UNIVERSITY OF KWAZULU-NATAL

Mechanisms of Cardiovascular Effects of Oleanolic Acid and Related Synthetic Oleanane Derivatives: An Experimental Study

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Mechanisms of cardiovascular effects of oleanolic acid and related synthetic oleanane derivatives: an experimental study

by

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Dedication

This PhD thesis is dedicated to the entire Madlala family which include my grandparents, aunts, brothers, sisters, cousins and nephews. I also dedicate it to Ncobeni family including my cousins, then the Danisa family which include all my in-laws especially my mother and granny. I especially dedicate this work to my parents whom I know are the most proud about my academic achievements. Also a special dedication to my fiancée and our son Monde who persevered through a difficult time during my absence in the course of the overseas experiments.

// ACKNOWLEDGEMENTS heading kept inside as it's the acknowledgements section
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DECLARATION

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

"Cardiovascular effects of oleanolic acid and related synthetic oleanane derivatives: an experimental study

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

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PLAGIARISM DECLARATION

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PHD IN HUMAN PHYSIOLOGY 2012-2014

1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's own.

2. I have used the Harvard convention for citation and referencing. Each contribution to, and quotation in, this thesis from the works of other people has been attributed, and has been cited and referenced.

3. This thesis is my own work.

4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

Signature ____________ Date 11/03/2015

LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
ACTH	Adrenocorticotrophic hormone
ADH	Antidiuretic hormone
Ang II	Angiotensin II
ANOVA	Analysis of variance
AP	4-aminopyridine
AQP2	Aquaporin type 2
ARB	Angiotensin receptor blockers
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BDH	British Drug Houses
BH4	Tetrahydrobiopterin
Br-OA	Brominated oleanolic acid
BRU	Biomedical Resource Unit
BSA	Bovine serum albumin
BSC1	Bumetanide- sensitive cotransporter type 1
BUN	Blood urea nitrogen
C	Clearance
CA	California
cAMP	Cyclic adenosine monophosphate
CCB	Calcium channel blockers
CDDO	2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid
CDDO-Me	Methyl 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid

cGMP	Cyclic guanosine monophosphate
CO	Cardiac output
COX	Cyclooxygenase
Cr	Creatinine
CVD	Cardiovascular disease
DCM	Dichloromethane
DG	Diacylglycerol
DHE	Dihydroethidium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium
DPI	Diphenyleneiodonium
DSS	Dahl salt-sensitive
DV	Dual view
EA	Ethyl acetate
EAS	Ethyl acetate solubles
ECF	Extracellular fluid
ECG	Echocardiography
EDCF	Endothelium-dependent contracting factor
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ENaC	Epithelial sodium channel
eNOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
ESR	Electron Spin Resonance

FE	Fractional excretion
FT-IR	Fourier Transform Infrared
GC-MS	Gas chromatography-mass spectrometry
GFR	Glomerular filtration rate
Gli	Glibenclamide
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSSG	Oxidised glutathione
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
HR	Heart rate
I	Current
IBX	2-Iodoxybenzoic acid,
INDO	Indomethacin
iNOS	Inducible nitric oxide synthase
IP3	Inositol trisphosphate
IVC	Inferior vena cava
LDL	Low-density lipoprotein
LED	Light-emitting diode
L-FABP	Liver-type fatty acid-binding protein
L-NAME	*N*-nitro-L-arginine methyl ester
L-NMMA	Nγ-monomethyl-L-arginine
L-NoArg	L-NG-nitro arginine
LOOH	Lipid peroxidases
LV	Left ventricular

LVH	Left ventricular hypertrophy
MA	Maslinic acid
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinases
m-CPBA	*m*-chloroperoxybenzoic acid
MDA	Malonyldialdehyde
Me-OA	Oleanolic acid methyl ester
mp	Melting point
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NAC	N-acetyl-cysteine
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NCC	Sodium/chloride-cotransporter
NCX	Sodium/calcium-exchanger
NHE3	Sodium/hydrogen-exchanger type 3
NKCC2	Sodium/potassium/chloride-cotransporter type 2
NMR	Nuclear Magnetic Resonance
nNOS	Neural nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
NSB	Non-specific binding
O_2^-	Oxygen radical
OA	Oleanolic acic

OT	Oxytocin
PCR	Polymerase chain reaction
PD	Phenylenediamine dihydrochloride
PET	Positron emission tomography
PGI2	Prostaglandin/prostacyclin
PKC	Protein kinase C
PMSF	Phenylmethane-sulphonylfluoride
pNpp	*p*-Nitrophenyl phosphate
PPG	Photoplethysmography
PUFAs	Polyunsaturated fatty acids
PVN	Paraventricular nucleus
RAAS	Renin-angiotensin aldosterone system
RhoA	Ras homolog gene family member A
ROS	Reactive oxygen species
RVLM	Rostral ventro-lateral medulla
RyR	Ryanodine receptors
SEM	Standard error of means
SERCA	Sarcoplasmic reticulum Ca^{2+}-ATPase
SGLT	Sodium/glucose-cotransporter
SHR	Spontaneously hypertensive rats
SMA	Small mesenteric arteries
SOD	Superoxide dismutase
SPECT	Single-photon emission computed tomography
SR	Sarcoplasmic reticulum
STZ	Streptozotocin

SV	Stroke volume
SVC	Superior vena cava
SVR	Systemic vascular resistance
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TGF-β1	Transforming growth factor-β1
TLC	Thin-layer chromatography
TP	Thromboxane-prostanoid
TPR	Total peripheral resistance
TRAP	Total peroxyl radical-trapping antioxidant parameter
TTP	Time to peak
UA	Ursolic acid
UCG	Ultrasound cardiography
UKZN	University of KwaZulu-Natal
UV	Ultraviolet
VPR	Volume pressure recording
WHO	World health organization
8-OH-dG	8-Hydroxy-2-deoxyguanosine

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Figure 26: Concentration–response curves for OA, Me-OA, and Br-OA in endothelium-intact mesenteric arteries [A and B] or aortic rings [C and D] isolated from Wistar rats, pre-contracted with sub-maximal concentration of PHE (5 µM) [**A** and **C**] and KCl (50 mM) [**B** and **D**]. The values shown are means ± SEM (n = 7). ★ $p < 0.001$ vs control, ♦ vs OA). 110

Figure 27: Concentration–response curves for OA in endothelium-intact mesenteric arteries [**A** and **B**] or aortic rings [**C** and **D**] isolated from Wistar and DSS rats, pre-contracted with sub-maximal concentration of PHE (5 μM) [**A** and **C**] and KCl (50 mM) [**B** and **D**]. The values shown are means ± SEM (n = 7). ★ $p < 0.001$ vs control). 111

Figure 28: Concentration–response curves for Br-OA in mesenteric arteries (**A**) and aortic rings (**B**) isolated from Wistar rats pre-contracted with PHE (5 μM). Curves were obtained in endothelium-denuded and intact arteries. Some endothelium-intact vessels were incubated in the presence of INDO (10 μM) only or in combination with L-NoArg (100 μM) prior to addition of the drug. Values shown are means ± SEM (n = 7). ★ $p < 0.001$ vs control. 113

Figure 29: Concentration–response curves for Br-OA in denuded mesenteric arteries (**A**) and aortic rings (**B**) isolated from Wistar rats pre-contracted with PHE (5 μM). Curves in denuded arteries incubated in the presence of Gli (5 mM) or in combination with AP (1 mM) prior to the addition of the drug. Values shown are means ± SEM (n = 7). ★ $p < 0.001$ vs control. 115

Figure 30: Concentration–response curves for Br-OA in intact mesenteric arteries (**A**) and aortic rings (**B**) isolated from Wistar rats pre-contracted with PHE (5 μM). Curves in intact arteries incubated in the presence of INDO (10 μM) and Gli (5 mM) only or in combination with AP (1 mM) prior to the addition of the drug. Values shown are means ± SEM (n = 7). ★ $p < 0.001$ vs control. 116

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APPENDIX V: Conference presentation

Hlengiwe P. Madlala, Thomas Metzinger, Fanie R. van Heerden, Kanigula Mubagwa, Chantal Dessy and Cephas T. Musabayane. "Mechanisms of cardiovascular effects of oleanolic acid and novel synthetic derivatives" Institut de Recherche Expérimentale et Clinique (IREC) [Institute of Experimental and Clinical Research] 2nd PhD day, University of Louvain, Brussels, Belgium 26 September 2014. 172

APPENDIX VI: Draft manuscript

Hlengiwe P. Madlala, Fanie R. Van Heerden, Kanigula Mubagwa and Cephas T. Musabayane. Renal mechanisms of hypotensive effects of OA and related triterpene derivatives. 173

ABSTRACT

Introduction

Despite the various conventional treatments that are available to treat hypertension, this disease continues to be globally responsible for approximately 9.4 million deaths each year. The high mortality can partly be attributed to side effects of available drugs or to the inaccessibility of current synthetic drugs to communities from poor socioeconomic background because of their relative high cost. This problem has resulted in a growing interest in the use of medicinal plant products because they are considered to be cheap, believed to possess few side effects and are easily accessible to the general population in developing countries. Although traditional herbal remedies are widely used in Africa for the management of various disorders including cardiovascular diseases, very little reliable data is available on their therapeutic and pharmacological effects. In search for plants with therapeutic properties for the treatment of hypertension and complications, our laboratory has scientifically evaluated several plant species. In particular, we have isolated *Syzygium* spp-derived triterpenes and focused on the therapeutic effects of oleanolic acid (OA) and maslinic acid (MA). In the present study, we investigated the effects of *Syzygium aromaticum*-derived OA and related synthetic derivatives on arterial pressure and evaluated the underlying mechanisms in Wistar, spontaneously hypertensive rats (SHR) and Dahl salt-sensitive (DSS) animals.

Materials and methods

OA was extracted from dried flower buds of *Syzygium aromaticum* using a previously validated protocol in our laboratory. Oleanolic acid methyl ester (Me-OA) and a brominated derivative of OA (Br-OA) were synthesised according to methods described by Fu and Gribble. The structures of extracted OA and synthesised derivatives were confirmed using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and were comparable to previously reported data. Acute renal clearance studies investigated the influence of OA and derivatives on mean arterial pressure (MAP) and Na^{+} handling in the proximal tubule of anaesthetised Wistar rats using lithium clearance. Animals were given water with lithium (12 mmol L^{-1}) for 48 hours following which they were anaesthetised and cannulated using a previously validated standard protocol that has been reported from our laboratories. After a 3.5 h equilibration, animals were challenged with

hypotonic saline for 4 h of 1 h control, 1.5 h treatment and 1.5 h recovery periods. OA, Me-OA and Br-OA were added to the infusate during the treatment period followed by measurements of arterial pressure, fluid and electrolyte handling.

Sub-chronic study experiments were restricted to OA because of the low amounts of synthetic derivatives obtained during the synthetic studies to prepare the derivatives. Various doses of OA (30, 60, 120 mg kg^{-1}, p.o.) were administered to separate groups of male Wistar, SHR and DSS rats twice (8 h apart) every third day for nine weeks. Rats given dimethyl sulphoxide (DMSO)-saline (3 mL kg^{-1}, p.o.) acted as untreated controls. Mean arterial pressure (MAP) was monitored every third consecutive day using non-invasive tail cuff method with photoelectric sensors. Measurements of body weight, food and water intake, Na^{+}, K^{+}, Cl^{-}, urea and creatinine were taken 24 h after dosing. At the end of this 9 week study, the cardiac, renal and hepatic tissues harvested from hypertensive animals were evaluated for oxidative status by measuring malonyldialdehyde (MDA, lipid peroxidation marker) and antioxidant enzymes; superoxide dismutase (SOD) and glutathione peroxidase (GPx). In addition, we evaluated the effects of OA and derivatives on aldosterone and arginine vasopressin (AVP) secretion on plasma samples from both sub-chronic and acute experimental settings.

