mediated possibly in part via enhancement of the antioxidant status thus supporting previous reports of that extracts containing MA reduce the severity of kidney dysfunction in diabetes.[38] Furthermore, Yamabe et al.[39] reported that antioxidants and good control of diabetes led to improved renal function. Thus, it is supposed that oxidative stress could, at least partially, play a central role in cardio-renal dysfunction in diabetes. Against these observations are reports of the absence of improvement and even worsening of diabetic nephropathy with antioxidant treatment.[40,41]

STZ-induced rats exhibited marked weekly decreases in urinary Na^+ excretion, elevated plasma creatinine concentration and reduced GFR at the end of 5 weeks suggestive of the deterioration of kidney function. Previous observations show that ROS cause structural changes in the glomerulus and tubular components of the nephron.[42] MA reversed the inability of the kidney to excrete Na^+ in STZ-induced diabetic rats. Interestingly, there was anomaly in the time scale of the effects of MA on urinary Na^+ output as acute MA infusion increased Na^+ excretion within the 1.5 h infusion period whilst chronic MA treatment significantly elevated Na^+ output in the fourth week. The data of this study cannot explain this discrepancy; we partly attribute this observation to experimental design and/or data presentation. Na^+ excretion measurements in acute studies were from samples collected every 30 min over a 1.5 h period, while those of long-term experiments represent 24 h samples. Literature evidence indicates that extracts containing MA reduce the severity of kidney dysfunction in diabetes.[38]

This study also attempted to clarify the effect of MA on proximal tubule Na^+ handling using lithium clearance as a marker, a technique widely used in animal studies and clinical investigations to assess proximal tubular function.[43] This is a controversial technique as there is evidence that lithium can be reabsorbed by the distal nephron under certain conditions (e.g., Na^+ and K^+ depletion).[44] However, since the animals used in the study were Na^+ and K^+ replete it is reasonable to assume that Li^+ is a valid marker of proximal tubule function. MA increased Na^+ excretion accompanied by significant elevation of both FE_{Li} and $FE_{Na\ prox}$ in both non-diabetic and diabetic animals. This occurred without any measurable change in GFR in non-diabetic rats indicating that at least part of the overall reduction in Na^+ reabsorption occurred in

the proximal tubules. The results also point that MA increased $FE_{Na\ dist}$ of both non-diabetic and diabetic rats partly as an inherent response of reduced proximal tubular reabsorption. Although the effects of MA on renal fluid, Na^+, K^+ and Cl^- have been studied, renal handling of other ions such as Mg^{2+}, Ca^{2+} and PO_4^{2-} warrant investigations.

Our data revealed that there was marked reduction in the total body weight of the diabetic group compared to that of the control normal group. Treatment with MA stabilized body weight of STZ-induced diabetic rats possibly due to the hypoglycemic properties of MA. Indeed, MA improved glycemic control of STZ-induced diabetic rats in conformity with previous observations in various models of experimental diabetes.[6,7] However, MA had no significant effect on plasma insulin concentrations in non-diabetic and STZ-induced diabetic rats suggesting that hypoglycemic effect may be due to extra-pancreatic mechanisms. The plasma insulin concentrations in STZ-induced diabetic rats were significantly ($p < 0.05$) lower than in non-diabetic rats perhaps because of the destruction of pancreatic β-cells by STZ.[45] MA administration, however, slightly increased plasma insulin concentrations in non-diabetic rats to values that did not achieve statistical significance perhaps due to the masking effects on β-cells arising from the glucose load. The discrepancy can partly be attributed to the presence or absence of β-pancreatic cell activity.

