UNIVERSITY OF KWAZULU-NATAL

Hypoglycaemic And Renal Effects Of A Bioactive Plant Extract In Streptozotocin Induced Diabetic Rats.

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Hypoglycaemic and renal effects of a bioactive plant extract in streptozotocin induced diabetic rats

BY

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DECLARATION

I, **Rudo Fiona Mapanga**, hereby declare that the dissertation entitled

"Hypoglycaemic and renal effects of a bioactive plant extract in streptozotocin-induced diabetic rats"

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

Student:	**Rudo F. Mapanga**	**Signature**
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LIST OF ABBREVIATIONS

α	Alpha
AGE	Advanced glycation end-product
AMPK	Adenosine monophosphate protein kinase
ANOVA	One way analysis of variance
ATP	Adenosine triphosphate
β	Beta
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
DAG	Diacylglycerol
DCCT	Diabetes Control and Complications Trial
DEPT	Distortion Enhancement Proton Testing
DMSO	Dimethyl sulphoxide
EDHF	Endothelium derived hyperpolarizing factor
eNOS	endothelium nitric oxide synthase
ESRD	End-stage renal disease
ET-1	Endothelin-1
ETRA	Endothelin receptor A
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
GFAT	Glutamine:fructose-6-phosphate amidotransferase
GLP	Glucagon like peptide
GLUT-1	Glucose transporter-1
GLUT-2	Glucose transporter-2
GLUT-3	Glucose transporter-3
GLUT-4	Glucose transporter-4
GLUT-6	Glucose transporter-6
GLUT-8	Glucose transporter-8
GLUT-10	Glucose transporter-10

GLUT-12	Glucose transporter-12
GOx	Glucose oxidase
GSH	Glutathione
h	Hour
HbA_{1C}	Glycosylated haemoglobin
HBP	Hexosamine biosynthetic pathway
HDL	High density lipoprotein
HCl	Hydrochloric acid
HMQC	Heteronuclear multiple quantum coherence
H_2SO_4	Sulphuric acid
ICAM-1	Intracellular cell adhesion molecule-1
IR	Insulin receptor
K^+	Potassium
K_{ATP}	Adenosine-5'- triphosphate sensitive potassium channels
Kg	kilogram
LTD	limited
MAP	Mean arterial pressure
l	litre
LDL	Low density lipoprotein
M	Molar
MAPK	Mitogen activated protein kinase
MHz	millihertz
µ	micro
µg	microgram
µl	microlitre
mg	milligram
mmHg	Millimetres of mercury
mmol	millimole
NaCl	Sodium chloride
NAD^+	Oxidised nicotinamide dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrogen

NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NEI	National Eye Institute
NF-κβ	Nuclear factor-kappa beta
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
NPDR	Non-proliferative diabetic retinopathy
OA	oleanolic acid
OGTT	Oral glucose tolerance test
oxLDL	Oxidized low density lipoprotein
PAI-1	Plasminogen activator inhibitor-1
PI-3-K	Phosphatidyl inositol-3-kinase
PKC	Protein kinase C
PKC-α	Protein kinase C-alpha
PKC-β	Protein kinase C-beta
PKC-δ	Protein kinase C-delta
PKC-ε	Protein kinase C-epsilon
PKC-γ	Protein kinase C-gamma
PKC-ξ	Protein kinase-xi
pmol	picomole
p.o.	per os (by mouth, orally)
PPAR-α	Peroxisome proliferator alpha
PPAR-γ	Peroxisome proliferator gamma
ppm	parts per million
RAGE	Receptor for advanced glycation end product
sc	subcutaneous
SEM	Standard error of means
SGLT	Sodium glucose transporter
SGLT-1	Sodium glucose transporter-1
SGLT-2	Sodium glucose transporter-2
STZ	Streptozotocin
SU	Sulphonylurea

SUR-1	Sulphonylurea receptor-1
SUR-2	Sulphonylurea receptor-2
TGF-β	Transforming growth factor beta
TMB	3,3',5,5' Tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
TZD	Thiazolidinediones
UA	Ursolic acid
UDP	Uridine diphosphate
UDP-GlycNac	Uridine diphosphate acetylglucosamine
UK	United Kingdom
UKPDS	United Kingdom Prospective Diabetes Study
USA	United States of America
UKZN	University of KwaZulu-Natal
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
WHO	World Health Organisation

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ABSTRACT

Background

Evidence from our laboratories indicates that triterpene constituents of *Syzygium cordatum* (Hochst.) [Myrtaceae] crude leaf extracts can be used to treat diabetes mellitus. For the plant derived triterpenes to have further potential in diabetes management, they should, however, additionally alleviate or prevent some of the complications of diabetes mellitus such as impaired kidney function and cardiovascular disorders. Accordingly, this study was designed to isolate the triterpene, oleanolic acid (OA) from *S. cordatum* leaves and evaluate its effects on blood glucose, renal function and blood pressure in streptozotocin (STZ)-induced diabetic rats. OA was studied because it is the major constituent of many African plant species used in traditional medicine.

Materials and Methods

S. cordatum crude leaf ethyl acetate solubles (EAS) were obtained after defatting the leaves with hexane followed by dichloromethane before maceration with ethyl acetate. Preliminary experiments indicated that EAS contained triterpenes with hypoglycaemic properties. Solvent extraction and fractionation of EAS yielded mixtures of oleanolic acid/ursolic acid (OA/UA) and methyl maslinate/methyl corosolate. Recrystallisation of the OA/UA mixture using ethanol yielded OA, the structure of which was confirmed by NMR spectroscopy (^{1}H & ^{13}C). Oral glucose tolerance test (OGTT) responses to various doses of OA (40, 80 and 120 mg/kg) were monitored in separate groups of non-diabetic and STZ-induced diabetic rats given a glucose load (0.86 g/kg, p.o.) after an 18-h fast. Rats treated with deionized water (3 ml/kg p.o.), or standard drugs, (insulin, 200 µg/kg, s.c.; metformin, 500 mg/kg, p.o. and glibenclamide, 500 µg/kg, p.o.) acted as untreated and treated positive controls, respectively. To investigate the possible interaction between OA and standard drugs in lowering blood glucose, OGTT responses were studied in separate groups of animals simultaneously treated

with OA at either 40 or 80 mg/kg and insulin (100 or 200 μg/kg, s.c.), metformin, (250 or 500 mg/kg, p.o.) or glibenclamide (250 or 500 mg/kg, p.o.). Blood glucose was monitored at 15-min intervals for the first hour, and hourly thereafter for 3 h. Plasma insulin concentrations were measured in separate parallel groups of rats prepared as for OGTT studies to examine whether there was an association between OA treatment and pancreatic insulin secretion. Acute effects of OA on kidney function and mean arterial blood pressure (MAP) were investigated in anaesthetized rats challenged with hypotonic saline after a 3½-h equilibration for 4 h consisting of 1 h control, 1½ h treatment and 1½ h recovery periods. OA was added to the infusate during the treatment period. Short-term effects of OA were studied in individually-caged rats treated twice daily with OA (80 mg/kg, p.o.) for 5 weeks.

Results

OA decreased blood glucose concentrations of both non-diabetic and diabetic rats, as did some standard drugs except glibenclamide which did not exhibit any effects in STZ-induced diabetic animals. The blood glucose lowering effects were most potent in STZ-induced rats treated with combined OA and insulin by comparison with all other treatments. Short-term treatment of non-diabetic and STZ-induced diabetic rats with OA alone for 5 weeks decreased blood glucose concentrations, but the reduction in non-diabetic rats was to values that did not achieve statistical significance. Except for non-diabetic rats treated with insulin alone or in combination with OA, plasma insulin concentrations were not altered by treatment in non-diabetic and STZ-induced diabetic animals. Hepatic glycogen concentrations of non-diabetic and STZ-induced diabetic rats were significantly increased by all treatments at the end of 5 weeks.

Acute intravenous infusion of OA in anaesthetized rats significantly increased Na^+ excretion outputs of non-diabetic and STZ-induced diabetic rats without affecting urine flow, K^+ or Cl^- excretion rates. Similarly, daily OA treatment (80 mg/kg, p.o.) significantly increased Na^+ excretion rates of non-diabetic and STZ-induced diabetic rats throughout the 5 week experimental period without affecting urine flow, K^+ or Cl^- excretion rates. By comparison with respective control animals, Short-term administrations of OA significantly ($p<0.05$)

increased GFR of non-diabetic (2.88±0.14 vs 3.71±0.30 ml/min) and STZ-diabetic rats (1.81± 0.32 vs 3.07±0.16 ml/min, n=6 in all groups) with concomitant reduction of plasma creatinine concentrations. Acute and Short-term administrations of OA non-diabetic and STZ-induced diabetic rats reduced mean arterial blood pressure by comparison with respective control animals.

Discussion

The results suggest that *S. cordatum* leaf derived OA not only has the potential to lower blood glucose in diabetes, but also has beneficial effects on kidney function and blood pressure. We suggest that the hypoglycaemic effects of OA mimic those of metformin as evidenced by the fact that neither of these treatments altered plasma insulin concentration of non-diabetic rats. OA-evoked increases in urinary Na^+ outputs of STZ-diabetic rats and elevation of GFR suggest up-regulation of renal function by the triterpene. The findings are of considerable importance because they suggest the hypoglycaemic, renal and hypotensive effects of OA in the management of diabetes mellitus.

Conclusion

The results demonstrated that the oleanolic acid extracted from *S. cordatum* leaf has blood glucose-lowering effects comparable to standard anti-diabetic drugs in STZ-induced diabetic rats. Furthermore, OA augmented the hypoglycaemic effects of insulin in STZ-induced diabetic rats. These findings suggest that OA may have beneficial effects on some of the processes that are associated with renal derangement in STZ-induced diabetic rats. The results introduce the first *in vivo* evidence that OA ameliorates kidney function in STZ-induced diabetic rats.

Keywords: Renal function; diabetes mellitus; triterpenoids; oleanolic acid, hypoglycaemia

CHAPTER 1

1.0. INTRODUCTION/ LITERATURE REVIEW

1.1. Background

Diabetes mellitus is characterized by hyperglycaemia because glucose transport across the cells is impaired. The sustained hyperglycaemia leads to compromised kidney function and the development of microvascular and macrovascular complications through different mechanisms. Hence the goal in diabetes management is not only to maintain optimal blood glucose control, but also to avert these adverse outcomes. Current conventional diabetes therapy using blood glucose-lowering medications such as insulin or oral hypoglycaemic agents has limitations. For instance insulin therapy does not achieve glycaemic control in patients with insulin resistance, and oral hypoglycaemic agents may lose their efficacy after prolonged use. Alternative methods of lowering blood glucose are, therefore, needed. Herbal medicines have been used for many years by different cultures around the world, both for the prevention and management of diabetes (Kim, Kang, Park, Jung, Choi and Ku, 2007b). Previously the crude leaf extract of *Syzygium cordatum* (Hochst.) [Myrtaceae] has been shown to reduce blood glucose levels in streptozotocin (STZ)-induced diabetic rats perhaps due to its triterpene constituents. The study described in this dissertation was designed to isolate and investigate the blood glucose lowering and renal function effects of one of *S. cordatum* leaf derived triterpene constituents, oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid, OA) in STZ-induced diabetic rats. The effects of OA were investigated because it is the main triterpene that has been isolated from many medicinal plants, such as *Momordica charantia* (Linnaeus) [Curcubitaceae] (Kumar, Shetty and Salimath, 2008), *Olea europaea* (Linnaeus) [Oleaceae] (Chen, Liu, Zhang, Wu, Hua, Wu and Sun, 2006). The effects of OA on kidney function were also investigated to establish whether OA has beneficial effects on renal function in diabetes mellitus management.

1.2. Diabetes mellitus

Diabetes mellitus is a chronic metabolic disorder due to acquired or inherited causes leading to absolute or relative deficiency of insulin or due to insulin resistance (Evcimen and King, 2007). Diabetes can be classified into three main types: type 1, type 2 and gestational diabetes. Type 1 is due to autoimmune destructive lesions of β-cells of the pancreas leading to an absolute lack of insulin secretion. In type 2 which accounts for 90-95% of diabetic patients, there is a combination of decreased insulin secretion and insulin sensitivity (The Committee of the Japan Diabetes Society on the diagnostic criteria of diabetes mellitus, 2002; Jung, Park, Lee, Kang, Kang and Kim, 2006; World Health Organisation (WHO), 2006). The third type, gestational diabetes is defined as the first onset of diabetes mellitus in women during pregnancy precipitated by an excess production of glucocorticoids (American Diabetes Association, 2004).

According to WHO (2006), 180 million people have diabetes worldwide. Estimates are that by 2030 the number will more than double to about 366 million, making it an epidemic (Støvring, Andersen, Beck-Nielsen, Green and Vach, 2007). Chronic hyperglycaemia due to carbohydrate, lipid and protein metabolism disorders in diabetes mellitus leads to the development of macrovascular and microvascular complications (Rahimi, Nikfar, Larijani, Abdollahi, 2005). The hyperglycaemia occurs from alterations in the glucose homeostatic systems, resulting from impaired glucose transport across cells. The various ways through which glucose is transported are briefly described below.

1.2.1. Glucose transport

Glucose is transported across cell membranes through two main carrier proteins, sodium-glucose co-transporters (SGLT's) and glucose transporters (GLUT). The two main isoforms of SGLT, SGLT-1 and SGLT-2 are mostly expressed in the intestine and kidney, respectively (Barnard and Youngren, 1992; Kakuda and MacLeod, 1994; Bouché, Serdy, Kahn and Goldfine, 2004). They utilize the electrochemical sodium gradient to transport glucose against a concentration gradient. Inhibition of glucose uptake by the SGLT's using phlorizin

is one of the strategies used in glycaemic control (Panayotova-Heiermann, Loo, Kong, Lever and Wright, 1996; Link and Sorensen, 2000). On the other hand, GLUT transporters are a family of 13 hexose transporters that passively transport monosaccharides, including glucose, down a concentration gradient. According to Bouché, Serdy, Kahn and Goldfine, (2004), these can be classified as high affinity (GLUT-1, 3, 4, 6, 8, 10 and 12) and low affinity (GLUT-2). GLUT-2 is expressed in pancreatic β-cells, and tissues with high glucose fluxes like intestine, liver and kidney. GLUT-1 is expressed mostly in erythrocytes, kidney, colon and cells of blood-tissue barriers like the blood brain barrier and at low levels in liver, adipose tissues and muscles (Bouché, Serdy, Kahn and Goldfine, 2004; Wiernsperger, 2005). GLUT-1 and GLUT-3 are the primary GLUT transporters in foetal and neuronal tissues, respectively (Bouché, Serdy, Kahn and Goldfine, 2004). GLUT-4 mediates insulin-stimulated glucose uptake by skeletal muscles, heart, and white and brown adipose tissues. Insulin and exercise stimulate translocation of the GLUT-4 to the cell membrane from an intracellular pool (Barnard and Youngren, 1992; Bouché, Serdy, Kahn and Goldfine, 2004; Wiernsperger, 2005). In contrast, the GLUT-1 is expressed on the cell membrane in the absence of insulin. The activity of the GLUT transporters can, therefore, be insulin or non-insulin mediated, with GLUT-1 contributing to about 75% of the glucose transport. In type 2 diabetes mellitus, there are defects in GLUT-4 translocation due to insulin resistance and GLUT-1 is over-expressed (Miura, Itoh, Kaneko, Ueda, Ishida, Fukushima, Matsuyama and Seino, 2004; Wiernsperger, 2005). The high glucose influx in diabetes mellitus mediated via GLUT-1 transporters increases intracellular glucose metabolism leading to the development of complications.

1.3. Aetiology of diabetic complications

The metabolic pathways involved in the aetiology of diabetic complications are discussed in the following sections.

1.3.1. Polyol pathway

Aldose reductase, the first enzyme in the polyol pathway, catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of glucose to sorbitol which is oxidized to fructose by sorbitol dehydrogenase, an enzyme that uses NAD^+ as a cofactor (Brownlee, 2001; Sheetz and King, 2002; Lorenzi, 2007). This pathway is activated in diabetes mellitus in non-insulin sensitive tissues including the lense, peripheral nerve and the glomerulus (Brownlee, 2001; Sheetz and King, 2002; Lorenzi, 2007). Studies suggest that sorbitol evokes osmotic vascular damage and cataracts in the eyes (Sheetz and King, 2002; Lorenzi, 2007). The involvement of sorbitol in osmotic vascular damage, however, is often difficult to rationalize (Brownlee, 2001; Sheetz and King, 2002). Additionally, diabetic complications arise from advanced glycosylating agents formed by the breakdown of fructose (Brownlee, 2001; Lorenzi, 2007). Development of diabetic complications also involves decreasing the activity of enzymes that use NADPH/NADH as a cofactor. The enzymes include glutathione reductase which would cause redox imbalances due to depletion of reduced glutathione levels (Brownlee, 2001; Sheetz and King, 2002; Vincent, Russel, Low and Feldman, 2004; Lorenzi, 2007). Endothelial generation of nitric oxide is also impaired by the decreased NADPH levels. In addition there is inhibition of glyceraldehyde-3-phosphate dehydrogenase (GADPH), leading to an increase in triose phosphate. This increases the formation of methylglyoxal, a precursor of advanced glycation end products (AGE's) and diacylglycerol (DAG), which activates protein kinase C (PKC) (Brownlee, 2001). Despite the osmotic damage, studies suggest that oxidative stress is the main cause of cellular damage by the polyol pathway (Lorenzi, 2007).

Inhibition of aldose reductase by various synthetic drugs such as zenarestat and sorbinil and anti-diabetic plants has been effective in preventing and alleviating complications associated with diabetes mellitus (Brownlee, 2001; Lorenzi, 2007). Plant extracts of *Stelechocarpus cauliflorous* [Annonaceae] and its glycoside derivatives engeletin and astilbin have been reported to inhibit aldose reductase (Wirasathiena, Pengsuparpa, Suttisria, Uedab, Moriyasub and Kawanishib, 2007). Reno-protective effects were seen with berberine, one of the main constituents of *Coptis chinensis* (Franch) [Ranunculaceae] and *Phellodendron amurense*

(Ruprecht) [Rutaceae], and this was associated with a concomitant inhibition of oxidative stress (Wei-ha, Zi-qing, Hong, Fu-tian, He-qing, Xue-juan, Yan-hui, Shao-rui, Fen-fen, Wen-ge, Feng-ying and Pei-qing, 2008). Other isolated bioactive compounds from plants that inhibit aldose reductase activity include quercetin, silymarin, flavonoids and puerarin (Adaramoye and Adeyemi, 2005; Li, Dai, Yu, Li, Wu, Luan, Meng, Zhang and Deng, 2007). Reduced activity of aldose reductase prevents production of sorbitol and its downstream stimulation of the formation of AGE's and PKC pathways which are described in the next sections.

1.3.2. Advanced glycosylation end products (AGE's)

The formation and accumulation AGE's from dicarbonyl compounds is accelerated in diabetes. Major sources of the carbonyl group in the glycation reaction include glucose and carbonyl compounds, such as glyceraldehyde (Sakurai, Yonekura, Yamamoto, Watanabe, Tanaka, Li, Rahman, Myint, Kim and Yamamoto, 2003). The dicarbonyl compounds react irreversibly with amino groups of intracellular and extracellular proteins in the Maillard reaction to form AGE's (Brownlee, 2001; Sheetz and King, 2002; Sakurai *et al.*, 2003; Yonekura, Yamamoto, Sakurai, Watanabe, and Yamamoto, 2005; Thallas-Bonke, Thorpe, Coughlan, Fukami, Yap, Sourris, Penfold, Bach, Cooper and Forbes 2008). The structure and function of proteins, such as serum albumin, lens crystallin, intracellular proteins and collagen in the extra cellular matrix is altered by advanced glycation end products (AGE's) (Brownlee, 2001; Sheetz and King, 2002; Sakurai *et al.*, 2003; DeGroot, 2004). Altered cell function by AGE's occurs through the receptor for AGE's (RAGE) on endothelial cells, mesangial cells and macrophages (Brownlee, 2001; Sheetz and King, 2002; DeGroot, 2004; Yonekura, Yamamoto, Sakurai, Watanabe, and Yamamoto, 2005). Activation of the receptor has been reported to induce production of reactive oxygen species through activation of the nuclear transcription factor-κβ (Brownlee, 2001; Yonekura, Yamamoto, Sakurai, Watanabe, and Yamamoto, 2005). This occurs via a cascade of cellular signalling events, such as activation of mitogen-activated protein kinase (MAPK) or PKC, which can lead to cellular dysfunction (Sheetz and King, 2002). Additionally AGE's have been shown to reduce matrix protein flexibility through modification of extra-cellular matrix proteins leading to an

abnormal interaction with other matrix components and the receptors for matrix components on cells (Brownlee, 2001; Hartog, Voors, Bakker, Smit and van Veldhuisen, 2007). The activation of these events leads to development of diabetic complications like retinopathy, nephropathy and atherosclerosis. There are various strategies that can be used to prevent the effects of AGE's accumulation. These may involve glycaemic control which prevents the glucose-dependent first step in the Maillard reaction or use of antioxidants like flavonoid that inhibit the final Maillard step catalyzed by oxidative stress. In diabetic atherogenesis, blocking or genetically deleting the receptor for advanced glycation end product (RAGE) in experimental animals reverses atherosclerosis (Ihara, Egashira, Nakano, Ohtani, Kubo, Koga, Iwai, Horiuchi, Gang, Yamagishi and Sunagawa, 2007). Amino guanidine and pyridoxamine, AGE's formation inhibitors, have reno-protective effects in diabetic animals (Lassila, Seah, Allen, Thallas, Thomas, Candido, Burns, Forbes, Calkin, Cooper and Jandeleit-Dahm, 2004; Hartog, Voors, Bakker, Smit and van Veldhuisen, 2007). Furthermore, inhibition of AGE's effects can be achieved through breaking of the AGE's cross links by drugs such as alagebrium and also by inhibiting AGE signal transduction (incadronate disodium and cerivastatin) (Hartog, Voors, Bakker, Smit and van Veldhuisen, 2007). Additionally various medicinal plants and their derived compounds have been shown to prevent the complications in diabetes mellitus through inhibition of the AGE's or RAGE formation. The extracts of *Panax quinquefolium* (Linnaeus) [Araliaceae] and plant-derived bioactive compounds resveratrol, a phytoestrogen from *Vitis vinifera* (Linnaeus) [Vitaceae]; curcumin from *Curcuma longa* (Linnaeus) [Zingiberaceae] and glycosides from *Stelechocarpus cauliflorus* (R.E. Fr) [Annonaceae] have been reported to inhibit formation of AGE's or RAGE (Sheetz and King, 2002 Rahbar and Figarola, 2003; Kim, Kang, Yamabe, Nagai, Yokozawa, 2007a; Wirasathiena *et al.*, 2007).

1.3.3. Protein kinase C

PKC is a family of at least twelve enzyme isoforms which are involved in the development of diabetic complications (Brownlee, 2001; Sheetz and King, 2002; Evcimen and King, 2007). Nine of the PKC isoforms (including PKC-α, βI, βII, γ, δ, ε and ξ) are activated by the second messenger diacyl-glycerol (DAG), a critical signalling molecule that regulates many

vascular functions such as permeability, growth factor signalling, vasodilator release and endothelial activation (Brownlee, 2001; Aiello, 2002; Sheetz and King, 2002; Khan and Chakrabarti, 2007). DAG levels are increased both in vascular tissues, including those of the aorta, heart and renal glomeruli and also in non vascular tissues such as liver and skeletal muscles in diabetes mellitus (Brownlee, 2001; Sheetz and King, 2002; Evcimen and King, 2007). Multiple pathways have been shown to increase DAG levels in the diabetic state and one such is hydrolysis of phosphatidylcholine by phospholipase C (Brownlee, 2001; Sheetz and King, 2002; Evcimen and King, 2007). The generation of the reactive oxygen species, like hydrogen peroxide in the polyol pathway also activates PKC (Brownlee, 2001; Aiello, 2002; Sheetz and King, 2002; Vincent, Russel, Low and Feldman, 2004; Evcimen and King, 2007).

The activation of PKC is associated with changes in blood flow, basement membrane thickening, extra cellular matrix expansion, vascular permeability, angiogenesis, cell growth and enzymatic activity alterations (Evcimen and King, 2002). In early diabetes, activation of PKC has been implicated to impair retinal and renal blood flow possibly by increasing endothelin-1 levels (Brownlee, 2001). The effects of PKC activation on nitric oxide are unclear though there is evidence of reduced production of nitric oxide (Brownlee, 2001; Evcimen and King, 2007). PKC activation also increases directly the permeability of macromolecules across endothelial or epithelial barriers by phosphorylating cytoskeletal proteins or indirectly by regulating expression of various growth factors such as vascular endothelial growth factor (VEGF) (Brownlee, 2001; Aiello, 2002; Evcimen and King, 2007). Most of the manifestations due to PKC activation in diabetes mellitus are reversed with the use of PKC inhibitors (Evcimen and King, 2007). One such is ruboxistaurin, a PKC-β inhibitor which has been shown to reverse haemodynamic changes in retinopathy, nephropathy and neuropathy (Sheetz and King, 2002; Evcimen and King, 2007).

1.3.4. The hexosamine pathway

The hexosamine biosynthesis pathway (HBP) is a relatively minor branch of glycolysis. It involves conversion of fructose-6-phosphate to glucosamine-6-phosphate by the enzyme

glutamine: fructose-6-phosphate amidotransferase (GFAT). The major end-product is uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) which along with other amino-sugars generated by hexosamine biosynthetic pathway (HBP) provides essential building blocks for glycosyl side chains, of proteins and lipids (Buse, 2006). Shunting of excess intracellular glucose into the HBP may account for several manifestations of diabetic renal and vascular complications (Buse, 2006). This implies that under hyperglycaemic conditions there are increased amounts of fructose-6-phosphate diverted from glycolysis that provide substrates for reactions which require UDP-*N*-acetylglucosamine, such as proteoglycan synthesis and the formation of *O*-linked glycoproteins (Brownlee, 2001). The altered protein function due to *O*-linked GlcNAcylation results in diminished expression of sarcoplasmic reticulum Ca^{2+}-ATPase in cardiomyocytes and induction of TGF-β and plasminogen activator inhibitor –1 in vascular smooth muscle cells, mesangial cells and aortic endothelial cells (Buse, 2006). A study by Kolm-Litty, Sauer, Nerlich, Lehmann and Schleicher, (1998) showed that at high levels, D-glucosamine was more potent than D-glucose in increasing the matrix of mesangial cells. This is associated with an increase in TGF-β production which is converted to its active form TGF-β1 and subsequent production of matrix components heparan sulphate proteoglycan and fibronectin. Activation of the HBP is associated with reduced insulin mediated translocation of GLUT-4 transporters (Marshall, Garvey, Traxinger, 1991). Some of the effects of the HBP in diabetes include inhibition of endothelium nitric oxide synthase (eNOS) through hyperglycaemia-induced O-acetyl-glucosaminylation (Brownlee, 2001).

1.4. Diabetic complications

1.4.1. Macrovascular complications

Diabetes mellitus is associated with coronary, cerebral and peripheral arterial disease (Sobel and Schneider, 2005). Coronary and cerebral arterial diseases can result in myocardial infarction (MI) and stroke, respectively. These disorders, with peripheral arterial disease are defined as macrovascular diseases (Brownlee, 2001; Vinik and Flemmer, 2002).

1.4.1.1. Arterial diseases

Arterial disease is more associated with type 2 than type 1 diabetes mellitus, because in type 2 there is a metabolic syndrome characterised by hypertension, dyslipidaemia (increased triglycerides, decreased high density lipoproteins (HDL), and increased low density lipoproteins (LDL) and cholesterol) resulting in inflammation and impaired fibrinolysis. All these factors precipitate changes in the vasculature and create an environment conducive for accelerated atherosclerosis (Beckman, Creager and Libby, 2002; Vinik and Flemmer, 2002; Reusch, 2003). A greater risk to develop cardiovascular diseases, as well as increased morbidity and mortality is thus observed in diabetic patients due to the toxic metabolic milieu in the circulatory system (Beckman, Creager and Libby, 2002; Vinik and Flemmer, 2002; Reusch, 2003; Vinik and Vinik, 2003). The chief cause of cardiovascular disease in diabetes is atherosclerosis which is described below.

1.4.1.2. Atherosclerosis

Diabetes is associated with impaired function of the endothelium which contributes to atherosclerosis (Creager and Lüscher, 2003). The endothelial dysfunction is due to a disruption of the homeostatic factors. Homeostasis is maintained through integrity of the endothelium barrier and a balance of the vasodilators and vasoconstrictors (Vinik and Flemmer, 2002; Sadeghi, 2006). The vasodilators include nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) whereas the vasoconstrictors are endothelin-1 and angiotensin II (Brandes, Behra, Lebherz, Böger, Bode-Böger and Mügge, 1999; Vinik and Flemmer, 2002; Creager and Lüscher, 2003; Sadeghi, 2006). NO, however, represents a key marker in vascular health (Creager and Lüscher, 2003).

In diabetes mellitus the endothelium barrier is disrupted by the oxidative stress and increased activity of the metalloproteinases resulting in the entrapment of some of the excess atherogenic lipoproteins like very low density lipoproteins (VLDL), oxidized lipoprotein (oxLDL) and lipoprotein (a) (Vinik and Flemmer, 2002; Esposito, Francesco, Marfella, Giugliano, Giugliano, Ciotola and Quagliaro, 2002; Fan and Watanabe, 2003). This

infiltration triggers an inflammatory response thereby attracting monocytes and T-cells (Hansson, 2005; Sadeghi, 2006). The atherogenic lipoproteins also increase expression of the following adhesion molecules on the endothelium; the vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1), P-selectin and E-selectin (Vinik and Flemmer, 2002; Esposito *et al.*, 2002; Fan and Watanabe, 2003; Natarajan and Nadler, 2004). These trigger adhesion of the attracted monocytes and T-cells to the endothelium. After binding to the arterial wall the monocytes and T-cells then migrate into the sub-endothelial space where they differentiate into macrophages and foam cells. This migration is attributed to chemo-attractants like oxLDL, interleukin-1 and tumor necrosis factor alpha (TNF-α) which are produced due to activation of the transcription factors nuclear factor-κβ (NF-κβ) and activator protein 1 (Beckman, Creager and Libby, 2002; Esposito *et al.*, 2002; Fan and Watanabe, 2003).

The inflammatory reactions together with reduced amounts of NO precipitate atherosclerosis in diabetes. Reduced NO may be due to the fact that, the deformed endothelium exposes eNOS to uncoupling by the peroxynitrite formed from superoxide anion and nitric oxide making eNOS produce super oxide free radicals instead of NO (Beckman, Creager and Libby, 2002; Pacher, Obrosova, Mabley and Szabo, 2005). NO production may be inhibited by excessive liberation of free fatty acids from adipose tissue due to activation of the signalling enzyme protein kinase C which inhibits an eNOS agonist pathway phosphatidylinositol-3 (PI-3) kinase (Beckman, Creager and Libby, 2002). Thus, as NO bioavailability progressively decreases, concomitant increases in peroxynitrite further impair production of subsidiary vasodilators like the antiplatelet prostanoid prostacyclin (Beckman, Creager and Libby, 2002; Vinik and Flemmer, 2002; Creager and Lüscher, 2003). In the disordered endothelium there will be, therefore, an imbalance between the vasodilators and vasoconstrictors with an increase in the vasoconstrictors endothelin-1 and angiotensin II. Vasoconstriction exacerbated by paradoxical vasoconstriction due to the effect of acetylcholine on smooth muscle muscarinic receptors is observed in atherosclerosis (Beckman, Creager and Libby, 2002; Vinik and Flemmer, 2002; Vinik and Vinik, 2003). The processes discussed above together with hypertension and the impaired fibrinolytic capacity

in the prothrombotic milieu leads to atherosclerosis in the metabolic syndrome (Beckman, Creager and Libby, 2002; Fan and Watanabe, 2003).

Some of the anti-atherosclerotic drugs such as statins have been shown to lower the atherogenic lipoproteins and possess anti-inflammatory effects (Brandes *et al.*, 1999). These effects have also been observed with some oral anti-diabetic drugs (see pages 19-23). Atherosclerosis suppression also occurs with anti-diabetic plant extracts and their derivatives which possess anti-inflammatory and anti-hyperlipidaemic effects e.g. *Tamarindus indica* (Linnaeus) [Fabaceae], *Cortex Lycii radicis* (Miller) [Solanaceae] and *Annona squamosa* (Linnaeus) [Annonaceae] (Gupta, Kesari, Murthy, Chandra, Tandon and Watal, 2005; Maiti, Das and Ghosh, 2005; Gao, Li, Liu, Li, Liu, Fan, Li, Han and Li, 2007). Other plants contain derivatives with antioxidant effects, for example the dietary polyphenols and flavonoids such as quercetin which reduce atherogenic lipoproteins and ameliorate the oxidative stress in diabetes mellitus (Stoclet, Chataigneau, Ndiaye, Oak, El Bedoui, Chataigneau and Schini-Kerth, 2004; Machha, Achike, Mustafa and Mustafa, 2007). Prevention of atherosclerosis in diabetes mellitus is associated with a reduction in the development of macrovascular and microvascular complications.

1.4.2. Microvascular complications

The pathogenesis of microvascular complications is similar in type 1 and type 2 diabetes (Sheetz and King, 2002). Microvascular complications most common in type 1 diabetes mellitus include retinopathy, neuropathy, and nephropathy (Gerich, 2003; Pacher, Obrosova, Mabley and Szabo, 2005; Waisundara, Hsu, Huang and Tan, 2008).

1.4.2.1. Diabetic neuropathy

Diabetic neuropathy is defined as signs and symptoms of peripheral nerve dysfunction in a diabetic patient where other causes of peripheral nerve dysfunction have been excluded (Bansal, Kalita and Misra, 2006). It is one of the commonest complications of diabetes with about half of the patients having some degree of the disease as polyneuropathy or

mononeuropathy (Sheetz and King, 2002; Tesfaye, 2003). The disease can develop in all types of diabetes mellitus; more in type 1 than in type 2 (Little, Edwards and Feldman, 2007). Diabetic neuropathy leads to increased incidences of ulceration and limb amputations due to the irreversible progressive development of the disease, (Brown, Bird, Watling, Kaleta, Hayes, Eckert, Foyt, 2004; Bansal, Kalita and Misra, 2006). It accounts for silent myocardial infarction and shortens the lifespan of diabetic patients. The prevalence of diabetic neuropathy increases with the duration of the diabetic state (Bansal, Kalita and Misra, 2006; Little, Edwards and Feldman, 2007).

The cause(s) of diabetic neuropathy may include any of the following; oxidative stress, ischaemia and inflammation leading to dysfunction and loss of axons (Little, Edwards and Feldman, 2007). Oxidative stress can be due to increased activity of protein kinase C. The blood supply to neurones may be impaired by vascular damage and endoneural hypoxia due to oxidative stress (Sheetz and King, 2002; Vincent, Russell, Low and Feldman, 2004). Hypoxia further leads to capillary damage aggravating disturbances in axonal metabolism and nerve conduction (Bansal, Kalita and Misra, 2006). Distal symmetrical sensorimotor polyneuropathy characterized by thickening of axons of small myelinated and non-myelinated C-fibers is the most common type of diabetic neuropathy (Sheetz and King, 2002; Tesfaye, 2003; Bansal, Kalita and Misra, 2006; Little, Edwards and Feldman, 2007). Distal symmetrical sensorimotor polyneuropathy is manifested by paraesthesia, dysaesthesia, pain, impaired reflexes and decreased vibratory sensation (Sheetz and King, 2002; Bansal, Kalita and Misra, 2006).

Inhibition of the pathways involved in the aetiology of diabetic complications in addition to glycaemic control, antidepressants and analgesics may be used to manage diabetic neuropathy (Vincent, Russell, Low and Feldman, 2004; Wong, Chung and Wong, 2007). Studies indicate that plant derivatives such as alpha-lipoic acid, primrose oil and capsaicin have potential in the management of diabetic neuropathy (Halat and Denneby, 2003). With respect to the focus of this study, diabetic neuropathy influences renal function via changes in sympathetic input to various parts of the nephron, thereby modulating renin secretion and

the renal tubular reabsorption of sodium and water (Di Bona, 1985). Such a relationship may be a potential target for drugs or medicinal plants that alleviate diabetic nephropathy.