Additional *Ex vivo* studies to unravel the mechanisms of action of OA and derivatives were carried out in isolated ventricular cardiomyocytes and arteries. Measurements of cell shortening and Ca^{2+} currents were done in cells isolated from Wistar and DSS animals using edge detection and whole cell patch clamp techniques, respectively. Isometric tension measurements were done in endothelium-intact and denuded aortic rings and mesenteric arteries of Wistar and DSS rats. The influence of indomethacin (INDO), N-nitro-L-arginine methyl ester (L-NAME), glibenclamide (Gli) and 4-aminopyridine (AP) on the effects of OA and derivatives was investigated.

Results

The purity of the plant-derived OA was approximately 98% and the percentage yield varied from 0.79% to 1.72% of the dry plant material. The percentage yield of the synthetic derivatives, Me-OA and Br-OA was 65% and 30%, respectively, from the starting materials. Our results show that OA decreased MAP in both acute and sub-chronic experimental settings, and that the MAP

lowering effect was more marked in hypertensive animals compared to normotensive rats. Similarly, the two OA derivatives, used in acute settings, also decreased MAP. OA increased urinary Na^+ excretion rate under acute and sub-chronic conditions. A similar but quantitatively more marked increase was obtained with Br-OA derivative under acute conditions. Untreated hypertensive animals had elevated levels of plasma aldosterone and AVP in comparison to Wistar rats. OA treatment significantly reduced aldosterone secretion in these animals but had no influence on plasma concentrations of AVP. Compared with respective control rats, OA-treated animals exhibited significantly lower MDA levels and increased activity of the antioxidant enzymes, SOD and GPx in hepatic, cardiac and renal tissues. OA and derivatives had a positive inotropic effect on isolated ventricular cardiomyocytes in Wistar rats and had no influence on the contraction of cells from hypertensive animals. OA and derivatives caused relaxation in aortic rings and mesenteric arteries of both Wistar and DSS animals. This effect was partly inhibited by INDO and K^+ channel blockers when used independently. Addition of L-NAME did not further inhibit the cyclooxygenase (COX)-resistant relaxation. The combination of INDO, Gli and AP completely abolished OA or derivatives-evoked relaxation in endothelium-intact arteries indicating involvement of both endothelium-dependent and independent mechanisms.

Discussion

The present study investigated the effects of OA and related synthetic derivatives (Me-OA and Br-OA) on blood pressure and thereafter examined the possible underlying mechanisms. The absolute stereo-structure of *S. aromaticum*-derived OA and synthetic derivatives elucidated from the spectra using ^{1}H- and ^{13}C-NMR was comparable to previously reported data. Our results confirm the previously reported antihypertensive properties of OA in experimental models of hypertension. However, the present study demonstrates a more marked action in SHR and DSS by comparison with non-hypertensive animals, suggesting a specifically enhanced action in disease conditions. We noticed that urinary Na^+ excretion in control, untreated animals tended to spontaneously increase with time during the following 9 weeks post weanling. In both hypertensive models, no such increase with time was obtained, instead, urinary Na^+ excretion tended to decrease with time after weanling in the DSS model supporting Na^+ retention previously reported in these animals. To further support this theory we found increased aldosterone levels in the plasma of non-treated hypertensive rats. Our data show that OA decreased aldosterone secretion and increased urine Na^+

excretion, relatively larger increases were obtained in the hypertensive models. This suggests that treatment with the drugs was accompanied by alleviation of Na^+ retention in these animals. This increase in urinary Na^+ output is, at least in part, mediated via inhibition of proximal tubular Na^+ reabsorptions as indicated by increased Li^+ clearance. Indeed, we found a positive correlation between the increase in urinary Na^+ excretion rate and the decrease in MAP. However, despite the potent natriuretic effects of these triterpenes mediated by decreased aldosterone levels, the urine flow rate was not changed as supported by unchanged levels of AVP.

Our experiments indicated that OA induces vasorelaxation of aortic rings and small mesenteric arteries in both normotensive and hypertensive animals. The maximum relaxation evoked by the derivatives, particularly Br-OA, was significantly larger in the mesenteric vessels although these differences were not observed in the aorta. Glibenclamide and 4-aminopyridine used allowed a complete blockade of the relaxation to OA, Br-OA and Me-OA suggesting that ATP-dependent and voltage-activated K^+ channels opening mediate the endothelium-independent relaxation. Our experiments using isolated cardiomyocytes showed that OA and derivatives do not decrease but rather tend to increase myocyte shortening and had no influence on Ca^{2+} currents in Wistar rats. Increase cardiomyocyte contractility implies an increase in the force of cardiac tissue contraction and increase cardiac output which may be ideal for a drug which causes natriuresis. The results showed that OA-treated DSS animals had normal levels of MDA, SOD and GPx in comparison to untreated hypertensive rats. Therefore, we suggest that OA-evoked decreased reactive oxygen species (ROS) and increased antioxidant enzymes may have enhanced the production of prostanoids, thereby improving vasodilation. Hence, we speculate that antioxidant properties of OA could play a role in hypotensive mechanisms of this triterpene.

Conclusion

The results of this study introduce the first evidence that OA and its oleanane derivatives induce similar effects. These involve 1) increased urinary Na^+ output mediated by inhibition of proximal and distal tubular Na^+ reabsorption as indicated by increased Li^+ clearance and decreased aldosterone levels, respectively. 2) Modulation of oxidative status in cardiac, renal and hepatic tissues in hypertensive animals. As well as 3) decreased vascular resistance via endothelium-dependent COX/prostanoids pathway and endothelium-independent opening of ATP-dependent

and voltage-activated K^+ channels. The results of this study are novel and clinically relevant because OA and related triterpene derivatives exert multiple blood pressure lowering mechanisms while increasing the force of cardiac contraction hence balancing the fluid volume in the circulatory system so as to avoid a state of hypotension.

Limitations and direction for future studies

Due to low amounts obtained for the synthetic derivatives, we were unable to study their sub-chronic effects in conscious animals, therefore this should be explored in future. OA effects on renal function seem to mimic oxytocin-like activities i.e. increase Na^+ output without changing the urine flow rate, hence future studies should explore whether this hormone can have synergistic effects with OA and derivatives. This study did not evaluate the effects of these triterpenes on nitric oxide production or expression of eNOS and phosphorylation of K^+ channels.

Examiner 1

1. Specify name of Ca^{2+} channel.
   - Voltage-operated calcium channel pg 20.

2. Include section on the control of vascular tone.
   - This section has been included and comprises of various factors which initiate events that lead to contraction of smooth muscle cells based on receiving a relevant stimulus hence controlling the vascular tone. The mechanisms underlying vascular smooth muscle contraction have been discussed with particular mention of Ca^{2+} release being the mediator of contraction and the importance of channels involved including K^+ channels. 1.4.1 and 1.4.1.1 pg 27-29.

3. Add <u>also,</u> amend ROS scavengers, mention the role of uncoupled eNOS in oxidative stress.
   - Added <u>also</u>, deleted the sentence on 'Aprindine……..'; then mentioned currently used ROS scavengers (N-acetyl-cysteine (NAC), vitamins C and E) on pg 30. Mentioned the role of uncoupled eNOS on pg 31 section 1.4.3.

4. Mention classical techniques for monitoring oxidative stress.
   - This has been added on section 1.4.4.1 pg 33.

5. Amend section on therapeutic medications.
   - Vasodilatory effects of β-blockers mediate their function either by α-blocking action or activation of NOS thereby improving endothelial function in patients with hypertension pg 36.
   - ACE inhibitors mediate their anti-hypertensive properties via inhibition of Ang II-dependent vasoconstriction and production of aldosterone pg 36.
   - Statement about cocaine has been shifted to the last paragraph pg 38.

6. Opposing synthetic and natural drugs on the basis of the propency to induce side effects is incorrect.
   - I do not understand the meaning of propency.

7. Page 37 is missing.
   - Pg 37 is not missing on the electronic copy.

8. Change Ang 11 to Ang II.
   - This has been done on pg 7, 17, 27-29, 36.

Methods

1. Add 2.8.2.3 'in a wire myograph (DMT, Aarhus, Denmark)'; 2.8.2.3 were; and 2.8.2.3 software from DMT.
   - These have been added on pg 66.

Results

1. Rearrange structure.
    - Results now divided into 3 chapters (pgs 68, 74, 99) with the results for phytochemical studies having their own chapter. *In vitro* has been replaced with *ex vivo* (pgs 38, 47, 54, 63, 68, 97, 99).

2. Could the effects of derivatives be due to OA contamination?
    - No. The proton and carbon peaks obtained from NMR and IR spectra analysis for synthetic OA derivatives (Me- and Br-OA) were comparable to previously reported data and did not have any additional peaks which don't belong to the derivative's structure indicating that there was no contamination of the derivative products by any traces of OA pg 117/118.

3. Amend NO-dependent mechanisms of vasodilatory effects since this was not the finding of our study.
    - The results of our study suggest that OA and derivatives mediate their vasodilatory effects via additional mechanisms involving prostanoid pathway and action on K^+ channels pg 112, 121.

Examiner 2

1. Acknowledge the role of the central nervous system in blood pressure regulation as abnormalities may also be implicated in hypertension.
    - Dysfunction of the central nervous system has been acknowledged as one of the possible causes of abnormal blood pressure as this system plays a crucial role in the regulation of blood pressure pg 1.

2. Table 1 does not refer to types of hypertension.
    - *As indicated in table 1* has been deleted pg 3.

3. Neural effects for blood pressure long-term regulation.
    - I have included a section on the role of the CNS in the long-term regulation of blood pressure pg 6-7.

4. Pacemaker activity begins at the SAN not AVN.
    - *Atrioventricular* deleted pg 17.

5. Statement 'Reports suggest….' is not clear.
    - Sentence re-phrased pg 18 1st statement.

6. Mention tropomyosin.
    - Added 'and tropomyosin' pg 18 last statement.

7. Energy is only for Na^+/Ca^{2+} exchanger.
    - This has been specified on pg 18 3rd paragraph.

8. Literature on abnormalities of ryanodine receptor.
    - This section has been added on pg 18 1st paragraph.

9. Supra-threshold refers to any size above threshold, replace with that phrase.
    - Replaced with *voltage above threshold* pg 23.

10. Acknowledge other important references for electrophysiology techniques.
    - Hodgkin & Huxley; Neher & Sakmann have been acknowledge for their work in the discovery of electrophysiology techniques pg 24.

11. What is a triterpene.
    - this had been defined on pg 42.

12. Did you extract yourself?
    - Yes.

13. Real record of parameters measured pg 64.
    This is not possible since it can only be done with the Clampfit computer programme which is in the university computers in KU Leuven and I am now in SA (Real recording is shown on the results section (pg 100-102).

14. Discussion on results section?
    There cannot be a discussion in chapter 3 as I needed to separate between results and discussion and make sure not to discuss results on the results section but only to describe what they show.

15. Tyrode solution composition.
    - Tyrode solution containing (in mM: 135 NaCl, 5.4 KCl, 0.9 $MgCl_2$, 10 hepes, 10 glucose, pH adjusted to 7.45 with NaOH) was already mentioned on the methods section for cardiac studies pg 63.
    - Perfusion of cells replaced with superfusion pg 99, and on legends for Figs.18-24 (100-106).

16. Continuous trace of cardiomyocyte contraction.
    - Not possible because the software record traces on separate files as shown (pg 100-102).

17. Could the persistent increase during the wash period indicate that increase during treatment period is a coincidence (pg 100)?

- We think the increase is due to irreversible OA effects after 5 mins of washing the cells. We believe that a longer was period would have brought back the contraction to be the same as control period as there was already a slight decrease after 5 mins, however, for consistency we had to wash for 5 mins only as the drug was applied for that period. In addition, we got the same trend from 6 animals, we tested at least 5 cells for each animal and therefore this exclude the 'coincidental effect',

18. Was n of 2 used for Ca^{2+} current measurements? If so this should be stated explicitly.
    - We had mentioned on the description of results that these were preliminary studies, however, we have added that 'Due to time constraints, we could only perform the experiment on 2 animals, however, at least 3 cells were recorded for each of those two animals and for each drug pg 104.

19. Units for vascular results?
    - They are expressed as Log [drug], doses adopted from Rodriguez-Rodriguez *et al*. (2008) Figs 25-30, pg 108-116.