MA treatment decreased GLUT1 and GLUT 2 expression in the kidneys of diabetic rats to perhaps to reduce urinary glucose leak. In the absence of urinary glucose measurements, further studies are clearly needed to support this speculation. The renal reabsorption of glucose which occurs mostly in the proximal tubule involves luminal Na^+-glucose transporters (SGLTs) and facilitative diffusion through GLUT1 and GLUT2 in the brush border membrane.[46,47] Diabetes has been reported to stimulate insertion of GLUT2 into the brush border membrane of the proximal tubule thereby increasing facilitative glucose uptake.[46] Other possible mechanisms of the hypoglycemic effects of MA could arise from increased hepatic glycogen and skeletal muscle synthesis perhaps via inhibition of glycogen phosphorylase (GP).[48–50] Interestingly, MA treatment increased GLUT4 expression in the skeletal muscle of diabetic rats suggesting that the MA-mediated glycogen synthesis is insulin independent.

In conclusion, the current observations indicate MA-elicited improvement in glucose homeostasis in streptozotocin-induced diabetes could be linked to reduced renal glucose reabsorption, increased oxidative defense, increased glycogen synthesis and amelioration of kidney and liver functions. Considering that no single marketed anti-diabetic drug is capable of achieving long-lasting blood glucose control or compensating for metabolic derangements, MA may provide a strategy with a different mechanism of action and potential for effective diabetic therapy. The limitations of the study include the absence of assessment of the effects of MA in other segments of the nephrons as we only investigated the effects of MA on proximal tubular Na^+ handling and measurements of hormones that influence renal function. Further studies are expected to provide information of such effects.

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Declaration of interest

The authors declare that there is no interest that could be perceived as prejudicing the impartiality of the research reported. The authors are grateful to the NRF South Africa and the University of KwaZulu-Natal, Research Division for financial support.