1.4.2.2. Diabetic retinopathy

Diabetic retinopathy due to damage of the blood vessels of the retina, is the most common cause of blindness in diabetic patients (Khan and Chakrabarti, 2007; Kowluru and Chan, 2007). Nearly all people with type 1 and more than half with type 2 diabetes develop retinopathy 15-20 years after diagnosis (Williams, Airey, Baxter, Forrester, Kennedy-Martin and Girach, 2004). Retinal complications in chronic diabetes may culminate from microvascular dysfunction, neuroglial abnormalities, and the toxic metabolic environment (Khan and Chakrabarti, 2007). Diabetic retinopathy is a duration-dependent disease that develops in stages and which may not be detected in the first few years of diabetes (Kowluru and Chan, 2007).

Diabetic retinopathy can be described as non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (Khan and Chakrabarti, 2007; Williams *et al.*, 2004). Additionally NPDR has further been divided in progressive stages: mild, moderate and severe (Khan and Chakrabarti, 2007; Kowluru and Chan, 2007). NPDR is characterized by capillary basement membrane thickening, pericyte loss, micro-aneurysms, increased permeability, exudates deposits, and retinal micro-infarcts. The earliest sign of retinal damage during NDPR results from abnormal permeability and non-perfusion of capillaries, leading to the formation of micro-aneurysms. Visual acuity is impaired by macular oedema, following the leakage of fluid and solutes into the surrounding retinal tissue (Williams *et al.*, 2004). The later stages, sometimes called pre-proliferative retinopathy, show increased retinal damage as evidenced by increased retinal vascular blockage and infarcts. Proliferative retinopathy develops if the pre-proliferative retinopathy is not treated. This is characterized by abnormal proliferation of blood vessels on the retina. These vessels, however, are fragile and haemorrhage easily. The resulting accumulation of blood in the vitreous humour from these haemorrhaging vessels impairs vision; this impairment can be permanent due to

complications such as retinal detachment (Khan and Chakrabarti, 2007; Williams *et al.*, 2004).

The pathways previously described (see section 1.3) lead to the structural and functional changes that occur in diabetes mellitus, particularly the aldose reductase pathway (Khan and Chakrabarti, 2007). Diabetic retinopathy, like most other complications of diabetes mellitus, does not usually occur in isolation in the diabetic state. Therefore, drugs and medicinal plants that are used to inhibit the pathways involved in the development of diabetic complications may also prevent development of diabetic retinopathy (see section 1.3).

1.4.2.3. Diabetic nephropathy

Diabetic nephropathy a leading cause of end-stage renal disorder (ESRD), accounts for significant morbidity and mortality in diabetes (Mogensen, 2003; Schena and Gesualdo, 2005; Sarafidis, 2007; Bloomgarden, 2008). The pathophysiology of diabetic nephropathy involves interactions between the metabolic and haemodynamic factors. Some of the metabolic factors include increased formation of AGE's, polyols and activation of protein kinase C (Cooper, Gilbert and Epstein, 1998; Rao and Nammi, 2006). The involvement of these pathways in the development of diabetic nephropathy has been described above in section 1.3. Haemodynamic factors include systemic hypertension and the tone of both afferent and efferent arterioles (Cooper, Gilbert and Epstein, 1998). Diabetic nephropathy progresses from microalbuminuria to overt proteinuria and then renal failure. In the initial stages of diabetes, there is enlargement of the kidneys and increased glomerular filtration rate (GFR), then progressively the GFR decreases (Thomson, Vallon and Blantz, 2004; Rao and Nammi, 2006). The two main hypotheses that describe the initial events of diabetic nephropathy are the 'vascular hypothesis' and the 'tubular hypothesis' (Zerbini, Bonfanti, Meschi, Bognetti, Paesano, Gianolli, Querques, Maestroni, Calori, Maschio, Fazio, Luzi and Chiumello, 2006). The vascular hypothesis states that the initial hyperfiltration is due to an excessive production of vasodilator products like nitric oxide and prostaglandins and the increased osmolar load (Vinik and Vinik, 2003). Additionally, there is increased glomerular hydrostatic pressure associated with microalbuminuria (Mason and Wahab, 2003; Kumar,

Shetty and Salimath, 2008). These features result in basement membrane thickening, mesangial proliferation, and glomerulosclerosis as a compensatory mechanism to prevent electrolyte loss. In contrast, the tubular hypothesis contends that hyperglycaemia induces increased production of growth factors and cytokines which cause hyperplasia and hypertrophy of the nephron, particularly the proximal tubule (Zerbini *et al.*, 2006). As a result increased reabsorption of sodium occurs in the proximal tubule, consequently reducing the sodium load to the macula densa (Zerbini *et al.*, 2006). In experimental animals, it is, suggested that the vascular hypothesis is more applicable considering that the hyperfiltration is observed within 24 hours of diabetes induction.

The hypertrophy of the nephron in diabetic nephropathy occurs because of excessive deposition of extra cellular matrix proteins involved in the architecture of glomerular basement membrane (GBM). These include multifunctional glycoproteins, laminin, fibronectin and type IV collagen. At the same time, there is a decrease in production and under-sulphation of heparan sulphate proteoglycan. This enhances the permeability to macromolecules since the glycoproteins and heparan sulphate proteoglycan normally interact to form a barrier to charged molecules (Kumar, Shetty and Salimath, 2008). Some anti-diabetic plants, such as *Momordica charantia* (Linnaeus) [Curcubitaceae], have been shown to improve renal function and to delay the onset of diabetic nephropathy by preventing the decrease in heparan sulphate and its under-sulphation (Kumar, Shetty and Salimath, 2008). Endothelin-1 (ET-1), which increases five-fold in diabetic animal models has been implicated in causing glomerular hypertrophy mediated by TGF-β_1. Indeed, experimental evidence indicates that inhibition of ET-1 and TGF- β_1 and the endothelin-1 receptor A (ETRA) with plant extracts improves renal function and ameliorates glomerular injury (Khan, Farhangkhoee, Mahon, Bere, Gonder, Chan, Uniyal and Chakrabarti, 1999; Hargrove, Dufresne, Whiteside, Muruve, and Wong, 2000; Sorokin and Kohan, 2003; Nakagawa, Goto, Hikiami, Yokozawa, Shibahara and Shimada, 2007). The production of ET-1 is augmented by vasoconstrictor, profibrotic and inflammatory substances, all of which are increased in hyperglycaemic conditions. Additionally the activation of PKC favours ET-1 production, as this is the signalling pathway leading to up regulation of this hormone (Sorokin and Kohan, 2003). The effects of ET-1 are mainly directed at mesangial cells, and the proliferation of

these cells is due to direct or indirect stimulation of their mitogenesis (Sorokin and Kohan, 2003).

Irrespective of all the other structural and functional changes, the mesangial alterations appear to be the main cause of declining renal function in experimental diabetic animal models (Gnudi, Viberti, Raij, Rodriguez, Burt, Cortes, Hartley, Thomas, Maestrini and Gruden, 2003; Mason and Wahab, 2003; Kumar, Shetty and Salimath, 2008). Hyperfiltration can be attributed to increased production of the vascular permeability factor in response to stretching in the mesangium (Gruden, Thomas, Burt, Lane, Chusney, Sacks and Viberti, 1997). The decline in glomerular filtration rate is due to the expanded extracellular mesangial matrix which compresses the glomerular capillaries thereby reducing the filtration surface area (Gnudi *et al.*, 2003; Mason and Wahab, 2003; Kumar, Shetty and Salimath, 2008). The mesangial cells have also been shown to increase glucose uptake through the increased expression of GLUT-1 transporters (Schena and Gesualdo, 2005; Gnudi, Thomas and Viberti, 2007). Increased glucose uptake exacerbates intracellular hyperglycaemia and increased activity in the previously described pathways (see section 1.3.).

The increased levels of endothelin-1 and angiotensin II in diabetes mellitus lead to development of systemic and intrarenal hypertension (Vinik and Vinik, 2003). An enhanced tubuloglomerular feedback characterized by reduced sodium excretion in the kidney is the compensatory response to the elevated blood pressure (Zerbini *et al.*, 2006; Oh, Joo, Lee, Jeon, Lim, Han, Knepper and Na, 2007). Against this background are observations that mean arterial pressure (MAP) is not altered in experimental diabetic animals despite showing impaired fluid and electrolyte handling (Musabayane, Ndhlovu and Balment, 1995).

Several plants which include *Sclerocarya birrea* [(A. Rich) Hochst.] [Anachardiaceae], *Persea americana* (Miller) [Lauraceae] and *Ficus thonningii* (Blume) [Moraceae] have been shown to possess reno-protective effects (Musabayane, Gondwe, Kamadyaapa, Churtugoon and Ojewole, 2007; Gondwe, Kamadyaapa, Tufts, Chuturgoon and Musabayane, 2008 and Gondwe, Kamadyaapa, Tufts, Chuturgoon, Ojewole and Musabayane, 2008). For instance studies indicate that *Cornus officinalis* [Sieb et. Zucc] inhibits AGE formation and decreases

levels of RAGE (Yokozawa, Yamabe, Kim, Kang, Hur, Park, Tanaka, 2008). Polyphenols isolated from *Vigna angularis* [(Willdenow Ohwi & H. Ohashi)] [Fabaceae] were shown to attenuate glomerular expansion in STZ-induced diabetic rats, as well as suppressing the number of infiltrating macrophages (Sato, Yamate, Hori, Hatai, Nozawa and Sagai, 2005). Some plant extracts may, however, further compromise kidney function in diabetes mellitus by impairing renal fluid and electrolyte handling (Musabayane, Xozwa and Ojewole, 2005).

1.5. Diabetes management

Discussions in the preceding paragraphs indicate that hyperglycaemia is associated with impaired kidney and cardiovascular functions. The goal in the management of diabetes mellitus should, therefore, be to achieve near normal or improved glycaemia control to diminish the risk of long-term diabetic complications (DeFronzo, 1999; Robertson, Drexler and Vernillo, 2003; Krentz and Bailey, 2005; Yurgin, Secnik and Lage, 2008). The importance of blood glucose control in preventing microvascular complications of diabetes mellitus is now well recognized and the treatment regimen incorporates a controlled-energy diet, regular aerobic exercises and weight loss (Inzucchi, 2002; The Diabetes Control and Complications Trial Research Group (DCCT), 1993; Robertson, Drexler and Vernillo, 2003; Krentz And Bailey, 2005; Paterson, Rutledge, Cleary, Lachin and Crow, 2007; Holman, Paul, Bethel, Matthews and Neil, 2008). This, however, should include pharmacotherapy, since most patients fail to achieve adequate blood glucose control with lifestyle interventions alone (Inzucchi, 2002; Fowler, 2007) (see pages 18-24 below on pharmacotherapy). There are many standard anti-diabetic drugs used in the management of diabetes mellitus. These include various formulations of insulin and oral anti-diabetic agents which can be used as monotherapy or in combination to achieve better glycaemic regulation (Robertson, Drexler and Vernillo, 2003; Jung *et al.*, 2006). The oral anti-diabetic agents such as metformin with insulin, sulphonylureas and thiazolidinediones have been found to be more effective when used in combination than when singly administered. Indeed, single therapy has been found to be ineffective in maintaining normoglycaemia particularly as diabetes progresses (Kirpichnikov, McFarlane, Sowers, 2002; Krentz and Bailey, 2005). Recently, a 'polypill' treatment was suggested as a potential overall remedy for diabetes and its complications

(Gershell, 2005; Grundy, 2006). This combination pill includes an anti-hyperglycaemic, anti-inflammatory, anti-hypertensive, and anti-angiogenic agent. According to Krentz and Bailey, (2005), the anti-diabetic drugs can be classified as insulin secretagogues, insulin sensitizers and those that delay carbohydrate absorption. In addition to the use of synthetic drugs medicinal plants and their derivatives are also used in the management of diabetes mellitus. The following sections discuss the anti-diabetic mechanisms of the synthetic drugs and some of the medicinal plants.

1.5.1. Insulin

Insulin, discovered in 1921, is the major current hypoglycaemic agent used in the management of type 1 diabetes and late stage type 2 diabetes (Emilien, Maloteaux and Ponchon, 1999). Patients who do not achieve effective glycaemic control with oral agents or for whom other oral agents are contraindicated are also treated with insulin (Laube, 2002; Krentz and Bailey, 2005). Robertson, Drexler and Vernillo, (2003) classify insulin as human insulin and insulin analogues. Another form of classification based on the duration of action gives four main types; rapid-acting, short-acting, intermediate-acting and long-acting (Vázquez-Carrera and Silvestre, 2004; Bethel and Feinglos, 2005).

The short acting types have been designed to mimic bolus insulin secretion, while intermediate or long acting insulin analogues are designed to mimic basal glycaemic control (Vázquez-Carrera and Silvestre, 2004; Fonseca, 2006). Insulin is administered subcutaneously using multiple daily injections, or an external pump for continuous delivery (Hirsch, Bode, Garg, Lane, Sussman, Hu, Santiago and Kolaczynski, Insulin Aspart CSII/MDI Comparison Study Group, 2005). Other delivery routes include oral, inhaled, nasal, rectal, ocular, intravaginal and transdermal (Grover, Vats and Rathi, 2000; Takei and Kasatani, 2004). The disadvantages of insulin use include short shelf life, severe hypoglycaemia with overdosage, pain with subcutaneous injection and weight gain. A combination of insulin with oral anti-diabetic drugs has been shown to reduce some of these disadvantages, for example weight gain (DeFronzo, 1999; Robertson, Drexler and Vernillo, 2003; Takei and Kasatani, 2004; Laube, 2002; Massi-Benedetti and Orsini-Federici, 2008).

1.5.1.1. Insulin-like medicinal plants

Olea europaea (Linnaeus) [Oleaceae] fruit has been shown to increase glycogen concentration by inhibiting glycogen phosphorylase activity (Chen, Liu, Zhang, Wu, Hua, Wu and Sun, 2006). Puerarin, an isoflavone derived from the roots of *Pueraria lobata* (Willdenow Ohwi) [Fabaceae] has been shown to increase translocation of GLUT-4 transporters in the absence of insulin in isolated soleus muscles (Hsu, Liu, Kuo, Chen, Su and Cheng, 2003). Extracts of *Bauhinia* candicans (Benthamantha) [Caesalpiniaceae] and procyanidins from *Vitis vinifera* (Linnaeus) [Vitaceae] seeds have been shown to have insulin-mimetic effects on glucose uptake in insulin sensitive cell lines thereby increasing peripheral glucose utilization (Pinent, Blay, Bladé, Salvadó, Arola and Ardé, 2004; Fuentes and Alarcón, 2006). Maiti, Das and Ghosh, (2005) showed that the aqueous extract of the seed of *Tamarindus indica* (Linnaeus) [Fabaceae] reduced blood glucose levels by increasing hepatic glycogen concentrations.

1.5.2. Insulin sensitizers

1.5.2.1. Biguanides

Phenformin (phenethylbiguanide) and metformin (1,1 dimethylbiguanide hydrochloride) are examples of biguanides. Metformin was derived from *Galega officinalis* (Linnaeus) [Fabaceae] (French lilac), a plant rich in biguanides (Grover, Yadav and Vats, 2002; Krentz and Bailey, 2005; Modak, Dixit, Londhe, Ghaskadbi and Devasagayam, 2007). Metformin can be used alone or in combination with other drugs like sulphonylureas in diabetes management (Bailey, Path and Turner, 1996). Despite the mechanisms of the hypoglycaemic effects of biguanides being unclear, the end result is that they increase insulin sensitivity in type 2 diabetes (Bailey, Path and Turner, 1996) perhaps by enhancing insulin effects. It is known that the lowering of blood glucose by metformin not only involves suppression of gluconeogenesis and glycogenolysis, but also enhancement of insulin-stimulated glucose uptake in skeletal muscles (Rendell, 2004; Krentz and Bailey, 2005; Ajjan and Grant, 2006). Studies indicate that metformin activates 5'AMP-activated protein kinase (AMPK), a

heterotrimeric enzyme composed of a catalytic subunit (α) and two regulatory subunits (β and γ) (Ubl, Chen and Stucki, 1994; Krentz and Bailey, 2005). There are two isoforms of the catalytic subunit: AMPK α1, which is widely distributed, and AMPK α2, which is expressed in skeletal muscle, heart, and liver (Fryer, Parbu-Patel and Carling, 2002; Musi, Hirshman, Nygren, Svanfeldt, Bavenholm, Rooyackers, Zhou, Williamson, Ljunqvist, Efendic, Moller, Thorell, and Goodyear, 2002). Previous studies have shown that AMPK is activated following depletion of cellular ATP resulting in phosphorylation of AMPK to prevent breakdown of carbohydrates (Fryer, Parbu-Patel and Carling, 2002). The increase in AMPK activity results in the stimulation of glucose uptake in cells and the inhibition of hepatic glucose production, cholesterol and triglyceride synthesis, and lipogenesis (Musi *et al.*, 2002; Krentz and Bailey, 2005). Metformin also lowers blood glucose concentrations by decreasing intestinal absorption of glucose (Kirpichnikov, McFarlane and Sowers, 2002). Novel antihyperglycaemic mechanisms of metformin have been reported to involve enhancement of beta β-endorphin secretion from adrenal glands and stimulating opioid μ-receptors located in peripheral tissues. The stimulation of the opioid μ-receptors increases GLUT-4 transporters (Cheng, Huang, Liu, Tzeng and Chang, 2006).

The efficacy of glycaemic control achieved with metformin is similar to that achieved with sulphonylureas, although their modes of action differ. Metformin has beneficial effects on several cardiovascular risk factors (Krentz and Bailey, 2005). Furthermore, beneficial effects of metformin in type 2 diabetes, include weight reduction, improved lipid profiles, and enhanced endothelial function (Cheng, Huang, Liu, Tzeng and Chang, 2006; Skaer, Sclar, Robinson, 2006). The use of metformin, however, is contraindicated in conditions such as hypoxia, reduced perfusion of the heart in respiratory insufficiency and impaired renal function (Krentz and Bailey, 2005).

1.5.2.2. Plant insulin sensitizers

Some bioactive compounds have been shown to improve insulin sensitivity by primarily facilitating GLUT-4 translocation. Examples are flavonoids from *Cephalotaxus sinensis* [(Rehder & E.H. Wilson) H. L. Li] [Cephalotaxaceae] (Li, Dai, Yu, Li, Wu, Luan, Meng, Zhang and Deng, 2007). *Punica granatum* (Linnaeus) [Lythraceae] has been shown to enhance insulin secretion by increasing the number of pancreatic β-cells (Katz, Newman and Lansky, 2007). Jung, Ha, Shim, Choi, Yun-Choi and Lee, (2007) showed that *Campsis grandiflora* (K. Schumann) [Bignoniaceae] enhanced the effects of insulin on the signalling of the insulin receptor (IR). This was associated with an increase in insulin-mediated tyrosine autophosphorylation of the IR-β subunit as well as GLUT-4 translocation.

1.5.2.3. Thiazolidinediones

Thiazolidinediones (TZDs), troglitazone, pioglitazone and rosiglitazone are selective and potent agonists of peroxisome proliferator-activated receptor (PPAR)-γ (Chiarelli and Di Marzio, 2008). PPAR-γ and its isoforms PPAR-α and PPAR-δ, are members of the nuclear hormone receptors (Beckman, Creager and Libby, 2002; Kudzma, 2002; Chiarelli and Di Marzio, 2008). PPAR-γ receptors are found on insulin-sensitive tissues where they act as lipid sensors and regulate carbohydrate and lipid metabolism. Upon activation of PPAR-γ there is an improvement in insulin sensitivity thereby increasing glucose uptake in the skeletal muscles and reducing hepatic glucose output (Beckman, Creager and Libby, 2002; Kudzma, 2002; Chiarelli and Di Marzio, 2008). This effect has been attributed to be the cause of TZDs' ability to protect pancreatic β-cell function (Kudzma, 2002; Ajjan and Grant, 2006; Chiarelli and Di Marzio, 2008). TZDs have also been shown to modulate most risk factors for cardiovascular diseases (Kudzma, 2002; Krentz and Bailey, 2005). They modify lipid profiles, lowering blood pressure and prevent inflammation and atherosclerosis in vascular tissues (Kudzma, 2002).

1.5.2.4. Plant PPAR agonists

A study by Han, Choi, Lee, Song, Joe, Jung, and Hwang, (2008), showed that macelignan derived from *Myristica fragrans* (Gronov.) [Myrsticaceae] exerted anti-diabetic effects through dual activation of peroxisome proliferator activated receptors-α/γ in obese diabetic mice. *In vivo* and *in vitro* studies have shown that dietary soy isoflavones improve lipid metabolism and reduce blood glucose levels through activation of the PPAR receptors (Mezei, Banz, Steger, Peluso, Winters and Shay, 2003).

1.5.3. Insulin secretagogues

1.5.3.1. Sulphonylureas

The drugs in the sulphonylurea group include glibenclamide, chlopropamide, tolbutamide and gliclazide. Sulphonylureas (SUs) augment glucose-induced insulin release from the β-cells of the pancreas (Dhindsa, Davis and Donnelly, 2002; Krentz and Bailey, 2005). They block the opening of potassium channels (K_{ATP}) by binding to the pancreatic β-cell sulphonylurea receptors (SUR)1 and depolarise the membrane leading to calcium influx through opened voltage gated calcium channels (Rendell, 2004; Krentz and Bailey, 2005). Increased intracellular calcium levels mobilise calcium dependant insulin vesicles to fuse with the membrane and release insulin (Krentz and Bailey, 2005; Ajjan and Grant, 2006). SUR1 is the regulatory subunit on β-cell K_{ATP} channels whereas variants of SUR2 are on K_{ATP} channels of cardiac (SUR2A) and vascular smooth muscles (SUR2B) (Dhindsa, Davis and Donnelly, 2002). Tolbutamide and gliclazide block the SUR1 subunit, whereas glibenclamide and glimepiride have affinity for both SUR1 and SUR2 isoforms (Dhindsa, Davis and Donnelly, 2002). The binding of SUs to K_{ATP} channels of coronary vessels causes dilatation to enable the heart to adapt in ischaemic conditions (Ajjan and Grant, 2006). Sulphonylureas can cause hypoglycaemia for the release of insulin is initiated even when glucose concentrations are below the normal threshold for the release of the peptide (Krentz and Bailey, 2005).

1.5.3.2. Meglitinides

Meglitinides which include repaglinide and nateglinide enhance the initial surge of insulin release in response to meals by binding to specific SUR1 sites on pancreatic β-cell membranes (Gerich, 2003; Krentz and Bailey, 2005). Activity of meglitinides depends on the concentration of the blood glucose and the dose of the drug used (Gerich, 2003). The drugs are taken prior to meals to prevent postprandial hyperglycaemia because they have a short half life (Krentz and Bailey, 2005).

1.5.3.3. Plant insulin secretagogues

The aqueous extract of *Scoparia dulcis* (Linnaeus) [Scrophulariaceae] (Sweet broomweed) has been shown to increase insulin secretion in isolated pancreatic islet β-cells (Latha, Pari, Sitasawad and Bhonde, 2004). Similar effects were reported with various medicinal plants like *Viscum album* (Linnaeus) [Lorantaceae], *Sambucus nigra* (Linnaeus) [Adoxaceae] (elder) and *Smallanthus sonchifolius* [(Poepigg and Andlicher) H. Robinson] [Asteraceae] (yacon) (Gray and Flatt, 1999; Gray, Abdel-Wahab and Flatt, 2000; Aybar, Riera, Grau and Sánchez, 2001). Some bioactive compounds isolated from anti-diabetic plant extracts have also been shown to stimulate insulin secretion *in vitro* (Jayaprakasam, Vareed, Olson and Nair, 2005). An example is phanoside, a gypenoside isolated from *Gynostemma pentaphyllum* (Thunb. Makino) [Cucurbitaceae] which acts distal to the K_{ATP} and L-type calcium channels on the exocytotic machinery (Hoa, Norberg, Sillard, Van Phan, Thuan, Dzung, Jörnvall and Ostenson, 2004; 2007). *Asparagus racemosus* (Willdenow) [Asparagaceae] root extracts have been shown to increase insulin secretion in isolated β-cells and perfused pancreas via the cyclic adenosine monophosphate (cAMP) pathway (Hannan, Marenah, Ali, Rokeya, Flatt and Abdel-Wahab, 2007).

1. 5.4. α-Glucosidase inhibitors

α-Glucosidase inhibitor drugs such as acarbose, miglitol and voglibose inhibit intestinal glucose absorption (Krentz and Bailey, 2005; Ajjan and Grant, 2006; Modak, Dixit, Londhe,

Ghaskadbi and Devasagayam, 2007). α-Glucosidase a membrane-bound enzyme on the epithelium of the small intestine hydrolyses disaccharides and oligosaccharides (Ali, Jahangir, Hussan and Choudhary, 2002). Inhibition of α-glucosidase prevents a postprandial increase in blood glucose concentration due to delayed carbohydrate absorption (Ajjan and Grant, 2006). Acarbose has also been shown to increase the secretion of glucagon-like peptide (GLP)-1, although the relative contribution of this effect to the reduction in postprandial hyperglycaemia is unknown (Gerich, 2003).

1.5.4.1. α-Glucosidase inhibiting plants

Various medicinal plant extracts have been shown to lower blood glucose concentrations through inhibition of the α-glucosidase enzyme. The extracts include those from *Pinus densiflora* (Siebold and Zuccarini) [Pinaceae], *Syzygium cumini* (Linnaeus Skeels) [Myrtaceae], *Actinidia deliciosa* (C.F. Liang & A.R. Ferguson) [Actinidiaceae], *Malmea depressa* (Baillon) R.E. Fries [Annonaceae] and *Punica granatum* (Linnaeus) [Lythraceae]. Most of the plants that inhibit α-glucosidase have also been shown to inhibit α-amylase (Kim, Wang and Rhee, 2004; Li, Wen, Kota, Peng, Li, Yamahara and Roufogalis, 2005; Andrade-Cetto, Becerra-Jiménez and Cárdenas-Vázquez, 2008; Shinde, Taldone, Barletta, Kunaparaju, Hu, Kumar, Placido and Zito, 2008; Shirosaki, Koyama and Yazawa, 2008; Subramanian, Asmawi and Sadikun, 2008). The discussion in the preceding sections highlights blood glucose lowering effects of some synthetic anti-diabetic agents and plant derived extracts/agents. On this basis this study investigated the potential of a bioactive plant extract in diabetes mellitus management.

1.6. Traditional (indigenous/folk) medicine

Traditional medicine predominant in developing countries is established knowledge systems incorporating plant, animal and mineral-based medicines (Gilani and Rahman, 2005; Stangeland, Dhillion and Reksten, 2008). Examples of such systems include traditional African medicine, Chinese medicine, Indian ayurveda and Arabic unani medicine. In the sub-Saharan African region the majority of the population rely on traditional medicine (WHO

traditional medicine (WHO Report, 2002-2005). This is attributed to limited resources and poor access to modern health care systems, in contrast to traditional medicine which is easily available and affordable (WHO Expert Committee on Diabetes, 1980; WHO Report, 2002-2005). Plants have always been a source of drugs and many of the currently available drugs have been derived directly or indirectly from plant extracts (Gurib-Fakim, 2006). Drugs like aspirin, digoxin, ephedrine, reserpine and tubocurarine, in use today, were derived from plants (Stangeland, Dhillion and Reksten, 2008). The use of medicinal plants is also based on the belief that they have fewer side effects as compared to allopathic drugs (Marles and Farnsworth, 1994; Kim, Kang, Park, Jung, Choi and Ku, 2007b). Various disorders managed with medicinal plant extracts include diabetes mellitus, ulcers, cancers, cardiac diseases, renal diseases and some infectious diseases (Liu, 1995; Jouad, Haloui, Rhiouani, Hilaly and Eddouks, 2001).

1.6.1. Plant based management of diabetes mellitus

Diabetes mellitus is expected to increase the burden on health systems in the future, not only because of the absence of a known cure, but also due to its high prevalence and associated complications (Sobngwi, Mauvais-Jarvis, Vexiau, Mbanya and Gautier, 2001; Vinik and Vinik, 2003). Current conventional diabetes therapy using blood glucose-lowering medications such as insulin or oral hypoglycaemic agents has limitations. Alternative methods of lowering blood glucose are, therefore, needed. Herbal medicines have been used for many years by different cultures around the world, both for the prevention and management of diabetes mellitus (Jung *et al.*, 2006; Kim *et al.*, 2007b). Scientific investigations have established the potential of plant extracts in the management of diabetes mellitus and alleviation of associated complications (Marles and Farnsworth, 1994; Wang and Ng, 1999; Sharma, Nasir, Prabhu, Murthy and Dev, 2003; Li *et al.*, 2007). Indeed, extracts of *Trichosanthis radix* (Maximowicz) [Curcubitaceae], *Garcinia kola* (Heckel) [Clusiaceae], *Opunthia megacantha* (Miller) [Cactaceae], *Allium sativum* (Linnaeus) [Alliaceae], *Punica granatum* (Linnaeus) [Lythraceae] *and Syzygium aromaticum* [(Linnaeus) Merrill & Perry] [Myrtaceae] possess anti-diabetic properties (Bwititi and Musabayane, 1997; Bwititi, Musabayane and Nhachi, 2000; Adaramoye and Adeyemi, 2006;

Wei-ha *et al.*, 2008). The multiple beneficial activities like manipulating carbohydrate metabolism, and preventing and restoring integrity and function of β-cells suggest that medicinal plant extracts offer a useful source of hypoglycaemic drugs (Tiwari and Rao, 2002). Metformin was derived from the traditional approach of using the guanidine-rich *Galega officinalis* (Linnaeus) [Fabaceae] (French lilac) to treat diabetes mellitus (Parturier, 1957; Grover, Yadav and Vats, 2002; Krentz and Bailey, 2005; Modak, Dixit, Londhe, Ghaskadbi and Devasagayam, 2007). Bioactive compounds with hypoglycaemic effects isolated from plants include polysaccharides, flavonoids, xanthones, peptides and triterpenoids (Wang and Ng, 1999; Grover, Yadav and Vats, 2007).

Most of the work in our laboratory thus far has investigated the blood glucose lowering effects of crude plant extracts. The World Health Organisation recommends scientific evaluation of anti-diabetic plants and their bioactive compounds. Despite this, the establishment of the mechanisms through which medicinal plants exert their hypoglycaemic effects and proper scientific screening has been done for only a few ethnomedicinal plants. Scientific evaluation of ethnomedicinal plants may pave way for the discovery of natural products for developing new anti-diabetic drugs (Pushparaj, Tan and Tan, 2001; Sharma, Nasir, Prabhu, Murthy and Dev, 2003; Jung *et al.*, 2006). The bioactive compound of interest in this study was from *Syzygium cordatum* (Hochst.) [Myrtaceae]. Preliminary studies indicated that the crude extracts lowered blood glucose concentrations (Musabayane, Mahlalela, Shode and Ojewole, 2005). Compounds isolated from *S. cordatum* plant parts include pentacyclic triterpenoids (Candy, McGarry and Pegel, 1968; Bruneton, 1995)

1.6.2. *Syzygium cordatum* (Hochst.) [Myrtaceae]

S. cordatum an evergreen tree, grows to a height of 8-15meters along stream banks, on forest margins or in swampy spots from KwaZulu-Natal northwards to Zimbabwe and Mozambique (Van Wky, Van Oudtdhoorn and Gericke, 1997). The leaves are elliptic to circular, bluish green on top and a paler green below. The fruits (Figure 1) are oval berries, red to dark-purple when ripe with a slightly acidic taste. *S. cordatum* tree is known for its many uses. The fleshy fruit is used sometimes to make an alcoholic drink. The powdered bark is used as

a fish poison. In central Africa, the tree is used to treat stomach aches, diarrhoea, respiratory ailments and tuberculosis (Watt and Breyer-Brandwijk, 1962; Iwu, 1993). The crude leaf extract of *S. cordatum* has been shown to possess hypoglycaemic properties and increase muscle and hepatic glycogen concentrations (Musabayane, Mahlalela, Shode and Ojewole, 2005). The antihyperglycaemic effect of the leaf extract was attributed to its triterpenoid constituents which are discussed in the next section.

1.6.3. Triterpenoids

The triterpenoids are molecular structures with four or five rings composed of 30 carbon atoms formed by cyclization of squalene (Stangeland, Dhillion and Reksten, 2008) (see Figure 5 on page 49). Triterpenoids include oleanolic acid, maslinic acid, gallic acid, corosolic acid and ursolic acid (Liu, 1995; Shishodia, Majumdar, Banerjee and Aggarwal, 2003; Dzubak, Hajduch, Vydra, Hustova, Kvasnica, Biedermann, Markova, Urban and Sarek, 2006). Triterpenoids exist in food and medicinal herbs as free acids (aglycone or genin) or attached to carbohydrate residues, saponins or glycosides (Liu, 1995; Choi, You and Jeong, 2001). The triterpenoids can be classified in relation to the triterpene class (oleanane group), herbs that contain them (platycodon) and by therapeutic action (antitumor) (Connolly and Hill, 2002). The occurrences of the acids vary within the same plant and in different plants (Dzubak *et al.*, 2006).

The triterpenoids have a range of unique and potentially usable biological effects like anti-inflammatory, antitumor, anti-diabetic, antihypertensive and antimicrobial activity with varying potencies (Ghosh, Thejomoorthy and Veluchamy, 1981; Liu, 1995; Somova, Nadar, Ramnanan and Shode, 2003; Dzubak *et al.*, 2006; Lee, Nam, Kim and Lee, 2006; Jung *et al.*, 2007). The variations in the potencies are attributed to the different structures, such as the number of the hydroxyl groups. Thus, the pharmacokinetic and pharmacodynamic properties of the acids and ultimately the bioavailability and the biological effects are influenced by the structure of the acids (Liu, 2005; Jung *et al.*, 2007). Triterpenoids have been identified as the major components of many traditional medicinal plants and, therefore, a lot of interest has grown in elucidation of their biological roles (Choi, You and Jeong, 2001).

A

B

Figure 1 *S. cordatum* tree (A) and the fruit and leaves (B)

1.7. Basis of the present study

The previously reported antihyperglycaemic effect of crude *S. cordatum* leaf extract has been attributed to its triterpenoid constituents. Oleanolic acid (OA) is the most abundant triterpene from most medicinal plants. This study was, therefore, designed to isolate and investigate the acute and short-term term effects of oleanolic acid derived from *S. cordatum* on blood glucose, renal function and mean arterial blood pressure in streptozotocin (STZ)-induced diabetic rats.

1.7.1. Hypothesis

S. cordatum derived oleanolic acid possesses anti-hyperglycaemic and reno-protective effects in STZ-induced diabetic rats.

1.7.2. Aims

The overall aim was to study the anti-hyperglycaemic and renal effects of *S. cordatum* derived oleanolic acid in STZ-diabetic rats.

The specific research objectives were to:

a) isolate the oleanolic acid from *S. cordatum* leaves.
b) investigate the acute (1½ h) and short-term (5-weeks) effects of the oleanolic acid on blood glucose concentration in STZ-induced diabetic rats and compare them to those of allopathic anti-diabetic drugs.
c) investigate the combined effects of the oleanolic acid with allopathic anti-diabetic drugs on blood glucose concentration in STZ-induced diabetic rats.
d) investigate the acute (1½ h) effects of the oleanolic acid on renal fluid and electrolyte handling and blood pressure in STZ-induced diabetic rats.

CHAPTER 2

2.0. MATERIALS AND METHODS

2.1. Ethical consideration

Ethical clearance was obtained from the University of KwaZulu-Natal's Ethics committee (reference 037/07 Animal and 048/08 Animal, see Appendices I and II).