Examiner 3

1. Partly inhibition of vasodilatory effects by NOS should be omitted as it doesn't correspond with the results of this study.
   - This statement has been deleted pg xxx abstract, pg 113.

2. Amend conclusions based on new contribution of the study in the field.
   - This has now been clearly stated pg xxxi abstract, pg127.

3. Table 1 should be omitted.
   - This has already been deleted pg 3, comment from examiner 2.

4. Include statistics of hypertension in South Africa.
   - This section has been added on 1.1.2 pg 4.

5. Increase not decrease.
   - Amended on pg 5.

6. Include other mechanisms in which aldosterone increase Na^+ absorption.
   - Amended: 'The mechanism through which aldosterone mediates Na^+ reabsorption is by modulating the expression and activation of Na^+ channels in the plasma membrane as well as insertion of Na^+/K^+-pumps in the basolateral membrane' pg 12.

7. Add a reference for cardiomyopathies.
   - Reference added (Maron *et al*., 1986, Foley *et al*., 1996), pg 17 1st paragraph.

8. Remove cardiac glycoside, as it refers to digitalis drugs.
   - *Cardiac glycoside* deleted 1.5.2.1 pg 37.

9. Add a reference for discovery of reserpine.
   - Added on pg 37 (Gilani, 2005) 1st paragraph.

10. Herbal remedies are not always safe unless proven.
    - 'safety' deleted pg 39.

11. Include literature on effects of oxytocin under hormonal influence in the introduction section.
    - This section has been added 1.2.3 pg 13.

12. Add a review on cardiovascular, renal, antioxidant and vasodilatory effects of OA on section 1.6.1.2.
    - This section has been added and used as the basis of this study pg 44-45.

13. Some drugs and chemicals are missing on section 2.0.
    - The list of the missing drugs has been added on pg 47/48.

14. Where was the plant material obtained from?
    - 2.1.1 pg 49 added. 'The dried flower buds of *Syzygium aromaticum* [(Linnaeus) Merrill and Perry] (Myrtaceae) were purchased from the spice market in Durban, South Africa'.

15. Were dried flower buds milled?
    - Yes, amended on pg 49.

16. Add Elisa kits and cell isolation enzymes under drugs and chemicals 2.0
    - Added on pg 48.

17. General chemicals like NaCl need not be listed on 2.0
    - Removed but mentioned that it was also of analytical grade like other chemicals pg 48.

18. Mention spectroscopic technique used for confirmation of OA structure.
    - ^{1}H, ^{13}C NMR and infrared spectra recorded on a Bruker DRX-400 and Bruker Alpha FT-IR spectrometer pg 49/50.

19. Give credit to Fu & Gribble for the use of their method for derivatives synthesis.
    - Statement added on 2.1.3 'We are grateful to Fu and Gribble (2013) for having used their method below for the preparation of oleanane derivatives' pg 51.

20. Do not use brine or normal saline terms or give concentrations for bromination and sub-chronic studies.
    - Changed to NaCl pg 52 and 56.

21. Were the triterpenes soluble in polar solution without DMSO?
    - No, they were dissolved in DMSO 1st before being added into the polar solutions, this has been amended 2.5.1 pg 54; 2.8.1.2 pg 64.

22. Method for determination of urine and blood concentrations of electrolytes pg 54.
    - This is discussed in the next section 2.5.1.1, for more clarity this has been mentioned in the text pg 54.

23. Where were the dosage derived from for acute and sub-chronic studies.
    - The dosage for OA used in the acute studies was based on previous studies (Mapanga *et al*., 2009) and the same dosage for the derivatives was used for fair comparison of the effects pg 54.
    - The same study by Mapanga *et al*., (2009) used 60 mg/kg for sub-chronic studies hence our study decided to add a lower and a higher dose to determine the possibility of dose-dependent effects (Madlala *et al*., 2012). Added on pg 56.

24. Give the name of enzyme conjugate.
    - Aldosterone-alkaline phosphatase conjugate 2.6.1.1 pg 57.

25. Which company provided the ELISA kit.
    - Added: ELISA Kit (DRG International Inc, New Jersey, USA) 2.6.1.1 pg 57.

26. Aldosterone ELISA does not contain a vasopressin antibody.
    - Replaced with polyclonal rabbit antibody 2.6.1.1 pg 57.

27. Volume of assay buffer was 150 µL and not 150 mL.
    - Units changed to µL 2.6.1.2 pg 57.

28. Standards were not added to the Bo wells.
    - Amended on pg 58.

29. Assay was performed in duplicate or triplicate?
    - This was originally mentioned. 'Each determination was performed in duplicates for both standards and the test samples.' 2.6.2.2 pg 58.
    - This information has now been added for the determination of MDA, SOD and GPx 2.7.1 pg 60.

30. Rename kit components.

- Amended: ‘The kit components included a 96 well plate coated with goat anti-rabbit antibodies, vasopressin-alkaline phosphatase conjugate, rabbit anti-vasopressin antibody, assay buffer, wash buffer concentrate, vasopressin standards, p-nitrophenyl phosphatesubstrate (pNpp) and a stop solution’ 2.6.2.1 pg 58.

31. Edit the name of antibody.
    - Rabbit anti-vasopressin antibody pg 58.

32. Replace antigen with non-covalent forces.
    - Amended pg 58.

33. Vasopressin does not bind to conjugate but to the rabbit anti-vasopressin antibody
    - Amended pg 58

34. The wash does not remove enzyme-labelled antibody.
    - The wash removes enzyme-labelled vasopressin pg 58.

35. The plate was coated with what?
    - Already amended for comment 30 above (with goat anti-rabbit antibodies) pg 58.

36. What was used to blank the plate? And how was non-specific binding (NSB) determined?
    - Assay diluent was used as a blank. NBS was determined by adding a NSB solution in some wells and adding all other ingredients pg 58.

37. What is the composition of the isolation buffer?
    - 0.1 M Tris-HCl buffer, pH 7.4 pg 59.

38. How was the line of the standard curve fitted?
    - The BSA standard curve was plotted as a linear fit function of protein concentration pg 60.

39. Include units for Ca^{2+} concentration.
    - 1.8 Mm pg 63.

40. Was the pH adjusted?
    - Yes pH was adjusted to 7.45 with NaOH (was originally mentioned on pg 63).

41. How was the aorta and mesenteric arteries cut into rings?
    - Each aorting or mesenteric segment was cut with a sterile dissection scissor into rings of approximately 1.5 - 2 mm width pg 65.

42. What is meant by rubbing the lumen of the artery with hair?

- The piece of a large diameter straight black hair was inserted inside the vessel and the endothelium was removed by gentle moves back and forth inside the vessel (Gauthier *et al.,* 2008) pg 66.

43. PSS cannot contain both 50 and 94 mM KCl.
    - Amended, high KCl solution used to induce vessel contraction was 94 mM pg 66.

44. Was PSS-DMSO used as a solvent for various drugs used to test vasodilatory effects?
    - Yes, this has now been mentioned on pg 66.

45. Was acetylcholine or carbachol used to test vasodilatory action of drugs?
    - Charbachol, amended on pg 66.

46. What was used to block NOS?
    - L-NAME pg 66.

47. Include stat analysis for acute correlation between urinary Na^+ and MAP.
    - $p < 0.05$ pg 76.

48. Make asterisks visible on figures to indicate stat significance.
    - Made asterisks bigger on figure 9 pg 77.

49. Vertical bars indicating SEM are not visible, decrease side of line and symbols.
    - Amended for Figs 9, 10, 12, 15 pg 77ff.

50. Was total K^+ excretion significant on table 3?
    - Yes for Br-OA, asterisk placed pg 79.

51. Omit statement on referring to FE_{Cl} and FE_K data since it is not shown pg 81.
    * Phrase deleted

52. Give a reason for no dose-dependent MAP effects.
    * we suspect that we recruited maximal effects with the doses used and that more specificity could be demonstrated if sub-maximally effective doses were administered hence it would be recommended to look at lower OA doses for future studies pg 87.

53. Include the legend for Fig 15.
    * Legend is present on the electronic copy pg 91.

54. Do the bars in Fig 16 represent means of the 3 OA doses?
    * No, legend amended, only the data for median dose 60 mg/kg is shown pg 92.

55. Place the asterisks on the treated DSS rats for plasma Na^+ significance not on the value for the control group.
    * Asterisks placed on OA 30, 60, 120 mg/kg Table 7 pg 94.

56. Acute effects on aldosterone and AVP should be moved to section on acute results.
    * Hormone measurements are deliberately placed on their own to avoid having many small tables. In addition, we discriminated between acute and subchronic effects although placed in one table pg 96.

57. What is meant by …. As parameters measured above in this study?
    * Sentence re-phrased 'There was no dose dependent effects observed with the three doses of OA tested in this study' pg 97.

58. Refer to Figs 13 and 16A when talking about OA and derivative effects on ventricular cardiomyocytes.
    - This has been added on 5.1 pg 99.

59. Legend refers to asterisk indicating significance on Figs 18-20 but there is none on the fig itself.
    - Asterisks added on Figs 18-20 pg 100-102.

60. Shift protocol from results on how functional endothelium was tested into the method section.
    - Statement moved to pg 2.8.2.3 pg 66.

61. Add ' than OA and Me-OA'.
    - Phrase added 5.2.1 pg 109.

62. Replace DMSO with control Figs 25-30.
    - Replaced on Figs and legends pg 108-116.

63. Remove (M) on legends for Figs 26, 29, 30.
    - M removed on these Figs pg 110, 115, 116.

64. Show results of the effects of OA and Me-OA on vessel contraction.
    - Fig 26 pg 110 is showing the difference in vasodilatory effects both in the aorta and mesenteric arteries between OA and the two derivatives tested in this study.

65. Clearly state that Br-OA vasodilatory effects were blunted by indomethacin pre-treatment.
    - This fact has been explicitly stated on pg 112, paragraph 2, sentence 2.

66. Rephrase legend for Fig 28 'endothelium-intact vessels were incubated in the presence of INDO only or in combination with L-NoArg'.

- Legend amended pg 113.

67. Refer only to Br-OA and not OA and derivatives because only results for Br-OA are shown.
    - Reference to OA and derivatives deleted 5.2.3 pg 114.
68. Delete the statement 'larger endothelium-dependent relaxation observed with Br-OA as no comparison is shown for OA and Me-OA.
    - Statement removed pg 114.

69. Rephrase legend Fig 29 to 'denuded arteries incubated in the presence of Gli (5 mM) or in combination with AP'.
    - Statement amended pg 115.

70. Rephrase Fig 30 legend to 'intact arteries incubated in the presence of INDO (10 μM) and Gli (5 mM) only or in combination with AP'.
    - Statement amended pg 116.

71. Refer to the relevant result in the 1st paragraph of the discussion.
    - Amended accordingly pg 117.

72. Give a reference for Na^+ retention in DSS animals.
    - Stated Somova *et al*., 1999 pg 119.

73. Ref to Fig 11C should be omitted unless stats significance is proven.
    - There is a significance in the correlation of acute Na^+ excretion and MAP hence statement cannot be omitted pg 119. See comment 44 above.

74. Move the statement about 'OA mimic OT-like effects' from limitations and future studies to the discussion to try and explain the increase in Na^+ excretion without influence on urine flow.
    - A brief discussion about these findings are now mentioned pg 119/120.

75. Attempt to discuss how can OA increase urinary Na^+ output but not food intake or change plasma Na^+ levels or at least mention it for future studies.
    - Speculation on pg 120 and 127.

76. Na^+, K^+ and Cl^- use the same channels in the nephron so how do you explain increase Na^+ output without any change in K^+ and Cl^- ions?
    - A brief discussion for the possible explanation for this is on pg 120.

77. Discuss the finding about the involvement of COX/protanoid and not NOS pathway for vasodilatory effects of OA and this in disagreement with Rodriguez's findings.
    - This has been discussed on pg paragraph 2, pg 121.

78. Only refer to Br-OA for Glib and AP-mediated vasodilatory effects as the results for OA and Me-OA are not included.
    - Phrase deleted paragraph 1 pg 121.