References

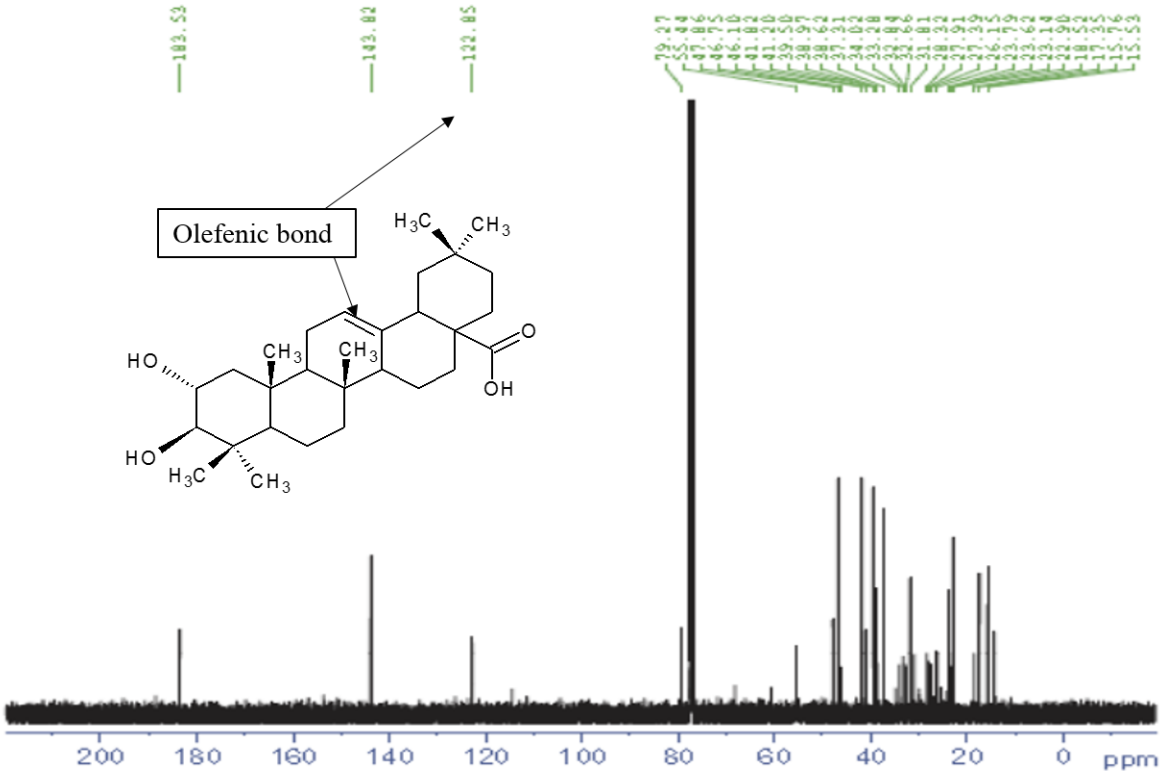
1. Dzubak P, Hajduch M, Vydra D, et al. Pharmacological activities of natural triterpenoids and their therapeutic implications. *Nat Prod Rep*. 2006;23:394 411.
2. Reyes Zurita FJ, Rufino Palomares EE, Lupiáñez JA, Cascante M. Maslinic acid, a natural triterpene from *Olea europaea L.*, induces apoptosis in HT29 human colon cancer cells via the mitochondrial apoptotic pathway. *Cancer Lett*. 2009;273:44 54.
3. Di Carli MF, Janisse J, Grunberger G, Ager J. Role of chronic hyperglycemia in the pathogenesis of coronary microvascular dysfunction in diabetes. *J Am Coll Cardiol*. 2003;41:1387 1393.
4. Parra A, Rivas F, Lopez PE, et al. Solution and solid phase synthesis and anti HIV activity of maslinic acid derivatives containing amino acids and peptides. *Bioorg Med Chem*. 2009; 17:1139 1145.
5. Allouche Y, Beltrán G, Gaforio JJ, Uceda M, Mesa MD. Antioxidant and antiatherogenic activities of pentacyclic triterpenic diols and acids. *Food Chem Toxicol*. 2010;48:2885 2890.
6. Liu J, Sun H, Duan W, Mu D, Zhang L. Maslinic acid reduces blood glucose in KK Ay mice. *Biol Pharm Bull*. 2007;30:2075 2078.
7. Tang XZ, Guan T, Qian YS, et al. Effects of maslinic acid as a novel glycogen phosphorylase inhibitor on blood glucose and hepatic glycogen in mice. *Chin J Nat Med*. 2008;6:53 56.
8. Mkhwanazi B, Masola B, van Heerden F, Musabayane C. Effects of *Syzygium aromaticum* derived maslinic acid on blood glu cose of streptozotocin induced diabetic rats. *Endocr Abs*. 2012;28:P214.
9. Kocak G, Aktan F, Canbolat O, et al. Alpha lipoic acid treatment ameliorates metabolic parameters, blood pressure, vascular reactiv ity and morphology of vessels already damaged by streptozotocin diabetes. *Diabetes Nutr Metab*. 2000;13:308 318.
10. Pari L, Latha M. Protective role of *Scoparia dulcis* plant extract on brain antioxidant status and lipidperoxidation in STZ diabetic male Wistar rats. *BMC Complement Altern Med*. 2004;4:16 24.
11. Azuma K, Minami Y, Ippoushi K, Terao J. Lowering effects of onion intake on oxidative stress biomarkers in streptozotocin induced diabetic rats. *J Clin Biochem Nutr*. 2007;40:131 140.
12. Musabayane CT, Ndhlovu CE, Balment RJ. Renal fluid and electrolyte handling in streptozotocin induced diabetic rats. *Ren Fail*. 1995;17:107 116.
13. Li Y, Higashiura K, Ura N, et al. Effects of the Chinese medicine, TSJN on insulin resistance and hypertension in fructose fed rats. *Hypertens Res*. 2000;23:101 107.
14. Krishnan R, Izatt S, Bargman JM, Oreopoulos D. Prevalence and determinants of erectile dysfunction in patients on peritoneal dialysis. *Int Urol Nephrol*. 2003;35:553 556.
15. Fujita H, Fujishima H, Chida S, et al. Reduction of renal superoxide dismutase in progressive diabetic nephropathy. *J Am Soc Nephrol*. 2009;20:1303 1313.
16. Kramer CK, Leitao CB, Pinto LC, et al. Risk factors for micro and macrovascular disease in black and white patients with type 2 diabetes mellitus. *Rev Assoc Med Bras*. 2009;55:308 314.
17. Weinberg JM. The cell biology of ischemic renal injury. *Kidney Int*. 1991;39:476 500.