2.2. Materials

2.2.1. Drugs and chemicals

Drugs and chemicals were sourced as indicated: glibenclamide (glyburide; *N-p*-[2-(5-chloro-2-methoxybenzamido] ethylbenzene-sulfonyl-*N'*-cyclohexylurea), metformin (1,1-dimethylbiguanide hydrochloride), streptozotocin, dimethyl sulphoxide (DMSO), inactin (5-ethyl-5-(1'-methylpropyl)-2-thiobarbiturate) (Sigma St Louis, MO, USA), D-glucose, citric acid, sodium citrate, sodium chloride, potassium hydroxide, hydrochloric acid, sulphuric acid, silica gel, ethyl acetate, dichloromethane, ethanol, methanol and chloroform (Merck, Waderville, RSA), porcine insulin (Intervet SA (PTY) Ltd, Isando, South Africa), heparin Novo (Novo Nordisk (Pty) Ltd, Johannesburg, RSA), halothane (Fluothane®, AstraZeneca Pharmaceuticals, Johannesburg, RSA). All chemical reagents were of analytical grade.

2.3. Plant material

Syzygium cordatum (Hochst.) [Myrtaceae] was identified and authenticated by Professor H. Baijnath, the former Chief Taxonomist/Curator of the University of KwaZulu-Natal's Department of Botany. A voucher specimen (collector number MH/05) of the plant has been deposited in the University's Botany Discipline Herbarium. Fresh leaves of *S. cordatum* were collected in and around Durban, South Africa, between February and

June, 2006 to eliminate seasonal variations of chemical constituents. The leaves were air dried at room temperature (±24°C) for approximately four weeks.

2.4. Bioassay-directed isolation of OA

Air-dried leaves of *S. cordatum* (1.65 kg) were milled using a Waring commercial blender. The powder was extracted thrice at room temperature for 24 h intervals sequentially in hexane, dichlomethane, ethyl acetate and methanol on each occasion with 3 litres, to give residues of hexane-solubles (HES), dichloromethane-solubles (DCMS), ethyl acetate-solubles (EAS), and methanol-solubles (MES), respectively. Filtering was done using 30 cm filter papers (Whatman, England). The filtrates were concentrated *in vacuo* at 60±1°C using a laboratory 4000 efficient rotary evaporator (supplied by Laboratory Consumables and Chemical Supplies, South Africa).

2.4.1. Thin layer chromatography

The crude extracts were analyzed by thin layer chromatography (TLC) on pre-coated aluminium plates using Merck Si gel F254 to reveal the type of chemical constituents they contained. This was done by spotting a diluted portion of the crude extract on a TLC plate with authentic oleanolic acid. The TLC plate was developed with ethyl acetate/hexane (8:2) in a TLC tank. The developed TLC plate was visualised initially by exposure to ultraviolet light at 254 to 366 nm wavelength and then sprayed with anisaldehyde/ sulphuric acid/ alcohol solution and heated at 110°C. Appearance of a blue or violet-blue colouration indicated the presence of triterpenoids (Hostettmann and Marston, 1995).

2.4.2. Isolation of OA

Since preliminary studies indicated that the EAS of *S. cordatum* contained triterpenoids, the solubles were, therefore, subjected to further purification processes. A portion of EAS (2 g) was fractionated on silica gel (70-230 mesh, 3.5 x 45 cm) by open column

chromatography using a step gradient of n-hexane-ethyl acetate 90:10 (250 ml), 80:20 (250 ml), and 70:30 (800 ml). Data from collected fractions analyzed by TLC were compared with authentic OA values. Fractions were pooled according to similar TLC profiles and concentrated *in vacuo* using a rotary evaporator at 55°C. The concentrates were reconstituted using minimal amounts of chloroform and allowed to air dry in pre-weighed vials. Measured eluates were collected and combined into fractions on the basis of their thin layer chromatography (TLC) similarities. The eluates with similar TLC profiles to oleanolic acid were combined and subjected to further chromatographic purification.

2.4.3. OA structure elucidation

Pure OA was obtained by recrystallization with ethanol and its structure (Figure 4) confirmed by spectroscopic analysis using 1D and 2D, ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) techniques.

2.5. Animals

Male Sprague Dawley rats (250-300 g) bred and housed in the Biomedical Resource Unit of the University of KwaZulu-Natal, Westville campus were used in the study. The animals were maintained under standard laboratory conditions of constant temperature (22±2 °C), CO_2 content of <5000 p.p.m, relative humidity of 55±5%, and illumination (12 h light/dark cycles) and the noise levels of <65 decibels. The animals had free access to standard rat chow (Meadows, Pietermaritzburg, South Africa) and water.

2.5.1. Induction of diabetes mellitus

Diabetes mellitus was induced in male Sprague Dawley rats with a single intraperitoneal injection of streptozotocin (STZ), at a dosage of 60 mg/kg dissolved in freshly prepared 0.1M citrate buffer (pH 6.3). Control animals were injected with the vehicle. Animals that exhibited glucosuria after 24 h, tested by urine strips (Rapidmed Diagnostics,

Sandton, South Africa) were considered diabetic. Blood glucose concentration of 20 mmol/l or above measured after one week was considered as a stable diabetic state before experimental procedures.

2.6. Experimental design

Oral glucose tolerance test (OGTT) responses were carried out in conscious non-diabetic and STZ-induced diabetic rats. The effects of short-(acute) administration of OA on renal fluid and electrolyte handling were evaluated in anesthetized non-diabetic and STZ-induced diabetic rats while the long-term (subchronic) effects of OA on renal parameters and blood glucose were assessed in conscious animals.

2.7. Acute studies

2.7.1. OGTT protocol

OGTT responses were carried out as previously described (Musabayane, Gondwe, Kamadyaapa, Chuturgoon and Ojewole, 2007) with slight modifications. The rats were divided into the following groups: non-diabetic control, non-diabetic treated, STZ-induced diabetic rats control and STZ-induced diabetic treated rats (n=6 in each group). An 18 h fast was allowed for all animals, followed by measuring blood glucose (time 0) and then loading glucose (0.86 g/kg, body weight, p.o.). OGTT responses were also monitored in separate groups of non-diabetic and STZ-induced diabetic rats OA was administered at various doses (40, 80 and 120 mg/kg, p.o.). OA was freshly dissolved in dimethyl sulphoxide (DMSO, 2 ml) and normal saline (19 ml) before use in each case (Musabayane, Mahlalela, Shode and Ojewole, 2005). Rats treated with deionized water (3 ml/kg, p. o.) served as control animals.

Separate groups of non-diabetic and STZ-induced diabetic rats administered insulin (200 µg/kg, s.c.) (Intervet, SA Pty Ltd, Isando, South Africa), metformin (1,1-dimethylbiguanide hydrochloride) (500 mg/kg, p.o.) (Sigma Aldrich, St. Louis, Missouri,

USA, or glibenclamide (glyburide; *N*-p-[2-(5-chloro-2-methoxybenzamido) ethylbenzene-sulfonyl-*N'*-cyclohexylurea] (500 µg/kg, p.o) (Sigma Aldrich, St. Louis, Missouri, USA), acted as positive control animals. This was to establish whether OA possesses hypoglycaemic effects comparable to standard anti-diabetic drugs.

The influence of standard drugs on OA-induced OGTT responses was studied in separate groups of animals that were similarly treated with OA at either 40 or 80 mg/kg followed by insulin (100 or 200 µg/kg, s.c.), metformin, (250 or 500 mg/kg, p.o.) and glibenclamide (250 or 500 mg/kg, p.o.). Blood samples were collected from the tail veins of the animals 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).

2.7.1.1. Effects of acute OA treatment on insulin secretion

Plasma insulin concentrations were determined in blood samples of separate groups of non-diabetic and STZ-induced diabetic rats (n=6 in each group) following separate administration of OA (80 mg/kg, p.o.), metformin (500 mg/kg, p.o.), insulin (200 µg/kg, s.c.) or OA combined with the standard anti-diabetic drugs for plasma insulin measurement. This allowed comparison of incremental changes in plasma insulin concentration following OA administration with levels observed in control rats and animals treated with insulin. In preliminary experiments, the plasma lowering effect was found to reach plateau within 30 min and maintained for 60 min in fasted rats after OA or insulin administration. Thus, plasma insulin concentration was determined using blood samples collected after 60 min.

The rats were anaesthetized in an anaesthetic chamber with halothane (Fluorothane®, Astra Zeneca pharmaceuticals (Pty) LTD) and blood was collected by cardiac puncture into pre-cooled heparinized tubes for insulin determination. The blood was centrifuged (Eppendorf centrifuge 5403, Germany) at 4 °C, 3000rpm for 15 minutes and separated plasma was stored at -70 °C in a Bio Ultra freezer (Snijers Scientific, Holland) until

insulin assay.

2.7.2. Acute effects of OA on renal function

Acute effects of OA on renal function and electrolyte handling were assessed in separate groups of anaesthetized non-diabetic and STZ-induced diabetic rats (n=6 in each group) using a modified procedure that has been previously described (Musabayane, Gondwe, Kamadyaapa, Chuturgoon and Ojewole, 2007) (see Figure 2 page 38). The animals were anaesthetized by an intraperitoneal injection of inactin (5-ethyl-5-(1'-methylpropyl)-2-thiobarbiturate, Sigma–Aldrich, St Louis, MO, USA) at 0.11 g/kg body weight. Tracheostomy was performed to maintain clear airway. The right jugular vein was cannulated with polyethylene tubing (internal diameter, (i.d.) 0.86 mm; external diameter, o.d. 1.27 mm; Portex, Hythe, Kent, UK) to allow intravenous infusion of 0.077M NaCl at 9 ml/h using Harvard Apparatus Syringe Infusion Pump 22 (Harvard Apparatus, Holliston, Massachusetts, USA). The urinary bladder of each rat was exposed after an abdominal incision and cannulated with similar calibre polythene tubing to facilitate timed collection of urine samples. The body temperature of each animal was maintained at 37±1 °C with a heated table. Non-diabetic and STZ-induced diabetic control groups were placed on a continuous infusion of 0.077M NaCl at 9 ml/h. Following an initial equilibration period of 3½ h, eight consecutive urine collections were made into pre-weighed plastic vials at 30 min intervals over the subsequent 4 h for measurements of urine flow, and Na^+, Cl^- and K^+ excretion rates. The control group was designed to check the stability of renal function.

In those animals in which the effects of OA were studied, OA was added to the infusate at 90 µg/ h for 1½ h (treatment period), resulting in a total dose of 0.45 mg/ kg (for a 300 g rat), before the animals were returned to infusate alone for the last 1½ h (recovery period). Depth of anaesthesia was monitored throughout the experiments, and additional i.v. bolus doses of inactin (0.05 g/kg body weight) were administered when necessary.

2.7.2.1. Acute effects of OA on blood pressure

The acute effects of OA on mean arterial blood pressure (MAP) were assessed in parallel groups of rats (n=6 in each group) prepared as for renal studies, except that a heparinized cannula (Portex i.d. 0.86 mm; o.d. 1.27 mm, Hythe, Kent, UK) was inserted into the left common carotid artery and attached to a blood pressure transducer (Stratham MLT 0380/D, AD Instruments Inc., Bella Vista, NSW, Australia) compatible with the PowerLab System ML410/W, (AD Instruments Inc., Bella Vista, NSW, Australia) (see Figure 2). This permitted recording of mean arterial blood pressure at 30 minute intervals following equilibration for the subsequent 4 h experimental period.

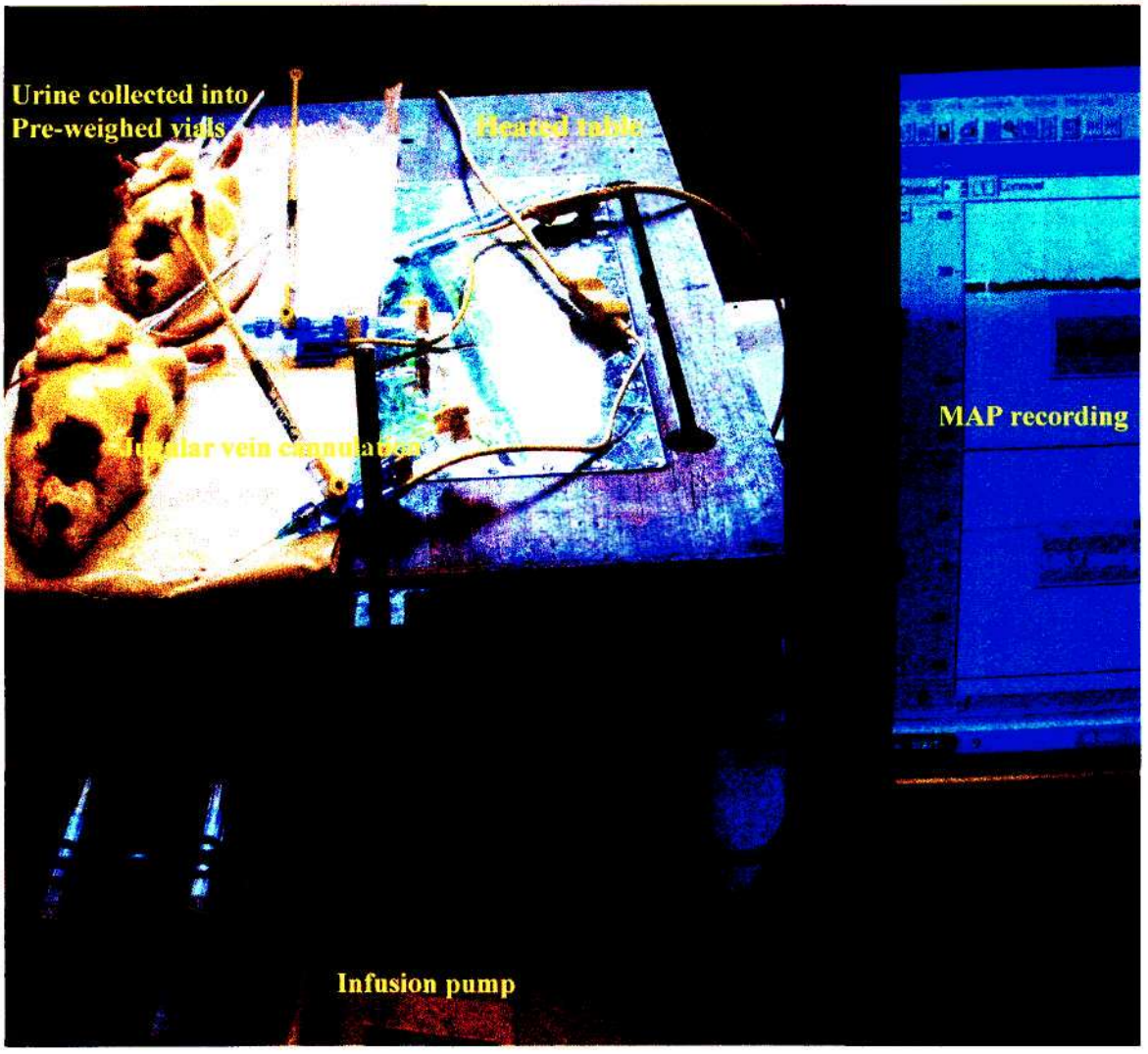


Figure 2: Layout of the Harvard Apparatus infusion pump and Power lab transducer systems used for investigations of acute effects of OA on renal function and mean arterial blood pressure.

2.8. Short-term studies

2.8.1. Physico-metabolic changes

Groups of treated and control non-diabetic and STZ-induced diabetic male Sprague-Dawley rats (250-300g body weight) were housed individually in Makrolon polycarbonate metabolic cages (Techniplats, Labotec, South Africa) for five weeks at the Biomedical Resource Unit, University of KwaZulu Natal, (n=6 in each group). The animals were maintained on a 12 h dark/light cycle with free access to water and standard rat chow (Meadows, Pietermaritzburg, South Africa). Effects of OA were investigated in rats orally administered OA (80 mg/kg) twice every third day at 09h00 and 15h00 by means of a bulbed steel tube for 5 weeks. Rats similarly treated with deionized water (3 ml/kg, p.o.) and standard anti-diabetic drugs (metformin, 500 mg/kg, p.o., insulin, 200 µg/kg, s.c) acted as untreated and treated positive controls, respectively. The hypoglycaemic effects of the administration of OA (80 mg/kg, p.o.) followed by insulin (200 µg/ kg, s.c.) were also monitored in separate groups of animals.

24 h urine volume and urinary outputs of glucose, Na^+, K^+ and Cl^- were determined for all groups every third day. The amounts of food and water consumed were recorded daily at 09h00. The weights of the animals were assessed every third day at 09h00.

2.8.2. Short-term effects of OA on MAP

Mean arterial blood pressure (MAP) was monitored once every week at 09h00 in conscious Sprague Dawley rats for five weeks using the non invasive tail cuff method (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, CA) as previously described (Musabayane, Gondwe, Kamadyaapa, Chuturgoon and Ojewole, 2007). The unit utilizes IITC hardware system employing an automatic scanner pump, sensing cuff and amplifier to measure blood pressure in the animals' tail and the results are displayed on the computer screen (see Figure 3). The equipment was calibrated before each day of mean arterial blood pressure measurements. The animals

were warmed in an enclosed chamber (IITC Model 303sc Animal Test Chamber, IITC Life Sciences, Woodland Hills, California, USA) for 30 minutes at ±30°C before taking three blood pressure recordings of each animal (Gondwe, Kamadyaapa, Tufts, Chuturgoon, Ojewole and Musabayane, 2008).

Figure 3: Layout of the IITC Model 303sc Animal Test Chamber apparatus used for investigations of short-term measurement of mean arterial blood pressure in non-diabetic and STZ-induced diabetic rats.

2.8.3. Terminal studies

Blood samples were collected by cardiac puncture 24 h after the last treatment from all non fasted groups of animals for glucose, electrolytes, urea, creatinine, osmolality and insulin assay at the end of the 5-week treatment period. The animals were initially anaesthetized by placing them in an anaesthetic chamber with 100 mg/kg of halothane (Fluorothane®, Astra Zeneca 2002) pharmaceuticals (Pty) LTD) for 3 minutes. Blood was centrifuged in an Eppendorf centrifuge 5403, Germany for 15 minutes at 3500 rpm g x 100 at 4 °C to separate the plasma. Separated plasma samples for insulin measurements were stored in a Bio Ultra freezer (Snijers Scientific, Holland) at -70 °C until assayed. Kidneys were removed, weighed and snap frozen in liquid nitrogen. The liver and gastrocnemius muscles were removed and weighed gravimetrically (Mettler balance PC 180-insruments, Protea Laboratory Services, South Africa) and then for glycogen measurement placed immediately on ice (Ong and Khoo, 2000). The plasma, liver and muscle samples were stored in a BioUltra freezer (Snijers Scientific, Holland) at -70 °C until assayed.

2.9. Laboratory analyses

2.9.1. Biochemical measurements

Urine volume was determined gravimetrically using a balance (Mettler balance PC 180-instruments, Protea Laboratory Services, South Africa). Na^+, K^+, Cl^-, urea and creatinine concentrations were determined by ion activity using the Beckman Coulter (Synchron LX20 Clinical Systems, USA). Creatinine estimation employed the reaction of creatinine and sodium picrate to form creatinine picrate. Urea estimation employed the hydrolytic degradation of urea in the presence of urease. The methods used reagent kits from Beckman Coulter, Dublin, Ireland. Osmolalities of the plasma and urine were measured on 50 µl samples by freezing point depression using an osmometer (Osmomat 030, Lasec Laboratory and Scientific Company, Berlin, Germany). 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 5^{th} week.

2.9.2. Insulin assay

Plasma insulin was measured using ultra sensitive rat insulin ELISA kit (DRG diagnostics EIA-2746 GmbH, Marburg, Germany). The kit contained a 96 well plate coated with mouse monoclonal anti-insulin, standards, enzyme conjugate, enzyme conjugate buffer, wash buffer, substrate 3,3′,5,5′-tetramethylbenzidine (TMB) and a stop solution. The principle of the assay is a solid phase two-site enzyme immunoassay based on the direct sandwich technique in which two monoclonal antibodies are directed towards separate antigenic determinants on the insulin molecule. During the incubation period insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microtitration well. The washing step removes unbound enzyme labeled antibody, leaving the bound conjugate which reacts with TMB. This reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

Each determination was performed in duplicate for standards and samples. The insulin assay procedure was as follows: 50 µl of each insulin standard (0.02, 0.05, 0.15, 0.4 and 1 µg/l) were added to anti-insulin wells. 50 µl of the plasma samples were then added to the remaining wells followed by 50 µl enzyme conjugate to all wells of standards and plasma samples. The plates were incubated at room temperature for 2 hours on a plate shaker (Velp® Scintifica, Milano, Italy) followed by aspirating the reaction volume, adding wash buffer (350 µl) and aspirating 6 times. After the final wash the plates were inverted firmly against absorbent paper to absorb all the liquid in the plates. 200 µl substrate TMB was added to all the wells and incubated for 30 minutes. The reaction was stopped by adding 50 µl of stop solution to all wells and mixing on a shaker for 5 minutes. The absorbance was measured at 450 nm using a Biotek microplate reader (Winooski, Vermont, USA). A standard curve was constructed from the absorbance values of the known concentrations of the standards using the spline regression curve in

Graph Pad Instat software (version 4.00). The respective insulin concentrations of the unknown samples were then extrapolated from the standard curve. The lower and upper limits of detection were 1.39 pmol/l and 960 pmol/l, respectively. The intra-assay analytical coefficient of variation ranged from 4.4 to 5.5% and the inter-assay coefficient variation from 4.7 to 8.9%.

2.9.3. Glycogen measurements

Hepatic and muscle glycogen concentrations were determined as described by Ong and Khoo, (2000). Briefly, the tissue samples were homogenized in 2 ml of 30% potassium hydroxide (300 g/l) and boiled at 100 ^{o}C for 30 min, and then cooled in ice saturated sodium sulphate.

2 µl of each sample were added to 2 µl of 95% ethanol mixed with 1.2 ml of distilled water. Blanks were made by adding 2 µl of distilled water followed by ethanol mixed with 1.2 ml of distilled water. 4 ml of anthrone reagent (0.5 g of anthrone powder was dissolved in 250 ml of sulphuric acid (H_2SO_4) to make the reagent) was pipetted into samples and blanks. Glycogen powder from oyster, type II was used to prepare standards (10-2000 mg/l). The samples together with the blanks were boiled for 10 minutes after vortexing. The glycogen was precipitated with ethanol, pelleted and resolubilized in deionized water. The glycogen content determined by treatment with anthrone reagent was measured at 620 nm using the Cary dual spectrophotometer (Biochrom Ltd, Cambridge, England) using Cary Win UV Simple Reads Application, software version 3.00 (182).

3.0. Data presentation

All data were expressed as means ± standard error of means (SEM). Data for untreated non-diabetic and STZ-induced diabetic rats were used as baseline. The data were treated and presented separately for the non-diabetic and STZ-diabetic rats. OGTT responses to oleanolic acid alone and/or standard anti-diabetic drugs were presented separately for the

non-diabetic and STZ-induced diabetic rats. This was to establish whether OA possesses pharmacological activities comparable to standard antidiabetic drugs already in use or influenced standard drugs' evoked OGTT responses. Renal excretion data for acute studies were presented graphically showing 30-minute collections over the 4-h post-equilibration period. The total amounts of fluid voided and electrolytes excreted during the 1½ h administration of the OA were calculated and compared to values in controls for the corresponding time. For chronic studies, calculating mean daily fluid voided and urinary amounts of electrolytes excreted assessed renal function. GFR was evaluated by creatinine clearance as assessed by 24-hour urinary excretion rates of creatinine in relation to plasma concentration. Statistical analyses were done with GraphPad InStat Software (version 4.00, GraphPad Software, San Diego, California, USA). Statistical comparison between groups was done using one way one-way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparison test. Values of $p < 0.05$ were taken to imply statistical significance.

CHAPTER 3

3.0. RESULTS

This chapter describes the following:

i) phytochemical and spectroscopic data elucidating the structure of OA;

ii) OGTT responses and acute and sub-chronic renal effects of various treatments and.

iii) sub-chronic effects on body weight, food and water intake

3.1. Structural elucidation of OA

The *S. cordatum* crude leaf extract was sequentially extracted with ethyl acetate to give ethyl acetate-solubles (EAS). The percentage yield of OA from EAS varied from 0.79% to 1.72%. Spectroscopic analyses of the white powder obtained after recrystallization with ethanol carried out using ^{1}H- and ^{13}C-NMR (1D and 2D) spectroscopy are shown in Figure 4. Figure 4A is the 1D ^{1}H NMR spectra showing all the hydrogens in the molecule. Figure 4B shows the 1D ^{13}C NMR spectra of all carbons in the molecule with two carbon signals at 143.6 and 122.7 ppm corresponding to the carbon-carbon double bond at carbon 12 and 13. Figure 4C and 4D are the two dimensional ^{1}H and ^{13}C NMR spectra of OA. In Figure 4C the bottom line shows all the carbons attached to hydrogen atoms; the middle line shows carbons that are attached to one hydrogen atom (CH groups) and in the uppermost line, signals pointing downwards are CH_2 and upwards are CH and CH_3. Table 1 compares the relative resonation frequencies of all the carbon atoms in the *S. cordatum*-derived OA with literature data (Mahato and Kundu 1994). Figure 5 shows OA structure as elucidated by ^{1}H- and ^{13}C-NMR.

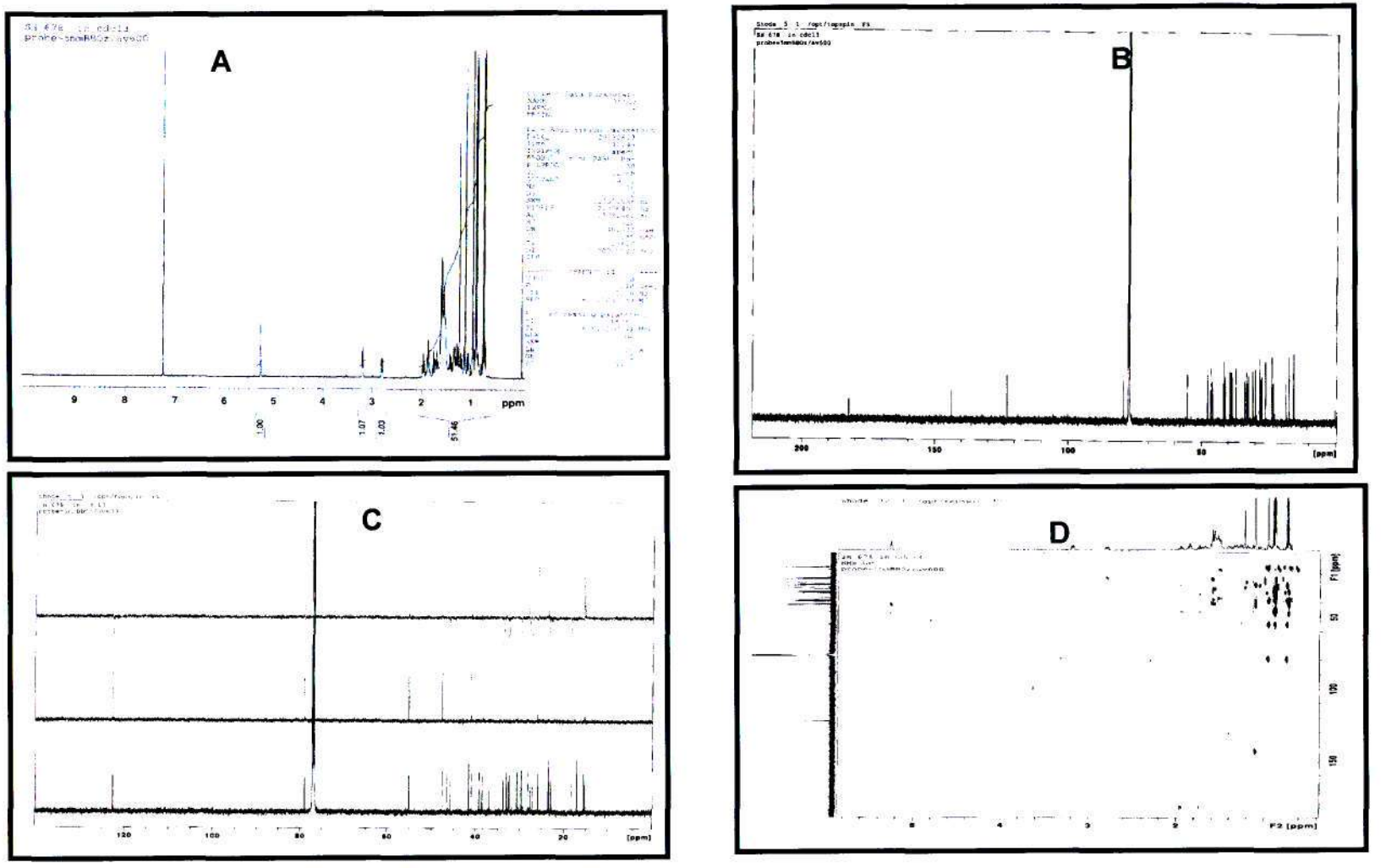
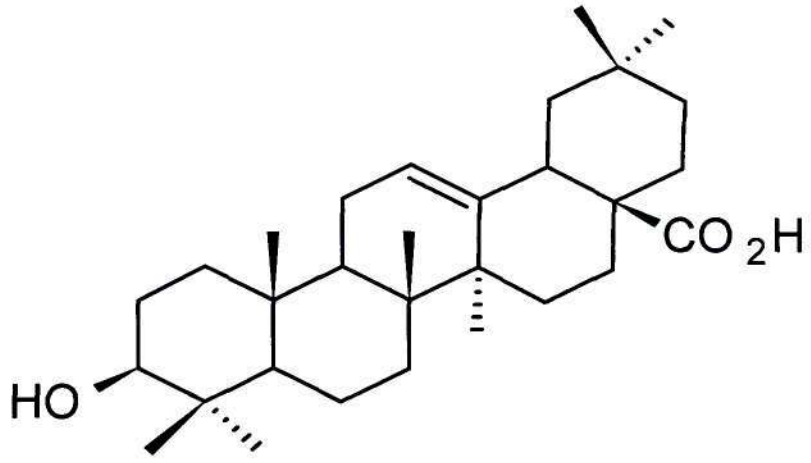


Figure 4: *Syzygium cordatum* leaf derived OA one dimensional ^{1}H and ^{13}C- NMR spectra (**A-B**) and two dimensional ^{1}H and ^{13}C- NMR spectra by Distortion Enhancement Proton Testing (DEPT) (**C**) and Heteronuclear multiple quantum coherence (HMQC) (**D**).

Table 1: ^{13}C (100.64 MHz) Bruker Avance III NMR spectral data of plant derived OA and reported OA (Mahato and Kundu 1994)

	Plant Derived OA	**Reported OA [Mahato and Kundu 1994]**
Carbon Position	$^{\delta}C$	$^{\delta}C$
1	38.4	38.5
2	27.2	27.4
3	79.0	78.7
4	38.8	38.7
5	55.2	55.2
6	18.3	18.3
7	32.6	32.6
8	39.3	39.3
9	47.6	47.5
10	37.1	37.1
11	23.0	22.9
12	122.7	122.5
13	143.6	143.5
14	41.6	41.6
15	27.7	27.7
16	23.4	23.4
17	46.5	46.5
18	41.0	40.9
19	45.9	45.9
20	30.7	30.6
21	33.8	33.8
22	32.4	32.4
23	28.1	28.1
24	15.5	15.5
25	15.3	15.3
26	17.1	17.1
27	25.9	25.9
28	182.2	183.5
29	33.07	33.1
30	23.6	23.6

A



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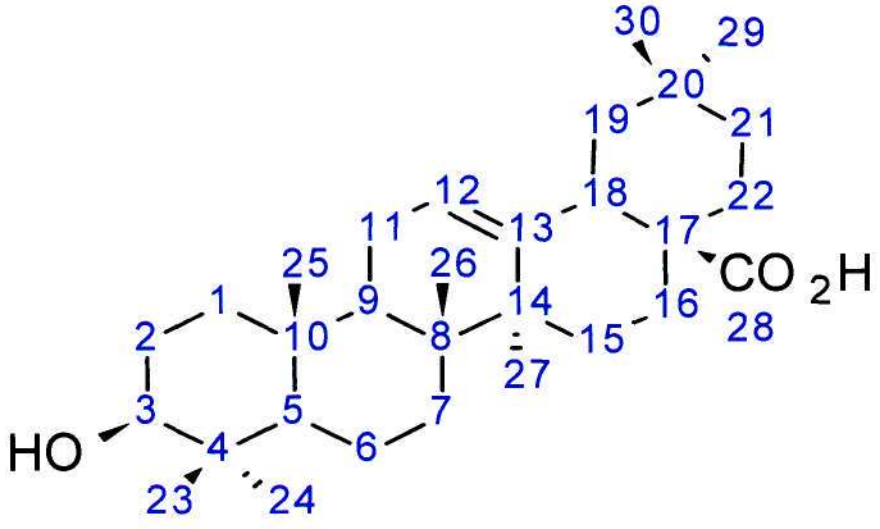

Figure 5: Structure (**A**) and numbering (**B**) of oleanolic acid (International Union of Pure and Applied Chemistry, IUPAC).

3.2. OGTT responses

Oral glucose tolerance (OGTT) responses to various doses of OA (40, 80 and 120 mg/kg) were monitored in separate groups of non-diabetic and STZ-induced diabetic rats following a glucose load (0.86 g/kg, p.o.) after 18-h fast. Rats treated with deionized water or standard anti-diabetic drugs (insulin, metformin and glibenclamide) acted as untreated and treated positive controls, respectively.

3.2.1. OGTT responses to standard anti-diabetic drugs

Figure 6 compares OGTT responses of separate groups of non-diabetic and STZ-induced diabetic rats to anti-diabetic drugs with respective control animals.

3.2.1.1. Non-diabetic rats

The blood glucose concentrations of control non-diabetic animals increased to peak levels (7 mmol/l) by 15 minutes following glucose load from preloading values of 4.2±0.1 mmol/l, but declined at the end of the 4 h experimental period to values slightly higher than the preloading values (5.0±0.1 mmol/l), though not statistically significant (Figure 6). All standard anti-diabetic drugs significantly ($p<0.05$) reduced blood glucose concentrations at all sampling time points by comparison with the control groups. The blood glucose lowering effects of insulin were most potent throughout the 4-h experimental period when compared with the effects of metformin and glibenclamide. Blood glucose concentrations of standard drug-treated animals, however, returned to pre-loading values at the end of the experiment.

3.2.1.2. STZ-induced diabetic rats

The mean fasting blood glucose concentration of STZ-induced diabetic rats was five-fold more than that of non-diabetic animals (Figure 6). Blood glucose concentrations of control STZ-induced diabetic rats increased to a peak value of 24.8±0.5 mmol/l after 15 minutes of glucose loading and declined to values below the pre-loading values by the end of the 4-h experimental period (Figure 6). OGTT responses of separate groups of STZ-induced diabetic rats to insulin and metformin

were lower at all the time points that blood was sampled when compared with control groups at the corresponding time periods. Glibenclamide, however, did not have any effects on blood glucose of these STZ-induced diabetic rats.

3.2.2. OGTT responses to OA

OGTT responses to various doses of OA (40, 80 and 120 mg/kg, p.o.) were monitored in non-diabetic and STZ-induced diabetic rats to determine the blood glucose lowering effects of the triterpene.

3.2.2.1. Non-diabetic rats

Figure 7 shows that OA decreased blood glucose concentrations of non-diabetic rats throughout the experimental period by comparison with control animals. The OGTT responses were, however, not dose-dependant although the highest dose (120 mg/kg) showed the most potent effects. The blood glucose concentrations of OA-treated non-diabetic rats returned to pre-loading values at the end of the 4-h experimental period.

The influence of standard drugs (insulin, metformin, and glibenclamide) on OA-induced OGTT responses was studied in separate groups of animals that were similarly treated with the OA followed by the standard drugs. OGTT responses to combined treatments were, however, not significantly different to responses evoked by each respective drug alone (Figures 8 and 9).