79. Plasma Na^+ concentration cannot be used as an explanation for vasodilatory effects as this parameter did not change in Wistar and SHR animals.
    - Have specified that this was only observed in DSS animals, pg 122.

80. Add a reference for reduction of plasma Na^+ and vasodilatory effects via decreased Ca^{2+} concentration.
    - Have stated that this is a speculation not a statement based on literature findings.

81. Discuss the difference in increase GFR during sub-chronic study and no change in this same parameter during acute study.
    - A possible reason which is a difference in experimental periods as well as dosages used, has been given on pg 119.

82. Intravascular fluid volume cannot be used to explain cardiomyocyte contraction since water intake and excretion were not changed.
    - Statement deleted pg 124.

83. Compare oxidative stress results with those obtained by Somova *et al.*, 2003.
    - Mentioned pg 125.

84. add a reference for anti-diuretic action of OT
    - Conrad *et al.*, 1986 pg 127.

85. Use reference style for National Library of Medicine.
    - The university requirement for references is Harvard style and therefore cannot be changed.

86. Improve ***editorial care*** quality on the following:

    i. Don’t define Greek letters.
    - α, β, δ, γ and μ deleted from the list.

    ii. Don’t define periodic table elements.
    - Br_2, C, Cl^-, Cs, H, H^+, K^+, Li^+ and Na^+ deleted from the list.

    iii. Don’t define chemical formula’s.
    - AcOH, $CaCl_2$, CH_3OH, CH_3ClN, $CHCl_3$, CO_2, Et_2O, H_2SO_4, HBr, $KBrO_3$, KCl, LiCl $MgCl_2$, $MgSO_4$, $Na_2S_2O_3$, Na_2SO_4, NaCl, $NaHCO_3$ and NaOH deleted from the list.

iv. Don't define standard units of measurements.

- °C, g, Hz, I kD L, M, m, m.p, m/v, MHz, mmHg, mmol, nmol, OD, ppm, % and s deleted from the list.

v. Add missing abbreviations to complete the list.

- C, Cr, COX, FE, GSSG, HEPES, L-NoArg, MA, MAP, PMSF, SVC, TBARS added in the list.

vi. Align text vertically i.e IBX.

- IBX aligned accordingly.

vii. Correct spelling mistakes.

- Monomethyl pg x, distal pg 11, interstitium pg 11, copper pg 60, cyclooxygenase pg 112 and weaning pg 53, 90, 119 corrected.

viii. Don't define standard abbreviations e.g Ltd.

- Ltd and Pty deleted from the list.

ix. Don't define standard prefixes for decimal submultiples.

- m deleted from the list.

x. Use commas appropriately.

- Comma next to *N*-nitro-L-arginine methyl ester deleted pg x.

xi. Use full stops appropriately.

- m.p. full stops deleted pg xi.

xii. Define abbreviations correctly.

- Reduced nicotinamide adenine dinucleotide phosphate (oxidase deleted) pg xi.

xiii. Number sections continuously.

- Section 1.3 inserted pg xv.

xiv. Space sections consistently.

- 2.3 and 2.4 separated with a space pg xvi.

xv. Space text consistently.

- n=6 changed to n = 6 pg xxii and xxiii.

xvi. Use South African English spelling consistently.

- Words spelled correctly: Recrystallisation pg 69; synthesised pgs 1, 29, 44, 76; anaesthetised pgs 54, 55, 68, 76, 81; characterised pgs 4, 16, 26, 123, 125; centres pg

4; normalised pg 64; localisation 23; polarisation pgs 24, 29; defence pgs 1, 31, 60, 124; oxidised pgs 35, 51, 52, 62; summarises 40; metres 42; colour pgs 33, 43, 57, 59, 60; deionised pgs 50, 59, 60 and favour pgs 112, 122.

xvii. Use capital letters correctly.

- structure xxi, rhomitoxin pg 37, PLoS 131, 150.

xviii. Define the word upon 1st use.

- Corrected: NMR pg 50, DMSO pg 47, NOS pg 29, ROS pg 27, COX pg 27, NHE3 pg 10, SR pg 17, ACh pg 29, UA pg 49, TLC pg 49.

xix. Stop defining abbreviations twice.

- Second definitions deleted: AVP pg 12, VPR pg 9, NCC pg 11, ADH pg 12, HPLC pg 32, ELISA pg 32, ACE pg 7, ARB pg 37 and iNOS pg 44.

xx. Use plural noun form appropriately.

- Corrected: Other physical factors pg 2.

xxi. Use South African English words correctly.

- Replaced epinephrine with adrenalin pg 5.

xxii. Use singular verb form correctly.

- OA induces pg xxxi, osmolarity activates pg 5, cascade leads pg 12 and resistance implies pg 125.

xxiii. Insert abbreviation upon 1st use of the word.

- RAAS abbreviated accordingly pg 6.

xxiv. Use the abbreviation once the word has been defined.

- Abbreviation used for: PPG pg 8, RAAS pg 7, CO pg 5, HR pg 15, SV pg 15, SR pg 17, NOS pg 29 and CCB pg 37.

xxv. Indicate ionic form of Na^+ correctly.

- Corrected superscript pg 10.

xxvi. Hyphenate words correctly.

- Na^+/K^+-pump (Na^+/K^+-ATPase) pg 11 and endothelium-dependant pg 107, 121 hyphenated correctly.

xxvii. Use Li^+ instead of lithium just like consistent use of Na^+.

- Lithium replaced with Li^+ pg 10, 14, 15, 47, 55, 82, 119, 120.

xxviii. Number sections sequentially.

- Sections numbered correctly 1.3…1.3.1 etc pg 15-16.

xxix. Add definite article appropriately.

- Article added: The SV multiplied……pg 15.

xxx. Add indefinite article appropriately.

- Article added: In addition, a plant extract…. Pg 17.

xxxi. Format superscript correctly.

- All Ca^{2+}-ATPase already formatted correctly pg 19.

xxxii. Finish your sentences.

- Sentence completed: the relaxation time constant obtained by fitting an exponential on the contraction curve pg 23.

xxxiii. Replace backslash with or in the text.

- Replaced: heart attack or stroke volume pg 26.

xxxiv. Replace letter G with a Greek letter γ.

- Replaced Nγ-monomethyl-L-arginine pg 30.

xxxv. Insert missing words or units.

- Inserted: urinary pg xxii, The pg 20, loss pg 35, compound pg 49, conditions pg 53, Ciocalteu pg 59, mean body weight pg 66, Wistar pg 75, solution pg 99, rats 104, mV 104, 105, 106, mg/kg BW pg 87, and pg 127.

xxxvi. Define abbreviations correctly.

- Angiotensin receptor blockers (ARB), deleted antagonists pg 36.

xxxvii. Use italics correctly.

- Italicised *Digitalis purpurea* and removed italics from willow bark pg 37.

xxxviii. Use commas appropriately.

- Comma inserted: Not easily accessible, especially…. Pg 39.

xxxix. Merge paragraphs of same ideas.

- Merged these statements: …aspirated. The process pg 58.

xl. Capitalize acronyms correctly.

- HEPES capitalized correctly not Hepes pgs 48, 63, 65.

xli. Abbreviate phenylephrine as PHE not Phe.

- Replaced Phe with PHE pgs 48, 67, 109-116.

xlii. Replace….*curves was constructed* with …*effects were measured.*

- Replaced pg 67.

xliii. Abbreviate indomethacin with INDO, glibenclamide with Gli and 4-aminopyridine with AP.

- Replaced pgs 47, 67, 112 - 116, 122.

xliv. Use the semicolon consistently.

- Semicolon inserted after liver pg 69.

xlv. Replace u with Greek letter μ in some figures.

- Replaced on Figs 11C pg 80, 18B pg 100, 19B pg 101, 20A & B pg 102, Figs 22 pg 104, 23 pg 105, 24 pg 106.

xlvi. Abbreviate body weight with BW and not b.wt.

- Replaced pg 83 - 86.

xlvii. Add full stops at the end of sentences.

- Full stop placed after model pg 90.

xlviii. Refer to Fig 16C instead of Fig 15.

- Changed accordingly pg 90.

xlix. Separate text with spaces.

- Text separated on legend for Fig 15 E, F pg 91.

l. Change relative pronoun *whose* to *which.*

- Changed accordingly pg 97.

li. Use the passive instead of active tense.

- Actual sentences not specified hence not too sure what is being referred to pgs 66 & 99.

lii. Change legend of Fig 25 & 28 to *denuded and intact endothelium.*

- Legends changed accordingly: pg108 & 113.

liii. Remove the last full stop from the numbering of sections.

- Last full stop removed: section 5.2 - 5.2.3 (pgs 107, 109, 112 and 114).

liv. Format exponents correctly.

- Exponent formatted 10^{-4} M pg 109.

lv. Remove Phe from the axis title.

- Phe replaced with PHE and KCl added to accommodate KCl contracted vessels Figs 26 - 30 (pgs 110 - 116).

lvi. Change Indom/nitroArg in the legend to INDO + L-NoArg.

- Legend changed accordingly Fig 28 pg 113.

lvii. Split long sentences.

- Sentences split: …. To excrete Na^+. Indeed….Na+ excretion. Significantly larger…. Pg 119 1st and 2nd paragraphs.

lviii. Format subscripts correctly.

- Formatted correctly: C_{cr} pg 123.

For the following comments, the numbers refer to references and not pages!

lix. Add volume number of journals.

- Volume numbers added on refs 65, 80, 135.

lx. Replace the square symbol with hyphen.

- Hyphen not resent on electronic copy refs 122, 224.

lxi. Add missing page numbers of journals.

- Page numbers added refs 67, 133, 147, 324, 327, 339.

lxii. Use a single not double full stop.

- Second full stop removed refs 72, 155, 171, 190, 230.

lxiii. Disregard hyphens and prefixes in given names.

- Hyphens removed refs 117, 120.

lxiv. Add a colon and a space after the volume number.

- Added ref 139.

lxv. Don’t repeat single numbers if a single page is cited

- Page numbers corrected ref 243.

lxvi. Use a single not a double colon.

- Second colon removed refs 226 and 375.

lxvii. Hyphenate and space page numbers consistently.

- Corrected refs 56 ff (too many to list).

CHAPTER 1

INTRODUCTION / LITERATURE REVIEW

1.0 Basis of the study

Despite conventional treatments available for elevated blood pressure in the market, hypertension continues to be associated with the deaths of approximately 9.4 million people each year globally. The high mortality can partly be ascribed to side effects and inaccessibility of current synthetic drugs especially to the communities from poor socioeconomic background because of high cost. This problem has resulted in a growing interest of many communities to use medicinal plant products because they are cheaper. Although the use of medicinal plant extracts by different cultures around the world, including rural Africa, dates back to the origin of the human being, only recently have scientific studies started to validate their therapeutic and pharmacological properties. In search for plants with potential beneficial effects in alleviating cardiovascular disorders, we have also scientifically evaluated several plant species. In particular, we have isolated triterpenes from *Syzygium* spp and focused on the therapeutic effects of oleanolic acid (OA) and maslinic acid (MA). Literature has reported diverse pharmacological properties of OA and this therapeutic importance has led to the use of this triterpene as a starting material for the synthesis of new oleanane derivatives. In this study, we synthesised oleanane derivatives and investigated their therapeutic activities on experimental models of hypertension. We also investigated the effects of OA and related synthetic derivatives, a methyl ester of OA (Me-OA) and a brominated derivative of OA (Br-OA) on arterial blood pressure in Wistar, and experimental models of hypertension; Dahl-salt sensitive (DSS) and spontaneously hypertensive rats (SHR). The potential mechanisms responsible for an abnormal blood pressure include a combination of abnormal renal electrolyte handling, myocardial, endothelial and central nervous system dysfunction. Accordingly, we investigated whether these oleananes influence kidney, cardiac and vascular functions of hypertensive animals. Studies report that hypertension is associated with oxidative stress which plays a major role in endothelial dysfunction. In this regard, we also evaluated the effects of OA on oxidative status of cardiac, renal and hepatic tissues by monitoring levels of malonyldialdehyde (MDA), a commonly known marker of oxidative stress, and the levels of antioxidant defence enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx) in SHR and DSS animals.