18. Dominguez JH, Camp K, Maianu L, Feister H, Garvey WT. Molecular adaptations of GLUT1 and GLUT2 in renal proximal tubules of diabetic rats. *Am J Physiol.* 1994;266:F283 F290.
19. Mapanga RF, Tufts MA, Shode FO, Musabayane CT. Renal effects of plant derived oleanolic acid in streptozotocin induced diabetic rats. *Ren Fail.* 2009;31:481 491.
20. Somova LO, Nada A, Rammana P, Shode FO. Cardiovascular, antihyperlipidemic and antioxidant effects of oleanolic and ursolic acids in experimental hypertension. *Phytomedicine.* 2003;10: 115 121.
21. Thomsen K, Shirley DG. The validity of lithium clearance as an index of sodium and water delivery from the proximal tubules. *Nephron.* 1997;77:125 138.
22. Shalmi M, Thomsen K. Alterations of lithium clearance in rats by different modes of lithium administration. *Ren Physiol Biochem.* 1989;12:273 280.
23. Madlala HP, Masola B, Singh M, Musabayane CT. The effects of *Syzygium aromaticum* derived oleanolic acid on kidney function of male Sprague Dawley rats and on kidney and liver cell lines. *Ren Fail.* 2012;34:767 776.
24. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem.* 1951; 193:265 275.
25. Ngubane PS, Masola B, Musabayane CT. The effects of *Syzygium aromaticum* derived oleanolic acid on glycogenic enzymes in streptozotocin induced diabetic rats. *Ren Fail.* 2011;33:434 439.
26. Musabayane CT, Tufts MA, Mapanga RF. Synergistic antihyper glycemic effects between plant derived oleanolic acid and insulin in streptozotocin induced diabetic rats. *Ren Fail.* 2010;32:832 839.
27. Musabayane CT, Mahlalela N, Shode FO, Ojewole JAO. Effects of *Syzygium cordatum* (Hochst.)[*Myrtaceae*] leaf extract on plasma glucose and hepatic glycogen in streptozotocin induced diabetic rats. *J Ethnopharmacol.* 2005;97:485 490.
28. Garcia Granados A, Martinez A, Moliz JN, Parra A, Rivas F. 2 a, 3 b Dihydroxyolean 12 en 28 oic acid (maslinic acid). *Molecules.* 1998;3(3):88.
29. Júlio CAT, Gentil JV, Cleuza CS. A new tormentic acid derivative from *Luehea divaricata* Mart. (Tiliaceae). *J Braz Chem Soc.* 2003; 14:475 478.
30. Lenzen S. The mechanisms of alloxan and streptozotocin induced diabetes. *Diabetologia.* 2008;51:216 226.
31. Kuramochi G, Homma S. Effects of furosemide on renal oxygen consumption after ischemia in normal and streptozotocin diabetic rats. *Nephron.* 1993;64:436 442.
32. Bhor VM, Raghuram N, Sivakami S. Oxidative damage and altered antioxidant enzyme activities in the small intestine of streptozoto cin induced diabetic rats. *Int J Biochem Cell Biol.* 2004;36:89 97.
33. Miyake Y, Yamamoto K, Tsujihara N, Osawa T. Protective effects of lemon flavonoids on oxidative stress in diabetic rats. *Lipids.* 1998;33:689 695.
34. Arivazhagan P, Thilakovathy T, Panneerselvam C. Antioxidant lipoate and tissue antioxidants in aged rats. *J Nutr Biochem.* 2000; 11:122 127.