3.2.2.2. STZ-induced diabetic rats

Figure 7 compares OGTT responses to various doses of OA of STZ-induced diabetic rats with control diabetic animals. All doses of OA significantly decreased blood glucose concentrations after 30 minutes with the highest dose showing most potent effects. The blood glucose concentrations were significantly low throughout the 4 h experimental period by comparison with control animals at corresponding times. Administration of OA in combination with standard anti-diabetic drugs (metformin, glibenclamide and insulin) reduced blood glucose of STZ-induced diabetic rats (Figures 8 and 9). The blood glucose concentrations, however, decreased to

hypoglycaemic levels (2.5±0.2 mmol/l, n=6 in each group) in animals treated with OA (80 mg/kg) followed by insulin (200 µg/kg) at the end of the experiment (Figure 9 A).

3.2.3. OA effects on insulin secretion

Plasma concentrations of insulin and glucose were monitored in separate groups of non-diabetic and STZ-induced treated with OA and/or standard hypoglycaemic drugs 60 min after a glucose load to assess the influence of OA on pancreatic insulin secretion.

3.2.3.1. Non-diabetic rats

Separate treatments with insulin alone or in combination with OA significantly increased plasma insulin concentrations in non-diabetic rats with concomitant reduction of blood glucose (Figure 10). Metformin and OA, however, reduced blood glucose without altering plasma insulin concentrations (Figure 10).

3.2.3.2. STZ-induced diabetic rats

The plasma insulin concentrations of STZ-induced diabetic rats were significantly low by comparison with levels of non-diabetic rats (Figure 10). The plasma insulin concentrations were not altered by any other treatment in STZ-induced diabetic rats except for animals administered insulin alone or in combination with OA. Blood glucose concentrations were reduced by all treatments.

In summary OA, like standard drugs, decreased blood glucose concentrations in both non-diabetic and STZ-induced diabetic rats without altering insulin secretion. The hypoglycaemic effects were most significant in STZ-induced rats administered OA combined with insulin by comparison with all other treatments. OA alone did not alter insulin secretion of non-diabetic rats.

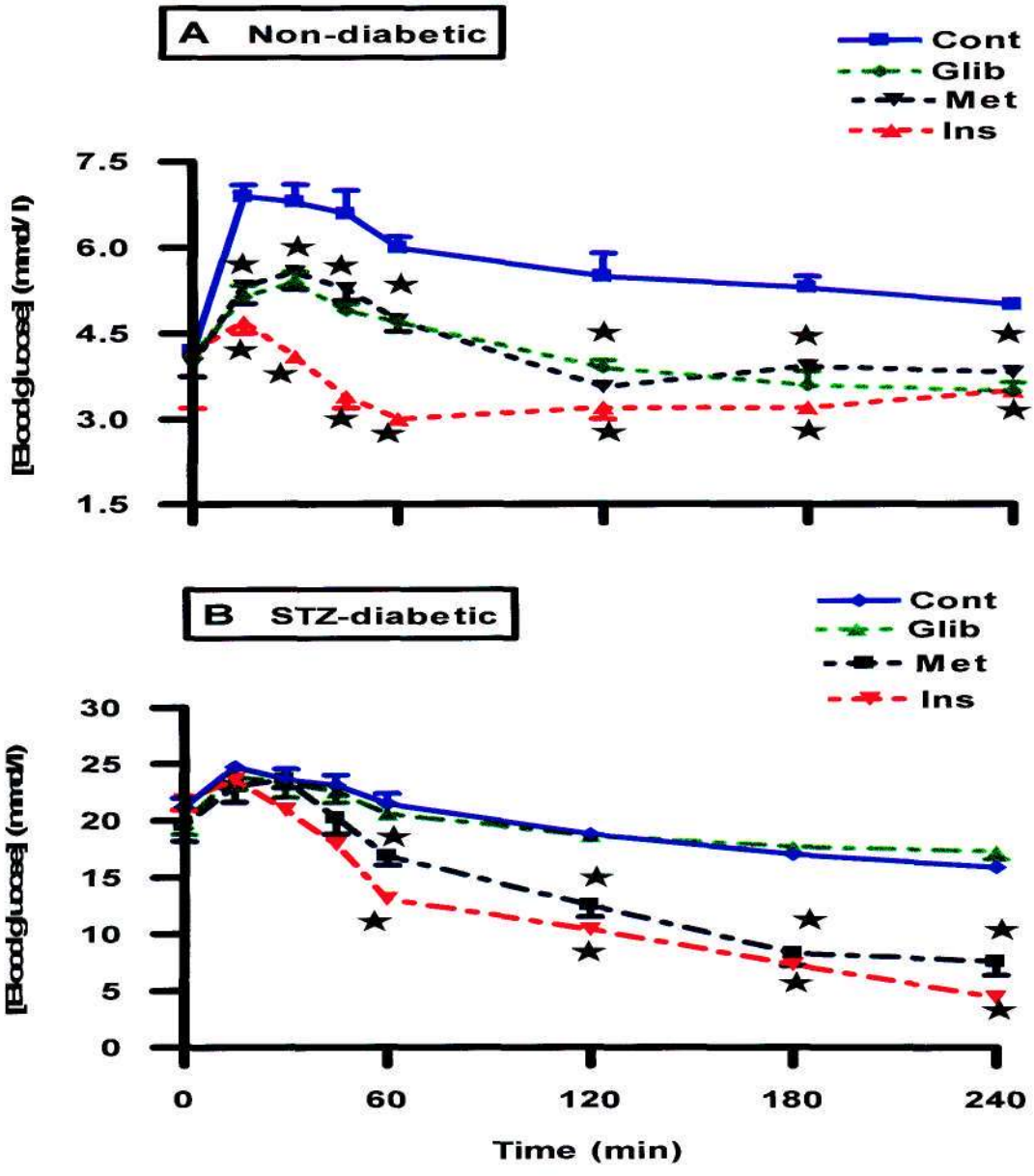

Figure 6: Comparison of OGTT responses to standard anti-diabetic drugs in separate groups of non-diabetic rats **(A)** and STZ-induced diabetic **(B)** rats with respective control animals. Values are presented as means, and vertical bars indicate SEM (n=6 in each group).

★ $p<0.05$ by comparison with respective control animals

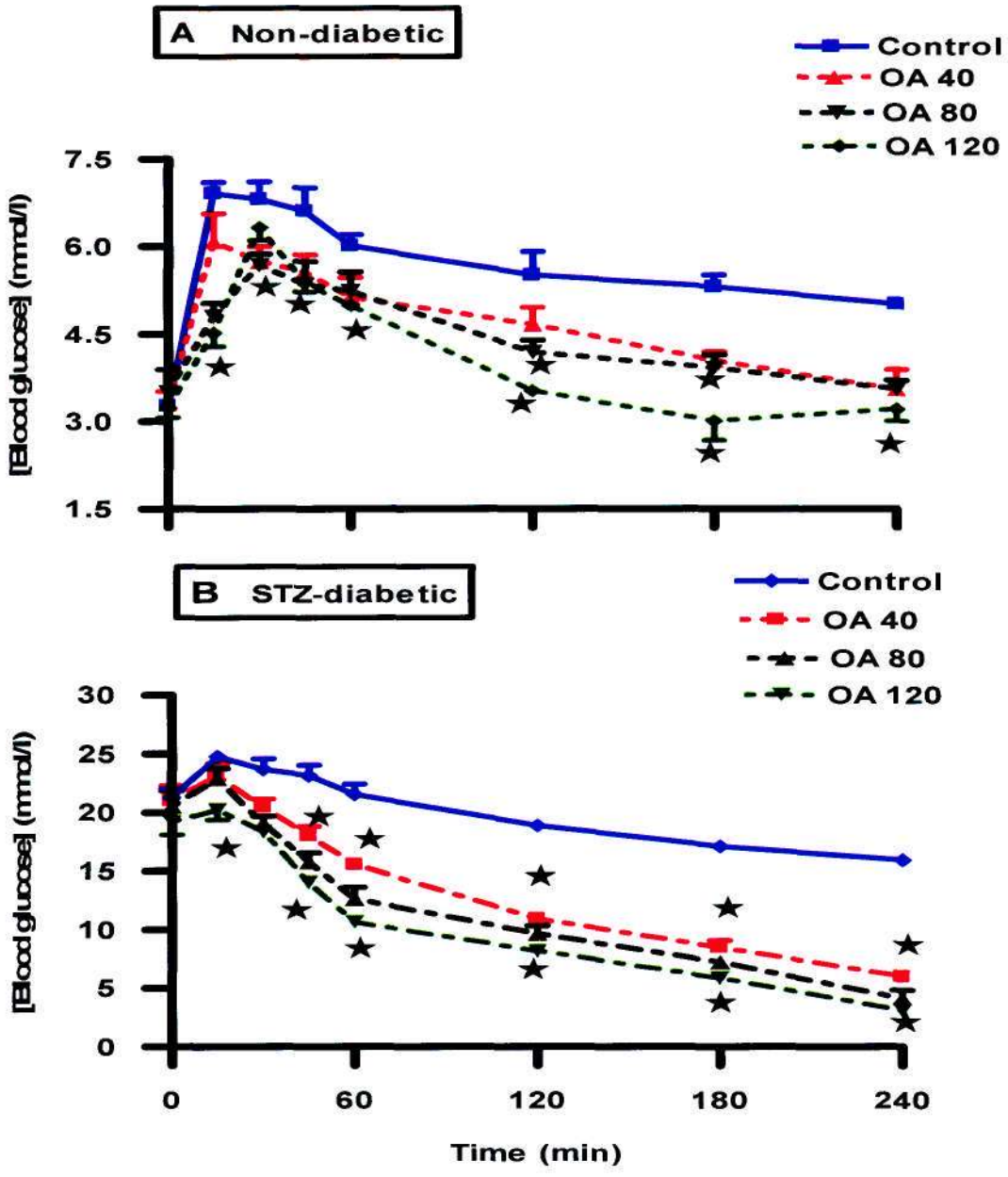

Figure 7: Comparison of OGTT responses to various OA doses in separate groups of non-diabetic rats **(A)** and STZ-induced diabetic **(B)** rats with respective control animals. Values are presented as means, and vertical bars indicate SEM (n=6 in each group).

★ $p<0.05$ by comparison with respective control animals

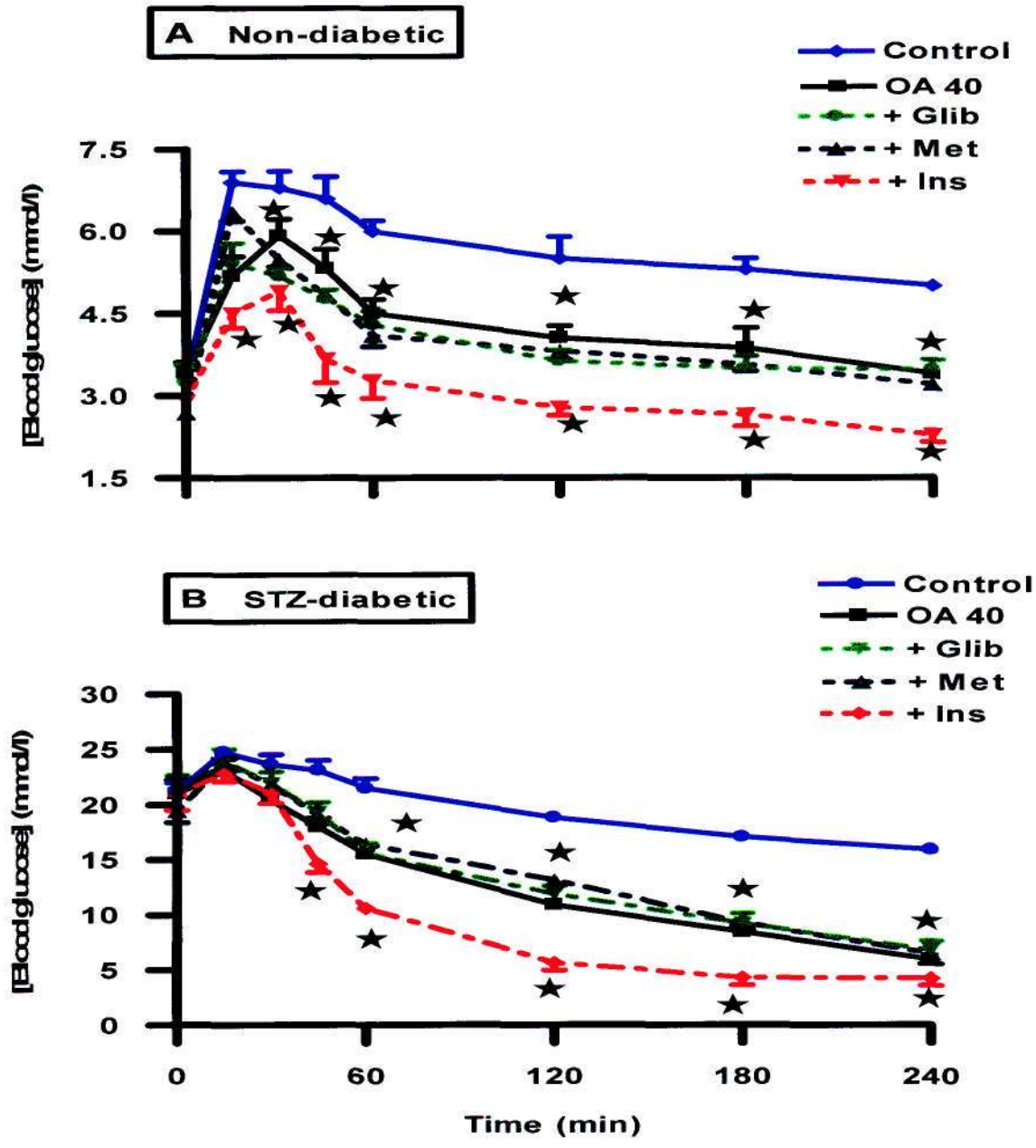

Figure 8: Comparison of OGTT responses to OA (40 mg/kg) alone and in combination with standard anti-diabetic drugs in separate groups of non-diabetic rats **(A)** and STZ-induced diabetic **(B)** rats with respective control animals. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ $p<0.05$ by comparison with respective control animals

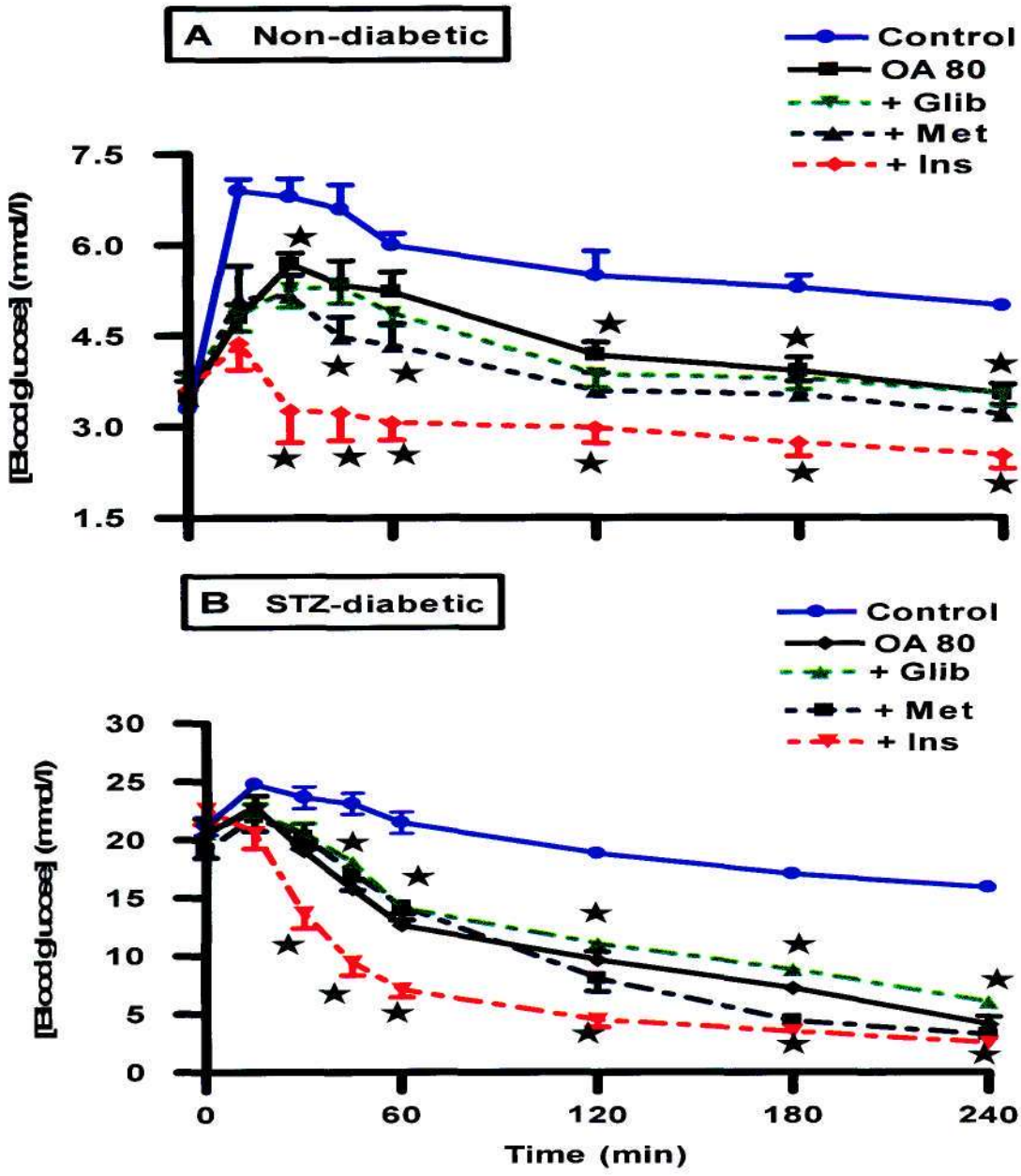

Figure 9: Comparison of OGTT responses to OA (80 mg/kg,) alone and in combination with various standard anti-diabetic drugs in separate groups of non-diabetic rats **(A)** and STZ-induced diabetic **(B)** rats with respective control animals. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ $p<0.05$ by comparison with respective control animals

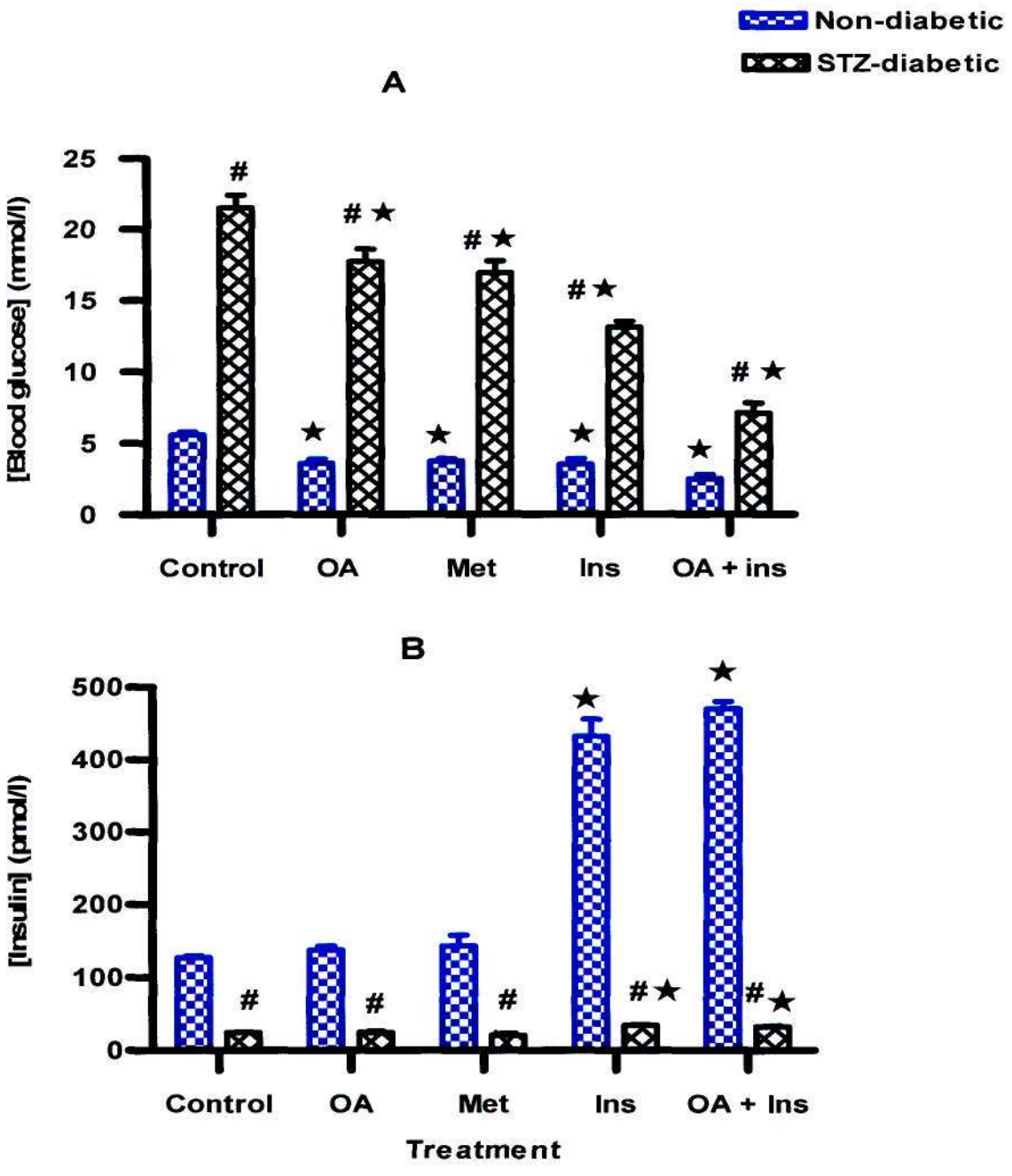

Figure 10: Comparison of the effects of OA and standard anti-diabetic drugs on plasma insulin and glucose concentrations in non-diabetic and STZ-induced diabetic rats with respective control groups. Blood samples were collected 60 min after glucose load. Values are presented as means, and vertical bars indicate 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

3.3. Acute renal effects of OA

Acute effects of OA on kidney function were investigated in anaesthetized non-diabetic and STZ-induced diabetic rats challenged with hypotonic saline after a 3½ h equilibration for 4 h of 1 h control, 1½ h treatment and 1½ h recovery periods.

3.3.1. Non-diabetic rats

Figure 11 shows stable urine flow and Na^+, K^+ and Cl^- excretion rates of control non-diabetic rats during the 4 h post equilibration period. Acute intravenous infusion of OA during the 1½ h treatment period increased Na^+ excretion rate from a pre-treatment mean value of 718±25 μmol/h to 880±25 μmol/h (n=6 in each group) without affecting K^+, Cl^- and urine flow rates (Figure 11). The increase in urinary Na^+ output was reflected as the increased mean total amount of Na^+ excreted during the treatment period (Table 2).

3.3.2. STZ-induced diabetic rats

The mean urine flow, Na^+, K^+ and Cl^- excretion rates of anaesthetized, control and OA-treated STZ-induced diabetic rats during the 4 h post-equilibration period are compared in Figure 12. STZ-induced diabetic control rats exhibited lower urine flow and electrolyte excretion rates by comparison with non-diabetic rats. OA infusion increased the mean Na^+ excretion rate from a pre-treatment value of 297±29 μmol/h to 420±38 μmol/h by the cessation of 1½ h treatment. The increase in Na^+ excretion was maintained during the recovery period. OA, however, did not influence K^+, Cl^- and urine excretion rates by comparison with control animals (see Figure 12). The mean total amounts of Na^+ excreted during the 1½ h OA treatment was significantly more than that of control animals at the corresponding time. The total amounts of fluid voided and K^+ and Cl^- excreted were, however, not altered by OA treatment.

In summary acute OA treatment increased Na^+ excretion in non-diabetic and STZ-induced diabetic rats without altering urine volume or urinary outputs of K^+ and Cl^-.

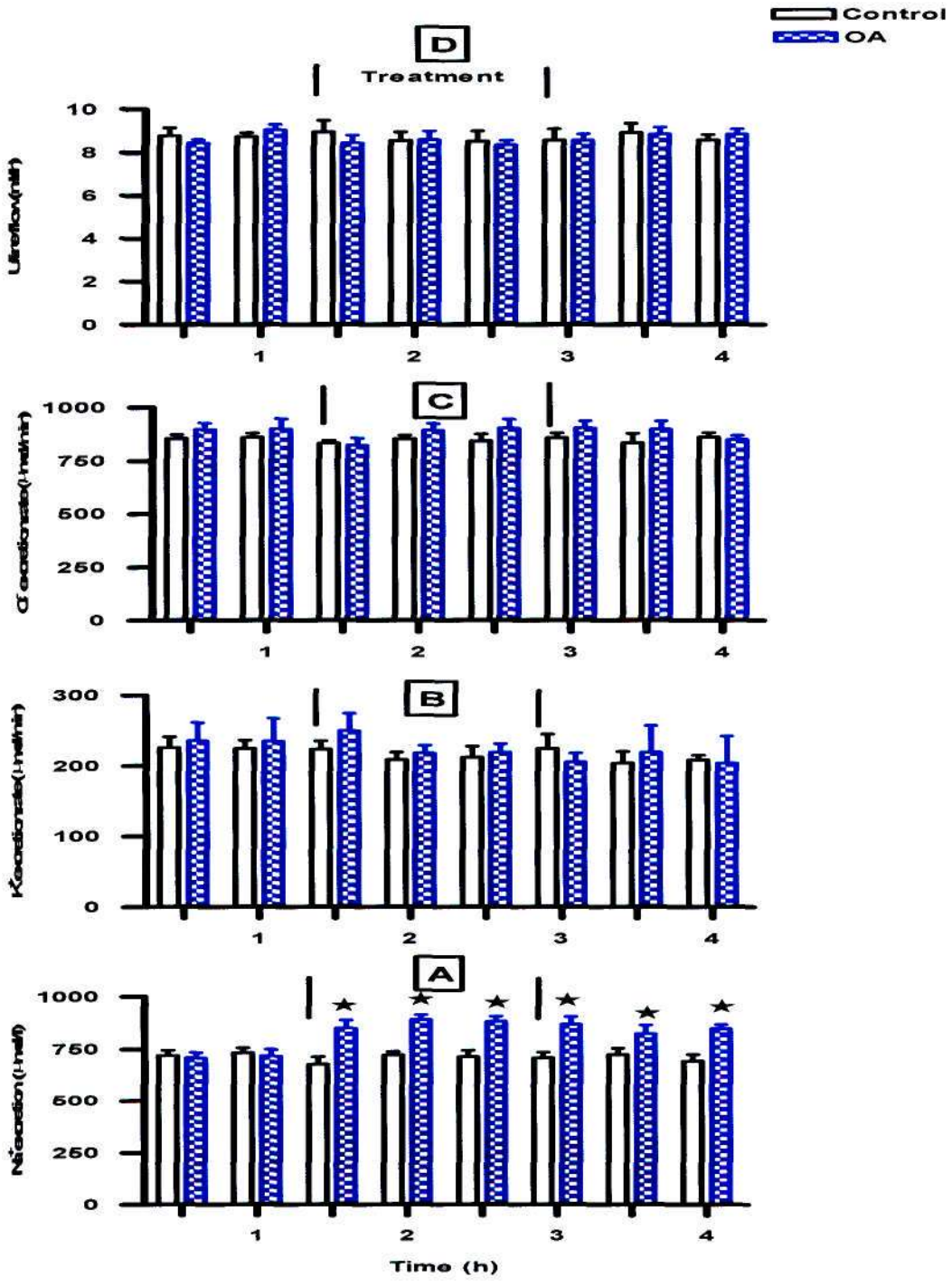

Figure 11: Comparison of the effects of 1½ h OA infusion on Na^+ (**A**), K^+ (**B**) and Cl^- (**C**) excretion and urine flow (**D**) rates with control animals in anaesthetized non-diabetic rats (n=6 in each group). Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ $p<0.05$ by comparison with control animals at each corresponding time

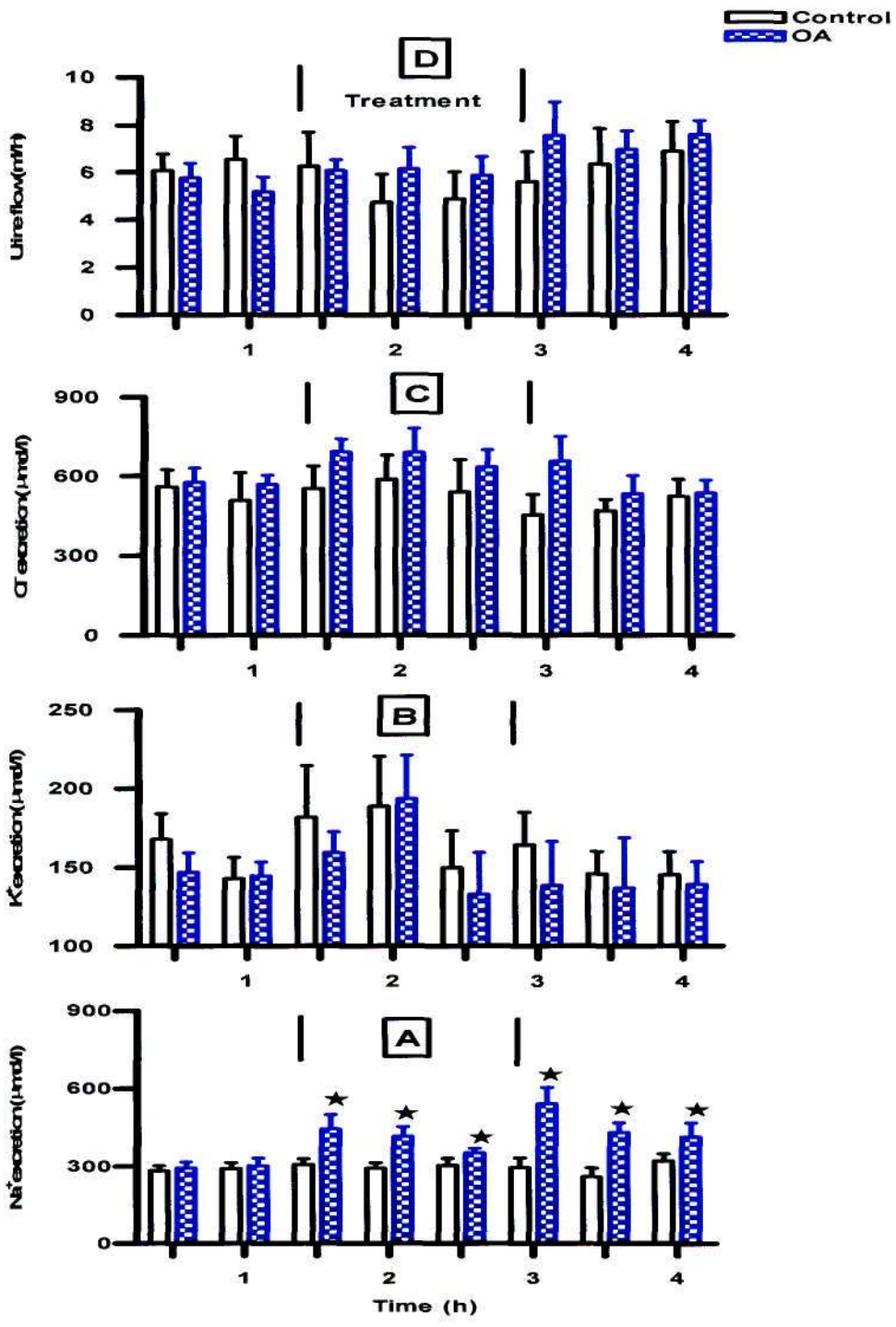

Figure 12: Comparison of the effects of 1½ h OA infusion with control animals on Na^+ (**A**), K^+ (**B**) and Cl^- (**C**) excretion and urine flow (**D**) rates in anaesthetized STZ-induced diabetic rats (n=6 in each group). Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ $p<0.05$ by comparison with control animals at each corresponding time

Table 2: Comparison of total mounts of urine volume, Na^+, K^+ and Cl^- excreted during the treatment period by anaesthetized OA-treated non-diabetic and STZ-induced diabetic rats with respective control animals (n = 6 in all groups). Data are expressed as means ± 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

		Urine (ml)	Na^+ (µmol)	K^+(µmol)	Cl^-(µmol)
Non diabetic	Control	13.1±0.6	1056±25	322±7	1264±14
	Treated	12.7±0.8	1394±15*	344±15	1388±43
Diabetic	Control	10.4±0.5	425±23$^{\#}$	260±42	1031±92
	Treated	10.1±0.6	622±41*$^{\#}$	243±16	1011±70

3.4. Short-term effects on body weight, food and water intake

Table 3 compares the effects of OA and/or standard anti-diabetic drugs on mean weight changes, food and water intake in separate groups of non-diabetic and STZ-induced diabetic rats over a 5-week period with respective control animals. Non-diabetic control rats progressively gained weight while STZ-induced diabetic rats exhibited severe wasting throughout the 5-week period. OA administration reduced the body weight loss of STZ-induced diabetic rats in the 5th week, whereas treatment with metformin, insulin or combined insulin and OA stabilized body weight of STZ-induced diabetic rats from the 3rd to the 5th week.

The total amounts of food taken per day by control non-diabetic and STZ-induced diabetic rats and respective treated groups did not significantly differ, except for metformin which significantly decreased food intake of STZ-induced diabetic rats in the 5th week.

Control STZ-induced diabetic rats exhibited polydipsia by comparison with the respective non-diabetic animals as evidenced by the fact that STZ-induced diabetic rats drank four to five times more water than non-diabetic rats. OA administration caused significant reduction of water intake

by STZ-induced diabetic rats in week 5 by comparison with respective control animals at the corresponding period. On the other hand, separate treatment of STZ-induced diabetic rats with metformin, insulin alone or in combination with OA significantly ($p<0.05$) reduced water intake from the first week up to the end of the five weeks.

In summary, treatment with OA alone or in combination with insulin stabilized body weight of STZ-induced diabetic rats without altering food intake.

Table 3: Comparison of the effects of 5-week treatment with OA and/or standard anti-diabetic drugs of non-diabetic (ND) and STZ-induced diabetic animals on food intake, water intake and body weight changes with the respective control animals (n=6 in each group). Values are presented as means ± SEM. * $p<0.05$ by comparison with control animals

Parameter	Treatment		week 1	week 2	week 3	week 4	week 5
Food intake (g/day)	Control	ND	30 ±1	32 ±1	31±1	30±1	26 ± 1
		STZ	35 ±1	33 ±1	32±2	28±2	34 ± 2
	OA	ND	28 ±1	29 ±1	29±1	29±2	28 ± 1
		STZ	34 ±1	32 ±1	31±3	32±1	33 ± 2
	Metformin	ND	29 ±2	30 ±1	29±2	25±1	28 ± 1
		STZ	33 ±1	28 ±1	27±1	28±1	27 ± 1*
	Insulin	ND	30 ±1	26 ±2	23±1	30±1	26 ± 1
		STZ	34 ±1	33 ±1	32±1	30±1	36 ± 1
	OA+ insulin	ND	32 ±1	27 ±1	28±1	30±2	30 ± 1
		STZ	38 ±1	35 ±1	33±2	32±1	33 ± 2
Water intake (ml/day)	Control	ND	33 ±2	36±3	33±1	34±2	31 ±1
		STZ	123±4	131±9	136±9	133±10	135±13
	OA	ND	33 ±2	34±1	36±1	33±1	29 ± 2
		STZ	125±4	141±7	132±3	127±8	107±7*
	Metformin	ND	34 ±2	40±1	33±2	29±2	31 ±1
		STZ	107±3*	107±12*	106±8*	100±6*	94 ±8*
	Insulin	ND	33 ±3	30±2	31±2	35±2	36 ±2
		STZ	115±2*	111±6*	113±5*	102±10*	101±11*
	OA+ insulin	ND	37 ±1	31±2	38±2	35±3	33 ±1
		STZ	109±4*	110±3*	109±11*	104±6*	102±6*
%b.wt change/week	Control	ND	8±1	11 ±2	18±1	22±1	23 ± 2
		STZ	-6±2	-4±2	-5±2	-4±2	-5 ± 2
	OA	ND	5±1	12 ±1	17±1	21±1	24 ± 1
		STZ	-5±2	-2 ±2	-2±3	-3±3	-1 ± 3*
	Metformin	ND	6±1	11 ±1	18 ± 2	21 ± 1	24 ± 1
		STZ	-8±1	-7 ±2	-2±1*	-2±2*	1 ± 2*
	Insulin	ND	7±1	15 ±1	21±1	23±1	27 ± 1*
		STZ	-7±1	-3 ±1	-2±1*	-1±2*	-1 ± 2*
	OA+ insulin	ND	8±1	14 ±1	20±3	24±3	25±1
		STZ	-4±2	-3 ±1	-1±1*	1±2*	2 ± 2*

3.5. Short-term renal effects of OA

The effects of OA on renal function were studied in individually-caged non-diabetic and STZ-induced diabetic rats that were treated with OA (80 mg/kg, p.o.) twice every third day for five weeks.