1.1 Introduction / Literature review

1.1.1 Blood Pressure

Blood pressure (arterial) can be defined as the pressure exerted by circulating blood on the walls of blood vessels. During each heartbeat, blood pressure varies between a maximum level (called systolic pressure, and occurring during cardiac contraction) and minimum level (called diastolic pressure, associated with cardiac relaxation. The mean arterial pressure is an average of the pressure over the whole cardiac cycle (Janssen, De Celle, Debets, Brouns, Callahan and Smith, 2004). Determinants of the mean arterial pressure are the cardiac output (CO) and the total peripheral resistance (TPR) (Hsiu, Chao, Hsu, Jan, Wang and Wang, 2008), and an abnormal change in blood pressure is often an indication of a problem affecting the cardiovascular system. Hence, blood pressure monitoring is critical for assessment of any pathology related to CO and blood vessel resistance (Calhoun, Jones, Textor, Goff, Murphy, Toto, White, Cushman, White and Sica, 2008). These parameters are influenced by physical factors such as dietary salt intake, exercise, disease, alcohol, stress and obesity (Writing Group of the Premier Collaborative Research Group, 2003). This thesis investigated how therapeutic interventions modulate the effects of diet and genetic factors on the development of high blood pressure in experimental animals.

Other physical factors such as blood viscosity also affect blood pressure by their effect on the resistance to flow (Walsh, Culleton, Tonelli and Manns, 2005). Under normal conditions, changes in blood pressure have an effect of the activity of the autonomic nervous system, which then triggers corrective mechanisms that will bring blood pressure back to normal levels (Guyenet, 2006). Despite the involvement of nervous and other different organs systems in the regulation of blood pressure, chronic disease conditions can result in dysfunctional regulatory mechanisms which can cause blood pressure to become too low or high. This study was interested in pathologically high blood pressure referred to as hypertension which is defined as systolic and diastolic pressures of > 140 and 90 mmHg, respectively.

1.1.2 Hypertension

Hypertension, commonly known as high blood pressure, is a chronic medical condition in which the blood pressure in the arteries is elevated. The most common form of hypertension is classified as primary (essential) hypertension which contributes about 90-95% of hypertension cases with unknown underlying medical cause (Rosario and Wesson, 2006). The remaining cases (5-10% of hypertension cases) constitute secondary hypertension and are due to underlying diseases that affect the kidneys, arteries, heart and endocrine system (Kearney, Whelton, Reynolds, Muntner, Whelton and He, 2005). Gestational hypertension is also a case of secondary hypertension and occurs in approximately 8-10% of pregnancies. Most women with high blood pressure in pregnancy are at risk of developing pre-eclampsia which occasionally progresses to life-threatening eclampsia (Dildy and Belfort, 2010). Eclampsia is a hypertensive emergency and has several serious complications including vision loss, cerebral oedema, seizures or convulsions, renal failure and pulmonary oedema (Duley, 2009). In the present study we used the accepted experimental models of hypertension, Dahl-salt sensitive (DSS) and spontaneously hypertensive (SHR) rats to evaluate the effects of plant-derived products as potential alternative treatment for hypertension.

Over 1 billion people are living with high blood pressure and this number is continuing to grow despite the use of conventional therapeutic drugs to control this condition (WHO, 2013). The global prevalence of hypertension in 2008, in adults aged 25 and above was around 40% (WHO, 2013). Arterial hypertension is a major risk factor for renal failure and cardiovascular disease (CVD) such as heart attacks, stroke, left ventricular hypertrophy and heart failure. An estimated 17.3 million people died from CVDs in 2008 of which over 80% of this mortality occurred in low- and middle-income countries where health systems are weak (Meyer, Yoon, Kaufmann and Office for State, 2013). The number of annual CVD mortalities is estimated to reach 23 million by 2030, hence hypertension poses a heavy health burden in affected countries (Mathers and Loncar, 2006).

Studies reported a significant burden from hypertension, referred to as the silent epidemic in South Africa, due to under-diagnosis or inadequately treatment which results in extensive target-organ damage and premature death (Hasumi and Jacobsen, 2012). In population-based surveys, high rates of hypertension were found among older adults in South Africa, 44.0-52.0% among men and 51.6-60.4% among women in 1998 (Steyn, Gaziano, Bradshaw, Laubscher and Fourie, 2001). These have probably increased due to a rise in the predisposing factors but recent data on the national prevalence of hypertension are, unfortunately, lacking. Data from the South African Dialyses and Transplantation Registry have shown that hypertension was responsible for 35% of end-stage renal failure in blacks and that malignant hypertension was diagnosed in 57% of the black patients with essential hypertension (Veriava, Du Toit, Lawley, Milne and Reinach, 1990). Elevated blood pressure triggers regulatory mechanisms which are responsible for maintaining normal blood pressure levels.

1.1.3 Regulation of blood pressure

The endogenous regulation of arterial pressure is not completely understood. However, the mechanisms of arterial pressure regulation outlined in the following sections have been well-characterised. Based on the time scale of their effects, one can distinguish two types of mechanisms that mediate regulation of blood pressure. These are short- and long-term regulatory mechanisms.

1.1.3.1 Short-term regulation

Short-term mechanisms are largely neural and regulate blood pressure by influencing blood vessel diameter, heart rate (HR) and contractility (Janssen, Leenders and Smits, 2000). When arterial blood pressure changes, the baroreceptors in the high pressure receptor zones detect these changes and send impulses to the medulla of the brain stem, particularly the rostral ventro-lateral medulla (RVLM) (Kishi, Hirooka, Kimura, Ito, Shimokawa and Takeshita, 2004). The most important arterial baroreceptors are located in the left and right carotid sinuses and in the aortic arch. A decrease in mean arterial pressure decreases the stretch of baroreceptors, which then transmit less afferent inputs to the medulla. As afferent signals normally inhibit centres in the medulla, the decreased pressure results in an increased activity of sympathetic efferent signals and a decreased

activity of parasympathetic efferences. The combination of increased sympathetic activity and decreased parasympathetic activity adjusts the mean arterial pressure by increasing both the force and rate of the heart contractions, hence increasing the CO, and by causing peripheral vasoconstriction. In contrast, an increase in mean arterial pressure increases the stretch of baroreceptors, which then transmit more afferent signals to the medulla, and results in a decreased sympathetic efferences and increased parasympathetic efferences. The combination of a decreased sympathetic tone and an increased parasympathetic activity will adjust the mean arterial pressure by decreasing both the force and rate of the heart contractions, hence decreasing the CO (Freeman, Dewey, Hadley, Myers and Froelicher, 2006). In addition, the decreased sympathetic activity causes relaxation of vascular smooth muscles and increases the arterial diameter hence decreasing total peripheral resistance (Munir, Jiang, Guilcher, Brett, Redwood, Marber and Chowienczyk, 2008).

Whereas the above effects are neutrally mediated, they are also enhanced by changes of the hormonal release of adrenalin and noradrenalin by the adrenal gland (Morrison and Cao, 2000). In addition, other changes that are associated with those of blood pressure may themselves constitute additional trigger mechanisms for responses that have an effect on blood pressure. Decreased blood volume (which can be the cause of the decrease in blood pressure) constitutes itself a trigger mechanism for responses that involve increased thirst (leading the individual to drink) and increased arginine vasopressin (AVP) secretion (causing increased water reabsorption in the kidneys). Increased blood osmolarity also activates the thirst centre in the hypothalamus (Heusser, Tank, Engeli, Diedrich, Menne, Eckert, Peters, Sweep, Haller and Pichlmaier, 2010). Conditions that cause dehydration such as diarrhoea result in increased osmolarity which is sensed by the hypothalamus which in turn stimulates the release of antidiuretic hormone (ADH) by the posterior pituitary gland. ADH mediates water retention through V_2-type receptors by causing insertion of water channels (aquaporin-2) in the apical membrane of the principle cells via activation of adenylate cyclase in the late distal tubules and collecting ducts (Schrier, Chen and Cadnapaphornchai, 2004).

Therapeutic drugs such as alpha-adrenergic receptor blockers and beta-adrenergic receptor blockers (β-blockers) exert part of their effect by interfering with the neural regulation of the cardiovascular system. Alpha-blockers are used to reduce vascular restistance, whereas β-blockers are used to reduce force and rate of heart contraction. Plant extracts such as reserpine also interfere with the neural regulation. Extracts of *Mentha villosa*, *Orthosiphon aristatus, Petasites formosanus* and a bioactive compound, verapamil isolated from *Sideritis raeseri* have been shown to possess blood pressure lowering effects mediated by reduction of the force and the rate of contraction in the heart as well as vasorelaxant effects on smooth muscles (Matsubara, Bohgaki, Watarai, Suzuki, Ohashi and Shibuya, 1999; Wang, Shum, Lin, Liao, Wu, Ren and Chen, 2001; Guedes, Silva, Barbosa-Filho and Medeiros, 2004; McNeill and Jurgens, 2006). The effects of the latter extracts are likely mediated by a direct action on cardiac and vascular cells. This study investigated whether plant-derived triterpenes influence the force of contraction of cardiomyocytes and vascular smooth muscle cells to probably modulate the blood pressure.

1.1.3.2 Long-term regulation

Long-term regulation of blood pressure is mainly achieved by altering the blood volume. Incidents such as haemorrhage, or donating blood lower blood pressure and therefore trigger processes that restore blood volume or pressure back to normal levels. Long-term regulatory processes stimulate the preservation of body fluids by neural and renal mechanisms which normalise blood volume or pressure via the sympathetic or parasympathetic systems and the renin-angiotensin aldosterone system (RAAS), respectively (Bie, 2009). Neural regulation of arterial blood pressure is maintained by baroreceptors located at the carotid sinus and aortic arch that transmit their signals to integrative medullary areas (Chapleau, Li, Meyrelles, Ma and Abboud, 2001; Fisher and Paton, 2012). Thus, the balanced activity of the efferent autonomic nervous system and arterial baroreceptors is essential for the control of the cardiovascular system to achieve optimal blood flow to the organs of the body. Autonomic neural regulation of the circulation that provides optimal perfusion of every organ in accordance to its metabolic needs. The baroreceptor reflexes activated during a rise in pressure and the chemoreceptor reflexes activated with hypoxia and acidosis are powerful triggers of essential circulatory and ventilatory adjustments (Snitsarev, Whiteis, Abboud

and Chapleau, 2002). Abnormalities in this coordinated neural effects of blood pressure control may result in abnormalities which could cause cardiovascular diseases including hypertension. Renal mechanisms that mediate arterial pressure regulation involves RAAS which is activated when the juxtaglomerular cells in the kidney sense the decline in blood pressure and release renin into the blood stream. The renin activates the inactive angiotensinogen, a plasma protein released by the liver, into angiotensin 1. Angiotensin 1 is converted to angiotensin II (Ang II) by an angiotensin-converting enzyme (ACE) in the lung capillaries (Weir and Dzau, 1999). Ang II is a vasoconstrictor and therefore contributes to the changes in vascular peripheral resistance. Ang II reaches the adrenal gland via the blood stream and stimulates the adrenal cortex cells to release aldosterone.

Aldosterone stimulates sodium retention and potassium excretion by the distal tubules of the kidney. Since sodium is the main ion that determines the extracellular fluid (including vascular fluid) volume, aldosterone increases indirectly arterial pressure (Meneton, Loffing and Warnock, 2004). When the blood pressure is increased such as in hypertension, the opposite is true for the events mentioned above. Therapeutic drugs such as ACE inhibitors as well as diuretics like thiazides are used to lower the blood pressure in hypertension (Wing, Reid, Ryan, Beilin, Brown, Jennings, Johnston, McNeil, Macdonald and Marley, 2003). Plant extracts of *Rosmarinus officinalis, Centaurium erythraea, Carum carvi, Tanacetum vulgar* and *Tropaeolum majus* have been shown to block RAAS and possess diuretic properties (Haloui, Louedec, Michel and Lyoussi, 2000; Lahlou, Tahraoui, Israili and Lyoussi, 2007; Gasparotto Junior, Boffo, Lourenço, Stefanello, Kassuya and Marques, 2009). To elucidate the mechanisms by which plant-derived potential therapeutic products tested in this study influence the blood pressure, we investigated the effects of these drugs on fluid and electrolyte handling by the kidney in hypertensive animals.