35. McCord JM. The evolution of free radicals and oxidative stress. *Am J Med.* 2000;108:652 658.
36. Montilla MP, Agil A, Navarro MC, et al. Antioxidant activity of maslinic acid, a triterpene derivative obtained from *Olea europaea*. *Planta Medica.* 2003;69:472 474.
37. Narasimhanaidu K, Ponnaian SMP. Antihyperglycaemic and anti oxidant effect of rutin, a polyphenolic flavonoid, in streptozotocin induced diabetic Wistar rats. *Basic Clin Pharm Toxicol.* 2006;98: 97 103.
38. Xu Q, Chen G, Fan J, et al. Chemical constituents of roots of *Boehmeria nivea*. *Zhongguo Zhong Yao Za Zhi.* 2009;34: 2610 2612.
39. Yamabe N, Yokozawa T, Oya T, Kim M. Therapeutic potential of ( ) epigallocatechin 3 O gallate on renal damage in diabetic nephropathy model rats. *J Pharmacol Exp Ther.* 2006;319: 228 236.
40. Agardh CD, Eckert B, Agardh E. Irreversible progression of severe retinopathy in young type I insulin dependent diabetes mellitus patients after improved metabolic control. *J Diabetes Complications.* 1992;6:96 100.
41. Soulis Liparota T, Cooper ME, Dunlop M, Jerums G. The relative roles of advanced glycation, oxidation and aldose reductase inhibition in the development of experimental diabetic nephropathy in the Sprague Dawley rat. *Diabetologia.* 1995;38:387 394.
42. Obrosova IG, Fathallah L, Liu E, Nourooz Zadeh J. Early oxidative stress in the diabetic kidney: effect of DL alpha lipoic acid. *Free Radic Biol Med.* 2003;34:186 195.
43. Boer WH, Fransen R, Shirley DG, Walter SJ, Boer P, Koomans HA. Evaluation of the lithium clearance method: direct analysis of tubular lithium handling by micropuncture. *Kidney Int.* 1995;47: 1023 1030.
44. Shirley DG, Walter SJ. Renal tubular lithium reabsorption in potassium depleted rats. *J Physiol.* 1997;501:663 670.
45. Szkudelski T. The mechanism of alloxan and streptozotocin action in β cells of the rat pancreas. *Physiol Res.* 2001;50:537 546.
46. Marks J, Carvou NJ, Debnam ES, Srai SK, Unwin RJ. Diabetes increases facilitative glucose uptake and GLUT2 expression at the rat proximal tubule brush border membrane. *J Physiol.* 2003;553: 137 145.
47. Thorens B, Lodish HF, Brown D. Differential localization of two glucose transporter isoforms in rat kidney. *Am J Physiol.* 1990;259: C286 C294.
48. Chen J, Liu J, Zhang L, et al. Pentacyclic triterpenes. Part 3: synthesis and biological evaluation of oleanolic acid derivatives as novel inhibitors of glycogen phosphorylase. *Bioorg Med Chem Lett.* 2006;16:2915 2919.
49. Henke BR, Sparks SM. Glycogen phosphorylase inhibitors. *Mini-Rev Med Chem.* 2006;6:845 857.
50. Wen X, Zhang P, Liu J, et al. Pentacyclic triterpenes. Part 2: synthesis and biological evaluation of maslinic acid derivatives as glycogen phosphorylase inhibitors. *Bioorg Med Chem Lett.* 2006; 16:722 726.