3.5.1. Non-diabetic rats

The mean daily Na^+ excretion rate of control non-diabetic rats ranged from 547±21 mmol/day to 678±68 mmol/day throughout the 5 week experimental period (Figure 13). OA administration significantly increased the mean daily Na^+ output from 1st week value of 645±28 mmol/day to 837±49 mmol/day by the end of the 5th week, but the volume of urine voided and urinary outputs of K^+ and Cl^- were not altered. The OA-elicited natriuresis was not reflected in plasma collected after 5 weeks treatment as the plasma Na^+ concentrations compared with those of control animals at the corresponding time (Table 4). OA administration for 5 weeks significantly reduced plasma creatinine concentrations of non-diabetic rats with concomitant elevation of GFR. In contrast, plasma urea concentrations of non-diabetic animals were not altered by OA treatment. OA administration caused slight, but statistically insignificant decreases of plasma and increases of urine osmolalities of non-diabetic rats (Table 4). Administration of OA did not affect kidney masses of non-diabetic rats (Table 4).

3.5.2. STZ-induced diabetic rats

The mean daily urine volume of untreated STZ-induced diabetic rats was greatly elevated throughout the 5-week compared to non-diabetic control animals, although the urinary Na^+, K^+ and Cl^- outputs were enormously reduced (Figure 14). Administration of OA increased urinary Na^+ outputs of STZ-induced diabetic rats from 35±3 mmol/day in the first week to 48±2 mmol/day in the 5th week. The mean daily urine volume, urinary K^+ and Cl^- outputs were, however, not altered by OA administration.

As described for non-diabetic rats, Table 4 shows that OA administration in STZ-induced diabetic rats did not affect plasma Na^+, K^+ and Cl^- by the end of the 5-week experimental period. In contrast with non-diabetic rats, OA administration significantly reduced ($p<0.05$) plasma osmolality of STZ-treated diabetic rats with concomitant increase in urine osmolality. Plasma creatinine and urea concentrations of STZ-induced diabetic rats were elevated when compared with non-diabetic rats. OA treatment did not alter plasma urea concentrations unlike plasma creatinine levels which were significantly reduced with a concomitant elevation of GFR (Table 4). Kidney mass which was significantly increased in STZ-induced diabetic rats by comparison with non-diabetic animals was not altered by sub-chronic OA treatment (Table 4).

3.5.3. Haemodynamic changes

Acute infusion of hypotonic saline to control non-diabetic rats did not cause any significant variations in the mean arterial blood pressure (MAP) throughout the 4-h post-equilibration period (Figure 15). Acute intravenous infusion of OA at 90 μg/h for 1½ h, however, reduced the mean arterial blood pressure from a pre-treatment value of 115±1 mmHg (n=6) to 105±1 mmHg (n=6) by the end of 1½ h treatment period. The hypotensive effect of OA persisted during the post-treatment period to a mean value of 108±2 mmHg at the end of the experiment (Figure 15A). Similar trends were observed in STZ-induced diabetic rats. The MAP changes due to long-term (sub-chronic) OA treatments are shown in Figure 15. Treatment of the rats with OA (80 mg/kg daily for 5 weeks, p.o.) caused significant decreases ($p<0.05$) in MAP in non-diabetic and STZ-treated diabetic rats throughout the study period by comparison with control rats at the corresponding time periods (Figure 15).

3.5.4. Summary of the sub-chronic effects of OA

Sub-chronic OA treatment significantly increased Na^+ excretion rates of non-diabetic and STZ-induced diabetic rats without affecting the volumes of urine voided and urinary concentrations of K^+ and Cl^-. OA treatment decreased plasma concentrations of creatinine with concomitant elevation of creatinine clearance Ccr. Significant hypotensive effects of OA were also observed in non-diabetic and STZ-induced diabetic rats.

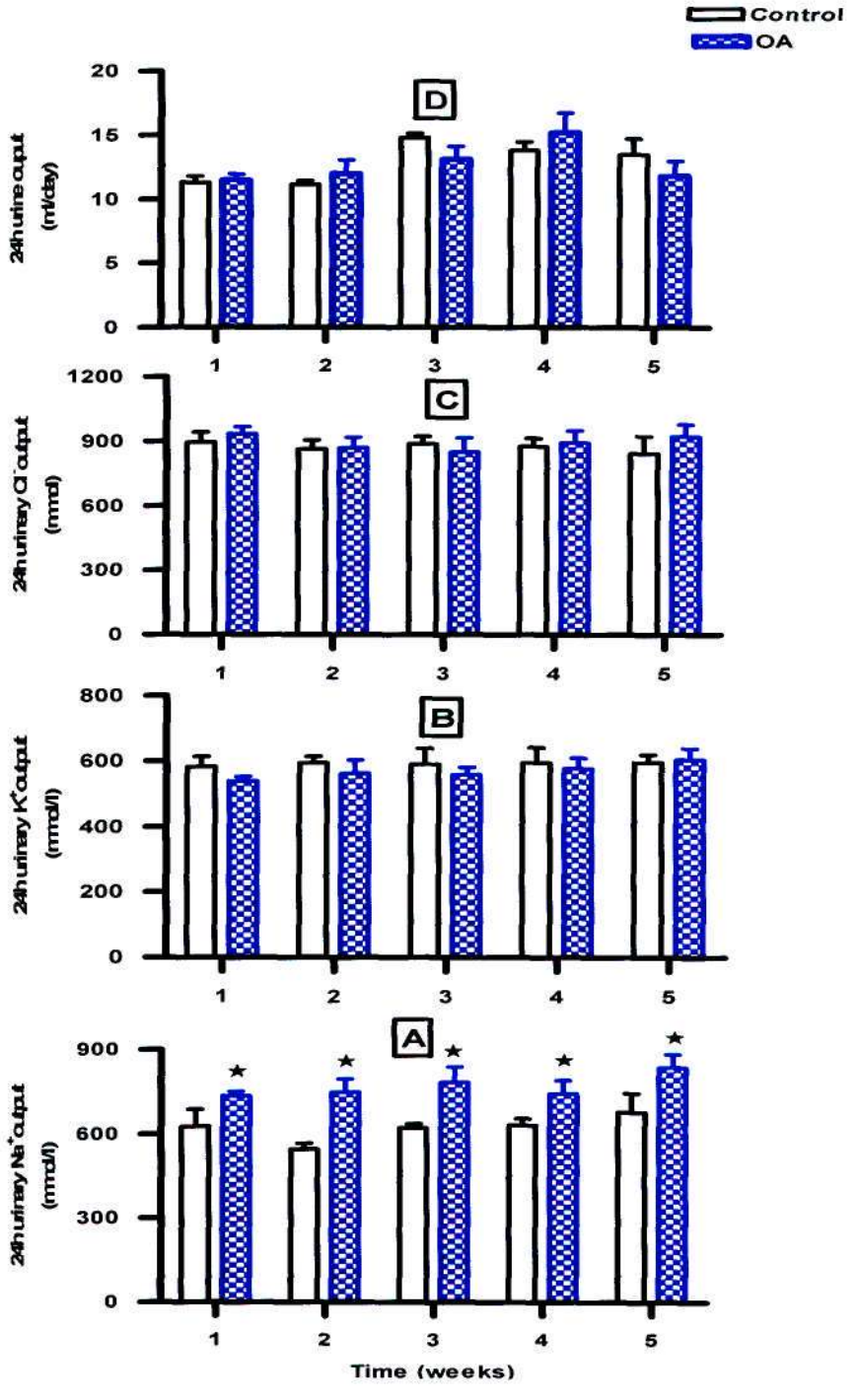

Figure 13: Comparison of 24h Na^+(**A**), K^+ (**B**), Cl^- (**C**) excretion and urine flow (**D**) rates in non-diabetic rats treated with OA twice every third day with control animals. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ $p<0.05$ by comparison with control animals at each corresponding time

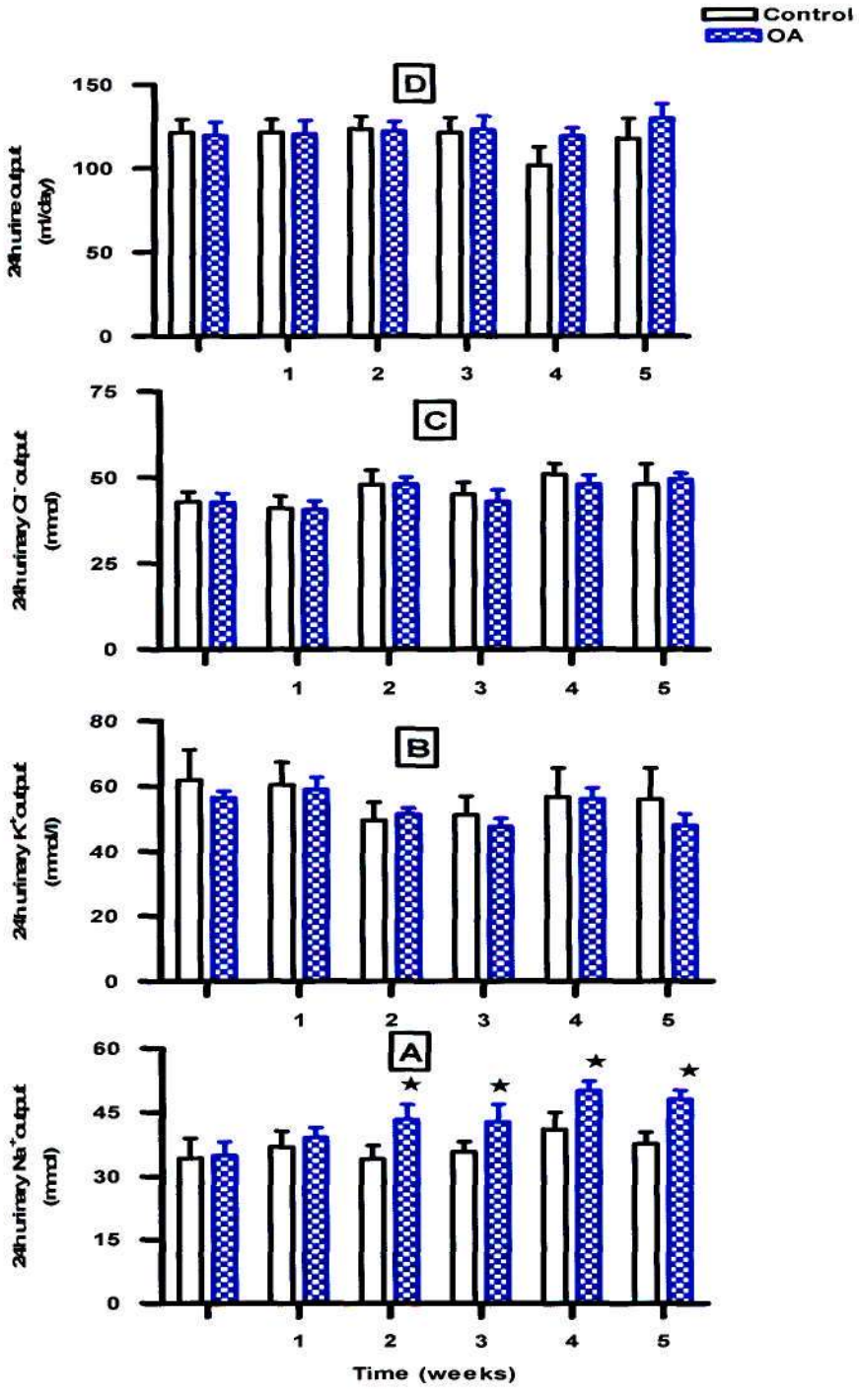

Figure 14: Comparison of 24h Na^+(**A**), K^+ (**B**), Cl^- (**C**) excretion and urine flow (**D**) rates in STZ-induced diabetic rats treated with OA twice every third day with control animals. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★$p<0.05$ by comparison with control animals at each corresponding time

Table 4: Plasma biochemical parameters of non-diabetic and STZ-diabetic control and rats administered OA twice every third consecutive day for 5 weeks (n = 6 in all groups).

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

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

Parameter	Non diabetic		STZ-diabetic	
	Control	Treated	Control	Treated
Na^+ (mmol/l)	142±1	142±1	129±6	124±3
K^+ (mmol/l)	3.68±0.25	3.59±0.17	3.76±0.21	3.53±0.20
Cl^- (mmol/l)	101±1	103±1	102±2	96±4
Urea (mmol/l)	7.3±0.9	8.3±0.7	10.7±1.7#	12.1±1.8#
Creatinine(µmol/l)	25±2	19±1*	31±3#	23±2*#
Plasma osmolality ($mOsm/kgH_2O$)	263±27	247±15	382±10#	316±16#
GFR (ml/min)	2.88±0.14	3.71±0.30*	1.81± 0.32#	3.07±0.16*#
Kidney mass (g/100g b.wt)	0.46±0.02	0.44±0.02	0.78±0.07#	0.73±0.04#
Urine osmolality ($mOsm/kgH_2O$)	2512±99	2661±78	991±92#	1115±56*#

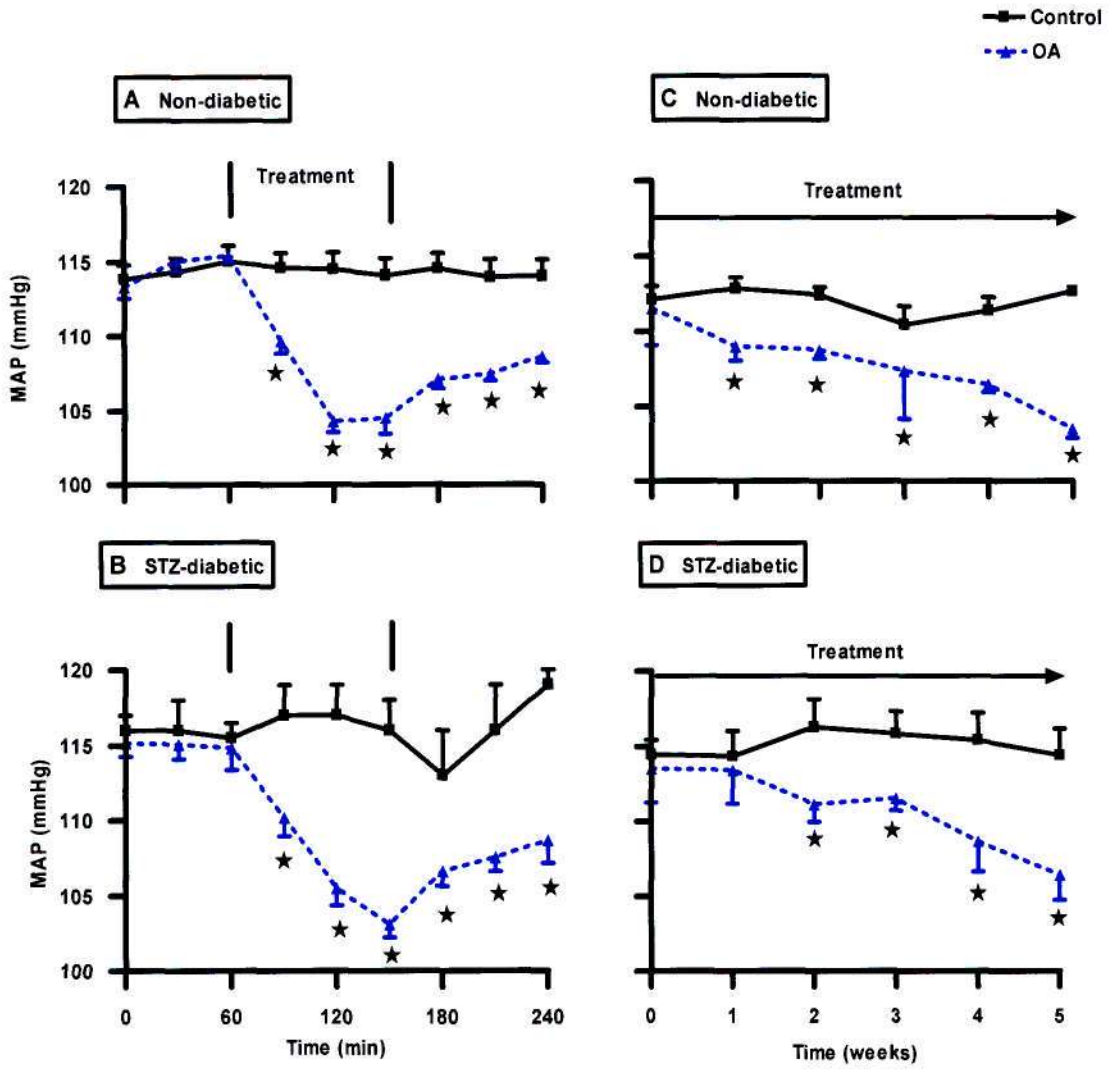

Figure 15: Comparison of acute effects of OA infusion on MAP in non-diabetic and STZ-induced diabetic rats (**A-B**) and sub-chronic short-term administration in non-diabetic and diabetic rats (**C-D**) with respective control animals (n=6 in each group). Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ $p<0.05$ by comparison with respective control animals at each corresponding time

3.5.5. Effects of OA on terminal blood glucose and plasma insulin concentrations

Figure 16 shows the short-term effects of treatment with OA and standard anti-diabetic drugs on blood glucose concentrations and plasma insulin concentrations in non-diabetic and STZ-induced diabetic rats measured 24 h after the last treatment. Treatment of non-diabetic rats with OA alone or standard anti-diabetic drugs reduced blood glucose concentrations by amounts that did not achieve statistical significance. Combined OA and insulin treatment, however, significantly decreased blood glucose concentrations of non-diabetic animals by comparison with respective control animals. The administrations of OA alone and/or standard anti-diabetic drugs significantly ($p<0.05$) reduced blood glucose concentrations of STZ-induced diabetic rats by comparison with the respective control animals. Figure 16 shows that the circulating plasma insulin concentrations of control STZ-induced rats were significantly low by comparison with non-diabetic animals. OA treatment did not alter plasma insulin concentrations of non-diabetic or STZ-induced diabetic rats. Significant increases in plasma insulin concentrations were observed only in non-diabetic and STZ-induced diabetic rats treated with combined insulin and OA or insulin alone. Figure 17 shows that treatment with OA alone significantly ($p<0.05$) decreased the excretion of glucose of STZ-induced diabetic rats throughout the 5-week period by comparison with the respective control rats.

3.5.5.1. Effects of OA on hepatic and muscle glycogen concentrations

Hepatic and muscle glycogen concentrations were measured in separate groups of non-diabetic and STZ-induced diabetic rats after 5 weeks of treatment with OA alone and/or standard anti-diabetic rats in an effort to establish in part the mechanism(s) of the hypoglycaemic effects of OA. All treatments increased hepatic glycogen concentrations of non-diabetic and STZ-induced diabetic rats (Table 5) whereas muscle glycogen concentrations were elevated by these treatments only in non-diabetic rats.

In summary, sub-chronic OA treatment like standard anti-diabetic drugs decreased blood glucose concentrations in rats without influencing pancreatic insulin secretion. The hypoglycaemic effects were most significant in animals co-administered OA and insulin. Muscle glycogen concentrations of STZ-induced diabetic rats were increased by combined insulin and OA treatment.

3.6. Overall summary of the results of this study

- The absolute stereostructure of *S. cordatum* derived OA was elucidated using 1D and 2D ^{1}H NMR and ^{13}C NMR techniques on the basis of chemical and physicochemical evidence.

- OGTT responses elicited by *S. cordatum* leaf derived OA in non-diabetic and STZ-induced diabetic rats were comparable to those of standard anti-diabetic drugs.

- OA augments the insulin-elicited OGTT responses in STZ-induced diabetic rats.

- OA treatment improved renal function of STZ-induced diabetic rats as shown by increased urinary Na^{+} outputs, reduced plasma creatinine with concomitant increase in GFR.

- OA evoked potent hypotensive effects in STZ-induced diabetic rats.

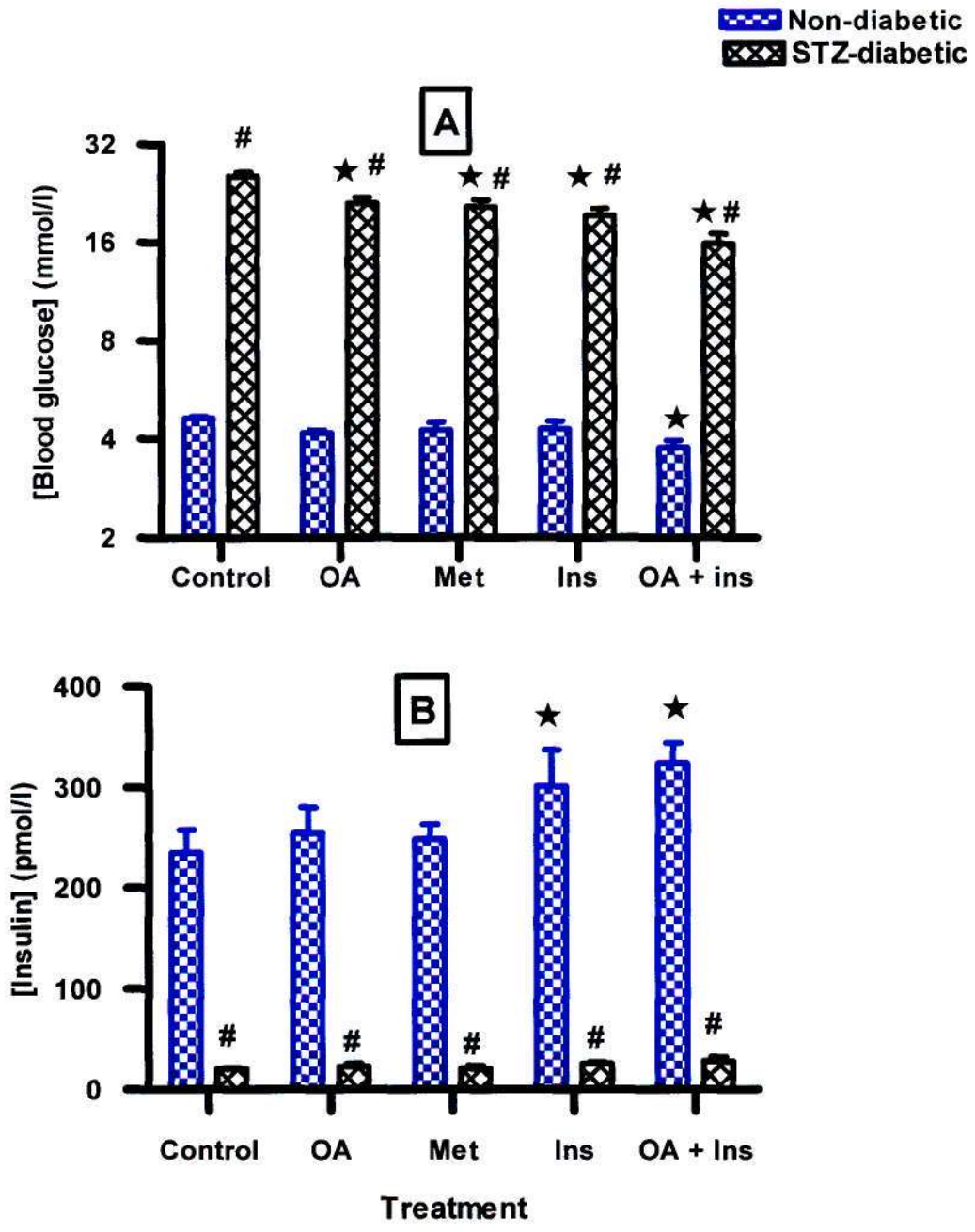

Figure 16: Comparison of the sub-chronic effects of OA and standard hypoglycaemic drugs on blood glucose (**A**) and plasma insulin (**B**) concentrations in non-diabetic and STZ-induced diabetic rats with respective control groups. Blood samples were collected 24 h after the last treatment. Values are presented as means, and vertical bars indicate SEM (n=6 in each group).

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

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

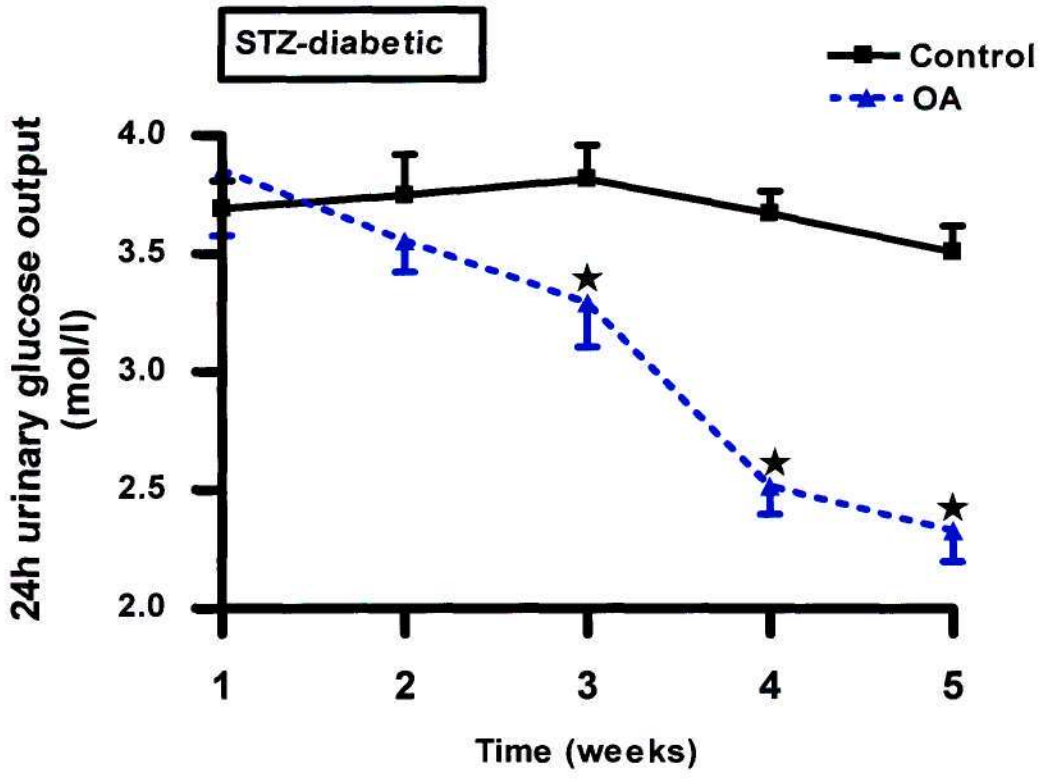

Figure 17: Comparison of mean daily urinary glucose outputs of control STZ-induced diabetic rats with STZ-induced diabetic rats treated with OA twice every third day for 5 weeks (n=6 in each group).

★ $p<0.05$ by comparison with respective control animals

Table 5: Comparison of the effects of 5-week treatment with OA alone and/or standard anti-diabetic drugs on hepatic and muscle glycogen concentrations in non-diabetic and STZ-induced diabetic rats with respective control groups. Data are expressed as mean ± SEM (n=6 in each group).

* $p<0.05$ by comparison with respective control group

Treatment		**[Hepatic glycogen] (mg/100mg tissue/ 300g b.wt)**	**[Muscle glycogen] (mg/100mg tissue)**
Non-diabetic rats (n=6 in each group)	Control	28.4±1.9	2.6±0.3
	OA	37.9±3.7*	3.5±0.3*
	Metformin	39.5±2.0*	5.2±0.7*
	Insulin	66.3±2.7*	5.2±0.4*
	OA + insulin	74.0±6.1*	5.0±0.3*
STZ-induced diabetic rats (n=6 in each group)			
	Control	17.1 ± 0.4	1.1 ± 0.1
	OA	30.3 ± 0.6*	2.0 ± 0.4
	Metformin	36.0 ± 0.7*	2.1 ± 0.4
	Insulin	42.3 ± 0.7*	2.4 ± 0.3
	OA + insulin	56.4 ± 0.5*	3.1 ± 0.3*

CHAPTER 4

4.0. DISCUSSION

4.1. General

The aim of the present study was to investigate the effects of *S. cordatum* leaf-derived OA on blood glucose concentrations and renal function of STZ-induced diabetic rats. The results suggest that OA not only has the potential to lower blood glucose and blood pressure in diabetes, but may also have beneficial effects on kidney function. The findings are of considerable importance because they contextualize the hypoglycaemic, renal and hypotensive effects of OA in the management of diabetes. This is clinically relevant considering the prevalence of microvascular and macrovascular complications in diabetic patients (The Diabetes Control and Complications Trial Research Group, 1993; Vinicor, 1994). Management of diabetes mellitus with OA may, therefore, be associated not only with the regulation of blood glucose, but also the prevention or alleviation of these complications.

4.2. Structural elucidation of OA

The absolute stereostructure of OA from the crude ethyl acetate extract of *S. cordatum* leaves was established on the basis of chemical and physicochemical evidence. The spectra of the plant-derived OA elucidated using ^{1}H- and ^{13}C-NMR was compared with previously reported data (Mahato and Kundu 1994). The spectral data of OA, particularly the presence of the olefenic bond between carbon 12 and 13 which is peculiar to triterpenoids, was identical with that of previously reported OA (Mahato and Kundu, 1994). The identification of OA from *S. cordatum* leaves confirms previous reports that the *S. cordatum* leaf crude ethyl acetate extract contains triterpenoid mixtures (Musabayane, Mahlalela, Shode and Ojewole, 2005). Other investigators have also isolated the triterpenes OA and ursolic acid from various medicinal plants following

sequential extraction with methanol (Jung *et al.*, 2007; Begum, Zehra and Siddiqui, 2008).

4.3. Hypoglycaemic effects

The experimental evidence presented in this study extends previous observations in our laboratories that the *S. cordatum* crude leaf extract lowers blood glucose of STZ-induced diabetic rats (Musabayane, Mahlalela, Shode and Ojewole, 2005) by indicating that the antihypeglycaemic effect is elicited by triterpene constituent, OA.

The fasting blood glucose concentrations of STZ-induced diabetic rats were significantly higher by comparison with the non-diabetic animals. The rise in blood glucose concentrations in untreated STZ-induced diabetic rats can be attributed to the absence of insulin, since streptozotocin induces permanent diabetes mellitus by direct pancreatic ß-cell cytotoxicity (Junod, Lambert, Stauffacher and Renold, 1969; Pinent, Blay, Bladé, Salvadó, Arola and Ardé, 2004). Streptozotocin first isolated from *Streptomyces achromogenes* is an antibiotic that selectively destroys pancreatic β-cells thereby inducing type 1 diabetes mellitus (Ruiz, Alegria, Barbera, Farre and Lagarda, 1994; Coskun, Kanter, Korkmaz and Oter, 2005; Li *et al.*, 2007; Srinivasan and Ramarao, 2007). Indeed, plasma insulin concentrations were significantly lower in STZ-induced diabetic rats compared with non-diabetic rats, thereby contributing to high glucose concentrations. This is consistent with the mechanism of action of streptozotocin on the beta-cells of the pancreas (Junod, Lambert, Stauffacher and Renold, 1969; Pinent, Blay, Bladé, Salvadó, Arola and Ardé, 2004). The hypothesis is further supported by the observation of a steady, gradual fall in blood glucose concentrations of control non-diabetic rats following glucose load, presumably due to increased pancreatic insulin secretion. In addition, glibenclamide was associated with blood glucose reduction only in non-diabetic rats and not STZ-induced animals. The hypoglycaemic effect of glibenclamide is seen only when pancreatic beta-cell activity is present, as glibenclamide action involves augmentation of insulin secretion (Luzi and Pozza, 1997). The blood glucose-lowering effects of OA at all doses (40, 80 and 120 mg/kg) in non-diabetic rats

could not be separated at any time. OGTT responses of STZ-induced diabetic rats to OA at 40 and 80 mg/kg were similar but, significantly lower than those of OA at 120 mg/kg throughout (Figure 7). The reasons for the discrepancy of OGTT responses to three doses of OA in non-diabetic and STZ-induced diabetic rats remain to be determined. We suggest that endogenous insulin masked the differences in hypoglycaemic effects evoked by the small range of OA used in non-diabetic rats.

OA alone and/or insulin and metformin decreased plasma glucose concentrations in non-diabetic and STZ-diabetic rats following sub-chronic treatment for 5 weeks. Blood glucose concentrations of STZ-induced diabetic rats following long-term treatment were significantly reduced by all treatments to levels that did not achieve euglycaemia. The reduction of blood glucose concentrations of non-diabetic rats did not reach statistical significance. The lack of pronounced OA blood glucose-lowering effects observed may be attributed to experimental design and data presentation. The blood glucose concentrations were measured 24 h after the last dosing and by that time the administered OA or standard anti-diabetic drugs may have been metabolized. The peak hypoglycaemic effect of triterpene containing herbal extracts occurs 2-4 hours after administration, with blood sugar returning to pre-treatment levels in 6-10 hours (Kakuda, Sakane, Takihara, Ozaki, Takeuchi and Kuroyanagi, 1996).

Plasma insulin concentrations were significantly elevated in non-diabetic rats administered insulin alone or in combination with OA by comparison with STZ-induced diabetic rats similarly treated. Therefore, insulin involvement in the hypoglycaemic effects of OA is excluded, since plasma insulin concentrations of non-diabetic and STZ-induced diabetic rats were not altered by treatment with OA alone. OA, however, would not be expected to significantly affect insulin levels in STZ-induced diabetic rats as the pancreatic ß-cells would have been selectively destroyed by streptozotocin administration (Junod, Lambert, Stauffacher and Renold, 1969; Pinent *et al.*, 2004). It is possible that OA alters glucose metabolism in the liver of diabetic animals, thus reducing blood glucose concentrations by increasing hepatic glycogenesis (Table 5). This has been previously reported for other plant extracts (Gondwe et al., 2008). It is suggested that the

hypoglycaemic effects of OA mimic those of metformin as evidenced by the fact that both these treatments did not alter plasma insulin concentrations (Figure 10 and 16). Metformin improves insulin sensitivity in peripheral target tissues and suppresses hepatic glucose output to reduce blood glucose (Lee and Morley, 1998; Krentz and Bailey, 2005). The possibility of lowering blood glucose concentrations through other mechanisms cannot be totally excluded.

Hypoglycaemic effects were most pronounced in STZ-induced diabetic rats treated with combined OA and insulin (Figures 8 and 9). Since the current data suggest that OA possesses metformin-like blood glucose lowering properties, it is possible that OA augmented the hypoglycaemic effects of insulin by increasing the sensitivity to insulin. This is in agreement with previously reported effects of other triterpenoids, ursolic acid and corosolic acid (Miura *et al.*, 2004; Jung *et al.*, 2007). It is also possible that OA might have interfered with gastric emptying and glucose absorption, as been previously reported for metformin (Lee and Morley, 1998). Indeed some triterpenoids have been shown to lower blood glucose concentrations in a similar way (Matsuda, Li, Yamahara and Yoshikawa, 1998; Matsuda, Li, Mukarami, Yamahara and Yoshikawa, 1999; Katz, Newman and Lansky, 2007). The ability of OA to prevent hyperglycaemia indicates that OA may be useful in preventing some of the complications associated with diabetes mellitus.