1.1.4 Techniques used for blood pressure measurement

The auscultatory technique and mercury sphygmomanometer used to be the method of choice for blood pressure measurement. However, due to toxicity of mercury, new devices known as hybrid sphygmomanometers have been developed to replace the mercury column by electronic pressure

gauge similar to oscillometric devices (Pickering, 2003). The major difference with oscillometric technique is that the oscillations of pressure in a sphygmomanometer cuff are recorded during gradual deflation, the point of maximal oscillation represents the mean intra-arterial pressure (Ogedegbe and Pickering, 2010). In addition to these two methods is the ultrasound technique which uses a transmitter and receiver positioned over the brachial artery under a sphygmomanometer cuff. This method also perceives the onset of blood flow at systolic pressure, which has been found to be of particular significance for measuring pressure in infants and children (Clothier, Rigby and Sinha, 2012). This technique is beneficial for patients with very faint Korotkoff sounds since placing a Doppler probe over the brachial artery may help to detect the systolic pressure. This experimental study, however, was conducted on animals and various techniques that are currently used for blood pressure measurements particularly in rodents are outlined below.

1.1.4.1 Invasive and non-invasive techniques

Direct blood pressure measurement using invasive surgical procedures is the gold standard in terms of accuracy in comparison to the non-invasive blood pressure technologies. Radio telemetry is a highly invasive surgical procedure which involves implanting radio transmitters in the rodent's body (D'eon, Serrouya, Smith and Kochanny, 2002). The non-invasive blood pressure methodology consists of placing a cuff on the animal's tail to block the blood flow. The three types of non-invasive blood pressure sensor technologies include photoplethysmography (PPG), piezoplethysmography and volume pressure recording (VPR).

PPG is based on light and detects the first presence and absence of the pulses which corresponds to deflation and inflation of the occlusion cuff, respectively. The sensor is responsible for Illumination of the tail with the light and for measuring tiny changes in light intensity normally caused by variations in perfusion volume of the tail (Maeda, Sekine and Tamura, 2011). Piezoplethysmography records the presence of a pulse in the tail which corresponds to systolic blood pressure and HR. This technology is based on piezoelectric ceramic crystals which are responsible for detection of the pulse signal. VPR is a non-invasive technique which records blood

volume by means of differential pressure apparatus. VPR measures systolic, diastolic, mean blood pressure, heart pulse rate, tail blood volume and tail blood flow (Daugherty, Rateri, Lu and Balakrishnan, 2009). This technique is reliable due to absence of light illumination which is normally associated with ambient light artifacts. This study used both PPG and VPR non-invasive tail cuff measurement techniques to monitor blood pressure in experimental hypertensive animals.

We investigated the effects of the triterpenes on kidney function, isolated cardiac cells and vascular smooth muscles to determine the mechanisms of their hypotensive effects. The next paragraphs will outline the roles of kidney, cardiac and vascular function in the regulation of blood pressure and how their normal function is influenced by hypertension. In addition, the section discusses how therapeutic drugs and plant-derived herbal extracts alleviate abnormalities caused by hypertension as well conventional methods that are used to assess the functional status of these body systems.

1.2 Kidney function

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

1.2.1 Importance of Na^+ handling

Na^+ is the major cation that controls osmolality in the extracellular fluid (ECF) volume (DeFronzo, Cooke, Andres, Faloona and Davis, 1975) and hence Na^+ reabsorption is vital for maintenance of homeostasis in the ECF (Edwards, 2010; DeFronzo, Davidson and Prato, 2011). Na^+ present in the ECF is filtered and reabsorbed in the largest amount in the kidneys. Reabsorption of water and many other solutes is linked to Na^+ reabsorption as some substances depend on co-transport with Na^+ for their uptake (Hackenthal, Paul, Ganten and Taugner, 1990; Klar, Vitzthum and Kurtz, 2004). However, the normal blood volume brought about by Na^+ reabsorption is disturbed in hypertension since this condition is associated with Na^+ and water retention. Na^+ reabsorption consumes a tremendous amount of adenine triphosphate (ATP) and oxygen and this is why the kidney receives approximately 25% of CO (Lewy, Quintanilla, Levin and Kessler, 1973; Ljungman, Laragh and Cody, 1990; Hallows, Mount, Pastor-Soler and Power, 2010). Two thirds of the filtered Na^+ is handled in the proximal tubule through transport processes explained below. Proximal tubular Na^+ handling can be quantitatively estimated through lithium (Li^+) clearance and hence this study employed this technique to establish OA and derivatives' effects on Na^+ handling in the proximal tubule.

1.2.2 Transport processes in the renal tubule

The most important process in the proximal tubule is the reabsorption of two thirds of the filtered Na^+ by an active process using transport proteins like the apical Na^+/H^+-exchanger type 3 (NHE3) and the basolateral Na^+/K^+-ATPase (Auriemma, Galdiero, De Martino, De Leo, Grasso, Vitale, Cozzolino, Lombardi, Colao and Pivonello, 2010). In the loop of Henle, the countercurrent multiplication system preserves inner medullary interstitial solute gradient (Yuan and Pannabecker, 2010). The transport proteins that play a role in this process include the $Na^{+/}K^+/Cl^-$ cotransporter type 2 or bumetanide-sensitive cotransporter type 1 (NKCC2/BSC1) and the NHE3 (Pannabecker and Dantzler, 2006; Pannabecker and Dantzler, 2007). In the early part of distal tubule, a thiazide-sensitive Na^+/Cl^-cotransporter (NCC) plays a major role, whereas in more distal parts and in the initial part of the collecting tube amiloride-sensitive Na^+ channels are involved. In

all segment the transport of Na^+ from the tubular cells to the peritubular interstitium is mainly via the Na^+/K^+-pump (Na^+/K^+-ATPase).

Hypertension is associated with increased Na^+ reabsorption probably due to increase function of the NHE3 in the proximal tubule and this is accompanied by water retention. The function of Na^+ transporters like NKCC2/BSC1, NHE3 and NCC can be inhibited by drugs such as furosemide, bumetanide, thiazides, hydrochlorothiazide and bendroflumethiazide; potassium sparing, amiloride and carbonic anhydrase inhibitors, acetazolamide (Martinez-Maldonado and Cordova, 1990; Kim, 2004; Pannabecker and Dantzler, 2007). This inhibition can be beneficial in hypertension by causing natriuresis and diuresis which may decrease blood volume (Martinez-Maldonado and Cordova, 1990; Kim, 2004). Some plant extracts such as those from *Foeniculum vulgare* L. (fennel, Apiaceae) have been shown to increase water and sodium excretion (Bardai, Lyoussi, Wibo and Morel, 2001). Furthermore, aqueous extracts from the roots of *Carissa edulis* (forssk) vahl (Apocynaceae), *Ananas comosus* (Linn.) Merr. and *Carica papaya* Linn. (Pawpaw, Melon tree) have been shown to possess diuretic properties (Sripanidkulchai, Wongpanich, Laupattarakasem, Suwansaksri and Jirakulsomchok, 2001; Nedia, Mekonnena and Urgab, 2004). Transport processes occurring in the late distal tubules and collecting ducts are mostly influenced by hormones.

1.2.3 Hormonal influence

Late distal tubules and collecting ducts have principal and α/β-intercalated cells which play a role in acid-base homeostasis (Prasad, Narra, Shah, Humphrey, Jagirdar, Catena, Dalrymple and Siegel, 2007). Principal cells play a role in the reabsorption of Na^+ which is associated with secretion of K^+. Na^+ reabsorption is increased by aldosterone whose effects are mediated through Na^+ pumps and transporters (Abdallah, Schrier, Edelstein, Jennings, Wyse and Ellison, 2001; Arroyo, Ronzaud, Lagnaz, Staub and Gamba, 2011). Principal regulators of aldosterone biosynthesis are the RAAS, extracellular potassium concentration and adrenocorticotrophic hormone (ACTH) (Hackenthal, Paul, Ganten and Taugner, 1990; Klar, Vitzthum and Kurtz, 2004;

Connell, MacKenzie, Freel, Fraser and Davies, 2008; Arroyo, Ronzaud, Lagnaz, Staub and Gamba, 2011).

The major action of aldosterone on epithelial cells is to cause the reabsorption of Na^+ thereby also influencing the transport of water across the cell apical membrane (Hackenthal, Paul, Ganten and Taugner, 1990; Klar, Vitzthum and Kurtz, 2004; Arroyo, Ronzaud, Lagnaz, Staub and Gamba, 2011). Aldosterone-induced reabsorption of Na^+ in the distal tubule is mediated via Na^+/K^+-ATPase and amiloride-sensitive epithelial sodium channel (ENaC) (McCormick, Bhalla, Pao and Pearce, 2005). Reports indicate that high-salt diet elevate ENaC expression in the kidney of salt-sensitive hypertensive rats thereby causing the disturbance of Na^+ balance (Aoi, Niisato, Miyazaki and Marunaka, 2004). The flavonoid, quercetin, which is abundant in plants has been shown to possess antihypertensive action in high-salt-induced hypertension by diminishing the elevated expression of ENaC messenger ribonucleic acid (mRNA) in the kidney (Aoi, Niisato, Miyazaki and Marunaka, 2004). The mechanism through which aldosterone mediates Na^+ reabsorption is by modulating the expression and activation of Na^+ channels in the plasma membrane as well as insertion of Na^+/K^+-pumps in the basolateral membrane (Geering, Beguin, Garty, Karlish, Fuzesi, Horisberger and Crambert, 2003; Klar, Vitzthum and Kurtz, 2004).

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

Oxytocin (OT) is another hormone involved in regulation of fluid balance, blood pressure and cardiac function (McCann, Antunes-Rodrigues, Jankowski and Gutkowska, 2002; Michelini, Marcelo, Amico and Morris, 2003). This non-peptide hormone is produced in neurons originating from the paraventricular nucleus (PVN) and projecting to many brain areas with receptors present in the vasculature, heart, and kidney (Petersson, Alster, Lundeberg and Uvnäs-Moberg, 1996; Rigatto, Puryear, Bernatova and Morris, 2003). Studies report that OT enhances natriuresis and does not influence urine flow rate but exert antidiuretic effects via V2 AVP receptor at high doses (Conrad, Gellai, North and Valtin, 1986; Chou, DiGiovanni, Luther, Lolait and Knepper, 1995). Natriuretic action of OT is caused by NO mediated generation of cyclic guanosine monophosphate (cGMP) and release of atrial natriuretic peptide (ANP) which further increase cGMP levels responsible for decreased Na^+ reabsorption via blockade of Na^+ channels (Haanwinckel, Elias, Favaretto, Gutkowska, McCann and Antunes-Rodrigues, 1995; Soares, Coimbra, Martins, Pereira, Carnio, Branco, Albuquerque-Araujo, de Nucci, Favaretto, Gutkowska, McCann and Antunes-Rodrigues, 1999).

1.2.4 Methods used to assess kidney function

1.2.4.1 Plasma and urine biomarkers

Kidney biomarkers are used to assess exposure to illness and to detect biological abnormalities, but are more often used to diagnose and measure a pathological condition (Madero, 2006; Endre and Westhuyzen, 2008; Tesch, 2010; Fassett, Venuthurupalli, Gobe, Coombes, Cooper and Hoy, 2011). There are a number of plasma and urine biomarkers that can be used to assess the functional status of the kidney and these biomarkers have to fulfill a certain number of criteria in order to be considered reliable. Blood urea nitrogen (BUN) and creatinine clearance are well established biomarkers of renal function that can be measured cheaply and easily using an enzyme/oxidation reaction assay and high performance liquid chromatography (HPLC), respectively (Mouton and Holder, 2006).