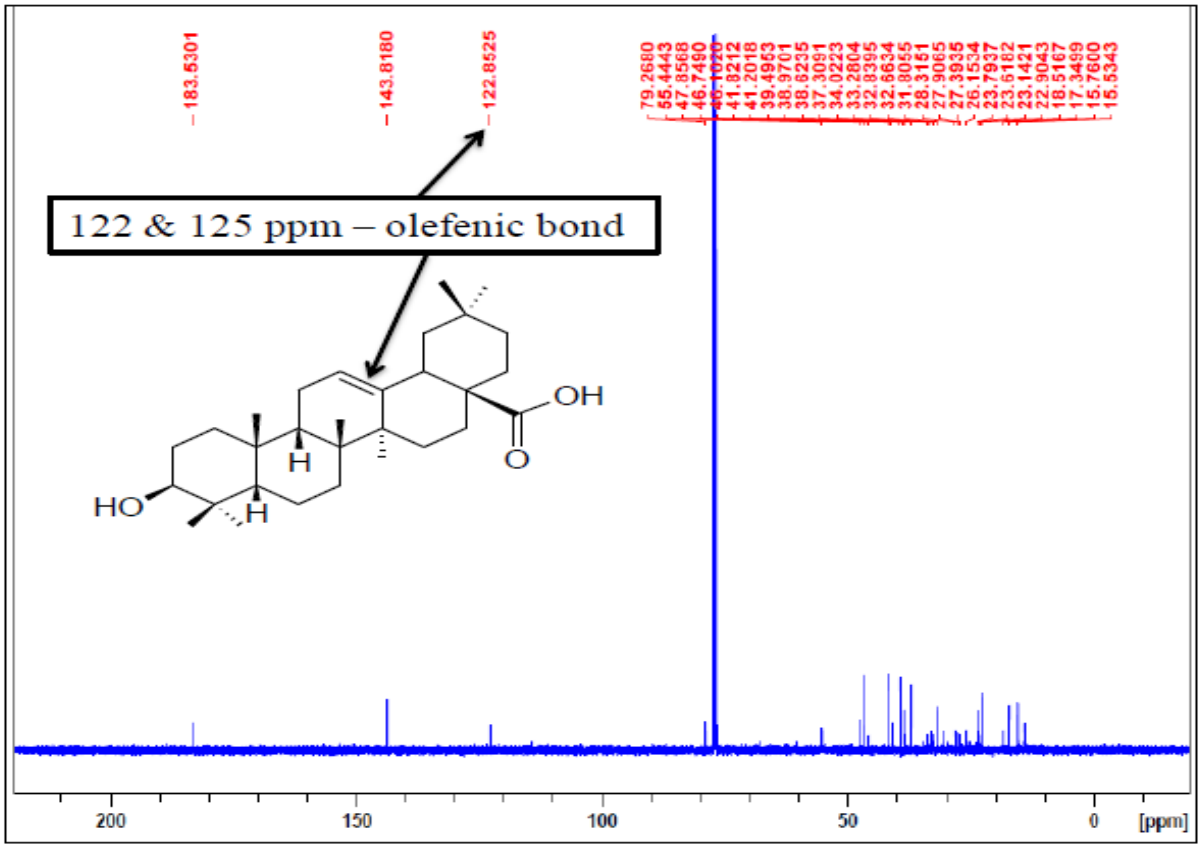
APPENDIX V

NMR Spectra

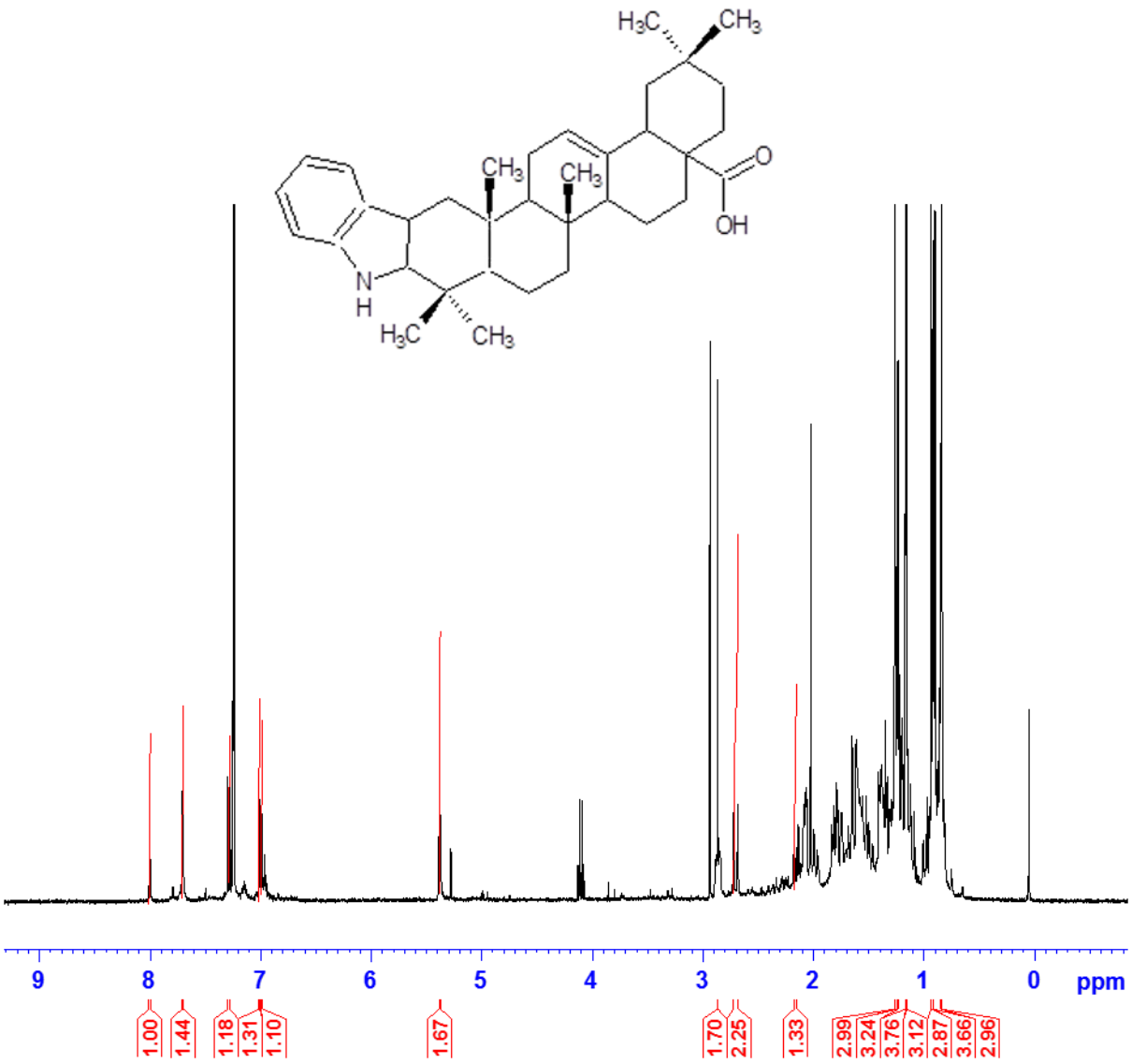
^{13}C NMR OF MA



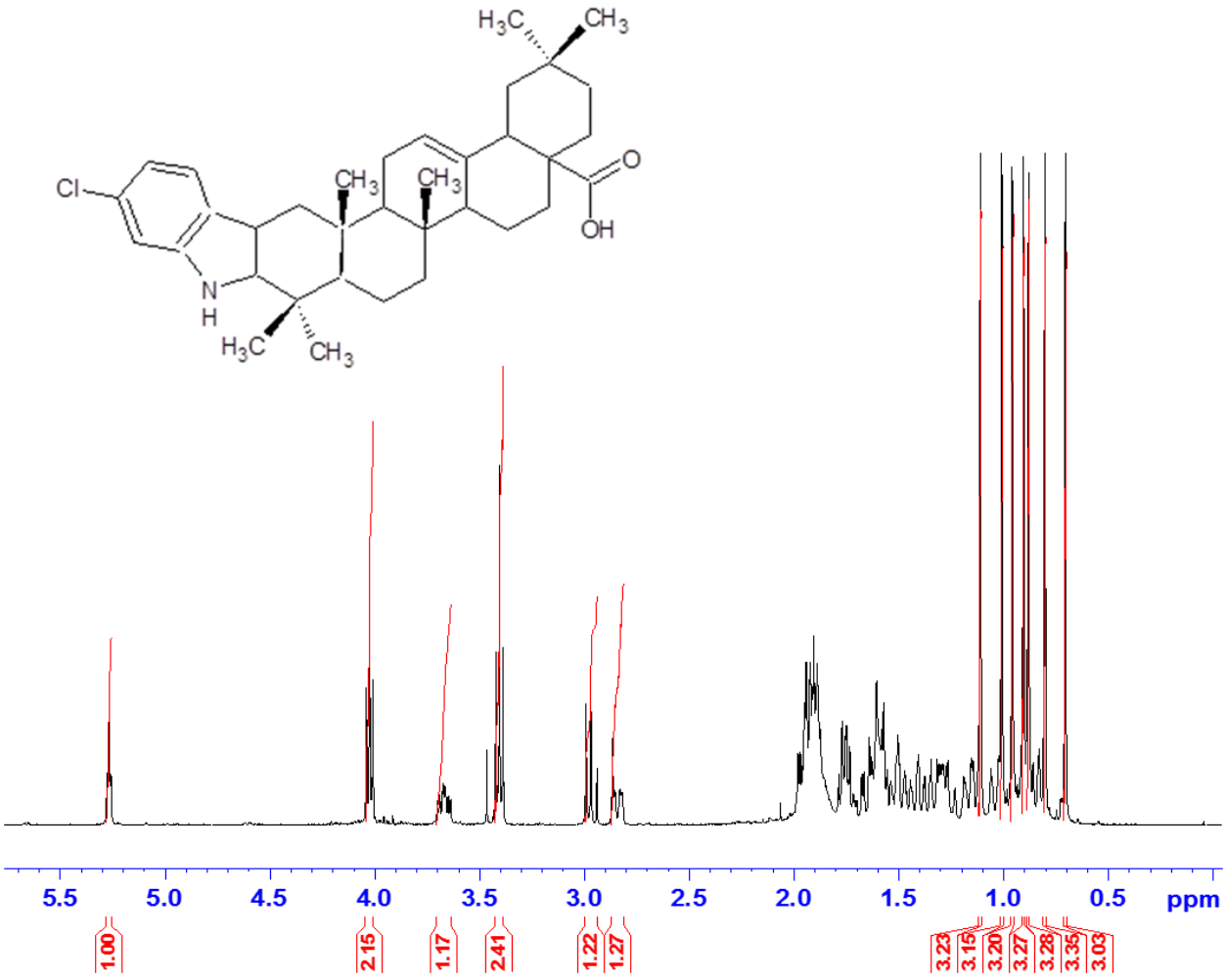
^{13}C NMR OF OA


183.5301
143.8180
122.8525
79.2680
55.4443
47.8568
46.7490
46.1020
41.8212
41.2018
39.4953
38.9701
38.6235
37.3091
34.0223
33.2804
32.8395
32.6634
31.8055
28.3151
27.9065
27.3935
26.1534
23.7937
23.6182
23.1421
22.9043
18.5167
17.3499
15.7600
15.5343
122 & 125 ppm – olefenic bond
OH
O
H
HO
H
200
150
100
50
0
[ppm]

[1]HNMR of phenyl hydrazine-MA derivative



[1]HNMR of chloro phenyl hydrazine derivative



[1]HNMR for F-phenly hydrazine derivative