OA like metformin and insulin increased hepatic glycogen concentrations of non-diabetic and STZ-induced diabetic rats by comparison with respective controls (Table 4). Hepatic glycogen concentrations were, however, significantly lower in STZ-induced diabetic rats as compared to the non-diabetic rats. Glycogen is the primary storable form of glucose and diabetes mellitus is associated with reduced capacity to store glycogen (Chandramohan, Ignacimuthu and Pugalendi, 2008). This is in agreement with previous observations that the crude leaf extract of *S. cordatum* increases hepatic glycogen concentrations, thereby reducing blood glucose levels (Musabayane, Mahlalela, Shode and Ojewole, 2005). On the contrary, muscle glycogen concentration of STZ-induced diabetic rats was not altered by treatment with OA alone, presumably due to the lack of

insulin. The observed difference between non-diabetic and STZ-induced diabetic rats muscle glycogen concentration may be due to the fact that the conversion of glucose into glycogen in skeletal muscle is dependent on insulin (Saltiel and Kahn, 2001; Hsu, Liu, Kuo, Chen, Su and Cheng, 2003; Jensen, Jebens, Brennesvik, Ruzzin, Soos, Engebretsen, O'Rahilly and Whitehead, 2006). In contrast, hepatic glycogen concentrations are determined by the extracellular glucose concentrations and glycogenic enzyme activities (Tana, Tanb and Pushparaj, 2005). It is possible; therefore, that OA-induced hepatic glycogen synthesis in STZ-induced diabetic rats was due to increased activities of GLUT-2 and glycogen synthase (Buschiazzo, Exton and Park, 1970; Libal-Weksler, Gotlibovitz, Stark and Madar, 2001; Tana, Tanb and Pushparaj, 2005). The activity of the GLUT-2 transporters is non-insulin dependent. Glycogen synthesis also involves inhibition of glycogen phosphorylase, decreased glucose output, enhancement of GLUT-4 translocation, insulin receptor autophosphorylation and inhibition of protein tyrosine phosphatase B as been reported for other triterpenoids (Chen *et al.*, 2006; Jung *et al.*, 2007). It is possible to speculate that the effects of the combined treatment could be due to OA sensitizing responses to exogenous insulin.

The non-diabetic rats showed weight gain throughout the study, whilst untreated STZ-induced diabetic rats showed progressive weight losses. The discrepancy between the groups may be attributed to the anabolic effects of insulin (Chandramohan, Ignacimuthu and Pugalendi, 2008). Treatment with OA alone or in combination with insulin, however, stabilized body weight of STZ-induced diabetic rats without altering food intake. Weight gain may be attributed to increase in the incorporation of glucose into tissues, as reported earlier in STZ-treated diabetic mice (Naik, Barbosa-Filho, Dhuley and Deshmukh, 1991; Okine, Nyarko, Osei-Kwabena, Oppong Barnes and Ofosuhene, 2005). Interestingly, polydipsia and increased water intake as a result of enhanced proteolysis associated with hyperosmotic dehydration has been shown to be associated with weight losses in STZ-induced diabetic rats (Rebsomen, Pitel, Boubred, Buffat, Feuerstein, Raccah, Vague and Tsimaratos, 2006; Narendhirakannan, Subramanian and Kandaswamy, 2006).

4.4. Renal effects

Renal function studies were designed to investigate whether *S. cordatum* derived OA can reverse the previously reported inability of the kidney to excrete Na^+ in STZ-induced diabetic rats (Musabayane, Ndhlovu and Balment, 1995). The hypotonic saline used has been shown to suppress endogenous arginine vasopressin secretion in rats (Balment, Brimble, Forsling and Musabayane, 1984). Long-term diabetes mellitus complications often include compromised kidney function and elevated blood pressure (Haller, Drab and Luft, 1996; Stengel, Billon, van Dijk, Jager, Dekker, Simpson, Briggs, 2003). The results indicate that OA not only has the potential to lower blood pressure in diabetes, but may also have beneficial effects on kidney function. The results introduce the first *in vivo* evidence that oleanolic acid extracted from *S. cordatum* leaves ameliorates kidney function in STZ-induced diabetic rats.

Kidney weights of STZ-induced diabetic rats were significantly increased by comparison with non-diabetic animals possibly due to accumulation of extra cellular matrix proteins (Aybar, Riera, Grau and Sánchez, 2001; Kang, Kim, Yamabe, Nagai and Yokozawa, 2006). The extra-cellular matrix protein induced kidney hypertrophy causes structural changes of glomerular basement membrane thereby impairing kidney function (Hargrove, Dufresne, Whiteside, Muruve, Wong 2000; Mason and Wahab, 2003; Kumar, Shetty, Salimath. 2008). This perhaps explains why STZ-induced rats exhibited marked daily decreases in urinary Na^+ excretion and elevated plasma creatinine concentration with concomitant reduction in Ccr at the end of 5 weeks by comparison with non-diabetics. These changes were attenuated in the OA-treated groups indicating that oleanolic acid might have beneficial effects on renal function in diabetes. OA-evoked increases urinary Na^+ outputs of STZ-diabetic rats and elevation of GFR as assessed by creatinine clearance suggest up-regulation of renal function by the triterpene. GFR is an important marker of kidney function and treatment-related increases in creatinine clearance indicate an improvement of kidney function in experimental animals and man rather than looking at plasma concentrations of urea because the latter varies with dietary protein (Satirapoj, Supasyndh, Patumanond and Choovichian, 2006; Yang and Bankir, 2006; Almond,

Siddiqui, Robertson, Norrie and Isles, 2008; van Hoek, Lefebvre, Kooistra, Croubels, Binst, Peremans and Daminet, 2008). Our laboratory and several other authors have previously used creatinine clearance (Ccr) in rats as a fundamental parameter of evaluating renal tubular function (Rebsomen *et al.*, 2006; Kumar, Shetty and Salimath, 2008). Low GFR is associated with fluid and electrolyte imbalance in nephrotic syndrome and acute renal failure (Fernãndez-Llama, Andrews, Nielsen, Ecelbarger and Knepper, 1998; Kwon, Frøkiaer, Fernãndez-Llama, Knepper and Nielsen, 1999; Kim, Jeon, Lee, Kang, Kook, Ahn, Kim, Cho, Kim, Han and Choi, 2000). A correlation between increased urinary Na^+ excretion and elevated GFR has been reported in the rat (Marin-Grez, Fleming and Steinhausen, 1986). It is speculated that the effects of OA on renal Na^+ excretion can be dissociated from its influence on fluid handling. Indeed, some studies dissociate the arginine vasopressin evoked increase in Na^+ excretion via V_1 receptors (Musabayane, Forsling and Balment, 1997) from its V_2 mediated antidiuretic effects. It is speculated that OA administration significantly increased urinary Na^+ excretion perhaps in part mediated via elevation of GFR possibly due to increases of the filtered load of Na^+ in the tubular filtrate. In addition, OA may have inhibited increased proximal reabsorption of Na^+ which occurs in diabetes mellitus (Palm, Liss, Fasching, Hansell and Carlsson, 2001; Vallon, 2003; Rebsomen *et al.*, 2006; Kanetsuna, Hirano, Nagata, Gannon, Takahashi, Harris, Breyer and Takahashi, 2006). Hence, OA improved kidney function in diabetic rats despite the failure to reduce elevated plasma urea concentrations. Kidneys maintain optimum chemical composition of body fluids by removal of urea and creatinine and the concentrations of these metabolites increase in the blood during renal disease. The low plasma creatinine concentrations probably explain the observed increase of GFR of both non-diabetic and STZ-induced diabetic rats treated with OA (Table 4). The influence of kidney mass on GFR in non-diabetic and diabetic rats is unclear as evidenced in the current study. Indeed, there were no apparent differences in kidney mass in all animals though there were apparent differences in GFR between the control and OA-treated animals. It is, therefore, hypothesized that the differences observed in GFR between control and treated rats are probably due to OA treatment.

OA treatment had no effect on urine flow in non-diabetic and STZ-induced diabetic rats, though it reduced water intake by the STZ-induced diabetic rats. The mechanisms by which OA caused these effects could not be elicited in this study. The increased water intake by STZ-induced diabetic rats was perhaps due to polydipsia arising from polyuria. This hypothesis is supported by marked increases in plasma osmolality of STZ-induced diabetic rats compared with the non-diabetic rats. Treatment with OA reduced water intake and plasma osmolality of STZ-induced diabetic rats at the end of the 5-week period perhaps due to increased Na^+ excretion and improved glycaemic control. Indeed OA decreased urine glucose outputs of STZ-induced diabetic rats, suggestive of improved glycaemic control (Rebsomen *et al.*, 2006). The mechanisms involved in the reduction of urine glucose could not be elicited in this study.

OA reduced MAP of non-diabetic and STZ-induced diabetic rats. The potent hypotensive properties of OA confirm previous observations in Dahl salt sensitive rats (Somova, Shode, Ramnanan and Nadar, 2003). The mechanisms of this effect were, however, not elucidated in this study. We speculate this effect may possibly be modulation of the parasympathetic nervous system. The results are significant considering that diabetes is characterized by hypertension, and risks of cardiovascular morbidity and mortality (Palm, Liss, Fasching, Hansell and Carlsson, 2001; Vallon, 2003; Kanetsuna *et al.*, 2006).

CHAPTER 5

5.0. CONCLUSIONS

The results described in this study demonstrate that the *S. cordatum* leaf extract oleanolic acid (OA) has blood glucose lowering effects comparable to standard anti-diabetic drugs. Furthermore, OA augmented the hypoglycaemic effects of insulin in STZ-induced diabetic rats. The results also show that OA-elicited blood glucose-lowering effects in STZ-induced diabetic rats are in part mediated via increased hepatic glycogen synthesis. OA may, therefore, be useful as adjuvant treatment to enhance insulin effects in diabetes mellitus management. The administration of OA improved renal function of STZ-induced diabetic rats as shown by increased urinary Na^+ outputs, reduction of plasma creatinine with concomitant increase in GFR. OA evoked potent hypotensive effects in STZ-induced diabetic rats. The results introduce the first *in vivo* evidence that OA ameliorates kidney function in STZ-induced diabetic rats. This suggests that in addition to ameliorating hyperglycaemia and kidney function, OA has the potential to manage hypertension, a cardiovascular complication that frequently occurs as a result of diabetes mellitus. The findings described in this thesis are of considerable importance because they contextualize the hypoglycaemic, renal and hypotensive effects of OA in the management of diabetes mellitus.

5.1. Shortfalls of the study

The present study had some limitations to elucidate some of the mechanisms of the biological effects of OA. For instance the study did not investigate the effects of OA on reactive oxygen species generation in tissues, glycosylated haemoglobin, lipidaemia and glycogenic enzymes. Moreover, the effects of OA on renally active hormones such as aldosterone and vasopressin should have been established so as to relate to renal function parameters.

5.2. Recommendations for future studies

This study has established increased glycogen synthesis as one of the blood glucose-lowering mechanism of OA. Future studies should, therefore, investigate the effects of OA on glucose transporters as well as the enzymes involved in the glycogenic pathway. To establish the renal function effects of OA, hormones related to sodium homeostasis should be measured, such as aldosterone and vasopressin. Previous studies have indicated increased proximal reabsorption of sodium occurring in diabetes mellitus, with implications for the Na^+-glucose co-transporters, therefore effects of OA on sodium transporters may explain the mechanism of the natriuretic effects observed in this study.

CHAPTER 6

6.0. REFERENCES

1) Adaramoye, O.A., Adeyemi, E.O. (2006). Hypoglycaemic and hypolipidaemic effects of fractions from kolaviron, a biflavonoid complex from *Garcinia kola* in streptozotocin-induced diabetes mellitus rats. *Journal of Pharmacy and Pharmacology; 58: 121-128.*

2) Aiello, L.P. (2002). The potential role of PKC in diabetic retinopathy and macular oedema. *Survey of Ophthalmology; 47: Supplement 2.*

3) Ajjan, R.A., Grant, P.J. (2006). Cardiovascular disease prevention in patients with type 2 diabetes: the role of oral anti-diabetic agents. *Diabetes and Vascular Disease Research; 3:147-58.*

4) Ali, M.S., Jahangir, M., Hussan, S.S., Choudhary, M.I. (2002). Inhibition of α-glucosidase by oleanolic acid and its synthetic derivatives. *Phytochemistry; 60: 295-299.*

5) Almond, A., Siddiqui, S., Robertson, S., Norrie, J., Isles, C. (2008). Comparison of combined urea and creatinine clearance and prediction equations as measures of residual renal function when GFR is low. *Monthly Journal of the Association of Physicians; 101(8): 619-624.*

6) American Diabetes Association (2004). Gestational diabetes mellitus. *Diabetes Care; 27: S88-S90.*

7) Andrade-Cetto, A., Becerra-Jiménez, J., Cárdenas-Vázquez, R. (2008). Alfa-glucosidase-inhibiting activity of some Mexican plants used in the treatment of type 2 diabetes. *Journal of Ethnopharmacology; 116: 27-32.*

8) Aybar, M.J., Riera, A.N.S., Grau, A., Sánchez, S.S. (2001). Hypoglycaemic effect of the water extract of *Smallantus sonchifolius* (yacon) leaves in normal and diabetic rats. *Journal of Ethnopharmacology; 74: 125-132.*

9) Bailey, C.J., Turner, R.C. (1996). Metformin. *The New England Journal of Medicine; 334(9): 574-579.*

10) Bansal, V., Kalita, J., Misra, U.K. (2006). Diabetic neuropathy. *Postgraduate Medical Journal; 82: 95-100.*

11) Barnard, R.J., Youngren, J.F. (1992). Regulation of glucose transport in skeletal muscle. *Federation of American Societies for Experimental Biology Journal; 6: 3238-3244.*

12) Beckman, J.A., Creager, M.A., Libby, P. (2002). Diabetes and atherosclerosis: Epidemiology, pathophysiology and management. *The Journal of American Medical Association; 287(19).*

13) Begum, S., Zehra, S.Q., Siddiqui, B.S. (2008). Two New Pentacyclic Triterpenoids from *Lantana camara* LINN. *Chemical and Pharmaceutical Bulletin; 55(9): 1317-1320.*

14) Bethel, M.A., Feinglos, M.N. (2002). Insulin analogues: new therapies for type 2 diabetes mellitus. *Current diabetes reports; 2(5): 403-408.*

15) Bloomgarden, Z.T. (2008). Diabetic nephropathy. *Diabetes Care; 31(4).*

16) Bouché, C., Serdy, S., Kahn, C.R., Goldfine, A.B. (2004). The cellular fate of glucose and its relevance in type 2 diabetes. *Endocrine Reviews; 25(5): 807-830.*

17) Brandes, R.P., Behra, A., Lebherz, C., Böger, R.H., Bode-Böger, S.M., Mügge, A. (1999). Lovastatin maintains nitric oxide-but not EDHF-mediated endothelium-dependent relaxation in the hyper-cholesterolaemic rabbit carotid artery. *Atherosclerosis; 142: 97-104.*

18) Brown, M.J., Bird, S.J., Watling, S., Kaleta, H., Hayes, L., Eckert, S., Foyt, H.L. (2004). Natural progression of diabetic peripheral neuropathy in the Zenarestat study population. *Diabetes Care; 27: 1153-1159.*

19) Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature; 414.*

20) Bruneton, J. (1995). *Pharmacolognosy, Phytochemistry, Medicinal plants.* Intercept, Hampshire.

21) Buschiazzo, H., Exton, J.H., Park, C.R. (1970). Effects of glucose on glycogen sythetase, phosphorylase and glycogen deposition in the perfused rat liver. *Proceedings of the National Academy of Sciences of the United States of America; 65(2): 383-387.*

22) Buse, M.G. (2006). Hexosamines, insulin resistance and the complications of diabetes: current status. *American Journal of Physiology. Endocrinology and Metabolism; 290(1): E1-E8.*

23) Bwititi, P., Musabayane, C.T. (1997). The effect of plant extracts on plasma glucose levels in rats. *Acta Medica et Biologica (Japan); 45(4): 167-169.*

24) Bwititi, P., Musabayane, C.T., Nhachi, C.F.B (2000). Effects of *Opuntia megacantha* on blood glucose and kidney function in streptozotocin diabetic rats. *Journal of Ethnopharmacology; 69(3): 247-52.*

25) Candy, H.A., McGarry, E.J., Pegel, K.H. (1968). Constituents from *Syzygium cordatum*. *Phytochemistry; 7: 889-890.*

26) Chandramohan, G., Ignacimuthu, S., Pugalendi, K.V. (2008). A novel compound from *Casearia esculenta* (Roxb.) root and its effect on carbohydrate metabolism in streptozotocin-diabetic rats. *European Journal of Pharmacology; 590: 437-443.*

27) Chen, J., Liu, J., Zhang, L., Wu, G., Hua, W., Wu, X., Sun, H. (2006). Pentacyclic triterpenes. Part 3: Synthesis and biological evaluation of oleanolic acid derivatives as novel inhibitors of glycogen phosphorylase. *Bio-organic & Medicinal Chemistry Letters; 2915-2919.*

28) Cheng, J.T., Huang, C.C., Liu, I.M., Tzeng, T.F., Chang, C.J. (2006). Novel mechanism for plasma glucose-lowering action of metformin in streptozotocin-induced diabetic rats. *Diabetes; 55: 819-825.*

29) Chiarelli, F., Di Marzio, D. (2008). Peroxisome proliferator-activated receptor-γ agonists and diabetes: Current evidence and future perspectives. *Vascular Health and Risk Management; 4(2): 297-304.*

30) Choi, C.Y., You, H.J., Jeong, H.G. (2001). Nitric oxide and tumor necrosis factor-α production by oleanolic acid via nuclear factor-κβ activation in macrophages. *Biochemical and Biophysical Research Communications; 288: 49-55.*

31) Connolly, J.D., Hill, R.A. (2002). Triterpenoids. *Natural Products Report; 19(4): 494-513.*

32) Cooper, M.E., Gilbert, R.E., Epstein, M. (1998). Pathophysiology of diabetic nephropathy. *Metabolism; 47(12), Suppl 1: 3-6.*

33) Coskun, O., Kanter, M., Korkmaz, A., 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.*

34) Creager, M.A., Lüscher, T.M. (2003). Diabetes and Vascular disease: Pathophysiology, clinical consequences, and Medical Therapy: Part I. *Circulation; 108:1527-1532.*

35) DeFronzo, R.A. (1999). Pharmacologic therapy for type 2 diabetes mellitus. *Annals of Internal Medicine; 131: 281-303.*

36) DeGroot, J. (2004). The AGE of the matrix: chemistry, consequence and cure. *Current Opinion in Pharmacology; 4:301-305.*

37) Dhindsa, P., Davis, K.R., Donnelly, R. (2002). Comparison of the micro- and macro-vascular effects of glimepiride and gliclazide in metformin-treated patients with Type 2 diabetes: a double-blind, crossover study. *British Journal of Clinical Pharmacology; 55: 616-619.*

38) Di Bona, G.F. (1985). Neural control of renal function: role of renal alpha receptors. *Journal of Cardiovascular Pharmacology; 7 (Suppl 8): S18-S23.*

39) Dzubak, P., Hajduch, M., Vydra, D., Hustova, A., Kvasnica, M., Biedermann, D., Markova, L., Urban, M., Sarek, J. (2006). Pharmacological activities of natural triterpenoids and their therapeutic implications. *Natural Products Report; 23(3): 394-411.*

40) Emilien, G., Maloteaux, J.M., Ponchon, M. (1999). Pharmacological management of diabetes: recent progress and future perspective in daily drug treatment. *Pharmacology & Therapeutics; 81(1): 37-51.*

41) Esposito, K., Francesco, N., Marfella, R., Giugliano, G., Giugliano, F., Ciotola, M., Quagliaro, L. (2002). Inflammatory cytokine concentrations are acutely increased by hyperglycaemia in humans: Role of oxidative stress. *Circulation; 106: 2067-2072.*

42) Evcimen, N.D., King, G.L. (2007). The role of protein kinase C activation and the vascular complications of diabetes. *Pharmacological Research; 55: 408-510.*

43) Fan, J., Watanabe, T. (2003). Inflammatory reactions in the pathogenesis of atherosclerosis. *Journal of Atherosclerosis and Thrombosis; 10(2): 63-71.*

44) Fernãndez-Llama, P., Andrews, P., Ecelbarger, C.A., Nielsen, S., Knepper, M. (1998). Concentrating defect in experimental nephritic syndrome: altered expression of aquaporins and thick ascending limb Na^+ transporters. *Kidney International; 54(1): 170-9.*

45) Fonseca, V. (2006). Management of diabetes in the hospital. *Reviews in Cardiovascular Medicine; 7 (Suppl 2): S10-7.*

46) Fowler, M.J. (2007). Diabetes treatment, Part 2: Oral agents for glycaemic management. *Clinical Diabetes; 25(4).*

47) Fryer, L.G., Parbu-Patel, A., Carling, D. (2002). The anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signalling pathways. *The Journal of Biological Chemistry; 277(28): 25226-25232.*

48) Fuentes, O., Alarcón, J. (2006). *Bauhinia candicans* stimulation of glucose uptake in isolated gastric glands of normal and diabetic rabbits. *Fitoterapia; 77: 271-275.*

49) Gao, D., Li, Q., Liu, Z., Li, Y., Liu, Z., Fan, Y., Li, K., Han, Z., Li, J. (2007). Hypoglycaemic effects and mechanisms of action of *Cortex Lycii radicis* on alloxan-induced diabetic mice. *The Pharmaceutical Society of Japan; 127(10): 1715-1721.*

50) Gerich, J.E. (2003). Clinical significance, pathogenesis, and management of postprandial hyperglycaemia. *Archives of Internal Medicine; 163:1306-1316.*

51) Gershell, L. (2005). Type 2 diabetes market. Nature reviews. *Drug Discovery; 4(5): 367-8.*

52) Ghosh, D., Thejomoorthy, P., Veluchamy. (1981). Anti-inflammatory and analgesic activities of oleanolic acid 3-/3-glucoside (RDG-1) from *Randia dumetorum* (Rubiaceae). *Indian Journal of Pharmacology; 15(4): 331-342.*

53) Gilani, A.H., Rahman, A.U. (2005). Trends in ethnopharmacology. *Journal of Ethnopharmacology; 100(1-2): 43-49.*

54) Gnudi, L., Thomas, S.M., 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.*

55) Gnudi, L., Viberti, G., Raij, L., Rodriguez, V., Burt, D., Cortes, P., Hartley, B., Thomas, S., Maestrini, S., Gruden, G. (2003). Glut-1 over-expression Link between haemodynamic and metabolic factors in glomerular injury. *Hypertension; 42: 19-24.*

56) Gondwe, M., Kamadyaapa, D.R., Tufts, M.A., Chuturgoon, A.A., Ojewole, J.A.O., Musabayane, C.T. (2008). Effects of *Persea americana* (Mill) [Lauraceae] ["Avocado"] ethanolic extract on blood glucose and kidney function in streptozotocin-induced diabetic rats and on kidney cell lines of the proximal (LLC-

PK1) and distal tubules (MDBK). *Methods and Findings in Experimental and Clinical Pharmacology; 30(1): 25-35.*

57) Gray, A.M., Abdel-Wahab, Y.H.A., Flatt, P.R. (2000). The traditional plant treatment, *Sambucus nigra* (elder), exhibits insulin-like and insulin-releasing actions in vitro. *Journal of Nutrition; 130: 15-20.*

58) Gray, A.M., Flatt, P.R. (1999). Insulin secreting activity of the traditional antidiabetic plant *Viscum album* (mistletoe). *Journal of Endocrinology; 160: 409-414.*

59) Grover, J.K., Vats, V., Rathi, S.S. (2000). Anti-hyperglycaemic effect of *Eugenia jambolana* and *Tinospora cordifolia* in experimental diabetes and their effects on key metabolic enzymes involved in carbohydrate metabolism. *Journal of Ethnopharmacology; 73(3): 461-470.*

60) Grover, J.K., Yadav, S., Vats,V. (2002). Medicinal plants of India with antidiabetic potential. *Journal of Ethnopharmacology; 81: 81-100.*

61) Gruden, G., Thomas, S., Burt, D., Lane, S., Chusney, G., Sacks, S., Viberti, G. (1997). Mechanical stretch induces vascular permeability factor in human mesangial cells: Mechanisms of signal transduction. *Proceedings National Academy of Sciences (USA); 94: 12112-12116.*

62) Grundy, S.M. (2006). Does the metabolic syndrome exist? *Diabetes Care; 29(7): 1689-1692.*

63) Gupta, R.K., Kesari, A.N., Murthy, P.S., Chandra, R., Tandon, V., Watal, G. (2005). Hypoglycaemic and antidiabetic effect of ethanolic extract of leaves of *Annona squamosa* L. in experimental animals. *Journal of Ethnopharmacology; 99: 75-81.*

64) Gurib-Fakem, A. (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular aspects of Medicine; 27: 1-93.*

65) Halat, K.M., Denneby, C.E. (2003). Botanicals and dietary supplements in diabetic peripheral neuropathy. *The Journal of the American Board of Family Practice; 16: 47-57.*

66) Haller, H., Drabm, M., Luft, F.C. (1996). The role of hyperglycaemia and hyperinsulinaemia in the pathogenesis of diabetic angiopathy. *Clinical Nephrology; 46(4): 246-55.*

67) Han, K.L., Choi, J.S., Lee, J.Y., Song, J., Joe, M.K., Jung, M.H., Hwang, J. (2008). Therapeutic potential of peroxisome proliferators–activated receptor-α/γ dual agonist with alleviation of endoplasmic reticulum stress for the treatment of diabetes. *Diabetes; 57: 737-745.*

68) Hannan, J.M.A., Marenah, L., Ali, L., Rokeya, B., Flatt, P.R., Abdel-Wahab, Y.H. (2007). Insulin secretory actions of extracts of *Asparagus racemosus* root in perfused pancreas, isolated islets and clonal β-cells. *Journal of Endocrinology; 192: 159-168.*

69) Hansson, G.K. (2005). Mechanisms of disease: Inflammation, atherosclerosis, and coronary artery disease. *New England Journal of Medicine; 352:1685-1695.*

70) Hargrove, G.M., Dufresne, J., Whiteside, C., Muruve, D.A., Wong, N.C.W. (2000). Diabetes mellitus increases endothelin-1 gene transcription in rat kidney. *Kidney International; 58: 1534-1545.*

71) Hartog, J.W.L., Voors, A.A., Bakker, S.J.L., Smit, A.J., van Veldhuisen, D.J. (2007). Advanced glycation end-products (AGE's) and heart failure: Pathophysiology and clinical implications. *European Journal of Heart Failure; 9:*

1146-1155.

72) Hirsch, I.B., Bode, B.W., Garg, S., Lane, W.S., Sussman, A., Hu, P., Santiago, O.M., Kolaczynski, J.W., Insulin Aspart CSII/MDI Comparison Study Group. (2005). Continuous subcutaneous insulin infusion (CSII) of insulin aspart versus multiple daily injection of insulin aspart/insulin glargine in type 1 diabetic patients previously treated with CSII. *Diabetes Care; 28(3): 533-538.*

73) Hoa, N.K., Norberg, A., Sillard, R., Van Phan, D., Thuan, N.D., Dzung, D.T., Jörnvall, H., Ostenson, C.G. (2007). The possible mechanisms by which phanoside stimulates insulin secretion from rat islets. *Journal of Endocrinology; 192(2): 389-394.*

74) Holman, R.R., Paul, S.K., Bethel, M.A., Matthews, D.R., Neil, H.A.W. (2008). 10-year follow-up of intensive control in type 2 diabetes. *New England Journal of Medicine; 359.*

75) Hostettmann, K., Marston, A. (1995). *Chemistry and Pharmacology of Natural Products: Saponins.* Cambridge University Press, UK.

76) Hsu, F.L., Liu, I.M., Kuo, D.H., Chen, W.C., Su, H.C., Cheng, J.T. (2003). Antihyperglycaemic effect of puerarin in streptozotocin-induced diabetic rats. *Journal of Natural Products; 66: 788-792.*

77) Ihara, Y., Egashira, K., Nakano, K., Ohtani, K., Kubo, M., Koga, J., Iwai, M., Horiuchi, M., Gang, Z., Yamagishi, S., Sunagawa, K. (2007). Upregulation of the ligand-RAGE pathway via the angiotensin II type I receptor is essential in the pathogenesis of diabetic atherosclerosis. *Journal of Molecular and Cellular Cardiology; 43: 455-464.*

78) Inzucchi, S.E. (2002). Oral antihyperglycaemic therapy for type 2 diabetes. *Journal of the American Medical Association; 287: 360-372.*

79) Iwu, M.M. (1993). *Handbook of African Medicinal Plants.* CRC Press, Florida

80) Jayaprakasam, B., Vareed, S.K., Olson, L.K., Nair, M.G. (2005). Insulin secretion by bioactive anthocyanins and anthocyanidins present in fruits. *Journal of Agricultural and Food Chemistry; 53: 28-31.*

81) Jensen, J., Jebens, E., Brennesvik, E.O., Ruzzin, J., Soos, M.A., Engebretsen, E.M.L., O'Rahilly, S., Whitehead, J.P. (2006). Muscle glycogen inharmoniously regulates glycogen synthase activity, glucose uptake, and proximal insulin signaling. *American Journal of Physiology Endocrinology and Metabolism; 290: E154-E162.*

82) Jouad, H., Haloui, M., Rhiouani, H., Hilaly, J.E., Eddouks, M. (2001). Ethnobotanical survey of medicinal plants used for the treatment of diabetes, cardiac and renal diseases in the North centre region of Morocco (Fez-Boulemane). *Journal of Ethnopharmacology; 77: 175-182.*

83) Jung, S.H., Ha, Y.J., Shim, E.K., Choi, S.Y., Jin, J.L., Yun-Choi, H.S., Lee, J.R. (2007). Insulin-mimetic and insulin-sensitizing activities of a pentacyclic triterpenoid insulin receptor activator. *Biochemical Journal; 403: 243-250.*

84) Jung, M., Park, M., Lee, H.C., Kang, Y.H., Kang, E.S., Kim, S.K. (2006). Antidiabetic agents from medicinal plants. *Current Medicinal Chemistry 13: 1203-1218.*

85) Junod, A., Lambert, A.E., Stauffacher, W., Renold, A.E. (1969). Diabetogenic action of streptozotocin: relationship of dose to metabolic response. *Journal of Clinical Investigation; 48: 866-872.*

86) Kakuda, D.K., MacLeod, C.L. (1994). Na(+)-independent transport (uniport) of amino acids and glucose in mammalian cells. *Journal of Experimental Biology; 196: 93-108.*

87) Kakuda, T., Sakane, I., Takihara, T., Ozaki, Y., Takeuchi, H., Kuroyanagi, M. (1996). Hypoglycemic effect of extracts from *Lagerstroemia speciosa* L. leaves in genetically diabetic KK-AY mice. *Bioscience Biotechnology and Biochemistry; 60(2): 204-8.*

88) Kanetsuna, Y., Hirano, K., Nagata, M., Gannon, M.A., Takahashi, K., Harris, R.C., Breyer, M.D., Takahashi, T. (2006). Characterization of diabetic nephropathy in a transgenic model of hypoinsulinemic diabetes. *American Journal of Physiology, Renal Physiology; 291: F1315-F1322.*

89) Kang, K.S., Kim, H.Y., Yamabe, N., Nagai, R., Yokozawa, T. (2006). Protective Effect of Sun Ginseng against Diabetic Renal Damage. *Biological and Pharmaceutical Bulletin; 29(8): 1678-1684.*

90) Katz, S.R., Newman, R.A., Lansky, E.P. (2007). Mini Review *Punica granatum*: Heuristic treatment for Diabetes Mellitus. *Journal of Medicinal Food 10(2): 213-217.*

91) Khan, Z.A., Chakrabarti, S. (2007). Cellular signalling and potential new treatment targets in diabetic retinopathy. *Experimental Diabetes Research Volume.*

92) Khan, Z.A., Farhangkhoee, H., Mahon, J.L., Bere, L., Gonder, J.R., Chan, B.M., Uniyal, S., Chakrabarti, S. (1999). Endothelins: Regulators of extracellular matrix protein production in Diabetes. *Experimental Biology and Medicine; 231: 1022-1029.*

93) Kim, H.Y., Kang, K.S., Yamabe, N., Nagai, R., Yokozawa, T. (2007a). Protective effect of heat-processed American ginseng against diabetic renal damage in rats. *Journal of Agricultural and Food Chemistry; 55: 8491-8497.*

94) Kim, J.D., Kang, S.M., Park, M.Y., Jung, T.Y., Choi, H.Y., Ku, S.W. (2007b). Ameliorative anti-diabetic activity of *Dangnyosoko*, a Chinese herbal medicine in diabetic rats. *Bioscience, Biotechnology and Biochemistry; 71 (6): 1527-1534.*

95) Kim, Y., Wang, M., Rhee, H. (2004). A novel α-glucosidase inhibitor from pine bark. *Carbohydrate Research; 339: 715-717.*

96) Kim, S.W., Jeon, Y.S., Lee, J.U., Kang, D.G., Kook, H., Ahn, K.Y., Kim, S.Z., Cho, K.W., Kim, N.H., Han, J.S., Choi, K.C. (2000). Diminished adenylate cyclase activity and aquaporin 2 expression in acute renal failure rats. *Kidney International; 57(4): 1239-1417.*

97) Kirpichnokov, D., McFarlane, S.I., Sowers, J.R. (2002). Metformin: an update. *Annals of Internal Medicine; 137(1): 150.*

98) Kolm-Litty, V., Sauer, U., Nerlich, A., Lehmann, R., Schleicher, E.D. (1998). High glucose-induced transforming growth factor β1 production is mediated by the hexosamine pathway in porcine glomerular mesangial cells. *Journal of Clinical Investigations; 101: 160-169.*

99) Kowluru, R.A., Chan, P.S. (2007). Oxidative stress and diabetic retinopathy. *Experimental Diabetes Research Volume; 2007: 43603.*

100) Krentz, A.J., Bailey, C.J. (2005). Oral antidiabetic agents: Current role in type 2 diabetes mellitus. *Drugs; 65(3): 385-411.*

101) Kudzma, D.J. (2002). Effects of thiazolidinediones for early treatment of type 2 diabetes mellitus. *American Journal of Managed Care; 8: S472-S482.*

102) Kumar, G.S., Shetty, A.K., Salimath, P.V. (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.*

103) Kwon, T.H., Frøkiaer, J., Fernãndez-Llama, P., Knepper, M, A., Nielsen, S (1999). Reduced abundance of aquaporins in rats with bilateral ischemia-induced acute renal failure: prevention by alpha-MSH. *The American Journal of Physiology; 277(3 Pt 2): F413-427.*

104) Lassila, M., Seah, K.K., Allen, T.J., Thallas, V., Thomas, M.C., Candido, R., Burns, W.C., Forbs, J.M., Calkin, A.C., Cooper, M.E., Jandeleit-Dahm, K.A.M. (2004). Accelerated nephropathy in diabetic apolipoprotein E-knockout mouse: Role of advanced glycation. *Journal of the American Society of Nephrology; 15: 2125-2138.*

105) Latha, M., Pari, L., Sitasawad, S., Bhonde, R. (2004). Insulin-secretagogue activity and cytoprotective role of the traditional antidiabetic plant *Scoparia dulcis* (Sweet Broomweed). *Life Sciences; 75: 2003-2014.*

106) Laube, B.L. (2002). Advances in treating diabetes with aerosolized insulin. *Diabetes Technology & Therapeutics; 49(4): 515-518.*

107) Lee, A., Morley, J.E. (1998). Metformin decreases food consumption and induces weight loss in subjects with obesity with type II non-insulin dependent diabetes. *Obesity Research; 6(1): 47-53.*

108) Lee, H.K., Nam, G.W., Kim, S.H., Lee, S.H. (2006). Phytocomponents of triterpenoids, oleanolic acid and ursolic acid, regulated differently the processing of

epidermal keratinocytes via the PPAR-α pathway. *Experimental Dermatology: 66-73.*

109) Li, W., Dai, R.J., Yu, Y.H., Li, L., Wu, C.M., Luan, W.W., Meng, W.W., Zhang, X.S., Deng, Y.L. (2007). Antihyperglycaemic effect of *Cephalotaxus sinensis* leaves and GLUT-4 translocation facilitating activity of its flavonoid constituents. *Biological and Pharmaceutical Bulletin; 30(6): 1123-1129.*

110) Li, Y., Wen, S., Kota, B.P., Peng, G., Li, G.Q., Yamahara, J., Roufogalis, B.D. (2005). Punica granatum flower extract, a potent α-glucosidase inhibitor, improves postprandial hyperglycemia in Zucker diabetic fatty rats. *Journal of Ethnopharmacology; 99: 239-244.*

111) Libal-Weksler, Y., Gotlibovitz, O., Stark, A.H., Madar, Z. (2001). Diet and diabetic state modify glycogen synthase activity and expression in rat hepatocytes. *Journal of Nutritional Biochemistry; 12: 458-464.*

112) Link, J.T., Sorensen, B.K. (2000). A method for preparing C-glycosides related to phlorizin. *Tetrahedron Letters; 41: 9213-9217.*

113) Little, A.A., Edwards, J.L., Feldman, E.L. (2007). Diabetic neuropathies. *Practical Neurology; 7:82-92.*

114) Liu, J. (1995). Pharmacology of oleanolic acid and ursolic acid. *Journal of Ethnopharmacology; 49(2): 57-68.*

115) Liu, J. (2005). Oleanolic acid and ursolic acid: research perspectives. *Journal of Ethnopharmacology; 100(1-2): 92-94.*

116) Lorenzi, M. (2007). The polyol pathway as a mechanism for diabetic retinopathy: attractive, elusive, and resilient. *Experimental Diabetes Research; 2007: 61038.*

117) Luzi, L., Pozza, G. (1997). Glibenclamide: an old drug with a novel mechanism of action? *Acta Diabetologica; 34: 239-244.*

118) Machha, A., Achike, F.I., Mustafa, A.M., Mustafa, M.R. (2007). Quercetin, a flavonoid antioxidant, modulates endothelium-derived nitric oxide bioavailability in diabetic rat aortas. *Nitric Oxide; 16: 442-447.*

119) Mahato, S.B., Kundu, A.P. (1994). ^{13}C NMR Spectra of pentacyclic triterpenoids-a complication and some salient features. *Phytochemistry; 37:1517-1573.*

120) Maiti, R., Das, U.K., Ghosh, D. (2005). Attenuation of hyperglycaemia and hyperlipidaemia in streptozotocin-induced diabetic rats by aqueous extract of seed *Tamarindus indica. Biological and Pharmacology Bulletin; 28(7): 1172-1176.*

121) Marin-Grez, M., Fleming, J.T., Steinhausen, M. (1986). Atrial natriuretic peptide causes pre-glomerular vasodilation and post-glomerular vasoconstriction in rat kidney. *Nature; 324: 473-476.*

122) Marles, R., Farnsworth, N. (1994). *Plants as sources of antidiabetic agents.* In: Wagner, H., Farnsworth, N.R. (Eds.), Economic and Medicinal Plant Research, vol. 6. Academic Press Ltd., UK, pp. 149-187.