Creatinine clearance has been used by several authors to assess renal tubular function as a measure of GFR (Bursztyn, Ben-Ishay, Mekler and Raz, 1995; Travlos, Morris, Elwell, Duke, Rosenblum and Thompson, 1996; Girchev, Markova, Mikhov and Natcheff, 1998; Bertuzzi, Bensi, Mayer, Niebylski, Armario and Gauna, 2003; Gondwe, Kamadyaapa, Tufts, Chuturgoon and Musabayane, 2008; Mapanga, Tufts, Shode and Musabayane, 2009; Mkhwanazi, Serumula, Myburg, Van Heerden and Musabayane, 2014). GFR can also be estimated using exogenous filtration markers like inulin, iothalamate, ethylenediaminetetra-acetic acid (EDTA), diethylene triamine penta-acetic acid and iohexol (Dharnidharka, Kwon and Stevens, 2001; Rosner and Bolton, 2006; Stevens, Coresh, Greene and Levey, 2006). One of the effects of hypertension is increased plasma flow into the kidney which may affect the GFR. Therefore, this study used creatinine clearance to assess the sub-chronic effects of OA on GFR.

A recently emerged, reliable alternative biomarker of renal function is cystatin-C which is a cysteine protease inhibitor that is normally catabolized by kidney tubules without re-entering the blood stream and can be measured by an enzyme-linked immunosorbent assay (ELISA) (Endre and Westhuyzen, 2008; Tesch, 2010). Urine angiotensinogen, microalbuminuria, transforming growth factor-β1 (TGF-β1) and connective tissue growth factor are also potential biomarkers of kidney diseases and can be measured by ELISA (Tesch, 2010; Fassett, Venuthurupalli, Gobe, Coombes, Cooper and Hoy, 2011). Analysis of the urine sediment by quantitative polymerase chain reaction (PCR) or ELISA can determine mRNA or protein levels of podocyte-specific molecules like nephrin, podocin and podocalyxin as markers of podocyte injury (Wang, Lai, Lai, Chow, Li and Szeto, 2007; Wang, Lai, Tam, Li, Lai, Chow, Li and Szeto, 2007b). In addition to creatinine clearance, the kidney function biomarker of interest in this study was Li^+ clearance which is used to quantify the amount of Na^+ output from the proximal tubule.

1.2.4.2 Lithium clearance

Renal clearance of Li^+ has been used widely in animal studies and clinical investigations as a means of assessing proximal tubular function in the mammalian kidney (Koomans, Boer and Dorhout-Mees, 1989; Thomsen, 1990; Walter and Shirley, 1991; Shirley and Walter, 1993; Boer, Fransen,

Shirley, Walter, Boer and Koomans, 1995; Whiting, 1999; Madlala, Masola, Singh and Musabayane, 2012; Mkhwanazi, Serumula, Myburg, Van Heerden and Musabayane, 2014). Li^+ clearance measurements are based on the observation that Li^+ undergoes iso-osmotic reabsorption in the proximal renal tubule to the same extent as Na^+ and water, hence is regarded as the best available marker for proximal tubular reabsorption of Na^+ (Thomsen, 1984). Consequently, Li^+ clearance techniques can be used in both experimental and clinical studies to assess glomerulo-tubular function and handling of Na^+ by the proximal tubule in both health and disease states (Whiting, 1999).

However, studies have shown that Li^+ reabsorption also takes place in the distal nephron in Na^+/K^+ deprived rats (Thomsen and Shalmi, 1997; Shalmi, Jonassen, Thomsen, Kibble, Bie and Christensen, 1998; Emamifar, Shalmi, Thomsen and Christensen, 2000). On the other hand, clearance and micropuncture studies in rats indicate that using low concentrations of Li^+ prevents this previously reported distal reabsorption of this marker (Koomans, Boer and Dorhout-Mees, 1989; Shirley, Walter and Sampson, 1992). Hence this study employed Li^+ clearance to investigate the effects of OA on proximal tubular Na^+ handling since salt-induced hypertension results from the changes in this part of the nephron where most of Na^+ reabsorption takes place.

1.3 Cardiac function

The primary function of the heart is to pump the blood in order to generate and sustain an arterial blood pressure necessary to provide adequate perfusion of organs. The heart achieves this by contracting the muscular walls around a closed chamber to generate sufficient pressure to propel blood from the ventricle, through the aortic valve and into the aorta. Each time the heart beats, a volume of blood, stroke volume (SV), is ejected. The SV multiplied by the number of beats per minute (HR), equals the CO. In general, an increase in arterial blood pressure can be caused by either an increase in systemic vascular resistance (SVR) or an increase in CO (Debrah, Conrad, Jeyabalan, Danielson and Shroff, 2005). The SVR is determined by the vascular tone (i.e. state of constriction) of systemic resistance vessels, whereas the CO is determined by HR and SV (Lamia, Chemla, Richard and Teboul, 2005). Therefore, understanding the mechanisms that regulate both

SVR and CO during a single cycle of cardiac activity is necessary for understanding how arterial blood pressure can become elevated.

1.3.1 Cardiac cycle

Diastole represents the period of time when the ventricles are relaxed (not contracting). Throughout most of this period, blood is passively flowing from the left and right atrium into the left and right ventricle, respectively. The blood flows through atrioventricular valves (mitral and tricuspid) that separate the atria from the ventricles. The right atrium receives venous blood from the body through the superior vena cava (SVC) and inferior vena cava (IVC). The left atrium receives oxygenated blood from lungs through four pulmonary veins that enter the left atrium.

Contraction of both atria at the end of diastole forces an additional amount of blood into the ventricles. Systole represents the time during which the left and right ventricles contract and eject blood into the aorta and pulmonary artery, respectively. During systole, the aortic and pulmonic valves open to permit ejection into the aorta and pulmonary artery. The atrioventricular valves are closed during systole, therefore no blood is entering the ventricles; however, blood continues to enter the atria though the vena cava and pulmonary veins (Hundley, Kitzman, Morgan, Hamilton, Darty, Stewart, Herrington, Link and Little, 2001). Factors that influence the mechanisms that regulate both SVR and CO during cardiac activity may result in elevated arterial blood pressure. Disease conditions such as sustained hypertension result in decreased blood ejection from the heart (because of increased afterload) and may cause hypertrophic enlargement of the ventricles and complications such as dilated cardiomyopathy (Wang, Lee, Fabsitz, Devereux, Best, Welty and Howard, 2006).

Cardiomyopathy is characterised by reduced cardiac contractility which often progresses to heart failure and sudden death (Fang, Prins and Marwick, 2004; Piccini, Klein, Gheorghiade and Bonow, 2004; Diamant, Lamb, Smit, De Roos and Heine, 2005; Abhayaratna, Seward, Appleton, Douglas, Oh, Tajik and Tsang, 2006; Lacombe, Viatchenko-Karpinski, Terentyev, Sridhar, Emani, Bonagura, Feldman, Györke and Carnes, 2007). Cardiomyopathy refers to a weakened heart

muscle which occurs when the heart's muscle wall stretches or thickens and as a result fails to efficiently function as a pump. Cardiomyopathies can be the result of coronary artery disease, but can also be caused by untreated hypertension, viral infections, drinking too much alcohol, certain other toxins; including certain chemotherapy agents, and other diseases that may deposit abnormal proteins or iron in the heart muscle (Maron, Wolfson, Epstein and Roberts, 1986; Foley, Parfrey, Harnett, Kent, Murray and Barre, 1996).

Hypertension has been reported to be associated with left ventricular hypertrophy (LVH) which is a fundamental manifestation of preclinical cardiovascular disease that strongly predicts myocardial infarction, stroke and cardiovascular death in hypertensive patients (Devereux and Alderman, 1993). Reports suggest that interruption of RAAS activity with ACE inhibitors or Ang II receptor antagonists such as losartan and atenolol may regress hypertensive LVH most effectively (Devereux, Dahlöf, Gerdts, Boman, Nieminen, Papademetriou, Rokkedal, Harris, Edelman and Wachtell, 2004). Green tea extract was shown to block the development of cardiac hypertrophy via attenuation of hypertension in experimental renal failure (Priyadarshi, Valentine, Han, Fedorova, Bagrov, Liu, Periyasamy, Kennedy, Malhotra and Xie, 2003). In addition, a plant extract isolated from *Allium sativum* (garlic) has been shown to be effective in reducing both systolic and diastolic blood pressure (Ashraf, Khan, Ashraf and Qureshi, 2013) in experimental models of salt-induced hypertension.

1.3.2 Cardiac excitation-contraction coupling

An action potential is induced by pacemaker cells in the sinoatrial node and conducted from non-contractile cardiac myocytes to contractile cells through gap junctions. The action potential travels along T-tubules and triggers calcium release from terminal cisternae via L-type calcium channels causing a net flux of calcium ions into the cardiac myocyte (Green, 2004). The increase in intracellular calcium ions is detected by ryanodine receptors (RyR) in the membrane of the sarcoplasmic reticulum (SR) which transport calcium out into the cytosol in a positive feedback physiological response. Calcium is the central factor in myocardial contraction and is stored within calsequestrin, a Ca^{2+} binding protein located within the lumen of SR (Park, Park, Kim, Youn,

Fields, Dunker and Kang, 2004). Reports suggest that calsequestrin Ca^{2+} store is the main source of Ca^{2+} ions that are released during excitation-contraction (Bers, 2002). Studies have shown that cardiac diseases are associated with chronic stimulation of the β-adrenergic signaling pathway leads to hyperphosphorylation of RyR which leads to impaired contractility and increases the probability of cardiac arrhythmias (Marks, 2001; Wehrens and Marks, 2002). Compounds such as dantrolene, 1,4-benzothiazepines and flecainide modulate RyR via improved channel gating, ion channel translocation, RyR subunit composition or posttranslational modifications (Wehrens, Lehnart, Reiken, Deng, Vest, Cervantes, Coromilas, Landry and Marks, 2004; Kobayashi, Bannister, Gangopadhyay, Hamada, Parness and Ikemoto, 2005; Hilliard, Steele, Laver, Yang, Le Marchand, Chopra, Piston, Huke and Knollmann, 2010).

Contraction of the cardiac muscle is dependent on this calcium-induced calcium release phenomenon. Cytoplasmic calcium binds to troponin C, calcium-activated troponin undergoes a conformational change which moves the troponin complex away from the actin binding site allowing the myosin head to bind to the actin filament (Clapham, 2007). Using ATP hydrolysis, the myosin head pulls the actin filament to the centre of the sarcomere (contraction). Troponin complex that plays a major role in excitation-contraction coupling is made up of troponin C, I, and T. Troponin I is tightly bound to actin and blocks its interaction with myosin when Ca^{2+} levels are low during diastole (Clapham, 2007). For relaxation, intracellular calcium is taken up by the sarco/endoplasmic reticulum ATPase pump into the sarcoplasm, or ejected from the cell by the sodium-calcium exchanger or the plasma membrane calcium ATPase.

The energy for ion transport via Na^{+}/Ca^{2+}-exchanger (NCX) is indirectly derived from ATP hydrolysis via the sodium gradient that is established by the Na^{+}/K^{+}-ATPase that transports 3 Na^{+} out and 2 K^{+} into the cell per molecule of ATP (Therien and Bolstein, 2000). The Na^{+}/Ca^{2+}-exchanger transports 3 Na^{+}/Ca^{2+} and is the major Ca^{2+} extrusion mechanism of the cardiac myocyte and may contribute significantly to myocardial relaxation. When intracellular calcium concentration drops, troponin and tropomyosin complex returns over the active site of the actin filament thereby

inhibiting myosin-actin interaction and the muscle fiber relaxes. Altered function of troponin I due to either ischaemia, long term hypertension or depletion of cardiac troponin I can produce diastolic dysfunction and myocardial hypertrophy (Devereux, Dahlöf, Gerdts, Boman, Nieminen, Papademetriou, Rokkedal, Harris, Edelman and Wachtell, 2004).