123) Marshall, S., Garvey, W.T., Traxinger, R.R. (1991). New insights into the metabolic regulation of insulin action and insulin resistance: role of glucose and amino acids. *Federation of American Societies for Experimental Biology Journal; 5: 3031-3036.*

124) Mason, R.M., Wahab, N.A. (2003). Extra-cellular matrix metabolism in diabetic nephropathy. *Journal of American Society of Nephrology; 14: 1358-1373.*

125) Massi-Benedetti., M., Orsini-Federici, M. (2008). Treatment of type 2 diabetes with combined therapy. *Diabetes Care; 31(S2): S131-S135.*

126) Matsuda, H., Li, Y., Mukarami, T., Yamahara, J., Yoshikawa, M. (1999). Structure-related inhibitory activity of oleanolic acid glycosides on gastric emptying in mice. *Bio-organic & Medicinal Chemistry; 7(2): 323-327.*

127) Matsuda, H., Mukarami, T., Li, Y., Yamahara, J., Yoshikawa, M. (1998). Mode of action of ecsins Ia and II a and E, Z-senegin II on glucose absorption in gastrointestinal tract. *Bio-organic & Medicinal Chemistry; 6(7): 1019-1023.*

128) Mezei, O., Banz, W.J., Steger, R.W., Peluso, M.R., Winters, T.A., Shay, N. (2003). Biochemical and molecular actions of nutrients soy isoflavones exert antidiabetic and hypolipidemic effects through the PPAR pathways in obese Zucker rats and murine raw 264.7 cells1. *Journal of Nutrition; 133: 1238-1243.*

129) Miura, T., Itoh, Y., Kaneko, Y., Ueda, N., Ishida, T., Fukushima, M., Matsuyama, F., Seino, Y. (2004). Corosolic acid induces GLUT-4 translocation in genetically type 2 diabetic mice. *Biological and Pharmaceutical Bulletin; 27(7): 1103-1105.*

130) Modak, M., Dixit, P., Londhe, J., Ghaskadbi, S., Devasagayam, P.A.T. (2007). Indian herbs and herbal drugs used for the treatment of diabetes. *Journal of Clinical Biochemistry and Nutrition; 40(3): 163-173.*

131) Mogensen, C.E. (2003). Genetics and diabetic renal disease: still a black hole. *Diabetics Care; 26(5): 1631-1632.*

132) Musabayane, C.T., Forsling, M.L., Balment, R.J. (1997). Arginine vasopressin increases renal sodium excretion in the anaesthetized rat through v1 receptors. *Renal Failure; 19(1): 23-32.*

133) Musabayane, C.T., Gondwe, M., Kamadyaapa, D.R., Chuturgoon, A.A., Ojewole, J.A.O. (2007). Effects of *ficus thonningii* (Blume)[Moraceae] 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.*

134) Musabayane, C.T., Mahlalela, N., Shode, F.O., Ojewole, J.A.O. (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.*

135) Musabayane, C.T., Ndhlovu, C.E., Balment, R.J. (1995). Renal fluid and electrolyte handling in streptozotocin-diabetic rats. *Renal Failure; 17(2):107-116.*

136) Musabayane, C.T., Xozwa, K., Ojewole, J.A.O. (2005). Effects of *Hypoxis hemerocallidea* (Fisch. & C.A. Mey) [Hypoxidaceae] corm (African Potato) aqueous extract on renal electrolyte and fluid handling in the rat. *Renal Failure; 27(5): 763-770.*

137) Musi, N., Hirshman, M.F., Nygren, J., Svanfeldt, M., Bavenholm, P., Rooyackers, O., Zhou, G., Williamson, J.M., Ljunqvist, O., Efendic, S., Moller, D.E., Thorell, A., Goodyear, L.J. (2002). Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. *Diabetes; 51(7): 2074-2081.*

138) Naik, S.R., Barbosa Filho, J.M., Dhuley, J.N., Deshmukh, V. (1991). Probable mechanism of hypoglycaemic activity of basic acid, a natural product isolated from *Bumelia sartorum. Journal of Ethnopharmacology; 33(1-2): 37-44.*

139) Nakagawa, T., Goto, H., Hikiami, H., Yokozawa, T., Shibahara, N., Shimada, Y. (2007). Protective effects of keishibukuryogan on the kidney of spontaneously

diabetic WBN/Kob rats. *Journal of Ethnopharmacology; 110: 311-317.*

140) Narendhirakannan, R.T., Subramanian, S., Kandaswamy, M. (2006). Biochemical evaluation of anti-diabetogenic properties of some commonly used Indian plants on streptozotocin-induced diabetes in experimental animals *Clinical and Experimental Pharmacology and Physiology; 33(12): 1150-1157.*

141) Natarajan, R., Nadler, J.L. (2004). Lipid inflammatory mediators in diabetic vascular disease. *Arteriosclerosis, Thrombosis and Vascular Biology; 24: 1542-1548.*

142) Oh, Y.K., Joo, K.W., Lee, J.W., Jeon, U.S., Lim, C.S., Han, J.S., Knepper, M.A., Na, K.Y. (2007). Altered renal sodium transporter expression in an animal model of type 2 diabetes mellitus. *Journal of Korean Medical Science; 22: 1034-1041.*

143) Okine, L.K., Nyarko, A.K., Osei-Kwabena, N., Oppong, I.V., Barnes, F., Ofosuhene, M. (2005). The antidiabetic activity of the herbal preparation ADD-199 in mice: a comparative study with two oral hypoglycaemic drugs. *Journal of Ethnopharmacology; 97(1): 31-38.*

144) Ong, K.C., Khoo, H.E. (2000). Effects of myricetin on glycaemia and glycogen metabolism in diabetic rats. *Life Sciences; 67: 1695-1705.*

145) Pacher, P., Obrosova, I.G., Mabley, J.G., Szabo (2005). Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications: Emerging new therapeutic strategies. *Current Medicinal Chemistry; 12(3): 267-275.*

146) Palm, F., Liss, P., Fasching, A., Hansell, P., Carlsson, P.O. (2001). Transient glomerular hyperfiltration in the streptozotocin-diabetic Wistar Furth rat. *Upsala Journal of Medical Sciences; 106: 175-182.*

147) Panayotova-Heiermann, M., Loo, D.D.F., Kong, C., Lever, J.E., Wright, E.M. (1996). Sugar binding to Na^+/glucose co-transporters is determined by the carboxyl-terminal half of the protein. *The Journal of Biological Chemistry; 271(17): 10029-10034.*

148) Parturier, G. (1957). Galega, the liver and diabetes. *Revue Medico-Chirurgicale Des Maladies Du Foie; 26(12): 5-8.*

149) Paterson, A.D., Rutledge, B.N., Lachin, J.M., Crow, R.S. (2007). The effect of intensive diabetes treatment on resting heart rate in type 1 diabetes; The Diabetes Control and Complications Trial/epidemiology of diabetes interventions and complications study. *Diabetes Care; 30: 2107-2112.*

150) Pinent, M., Blay, M., Bladé, M.C., Salvadó, M.J., Arola, L., Ardé, A. (2004). Grape seed-derived procyanidins have an anti-hyperglycaemic effect in streptozotocin-induced diabetic rats and insulino-mimetic activity in insulin-sensitive cell lines. *Endocrinology; 145: 4985-4990.*

151) Pushparaj, P.N., Tan, B.K.H., Tan, C.H. (2001). The mechanism of hypoglycaemic action of the semi-purified fractions of *Averrhoa bilimbi* in streptozotocin-diabetic rats. *Life Sciences; 70: 535-547.*

152) Rahbar, S., Figarola, J.L. (2003). Novel inhibitors of advanced glycation end-products. *Archives of Biochemistry and Biophysics; 419: 63-79.*

153) Rahimi, R., Nikfar, S., Larijani, B., Abdollahi, M. (2005). Dossier: A review on the role of antioxidants in the management of diabetes and its complications. *Biomedicine & Pharmacology; 59: 365-373.*

154) Rao, N.K., Nammi, S. (2006). Antidiabetic and renoprotective effects of the chloroform extract of *Terminalia chebula* Retz. Seeds in streptozotocin-induced

diabetic rats. *BMC Complementary and Alternative Medicine; 6:17.*

155) Rebsomen, L., Pitel, S., Boubred, F., Buffat, C., Feuerstein, J.M., Raccah, D., Vague, P., Tsimaratos, M. (2005). C-peptide replacement improves weight gain and renal function in diabetic rats. *Diabetes Metabolism; 32: 223-228.*

156) Rendell, M. (2004). Advances in diabetes for the millennium: drug therapy of type 2 diabetes. *Medscape General Medicine; 6(3 Suppl): 9.*

157) Reusch, J.E.B. (2003). Diabetes, microvascular complications, and cardiovascular complications: What is it about glucose? *Journal of Clinical Investigations; 112: 986-988.*

158) Robertson, C., Drexler, A.J., Vernillo, A.T. (2003). Update on diabetes diagnosis and management. *Journal of American Dental Association; 134: 16S-23S.*

159) Ruiz, C., Alegria, A., Barbera, R., Farre, R., Lagarda, M. J. (1994). Lipid peroxidation and antioxidant enzyme activities in patient with type-I diabetes mellitus. *Scandinavian Journal of Clinical and Laboratory Investigation; 59: 99-105.*

160) Sadeghi, M.M. (2006). The pathobiology of the vessel wall: Implications for imaging. *Journal of Nuclear Cardiology; 13: 402-414.*

161) Sakurai, S., Yonekura, H., Yamamoto, Y., Watanabe, T., Tanaka, N., Li, H., Rahman, A.K.M.A., Myint, K.M., Kim, C.H., Yamamoto, H. (2003). The AGE-RAGE system and diabetic nephropathy. *Journal of American Society of Nephrologists; 14: S259-S263.*

162) Saltiel, A.R., Kahn, C.R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature; 414(6865): 799-806.*

163) Sarafidis, P.A. (2007). Thiazolidinediones and diabetic nephropathy: Need for a closer examination? *Journal of Cardiometabolic Syndrome; 2:297-301.*

164) Sato, S., Yamate, J., Hori, Y., Hatai, A., Nozawa, M., Sagai, M. (2005). Protective effect of polyphenol-containing azuki bean (*Vigna angularis*) seed coats on the renal cortex in streptozotocin-induced diabetic rats. *Journal of Nutritional Biochemistry; 16: 547-553.*

165) Satirapoj, B., Supasyndh, O., Patumanond, J., 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 (10):1584-1591.*

166) Schena, F.P., Gesualdo, L. (2005). Pathogenic mechanisms of diabetic nephropathy. *Journal of American Society of Nephrologists; 16: S30-S33.*

167) Sharma, S.B., Nasir, A., Prabhu, K.M., Murthy, P.S., Dev, G. (2003). Hypoglycaemic and hypolipidaemic effect of ethanolic extract of seeds of *Eugenia jambolana* in alloxan-induced diabetic rabbits. *Journal of Ethnopharmacology; 85: 201-206.*

168) Sheetz, M.J., King, G.L. (2002). Molecular understanding of hyperglycaemia's adverse effects for diabetes complications. The *Journal of the American Medical Association; 288: 2579-2588.*

169) Shinde, J., Taldone, T., Barletta, M., Kunaparaju, N., Hu, B., Kumar, S., Placido, J., Zito, S.W. (2008). α-Glucosidase inhibitory activity of *Syzygium cumini* (Linn.) Skeels seed kernel in vitro and in Goto–Kakizaki (GK) rats. *Carbohydrate Research; 343: 1278-1281.*

170) Shirosaki, M., Koyama, T., Yazawa, K. (2008). Anti-hyperglycemic activity of kiwifruit leaf (*Actinidia deliciosa*) in mice. *Bioscience, Biotechnology and Biochemistry; 72(4): 1099-1102.*

171) Shishodia, S., Majumdar, S., Banerjee, S., Aggarwal, B.B. (2003). Ursolic acid inhibits nuclear factor-kappa B activation induced by carcinogenic agents through suppression of IkappaBalpha kinase and p65 phosphorylation: correlation with down-regulation of cyclooxygenase 2, matrix metalloproteinase 9, and cyclin D1. *Cancer Research; 63(15): 4375-4383.*

172) Skaer, T.L., Sclar, D.A., Robinson, L.M. (2006). Trends in the prescribing of oral agents for the management of type 2 diabetes mellitus in the United States, 1990-2001: Does type of insurance influence access to innovation? *The Diabetes Educator; 32(6) November/December.*

173) Sobel, B.E., Schneider, D.J. (2005). Cardiovascular complications in diabetes mellitus. *Current Opinion in Pharmacology; 5: 143-148*

174) Sobngwi, E., Mauvais-Jarvis, F., Vexiau, P., Mbanya, J.C., Gautier, J.F. (2001). Diabetes in Africans part 1: epidemiology and clinical specificities. *Diabetes Metabolism (Paris); 27: 628-634.*

175) Somova, L.I., Shode, F.O., Ramnanan, P., Nadar, A. (2003). Antihypertensive, antiathersoclerotic and antioxidant activity of triterpenoids isolated from *Olea europaea*, subspecies *africana* leaves. *Journal of Ethnopharmacology 84: 299-305.*

176) Somova, L.O., Nadar, A., Rammanan, P., Shode, F.O. (2003). Cardiovascular, antihyperlipidemic and antioxidant effects of oleanolic and ursolic acids in experimental hypertension. *Phytomedicine 10:115-121.*

177) Sorokin, A., Kohan, D.E. (2003). Physiology and pathology of endothelin-1 in renal mesangium. *American Journal of Physiology. Renal Physiology; 285: F579-F589.*

178) Srinivasan, K., Ramarao, P. (2007). Animal models in type 2 diabetes research: An overview. *Indian Journal of Medical Research; 125: 451-472.*

179) Stangeland, T., Dhillion, S.S., Reksten, H. (2008). Recognition and development of traditional medicine in Tanzania. *Journal of Ethnopharmacology; 117: 290-299.*

180) Stengel, B., Billon, S., van Dijk, P.C.W., Jager, K.J., Dekker, F.W., Simpson, K., Briggs, J.D. (2003). Trends in the incidence of renal replacement therapy for end-stage renal disease in Europe, 1990-1999. *Nephrology Dialysis Transplantation; 18 (9):1824-1833.*

181) Stoclet, J., Chataigneau, T., Ndiaye, M., Oak, M., El Bedoui, J., Chataigneau, M., Schini-Kerth, V.B. (2004). Vascular protection by dietary polyphenols. *European Journal of Pharmacology; 500: 299-313.*

182) Støvring, H., Andersen, M., Beck-Nielsen, H., Green, A., Vach, W. (2007). Counting drugs to understand the disease: The case of measuring the diabetes epidemic. *Population Health Metrics, 5:2.*

183) Subramanian, R., Asmawi, M.Z., Sadikun, A. (2008). In vitro α-glucosidase and α-amylase enzyme inhibitory effects of *Andrographis paniculata* extract and andrographolide. *Acta Biochimica Polonica; 55(2): 391-398.*

184) Takei, I., Kasatani, T. (2004). Future therapy of diabetes mellitus. *Biomedicine & Pharmacotherapy; 58(10): 578-81.*

185) Tana, B.K.H., Tanb, C.H., Pushparaj, P.N. (2005). Anti-diabetic activity of the semi-purified fractions of *Averrhoa bilimbi* in high fat diet fed-streptozotocin-induced diabetic rats. *Life Sciences; 76: 2827-2839.*

186) Tesfaye, S. (2003). Diabetic neuropathy: achieving best practice. *British Journal of Diabetes & Vascular Disease; 3 (2): 112-117.*

187) Thallas-Bonke, V., Thorpe, S.R., Coughlan, M.T., Fukami, K., Yap, F.Y.T., Sourris, K.C., Penfold, S.A., Bach, L.A., Cooper, M.E., Forbes, J.M. (2008). Inhibition of NADPH oxidase prevents advanced glycation end product-mediated damage through a protein kinase C-α-dependent pathway. *Diabetes; 57:460-469.*

188) The Committee of the Japan Diabetes Society on the diagnostic criteria of diabetes mellitus (2002). Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. *Diabetes Research & Clinical Practice 55; 65-85.*

189) The Diabetes Control and Complications Trial Research Group (1993). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *New England Journal of Medicine; 329: 977-986.*

190) Thomson, S.C., Vallon, V., Blantz, R.C. (2004). Kidney function in early diabetes: the tubular hypothesis of glomerular filtration. *American Journal of Physiology. Renal Physiology; 286: F8-F15.*

191) Tiwari, A.K., Rao, J.M. (2002). Diabetes mellitus and multiple therapeutic approaches of phytochemicals: Present status and future prospects. *Current Science; 83(1).*

192) Ubl, J.J., Chen, S., Stucki, J.W. (1994). Anti-diabetic biguanides inhibit hormone-induced intracellular Ca^{2+} concentration oscillations in rat hepatocytes. *The Biochemical Journal; 304: 561-567.*

193) Vallon, V. (2003). Tubuloglomerular feedback and the control of glomerular filtration rate. *News of Physiological Sciences; 18: 169-174.*

194) van Hoek, I., Lefebvre, H.P., Kooistra, H.S., Croubels, S., Binst, D., Peremans, K., Daminet, S. (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(4): 879-885.*

195) Van Wyk, B.E., Van Oudtshoorn, B., Gericke, N. (1997). *Medicinal plants of South Africa.* 1st edition. Briza Publications, South Africa.

196) Vázquez-Carrera, M., Silvestre, J.S. (2004). Insulin analogues in the management of diabetes. *Methods and Findings in Experimental and Clinical Pharmacology; 26(6): 445-461.*

197) Vincent, A.M., Russell, J.W., Low, P., Feldman, E.L. (2004). Oxidative stress in the pathogenesis of diabetic neuropathy. *Endocrine Reviews; 25: 612-628.*

198) Vinicor, F. (1994). Is diabetes a public-health disorder? *Diabetes Care; 17(S1): 22-27.*

199) Vinik, A., Flemmer, M. (2002). Diabetes and macrovascular disease. *Journal of Diabetes and Its complications; 16: 235-245.*

200) Vinik, A.I., Vinik, E. (2003). Prevention of the complications of diabetes. *American Journal of Managed Care; 9: S63-S80.*

201) Waisundara, V.Y., Hsu, A., Huang, D., Tan, B.K. (2008). Scutellaria baicalensis enhances the anti-diabetic activity of metformin in streptozotocin-induced diabetic Wistar rats. *American Journal of Chinese Medicine; 36(3): 517-540.*

202) Wang, T.G., Ng, T.B. (1999). Natural Products with hypoglycemic, hypotensive, hypocholestrolemic, antiatherosclerotic and antithrombotic activities. *Life Sciences; 65(25): 2663-2677.*

203) Watt, J.M., Breyer-Brandwijk, M.G. (1962). *The medicinal and poisonous plants of Southern and Eastern Africa.* 2nd edition, Livingstone, London.

204) Wei-ha, L., Zi-qing, H., Hong, N., Fu-tian, T., He-qing, H., Xue-juan, L., Yan-hui, C., Shao-rui, C., Fen-fen, G., Wen-ge, H., Feng-ying C., Pei-qing, L. (2008). Berberine ameliorates renal injury in streptozotocin-induced diabetic rats by suppression of both oxidative stress and aldose reductase. *Chinese Medical Journal; 121(8): 706-712.*

205) WHO Expert Committee on Diabetes Mellitus: second report (1980). *World Health Organisation technical Report Series; 646: 1-80.*

206) Wiernsperger, N.F. (2005). Is non-insulin dependent glucose uptake a therapeutic alternative? Part 1: physiology, mechanisms and role of non-insulin dependent glucose uptake in type 2 diabetes. *Diabetes Metabolism; 31: 415-426*

207) Williams, R., Airey, M., Baxter, H., Forrester, J., Kennedy-Martin, T., Girach, A. (2004). Epidemiology of diabetic retinopathy and macular oedema: a systematic review. *Eye; 18: 963-983.*

208) Wirasathiena, L., Pengsuparpa, T., Suttisria, R., Uedab, H., Moriyasub, M., Kawanishib, K. (2007) Inhibitors of aldose reductase and advanced glycation end-products formation from the leaves of *Stelechocarpus cauliflorus* R.E. Fr.

Phytomedicine; 14: 546-550.

209) Wong, M., Chung, J.W.Y., Wong, T.K.S. (2007). Effects of treatments for symptoms of painful diabetic neuropathy: systematic review. *British Medical Journal; 335 (7610): 87.*

210) World Health Organisation (WHO), (2006).The World Health Report 2006: Working together for health.

211) Yang, B., Bankir, L. (2005). Urea and urine concentrating ability: new insights from studies in mice. *American Journal of Physiology, Renal Physiology; 288: F881-F896.*

212) Yokozawa, T., Yamabe, N., Kim, H.Y., Kang, K.S., Hur, J.M., Park, C.H., Tanaka, T. (2008). Protective effects of morroniside isolated from *Corni fructus* against renal damage in streptozotocin-induced diabetic rats. *Biological and Pharmaceutical Bulletin; 31(7): 1422-1428.*

213) Yonekura, H., Yamamoto, Y., Sakurai, S., Watanabe, T., and Yamamoto, H. (2005). Roles of the receptor for advanced glycation end products in diabetes-induced vascular injury. *Journal of Pharmacological Sciences; 97(3): 305- 311.*

214) Yurgin, N., Secnik, K., Lage, M.J. (2008). Obesity and the use of insulin: a study of patients with type 2 diabetes in the UK. *Journal of Diabetes and its Complications; 22(4): 235-240.*

215) Zerbini, G., Bonfanti, R., Meschi, F., Bognetti, E., Paesano, P.L., Gianolli, L., Querques, M., Maestroni, A., Calori, G., Maschio, A.D., Fazio, F., Luzi, L., Chiumello, G. (2006). Persistent renal hypertrophy and faster decline of glomerular filtration rate precede the development of microalbuminuria in type 1 diabetes. *Diabetes; 55: 2620-2625.*

216) Zheng, S., Noonan, W.T., Metreveli, N.S., Coventry, S., Kralik, P.M., Carlson, E.C., Epstein, P.N. (2004) Development of late-stage diabetic nephropathy in OVE26 diabetic mice. *Diabetes; 53: 3248-3257.*

APPENDIX I

UNIVERSITY OF
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RESEARCH OFFICE
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Reference: [illegible]

Prof. CT Musabayane
Head of School of Medical Sciences
Discipline Chair-Physiology
Department of Human Physiology
University of KwaZulu-Natal
WESTVILLE CAMPUS

Dear Prof. Musabayane

Renewal: Ethical Approval of Research Project using Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2007 on the following project:

Management of diabetes in streptozotocin (STZ)-induced diabetic rats: comparison of the effects of some medicinal plants on blood glucose, cardiovascular and renal functions in some experimental animal paradigms.

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

cc Registrar
Research Office
Dean of Faculty of Health Sciences

APPENDIX II

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23 July 2008

Reference: 048/08/Animal

Ms K Mapanga
School of Medical Sciences (Physiology)
University of KwaZulu-Natal
WESTVILLE CAMPUS

Dear Ms Mapanga

Ethical Approval of Research Project using Animals

I have pleasure in informing you that on recommendation of the review panel the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2008 on the following project:

"Hypoglycaemic and renal effects of some bioactive plant extracts in STZ-induced diabetic rats".

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar
Research Office
Head of School

APPENDIX III

RENAL EFFECTS OF PLANT-DERIVED OLEANOLIC ACID IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

By

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ABSTRACT

Previous studies from our laboratories indicate that the anti-diabetic effects of *Syzygium cordatum* (Hochst.) [Myrtaceae] leaf extract in streptozotocin-induced diabetic rats may be attributed in part to mixtures of triterpenes, oleanolic acid (3ß-hydroxy-olea-12-en-28-oic acid, OA) and ursolic acid (3ß -hydroxyl-urs-12-en-28-oic acid, UA). For the bioactive compounds to have potential in diabetes management, they should alleviate or prevent complications of diabetes mellitus: kidney function and cardiovascular disorders. This study was, therefore, designed to assess whether *S. cordatum* leaf derived OA influenced renal function evaluated by the ability to increase urinary Na^+ outputs parameters and creatinine clearance (Ccr) of streptozotocin (STZ)-induced diabetic rats. Extraction and fractionation of *S. cordatum* powdered leaf ethyl acetate-solubles (EAS) yielded mixtures of OA/UA and methyl maslinate/methyl corosolate. Recrystallisation of OA UA mixture using ethanol afforded OA whose structure was confirmed by NMR spectroscopy (^1H & ^{13}C). Acute effects of OA on kidney function and mean arterial blood pressure (MAP) were investigated in anesthetized 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. OA was added to the infusate during the treatment period. Chronic effects of OA were studied in individually-caged rats treated twice daily with OA (60 mg/kg, p.o.) for 5 weeks. By comparison with respective control animals administrations OA significantly increased Na^+ excretion rates of non-diabetic and STZ-induced diabetic rats without affecting urine flow, K^+ and Cl^- rates. At the end of 5 weeks, OA treatment significantly ($p<0.05$) increased Ccr in non-diabetic (2.88±0.14 *vs* 3.71±0.30 *ml/min*) and STZ-diabetic rats (1.81± 0.32 *vs* 3.07±0.16 *ml/min*) with concomitant reduction of plasma creatinine concentration (n=6 in all groups). OA also caused significant decreases in MAP in non-diabetic and STZ-induced diabetic rats. These findings suggest that OA may have beneficial effects on some processes associated with renal derangement of STZ-induced diabetic rats.

Keywords: *Renal function; diabetes mellitus; triterpenes; oleanolic acid; Syzygium cordatum.*

INTRODUCTION

Plants containing triterpene constituents, oleanolic acid (3ß-hydroxy-olea-12-en-28-oic acid, OA) and ursolic acid (3ß -hydroxyl-urs-12-en-28-oic acid, UA) have several traditional uses in folk medicines in terms of anti-inflammatory, hepatoprotective, analgesic, cardiotonic, sedative and tonic effects.[1-5] We have also recently reported that *Syzygium cordatum* (Hochst.) [Myrtaceae] leaf extract with constituent mixtures of OA and UA possesses blood glucose lowering properties in streptozotocin-induced diabetic rats. [6] Hyperglycemia is a major risk factor for the development and progression end stage renal failure; [7,8] thus preventing the progression of kidney damage based on specific alterations associated with nephropathy represents a serious medical challenge [9,]. Studies, however, indicate that there is no effective treatment for diabetic nephropathy. Current biomedical evidence indicates that some crude plant products and plant derived triterpenes ameliorate the deterioration of kidney function in experimental animals. [11-13] In the present study, we thus tested the hypothesis that *S. cordatum* derived triterpene OA improves renal fluid and electrolyte handling in experimental diabetic animals. The main objective of the present study was, therefore, to assess whether *S. cordatum* leaf derived OA influenced renal functional parameters such as urinary Na^+ outputs and creatinine clearance as a measure of GFR in the rats. [13-,el] Since glomerular filtration rate (GFR) determines the degree of electrolyte retention, [15] intervention trials in diabetes mellitus should adopt reversing a decline in GFR as an outcome measure. We investigated the effects of OA on renal function because it is the main triterpene that has been isolated from many medicinal plants. [16]

MATERIALS AND METHODS

Plant material

Syzygium cordatum (Hochst.) [Myrtaceae] leaves were collected around Durban, South Africa, between January and June, 2006. The plant was identified by Professor H. Bainath, the former Chief Taxonomist/Curator of the University of KwaZulu-Natal's Department of Botany. A voucher specimen (collector number MH905) of the plant was deposited in the University's Botany Discipline Herbarium.

Extraction

Milled air-dried leaves of *S. cordatum* (1.65 kg) were sequentially extracted thrice at 24h intervals with 3 litres on each occasion of hexane, dichloromethane, ethyl acetate and methanol. The solvents were removed under reduced pressure to give corresponding residues of hexane-solubles (HES) (10.1g), dichloromethane-solubles (DCMS) (29.3g), ethyl acetate-solubles (EAS) (27.3g), and methanol-solubles (MES) (145.7g), respectively. The crude ethyl acetate extract was subjected to further purification since previous studies indicated that EAS contained bioactive compounds. [8, 19]

Isolation of oleanolic acid (OA)

A portion of EAS (2g) was subjected to open column chromatography on silica gel (70-230 mesh, 3.5 x 45cm) using a step gradient of *n*-hexane-ethyl acetate 90:10 (250mL), 80:20 (250ml), and 70:30 (800mL). Fractions (65) of 20mL each were collected and monitored by TLC (SiO$_2$, n-hexane/ ethyl acetate 7:3; anisaldehyde/H_2SO_4 spray reagent). The *semi-polar* column fractions (*fractions* 28-60, ~640 mL) enriched in triterpenes were combined and submitted for further silica gel chromatography. OA (109mg) was obtained as white powder and its structure was determined by ^{1}H- and ^{13}C-NMR (1D and 2D) spectroscopy (Figures 1).

Animals

Male Sprague-Dawley rats (250–300 g body weight) maintained under laboratory conditions of temperature, humidity and 12 h light/12 h dark regime at the Biomedical Resource Unit, University of KwaZulu-Natal, were used. The rats were exposed to both food (Epol-diet 4700, Epol, South Africa) and water *ad libitum*. All experiments were performed in accordance with the University of KwaZulu-Natal Research Ethics Committee guidelines for experimental animals.

Induction of experimental diabetes

Diabetes mellitus was induced with 60 mg/kg of streptozotocin (Sigma Aldrich, St. Louis, Missouri, USA) which was administered once by intraperitoneal injection, dissolved in citrate buffer, pH 6.3 as previously described.[20] Vehicle (citrate buffer) - injected animals acted as controls. Animals that exhibited glucosuria after 24 hours, tested by urine test strips (Rapidmed Diagnostics, Sandton, South Africa), were considered diabetic. Blood glucose concentration of 20 mmol/l measured after one week was considered as a stable diabetic state before our experimental procedures.

Experimental design

The effects of short-(acute) administration of OA on renal fluid and electrolyte handling were evaluated in anesthetized male Sprague-Dawley non-diabetic and STZ-induced diabetic rats while the long-term (subchronic) effects of OA on renal parameters and blood glucose were assessed in conscious animals.

Acute studies

Non-diabetic and STZ-induced diabetic rats divided into groups of untreated control and treated rats (n=6 in each group) were anesthetized by intraperitoneal injection of inactin (5-ethyl-5-(1'-methylpropyl)-2-thiobarbiturate, 0.11 g/kg body weight (Sigma Aldrich, St. Louis, Missouri, USA) and placed on a thermally-controlled heating table (37±1°C). After tracheotomy, a catheter was inserted into the jugular vein for intravenous infusion of 0.077M NaCl at 9 ml/h (Harvard syringe infusion Pump 22, Harvard Apparatus, Holliston, Massachusetts, USA). An additional heparinized catheter was also inserted

into the left carotid artery for blood pressure measurement 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). Following a 3½ h equilibration period, measurements were recorded over the 4h post-equilibration period of 1h control, 1h 30 min treatment and 1h 30 min recovery periods.

In control groups, eight consecutive urine collections were made into pre-weighed plastic vials at 30 min intervals over the subsequent 4 h for measurements of urine flow, Na^+, K^+ and Cl^- excretion rates. In those animals in which the effects of OA were studied, OA was added to the infusate at 90 ug/ h for 1.5 h (treatment period), resulting in a total dose of 0.45 mg/ kg, respectively (for a 300 g rat) before the animals were returned to infusate alone for the last 1.5 h (recovery period). Depth of anesthesia was monitored throughout the experiments, and additional i.v. bolus doses of inactin (0.05 g/kg body weight) were administered when necessary.

Chronic studies

Non-diabetic and STZ-induced diabetic male Sprague-Dawley rats divided into groups of untreated and treated were used. The rats were given both food (Epol-diet 4700, Epol, South Africa) and water *ad libitum*. Separate groups of non-diabetic and STZ-induced diabetic rats were treated with OA (60 mg/kg, p.o.) twice every third day at 09h00 and 15h00 for 5 weeks by means of a bulbed steel tube. Each rat was housed individually in Makrolon polycarbonate metabolic cages (Tecniplast, Labotec, South Africa) kept at the Biomedical Resource Unit, University of KwaZulu-Natal to allow quantitative measurements every third day at 09h00 for 5 weeks of urine volume and analysis of Na^+, K^+ and Cl^-. Rats treated with deionized water (3 mL/kg, p.o.) acted as untreated controls.

Mean arterial blood pressure (MAP) was monitored every third consecutive day for 5 weeks at 09h00 using non-invasive tail cuff method with photoelectric sensors (IITC Model 31 Computerized Blood Pressure Monitor, Life Sciences, Woodland Hills, CA) in all groups of animals. At the end of the 5-week experimental period, the kidneys were removed weights determined. Blood was collected from all groups of animals by cardiac puncture 24h after the last treatment. Blood glucose was measured using

Bayer's glucometer Elite® (Elite (Pty) Ltd, Health Care Division, South Africa). The plasma concentrations, creatinine and urea were determined using reagents reagent kits from Beckman Coulter, Ireland, Inc., and measured using Beckman Coulter (Synchron LX20 Clinical Systems, USA). GFR values were calculated through the creatinine clearance (C_{Cr}) using the standard formulae following measurements of urine and plasma concentrations of creatinine. Osmolalities of the plasma and urine were measured on 50 µl samples by freezing point depression using an osmometer (Osmomat 030, Lasec Laboratory and Scientific Company, Berlin, Germany).

Data analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). The renal excretion data are presented graphically showing timed collections over the acute and subchronic experimental periods. The total amounts of fluid voided and electrolytes excreted during the 1h 30min administration of OA were calculated and compared to values in controls for the corresponding time. At the end of 5 weeks, renal function was evaluated with 24 h C_{Cr}, plasma electrolytes, and urea and creatinine concentrations. To determine the effects of OA, the data were treated and presented separately for the non-diabetic and STZ-diabetic rats. All statistical calculations were performed with GraphPad InStat Software (version 4.00, GraphPad Software, Inc., San Diego, California, USA). Comparison between groups was performed using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. A value of $p<0.05$ was considered significant.

RESULTS

Structural elucidation of compounds

Oleanolic acid spectral data identified by ^{1}H- and ^{13}C-NMR (1D and 2D) compared with literature data.[7,8] The purity (about 99%) of OA was elucidated on the basis of chemical and physicochemical evidence (Table 1).

Acute renal effects

The mean Na^+ excretion rates of *anaesthetized non-diabetic and STZ-induced diabetic rats* significantly increased during the 1/2h OA administration by comparison with respective control animals (Figure 3). Urine flow, K^+ and Cl^- rates, however, varied little during OA treatment period as evidenced by the fact that mean total volume of, fluid voided and the amounts of K^+ or Cl^- excreted did not significantly differ from those of respective control animals (Table 2). OA, however, significantly elevated urinary Na^+ in both non-diabetic and STZ-induced diabetic rats.

Subchronic renal OA effects

The mean weekly urine volume was elevated in untreated conscious STZ-induced diabetic rats throughout the 5-week although the urinary electrolyte concentrations were significantly reduced compared to non-diabetic control animals. Subchronic *administrations of OA (60 mg/kg, p.o., twice every third day) to non-diabetic and STZ-*induced diabetic rats caused significant increases in urinary Na^+ outputs by comparison with respective control animals at the corresponding time periods. The weekly urine volume, urinary K^+ and Cl^- were, however, not altered by OA administration. By the end of the 5^{th} week experimental period, plasma Na^+, $K^+$and Cl^- were similar in untreated and OA treated STZ-induced diabetic rats (Table 3). Conversely, plasma creatinine and urea concentrations were elevated in STZ-induced diabetic rats in comparison with non-diabetic rats. OA treatment, however, significantly reduced ($p< 0.05$) plasma creatinine concentration of STZ-induced diabetic rats with a concomitant elevation in GFR (Table 3).

Effects of OA on blood and urinary glucose concentrations

OA treatment (60 mg/kg, p.o.) twice every third day significantly ($p<0.05$) decreased blood glucose and urinary glucose output of STZ-induced diabetic rats (Figure 3 and Table 3). By the end of the 5-week study period, OA administration significantly reduced ($p<0.01$) plasma osmolality in STZ-treated diabetic rats with concomitant increase in urine osmolality. The increase in plasma osmolality of non-diabetic rats did not achieve statistical significance level (Table 3). Kidney mass which was significantly increased in STZ-induced diabetic rats by comparison with non-diabetic animals was not altered by subchronic OA treatment (Table 3).

Effects of OA on blood pressure

Acute infusion of hypotonic saline to control non-diabetic and STZ-induced diabetic animals did not show any significant variations in the mean arterial blood pressure throughout the 4-h post-equilibration period (Figure 4). OA administration, however, elicited immediate reduction of blood pressure which persisted during the post treatment period. Subchronic treatment of the rats with OA (60 mg/kg daily for 5 weeks, p.o.) also caused significant decreases in MAP in non-diabetic and STZ-treated diabetic rats throughout the study period by comparison with control rats at the corresponding period (Figure 4).

DISCUSSION

The experimental evidence presented in this study extends our previous observations that *S.cordatum* leaf extract lowers blood glucose of STZ-induced diabetic rats.[6] The results indicate that the *S. cordatum* leaf extracted triterpene, oleanolic acid (OA) not only has the potential to lower blood pressure in diabetes, but may also have beneficial effects on the kidney function. Long-term diabetes mellitus complications often include compromised kidney function [22] and elevated blood pressure [23]. The spectra of *S.cordatum* leaf extracted OA elucidated using ^{1}H- and ^{13}C-NMR compared with previously reported data of oleanolic acid.[21]

In this study, STZ-induced rats exhibited marked weekly decreases in urinary Na^+ excretion and elevated plasma creatinine concentration with concomitant reduction in

Cer at the end of 5 weeks. These changes were attenuated in the OA-treated groups indicating that oleanolic acid might have beneficial effects on renal function in diabetes. The OA-evoked increases urinary Na^+ outputs of STZ-diabetic rats and elevated GFR as assessed by creatinine clearance suggest up-regulation of renal function by the triterpene. We and several other authors have previously used Cer in rats as a fundamental parameter of evaluating renal tubular function to monitor GFR. [15,19,24,25] Low GFR is associated with fluid and electrolyte unbalance in nephrotic syndrome [27] and acute renal failure.[28-30] It is, therefore, speculated that OA administration significantly improved renal function of STZ-induced diabetic rats perhaps in part mediated via elevation of GFR. We suggest that elevated GFR possibly increased the filtered load of Na^+ in the tubular filtrate thereby facilitating urinary Na^+ excretion. A correlation between increased urinary Na^+ excretion and elevated GFR has been reported in the rat. [31] The increased Na^+ excretion perhaps accounted for decreased plasma osmolality and increased 24-h urine osmolality at the end of the 5-week period. The beneficial effects of OA on kidney function in the STZ-induced diabetic cannot be explained on glycaemic control as OA-treatment was unable to achieve an euglycaemic state and yet decreased urinary glucose output.

We suggest that the triterpene ameliorates kidney function in diabetes as marked increased in creatinine clearance despite the failure of OA to reduce elevated plasma urea concentrations of STZ-induced diabetic rats. Treatment-related increases in creatinine clearance indicate improvement of kidney function in experimental animals [32] and man [33] rather urea because the latter varies with dietary protein. [34,35] Plasma creatinine concentration can, therefore, be used as a clinical chemistry end point to detect OA treatment-related effects on the kidney. Kidneys maintain optimum chemical composition of body fluids by removal of urea and creatinine and the concentrations of these metabolites increase in the blood during renal disease.

Kidney weights of STZ-induced diabetic rats were significantly increased by comparison with non-diabetic animals. Kidney hypertrophy due to accumulation of extra cellular matrix proteins is directly related to a decline in kidney function perhaps due to structural changes of glomerular basement membrane. [36-38] Typically, elevation of GFR accompanies increased kidney size as a result of increased glomerular volume and capillary surface area[39]. We hypothesize that the differences observed in GFR between

control and treated rats are probably due to OA treatment. This is supported by the observation that kidney weights of treated non-diabetic and STZ-induced diabetic rats did not differ from those of respective control animals.

The potent hypotensive properties and amelioration of kidney function by OA of STZ-induced diabetic rats confirms previous observations in Dahl salt sensitive rats.[19] In conclusion, the results of the present study introduce the first *in vivo* evidence that oleanolic acid extracted from *S. coriatum* leaves ameliorates kidney function in STZ-induced diabetic rats. The possible mechanisms behind the OA-evoked amelioration of kidney function are yet to be studied. The results are significant considering that: impaired kidney function in diabetes is characterized by a progressive decline in GFR, hypertension, and a high risk of cardiovascular morbidity and mortality.

ACKNOWLEDGEMENTS

This study was partly funded by the University of KwaZulu-Natal, Research Division. The authors are grateful to the following: Prof. H. Baijnath for the identification of *S. coriatum*; Dr S Singh and Ms I. Bester of the Biomedical Research Unit and Mr OO Oyedeji, School of Chemistry, University of KwaZulu-Natal for assistance in phytochemical studies.

REFERENCES

1. Gan KH, Lin CN. Studies on the constituents of Formosan gentianaceous plants. XI. Constituentsof *Gentiana flavor-maculata* and *Tripterrospermum taiwanense* and the antihepatotoxic activity of ursolic acid derivatives. *Chin. Pharmaceut. J.* 1988; 40:77-84.

2. Singh GB, Singh S, Bani S, Gupta BD, Banerjee SK. Anti-inflammatory activity of oleanolic acid in rats and mice. *J. Pharm. Pharmacol.* 1992; 44:456-458.

3. Niikawa M, Hayashi H, Sato T, Nagase H, Kito H. Isolation of substances from glossy privet (*Ligustrum lucidum* Ait) inhibiting the mutagenicity of benzo(a)pyrene in bacteria. *Mutation Res.* 1993; 319:1-9.

4. Huang MT, Ho CT, Wang ZY, Ferraro T, et al. Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. *Cancer Res.* 1994; 54:701-708.

5. Liu J. Pharmacology of oleanolic acid and ursolic acid. *J. Ethnopharmacol* 1995; 49:57-68.

6. Musabayane CT, Mahlalela N, Shode FO, Ojewole JAO (2005) Effects of *Syzygium cordatum* leaf extract on plasma glucose and hepatic glycogen in streptozotocin-induced diabetic rats. *J. Ethnopharmacol.* 2005; 97 (3): 485-490.

7. Selby JV, FitzSimmons SC, Newman JM, Katz PP, Sepe S, Showstack J. The structural history and epidemiology of diabetic nephropathy. Implications for prevention and control. *J. Am. Med. Assoc.* 1990; 263:1954-1960.

8. Held PJ, Port FK, Webb RL, et al. The United States renal data system's 1991 annual data report: an introduction. *Am. J. Kidney. Dis.* 1991; 18:1-16.

9. Cooper ME. Pathogenesis, prevention and treatment of diabetic nephropathy. *Lancet* 1998, 40:213-219.

10. The Diabetes and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *New Engl. J. Med.* 1993; 329:977-986.

11. Grover JK, Vats V, Rathi SS, Dawar R. Traditional Indian anti-diabetic plants attenuate progression of renal damage in streptozotocin induced diabetic mice. *J. Ethnopharmacol* 2001; 76:233-238.

12. Li R, Zhang YY, Huang XY, Sun YN, Jia YF, Li D. Beneficial effect of triterpene on systemic lupus erythematosus induced by chromatin in BALB/c mice. *Eur. J. Pharmacol.* 2005; 512:231-237.

13. Abdel-Zaher AO, Abdel-Rahman MM, Hafez MM, Omran FM. Role of nitric oxide and reduced glutathione in the protective effects of aminoguanidine, gadolinium chloride and oleanolic acid against acetaminophen-induced hepatic and renal damage. *Toxicology* 2007; 234 (1-2):124-134.

14. Li Y, Higashiura K, Ura N, Torii, I, Agata J, Wang L, Togashi N, Shimamoto K. Effects of the Chinese medicine, TSJN on insulin resistance and hypertension in fructose-fed rats. *Hypertens. Res* 2000; 23 (2):101-107.

15. Bursztyn M, Ben-Ishay D, Mekler J, Raz I. Insulin-induced renal dysfunction in regular Sabra rats. *Clin. Exp. Pharmacol.* 1995; 22 (1):S32-S33.

16. Travlos GS, Morris RW, Elwell MR, Duke A, Rosenblum S, Thompson MB. Frequency and relationships of clinical chemistry and liver and kidney histopathology findings in 13-week toxicity studies in rats. *Toxicology* 1996; 107 (1):17-29.

17. O'Hare JA, Ferriss JB, Brady D, Twomey B, O'Sullivan DJ. Exchangeable sodium and renin in hypertensive diabetic patients with and without nephropathy. *Hypertension* 1985; 7 (Suppl 7): II - 43 - II - 48.

18. Wang B, Jiang ZH. Studies on oleanolic acid. *Chin. Pharmaceut. J.* 1992; 27:393-397.

19. Somova LI, Shode FO, Nadar A, Ramnanan P. Antihypertensive, antiatherosclerotic and antioxidant activity of triterpenoids isolated from *Olea europaea*, subspecies *africana* leaves. *J. Ethnopharmacol.* 2003; 84:299-305.

20. Musabayane CT, Gondwe M, Kamadyaapa DR, Chatrgoon AA, Ojewole JAO. Effects of *Ficus thonningii* (Blume) [Moraceae] 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* (USA) 2007; 29(4):389-397.

21. Mahato SB, Kundu AP. ^{13}C NMR Spectra of pentacyclic triterpenoids-a compilation and some salient features. *Phytochemistry* 1994; 37:1517-1575

22. Stengel B, Billon S, van Dijk PCW, et al. Trends in the incidence of renal replacement therapy for end-stage renal disease in Europe, 1990-1999. *Nephrol. Dial. Transplant.* 2003; 18 (9):1824-1833.

23. Haller H, Drab M, Luft FC. The role of hyperglycemia and hyperinsulinemia in the pathogenesis of diabetic angiopathy. *Clin. Nephrol.* 1996; 46 (4): 246-255.

24. Girchev R, Markova P, Mikhov D, Natcheff N. Renal excretory function in conscious Long Evans and vasopressin deficient (Brattleboro) rats after endothelin-A receptor inhibition. *Acta Physiol. Pharmacol. Bulg.* 1998; 23 (3-4): 73-77.

25. Bertuzzi, ML, Bensi N, Mayer N, Niebylski A, Armario A, Gauna HF. Renal mechanisms involved in stress-induced antinatriuresis and antidiuresis in rats. *Arch. Physiol. Biochem.* 2003; 111 (3): 259-264.

26. Gondwe M, Kamadyaapa DR, Tufts, MA, Chuturgoon, AA, Musabayane CT. *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 rat. *Phytomedicine* 2008. 15:699-709.

27. Fernández-Llama P, Andrews P, Nielsen S, Ecelbarger CA, Knepper MA. Impaired aquaporin and urea transporter expression in rats with adriamycin-induced nephrotic syndrome. *Kidney Int.* 1998; 53 (5):1417-1418.

28. Kwon TH, Frøkiaer J, Fernández-Llama P, Knepper MA, Nielsen S. Reduced abundance of aquaporins in rats with bilateral ischemia-induced acute renal failure: prevention by alpha-MSH. *Am. J. Physiol.* 1999; 277 (3Pt2): F413-F427.

29. Kim SW, Jeon YS, Lee JU et al. Diminished adenylate cyclase activity and aquaporin 2 expression in acute renal failure rats. *Kidney Int.* 2000; 57 (4):1643-1650.

30. Lee J, Kang DG, Kim Y. Increased expression and shuttling of aquaporin-2 water channels in the kidney in DOCA-salt hypertensive rats. *Clin. Exp. Hypertens.* 2000; 22 (5):531-541.

31. Marín-Grez M, Fleming JT, Steinhausen M. Atrial natriuretic peptide causes pre-glomerular vasodilation and post-glomerular vasoconstriction in rat kidney. *Nature* 1986; 324:473-476.

32. van Hoek I, Lefebvre HP, Kooistra HS, et al. Plasma clearance of exogenous creatinine, exo-iohexol, and endo-iohexol in hyperthyroid cats before and after treatment with radioiodine. *J. Vet. Intern. Med.* 2008; 22 (4):879-885.

33. Almond A, Siddiqui S, Robertson S, Norrie J, Isles C. Comparison of combined urea and creatinine clearance and prediction equations as measures of residual renal function when GFR is low. *QMJ* 2008; 101 (8):619-624.

34. Mitch WE. Conservative management of chronic renal failure. In: Brenner BM, Stein J (eds): *Contemporary Issues of Nephrology, Chronic Renal Failure*. New York, Churchill Livingstone. 1981, pp 116-152.

35. Satirapoj B, Supasyndh O, Patomanond J, Choovichian P. Estimating glomerular filtration rate in Asian patients with chronic kidney diseases from bioelectrical impedance analysis. *J. Med. Assoc. Thai.* 2006; 89 (10):1584-1591.

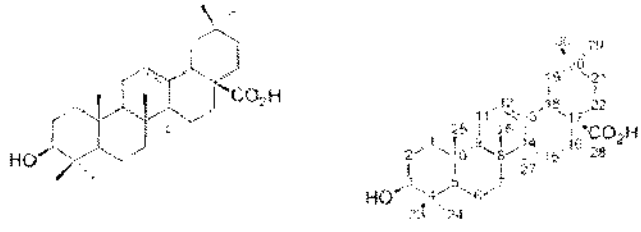
36. Hargrove GM, Dufresne J, Whiteside C, Muruve DA, Wong NCW. Diabetes mellitus increases endothelin-1 gene transcription in rat kidney. *Kidney Int.* 2000; 58:1534-1545.

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

38. Mason RM, Wahab NA. Extra cellular matrix metabolism in diabetic nephropathy. *J. Am Soc Nephrol.* 2003; 14:1358-1373.

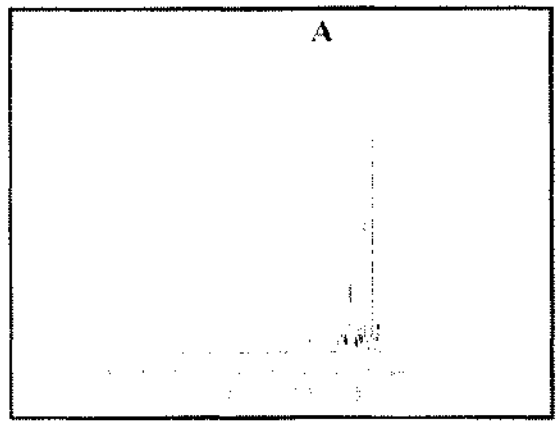
39. Anderson S, Vora JP. Current concepts of renal hemodynamics in diabetes. *J. Diabetes Complications* 1995; 9:304-307.

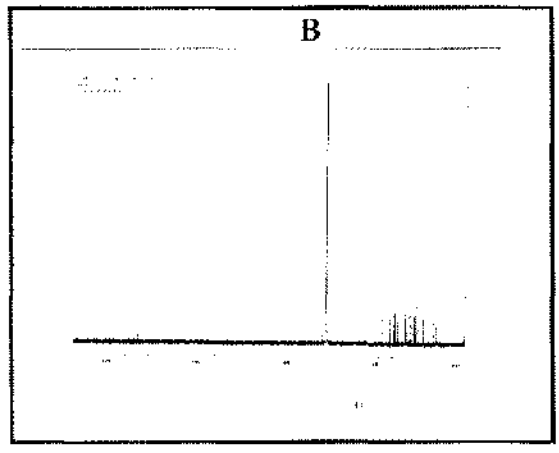
40. Gnudi L, Viberti G, Raij L, et al. Glut-1 overexpression: Link between haemodynamic and metabolic factors in glomerular injury. *Hypertension* 2003; 42:19-24.



Oleanolic acid IUPAC numbering of oleanolic acid

Figure 1. Chemical structure of oleanolic acid





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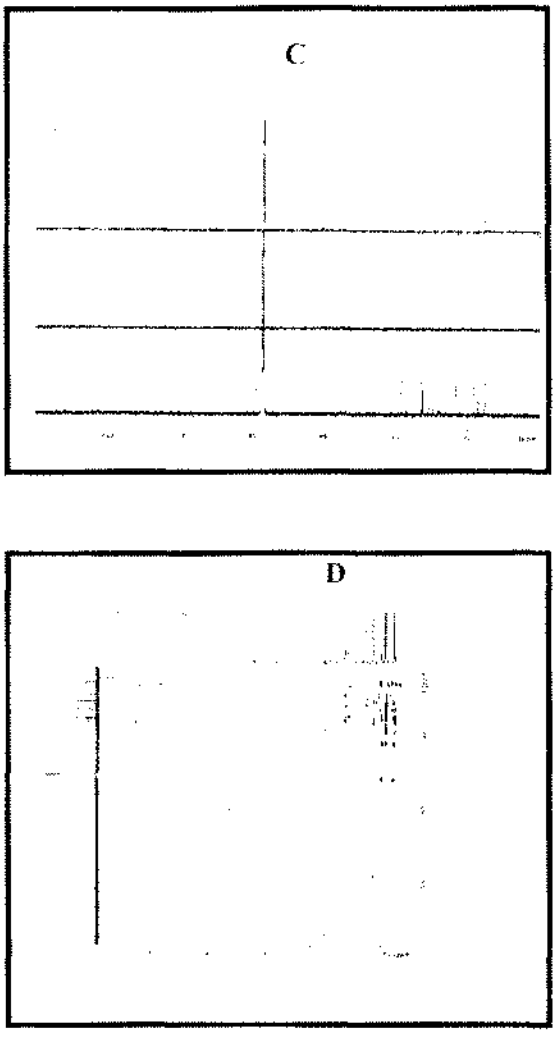

Figure 2. NMR spectra ^{1}H- (A), ^{13}C- (B), Distortion Enhancement Proton Testing (DEPT) (C) and HMQC (D) of *Syzygium corikanum* leaf derived OA.

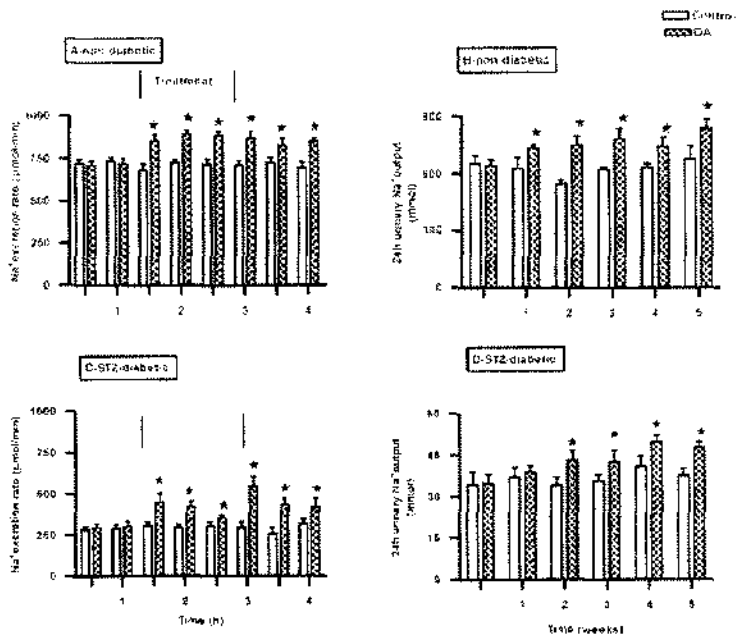

Figure 3. Comparison of the effects of OA treatment on Na^+ excretion rates non-diabetic (A-B) and STZ-induced diabetic rats **(C-D)** with respective control animals. Values are presented as means and vertical bars indicate SE of means (n=6 in each group).

★ p<0.05 by comparison with respective control animals.

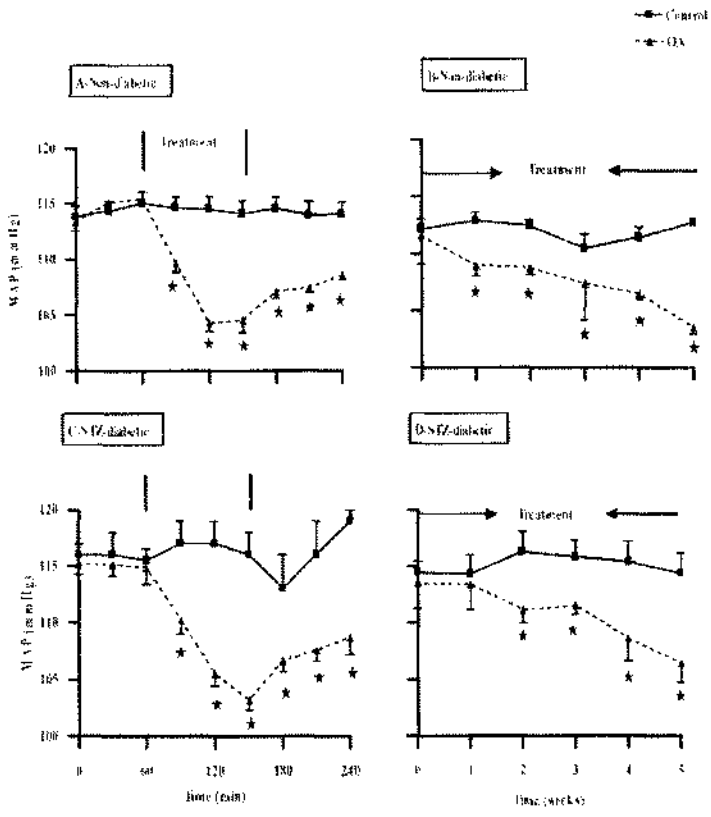

Figure 4. Comparison of the effects of OA treatment on mean arterial blood pressure (MAP) of non-diabetic **(A-B)** and STZ-induced diabetic rats **(C-D)** with the respective control animals. Values are presented as means and vertical bars indicate SE of means (n=6 in each group).

★ $p<0.05$ by comparison with respective control animals

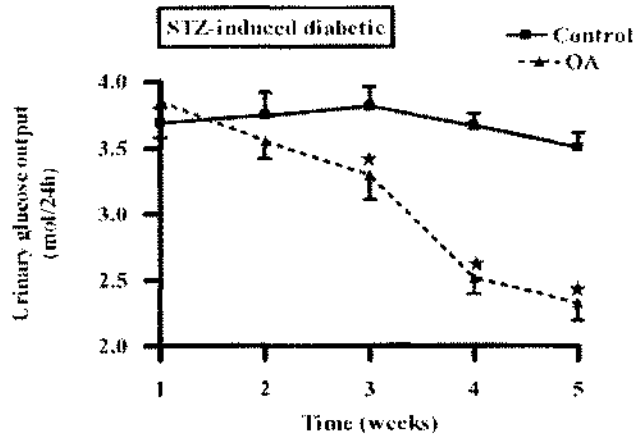

Figure 5. Comparison of mean weekly urinary glucose outputs of control STZ-induced diabetic rats and STZ-induced diabetic rats treated with OA (60 mg/kg, p.o.) twice every third day at 09h00 and 15h00 for 5 weeks.

★ $p<0.05$ by comparison with control animals

Table 1. ^{13}C (100.64 MHz) Bruker Avance III NMR spectral data of plant derived OA and reported OA.[21]

Carbon Position	Plant Derived OA δ_C	Reported OA [Mahato and Kundu 1994][21]. δ_C
1	38.4	38.5
2	27.2	27.4
3	79.0	78.7
4	38.8	38.7
5	55.2	55.2
6	18.3	18.3
7	32.6	32.6
8	39.3	39.3
9	47.6	47.5
10	37.1	37.1
11	23.0	22.9
12	122.7	122.5
13	143.6	143.5
14	41.6	41.6
15	27.7	27.7
16	23.4	23.4
17	46.3	46.5
18	41.0	40.9
19	45.9	45.9
20	30.7	30.6
21	33.8	33.8
22	32.4	32.4
23	28.1	28.1
24	15.6	15.5
25	15.3	15.3
26	17.1	17.1
27	25.9	25.9
28	182.2	183.3
29	33.07	33.1
30	23.6	23.6

Table 2. Total amounts of urine voided and Na^+, K^+, and Cl^- excreted by non-diabetic and STZ-diabetic control and rats during 12h OA treatment period (n = 6 in all groups).

Group	Treatment	Urine volume (ml)	Na^+ (μmol)	K^+ (μmol)	Cl^- (μmol)
Non diabetic	Control	13.1±1	1056±25	322±7	1264±14
Rats	OA	12.7±1	1394±15*,#	344±15	1388±43
STZ-induced	Control	10.4±1	425±23#	260±42#	1031±220#
diabetic rats	OA	10.1±1	622±41*,#	243±16#	1011±70#

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

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

Table 3. Effects of OA on solute excretion and plasma biochemical parameters in non-diabetic and STZ-diabetic control and rats administered OA twice every third consecutive day for 5 weeks. ($n = 6$ in all groups).

	Non-diabetic		STZ-induced diabetic	
Measure	**Control**	**OA-treated**	**Control**	**OA-treated**
Glucose (mmol/l)	4.9±0.1	4.2±0.1	26±1[a]	21±1[a]*
Na^+ (mmol/l)	142±1	142±1	140±2	137±3
K^+ (mmol/l)	3.68±0.25	3.59±0.17	3.76±0.21	3.53±0.20
Cl^- (mmol/l)	101±1	103±1	102±2	96±4
Urea (mmol/l)	7.3±0.9	8.3±0.7	10.7±1.7[a]	12.1±1.8[a]
Creatinine (μmol/l)	25±2	19±1*	31±3[a]	23±2*
Plasma osmolality (mOsm/kg H_2O)	263±27	247±15	382±10[a]	316±16*[a]
Urine osmolality (mOsm/kg H_2O)	2512±109	2061±78	991±92[a]	1115±56*[a]
GFR (ml/min)	2.88±0.14	3.71±0.30*	1.81±0.32[a]	3.07±0.16*[a]
Kidney weights (g/100g b.wt)	0.46±0.02	0.44±0.02	0.78±0.07[a]	0.73±0.04[a]

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

[a] $p<0.05$ by comparison with non-diabetic animals

APPENDIX IV (a)

19. - COMPETITION

PLANT DERIVED OLEANOLIC ACID AUGMENTS THE HYPOGLYCAEMIC EFFECTS OF EXOGENOUS INSULIN IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

APPENDIX IV (b)

RD/04/08

ANT-DERIVED OLEANOLIC ACID IN SPRAGUE DAWLEY RATS

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*ysiology and *Chemistry, University of KwaZulu-Natal, Westville Campus, an 4000, South Africa.*

the ethanolic crude *Syzygium cordatum* (Hochst.) [Myrtaceae] leaf extract etes mellitus management, perhaps due to triterpenoid constituents. ontrol of diabetes mellitus should include prevention or alleviation of renal tes is associated with impaired kidney function.

was to investigate the renal effects of *S. cordatum* leaf-derived oleanolic ats.

6kg) (milled) of *S. cordatum* was sequentially extracted with hexane, etate, and methanol to give hexane –solubles (HS), dichloromethane-tate-solubles (EAS), and methanol-solubles (MS). Preliminary experiments ned bioactive compounds. A portion of the EAS was subjected to column a gel. Gradient elution with n-hexane-ethyl acetate (9:1 to 6:4) produced re combined on the basis of their TLC similarities into three major sub-n-polar fraction (F1) was discarded and semi polar/polar fractions (F2 and d to silica gel column chromatography. F2 gave a mixture of oleanolic acid). F3 gave a mixture of methyl maslinate and methyl corosolate.)A/UA mixture afforded OA (mp > 300°C [*Lit.* 306-308°C] using ethanol ture was confirmed by NMR spectroscopy (^{1}H & ^{13}C) (Seo *et al.*, 1975).

dney function and mean arterial blood pressure (MAP) were investigated in ged with hypotonic saline after a 3.5-h equilibration for 4h of 1 h control, 1.5 overy periods. OA was added to the infusate during the treatment period. uced significant ($p<0.01$) decrease in Na^+, K^+ and Cl^- excretion rates without Furthermore, OA significantly ($p<0.05$) reduced the MAP from a mean pre- mmHg to reach 105±1 mmHg after the cessation of treatment. The blood ased by the end of the 4-h experimental period to values that did not t of control animals at the corresponding period.

at OA at the dose used may influence kidney function. Further studies are)A leads to impairment of renal electrolyte excretion.

nimal

APPENDIX IV (c)

Dear author,
Thank you for submitting your abstract entitled "RENAL EFFECTS OF PLANT-DERIVED OLEANOLIC ACID IN STREPTOZOTOCIN-INDUCED DIABETIC RATS". Your reference number for this submission is 0009 A copy of your submission is attached to this email.
The abstracts will be reviewed shortly and you will be informed by January 2009 whether or not your abstract has been accepted for presentation. Please remember to quote the conference name and the reference number in any communication with us.
With best wishes,
Conference Administrator
Society for Endocrinology BES, HARROGATE 2009

THE EFFECTS OF COMBINED PLANT DERIVED OLEANOLIC ACID AND INSULIN ON BLOOD GLUCOSE CONCENTRATION IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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We have previously attributed the hypoglycaemic effects of *Syzygium cordatum* leaf extract to crude ethyl acetate extract solubles (EAS) containing triterpenoid compounds. The present study was designed to isolate and characterize triterpene components EAS and investigate the antihyperglycaemic effects alone or in combination with insulin in non-diabetic and streptozotocin (STZ)-induced diabetic rats. EAS soluble extracts were obtained after defatting the *S. cordatum* leaves with hexane followed by dichloromethane before maceration with ethyl acetate. Column chromatography on silica gel using n-hexane-ethyl acetate yielded mixtures of oleanolic acid, ursolic acid, methyl maslinate and methyl corosolate. Oleonolic acid (OA) was obtained by recrystallisation and its structure was determined by ^{1}H- and ^{13}C-NMR spectroscopy. Oral glucose tolerance test (OGTT) responses were monitored in separate groups of non-diabetic and STZ-induced diabetic rats given glucose load after 18-h fast, followed by various OA doses (40, 80 and 120 mg/kg, p.o.). Rats treated with deionized water (3 ml/kg, p.o.), or insulin (200 µg/ kg, s.c) acted as untreated and treated positive controls, respectively. The influence of OA on insulin-induced OGTT responses were studied in separate groups of animals that were treated with combined OA (40 or 80 mg/kg) and insulin (100 or 200 µg/kg, s.c.). Blood glucose was monitored at 15-min intervals for the first hour, and hourly thereafter for 3h. All doses of OA as well as insulin significantly ($p<0.05$) decreased blood glucose concentrations of non-diabetic and STZ-induced diabetic rats. More notable, however, were the dramatic hypoglycaemic effects produced by combined OA and insulin administration. Compared with rats separately administered with insulin or OA, blood glucose concentrations were significantly ($p<0.05$) lower at all the time points that blood was sampled in animals treated with combined OA and insulin. We suggest that OA may be useful as adjuvant treatment to enhance insulin effects in diabetes mellitus management.

Key words: Diabetes mellitus; hypoglycaemic plants; *Syzygium cordatum*, triterpernoids; oleanolic acid.

APPENDIX IV (d)

Dear author,
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RENAL EFFECTS OF PLANT-DERIVED OLEANOLIC ACID IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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We have previously attributed the hypoglycaemic effects of *Syzygium cordatum* leaf extract to crude ethyl acetate extract solubles (EAS) containing triterpenoid compounds. The present study was designed to isolate and characterize triterpene components EAS and investigate the antihyperglycaemic effects alone or in combination with insulin in non-diabetic and streptozotocin (STZ)-induced diabetic rats. EAS soluble extracts were obtained after defatting the *S. cordatum* leaves with hexane followed by dichloromethane before maceration with ethyl acetate. Column chromatography on silica gel using n-hexane-ethyl acetate yielded mixtures of oleanolic acid, ursolic acid, methyl maslinate and methyl corosolate. Oleonolic acid (OA) was obtained by recrystallisation and its structure was determined by ^{1}H- and ^{13}C-NMR spectroscopy. Oral glucose tolerance test (OGTT) responses were monitored in separate groups of non-diabetic and STZ-induced diabetic rats given glucose load after 18-h fast, followed by various OA doses (40, 80 and 120 mg/kg, p.o.). Rats treated with deionized water (3 ml/kg, p.o.), or insulin (200 µg/ kg, s.c) acted as untreated and treated positive controls, respectively. The influence of OA on insulin-induced OGTT responses were studied in separate groups of animals that were treated with combined OA (40 or 80 mg/kg) and insulin (100 or 200 µg/kg, s.c.). Blood glucose was monitored at 15-min intervals for the first hour, and hourly thereafter for 3h. All doses of OA as well as insulin significantly ($p<0.05$) decreased blood glucose concentrations of non-diabetic and STZ-induced diabetic rats. More notable, however, were the dramatic hypoglycaemic effects produced by combined OA and insulin administration. Compared with rats separately administered with insulin or OA, blood glucose concentrations were significantly ($p<0.05$) lower at all the time points that blood was sampled in animals treated with combined OA and insulin. We suggest that OA may be useful as adjuvant treatment to enhance insulin effects in diabetes mellitus management.

Key words: Diabetes mellitus; hypoglycaemic plants; *Syzygium cordatum,* triterpernoids; oleanolic acid.