Diastolic dysfunction is evident when the time period during which the myocardium loses the ability to generate force and shorten or returns to an unstressed length is prolonged, slowed, or incomplete (Zile and Brutsaert, 2002). This results from changes in calcium homeostasis caused by (1) abnormalities in the sarcolemmal transporters responsible for short- and long-term extrusion of calcium from the cytosol, such as the sodium calcium exchanger and the calcium pump; (2) abnormal SR calcium re-uptake caused by a decrease in SR Ca^{2+}-ATPase; and (3) changes in the phosphorylation state of the proteins that modify SR Ca^{2+}-ATPase function, such as phospholamban, calmodulin, and calsequestrin (Zile and Brutsaert, 2002b). Changes in any of these processes can result in increased cytosolic diastolic calcium concentration, prolongation in the calcium transient, delayed and slowed diastolic decline in cytosolic calcium concentration (Zile and Brutsaert, 2002b). Reports suggest that diastolic dysfunction and myocardial hypertrophy are vital signs for development of systemic hypertension (Aeschbacher, Hutter, Fuhrer, Weidmann, Delacrétaz and Allemann, 2001). We investigated the effects of medicinal plant bioactive compound and its derivatives on the cell contraction (measured as cell shortening) and Ca^{2+} currents in isolated cardiomyocyte of hypertensive animals.

Reports suggest that SR Ca^{2+-}ATPase pump (SERCA) and sarcolemma NCX remove 70% and 28% of the cytosolic Ca^{2+}, respectively (Bers, 2000). However, the relative contributions of Ca^{2+} transporters are altered in heart failure preceded by hypertension, with a shift toward NCX and away from SERCA (Hasenfuss, 1998). This shift is detrimental due to reduction of Ca^{2+} stored in the SR, hence decreasing the amount of Ca^{2+} available for release during systole. In addition, the NCX system is energetically draining due to transportation of only one Ca^{2+} for each ATP consumed as opposed to the SERCA system which transports two Ca^{2+} per ATP (Villars, Hamlin, Shaw and Kanusky, 2004). Therapeutic upregulation of SERCA2 expression using replication deficient adenoviral expression vectors, pharmacological interventions using thyroid hormone

analogues, β-adrenergic receptor antagonists, and novel metabolically active compounds are currently under investigation for the treatment of uncompensated cardiac hypertrophy and heart failure (Zarain-Herzberg, 2006). Interestingly, plant-derived extracts such as astragaloside IV, a saponin isolated from *Astragalus membranaceus* have been reported to improve the expression of SERCA in isolated cardiomyocytes (Xu, Chen, Ji, Li, Bian, Yang, Xu, Bian and Zhang, 2008).

1.3.3 General structure of calcium channels

Voltage-operated calcium channel is composed of five different polypeptide subunits with different molecular masses (Takahashi, Seager, Jones, Reber and Catterall, 1987). The α_1 subunit (175 kD) forms the ion channel and contains Ca^{2+} antagonist binding sites and is considered to be the principal structural component of the Ca^{2+} channel (Tanabe, Takeshima, Mikami, Flockerzi, Takahashi, Kangawa, Kojima, Matsuo, Hirose and Numa, 1987; Cribbs, 2006). Alpha$_2$ subunit (143 kD) is associated with α_1 but does not contain any high-affinity binding site; and the remaining three low-molecular weight subunits include β (54 kD), γ (30 kD) and δ (27 kD) (Ruth, Rohrkasten, Biel, Bosse, Regulla, Meyer, Flockerzi and Hofmann, 1989; Jay, Ellis, McCue, Williams, Vedvick, Harpold and Campbell, 1990). The α_1 and β subunits contain phosphorylation sites for cAMP-dependent protein kinase. The α_2, γ, and δ subunits are heavily glycosylated, indicating that they are located extracellularly (Takahashi, Seager, Jones, Reber and Catterall, 1987).

The Ca^{2+} channels possess four homologous domains each having six trans-membrane regions (Catterall, 1988). These domains are predicted to span the cell membrane and to contribute to the outer portion of the channel pore. However, the short amino- and long carboxy-terminal segments of the α_1 subunit are positioned intracellularly (Yamakage and Namiki, 2002). Various types of Ca^{2+} channels are shown in Table 1 below. Ca^{2+} influx through ion channels and Ca^{2+} released from intracellular stores have been proposed as the major contributors of cardiac and smooth muscle contraction (Moosmang, Schulla, Welling, Feil, Feil, Wegener, Hofmann and Klugbauer, 2003). Ion channels play a key role in the electrophysiology of ventricular action potentials and

therefore are the targets of many important therapeutic agents, including anti-arrhythmic, Ca^{2+} channel antagonists and catecholamines. Calcium antagonists have been used extensively to treat hypertension for more than 15 years and are among the compounds listed as first-line treatment for the management of mild hypertension (Hansson, Hedner, Lund-Johansen, Kjeldsen, Lindholm, Syvertsen, Lanke, de Faire, Dahlöf and Karlberg, 2000). Medicinal plant extracts of *Marrubium vulgare* L. (horehound, Lamiaceae) and *Croton schiedeanus* Schlecht (Euphorbiaceae) possess hypotensive effects mediated via blockade Ca^{2+} channels which inhibit K^{+} induced contraction of smooth muscles (Bardai, Lyoussi, Wibo and Morel, 2001). This study assessed the effects of plant-derived compounds on L-type Ca^{2+} currents in isolated rat cardiomyocytes using a whole-cell patch clamp technique.

Table 1: Electrophysiological classification and characteristics of voltage-dependent Ca^{2+} channels

Channel type	Tissue distribution	Antagonists/Blockers	Reference
L-type (Long lasting)	brain, neuronal, endocrine, kidney cells, cardiac, smooth and skeletal muscles, fibroblasts and bone cells (osteocytes)	1,4-dihydropyridines, phenylalkylamines, benzothiazepines	(Chang and Hosey, 1988; Yamakage and Namiki, 2002; Cantí, Davies and Dolphin, 2003)
N-type (Neural/Non-L)	brain, neuronal cells	ω-conotoxin, agatoxin	(Witcher, De Waard, Sakamoto, Franzini-Armstrong, Pragnell, Kahl and Campbell, 1993; Liu, De Waard, Scott, Gurnett, Lennon and Campbell, 1996)
P/Q-type (Purkinje)	brain, neuronal cells, heart	funnel spider venom toxin	(Randall and Tsien, 1995)
R-type (Residual)	brain, neuronal cells	agatoxin	(Clozel, Ertel and Ertel, 1999)
T-type (Transient)	brain, kidney, liver, heart, bone	mibefradil, agatoxin	(Ertel and Ertel, 1997; Perez-Reyes, 2003)

1.3.4 Methods used to assess cardiac functional status

Two-dimensional (2D) echocardiography allows an evaluation of the valvular structure as well as the impact of the volume overload on the cardiac chambers by measuring aortic velocities, CO, left ventricular systolic and diastolic functions (Zoghbi, Enriquez-Sarano, Foster, Grayburn, Kraft, Levine, Nihoyannopoulos, Otto, Quinones, Rakowski, Stewart, Waggoner and Weissman, 2003). Disease conditions in which coronary flow reserve is decreased are potential sources of intolerance of increased systolic cardiac load (Hartley, Reddy, Madala, Michael, Entman and Taffet, 2008) and can be determined by measuring coronary flow using a 20 MHz Doppler ultrasound probe.

With the increasing use of small animals for research, molecular imaging must have sufficiently high spatial resolution to allow anatomic localisation of small coronary arteries (De Kemp, Epstein, Catana, Tsui and Ritman, 2010). Imaging methods based on electromagnetic radiation, such as positron emission tomography (PET), single-photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI) are increasingly applicable due to recent advances in novel scanner hardware, image reconstruction software and availability of novel molecules which have enhanced sensitivity in these methodologies (Gilson, Yang, French and Epstein, 2004; Kreissl, Wu, Stout, Ladno, Schindler, Zhang, Prior, Prins, Chatziioannou and Huang, 2006; Beekman and van der Have, 2007; Catana, Procissi, Wu, Judenhofer, Qi, Pichler, Jacobs and Cherry, 2008; Stegger, Heijman, Schäfers, Nicolay, Schäfers and Strijkers, 2009; De Kemp, Epstein, Catana, Tsui and Ritman, 2010).

Measurement of cell shortening can be performed by video edge detection technique which monitors the edges of the cell as it contracts. The parameters that can be measured with this technique include the amplitude of the shortening, the time to peak shortening and the relaxation time constant obtained by fitting an exponential on the contraction curve (Steadman, Moore, Spitzer and Bridge, 1988). To capture the contraction process of the isolated cardiac myocytes, cells must be placed in a chamber mounted on the stage of an inverted microscope. Rod-shaped myocytes with clear edges have to be selected for recording of mechanical properties. The cells need to be field stimulated with a voltage above threshold at a certain frequency for certain duration

of the stimulus. The stimulation is performed using a pair of platinum wires placed on opposite sides of the chamber connected to an electrical stimulator. The polarity of the stimulatory electrodes is reversed automatically to prevent electrode polarisation (Lim, Apstein, Colucci and Liao, 2000). Myocyte motion is then digitally recorded with a camera mounted on the microscope. For this study we employed this technique to investigate the effects of OA or derivatives on the cell contraction (shortening) in isolated cardiomyocytes.

We acknowledge collaborations of Hodgkin and Huxley for laying the foundation for understanding electrophysiology in terms of how voltage-gated ion channels give rise to propagating action potentials and for studying and analysing ion channel kinetics (Hodgkin, Huxley and Katz, 1952). Their work was extended by Neher and Sakmann who discovered the function of single ion channels in cells; and MacKinnon who studied structural and mechanistic studies of ion channels (Neher, Sakmann and Steinbach, 1978; Jiang, Lee, Chen, Ruta, Cadene, Chait and MacKinnon, 2003). Electrophysiology techniques are used to record changes in voltage and electric current of single ion channel proteins in isolated cells (Zimmermann, Schneiderbanger, Schubert, Didie, Münzel, Heubach, Kostin, Neuhuber and Eschenhagen, 2002). The preparations normally used for electrophysiology experiments include living organisms, excised tissue, dissociated cells from excised tissue, artificially grown cells or tissues (Vunjak-Novakovic, Tandon, Godier, Maidhof, Marsano, Martens and Radisic, 2009).

Conventional electrophysiology techniques are based on insertion of electrodes into membranes of various preparations of biological tissues. These electrodes are usually empty capillary glass tubes with a sharp tiny tip that has a resistance of few mega ohms (Clayton, Bernus, Cherry, Dierckx, Fenton, Mirabella, Panfilov, Sachse, Seemann and Zhang, 2011). The electrodes are filled with similar ionic composition to the intracellular fluid of the cell. The electrolyte is connected electrically to the amplifier and signal processing circuit by a chloride-based silver wire is inserted into the electrode. The sharp tip of the electrode is inserted on the cell membrane and to allow intracellular recording the membrane potential of a particular channel. The voltage of a reference electrode is compared to voltage measured by the silver chloride-coated electrode which is usually

in contact with the extracellular fluid around the cell (Keenan and Folch, 2008). The smaller the electrode tip, the higher the electrical resistance, so an electrode is a compromise between size and resistance (Piacentino, Weber, Chen, Weisser-Thomas, Margulies, Bers and Houser, 2003).

1.3.4.1 Voltage and current clamp

The voltage clamp technique is useful for determining the cell potential at a certain value to allow measurement of how much ionic current crosses a cell membrane at any given voltage (Kiss, Bennett, Uebele, Koblan, Kane, Neagle and Schroeder, 2003). This is necessary because most voltage-gated ion channels open when the membrane voltage is within a certain range. The digital subtraction of transient capacitive currents that permit the recording electrode and cell membrane are charged to alter the cell potential by voltage clamp measurements of current (Williams and Mitchell, 2008). The current clamp technique records the membrane potential by injecting current into a cell via the recording electrode (Hodgkin, Huxley and Katz, 1952; Prinz, Abbott and Marder, 2004). This technique is used to study the response of the cell to the entry of an electric current. For example, this is beneficial for understanding the response of neurotransmitters to opening membrane ion channels. In the voltage clamp mode, the membrane potential is held at a particular level determined, whereas in current clamp mode the membrane potential is free to change, and the amplifier records any voltage the cell produces due to stimulation (Gustafson, Gireesh-Dharmaraj, Czubayko, Blackwell and Plenz, 2006; Perkins, 2006).

1.3.4.2 Patch-clamp

Conventional intracellular recording explained above involves impaling a cell with a fine electrode; patch-clamp recording uses a micropipette with a relatively large tip microelectrode diameter (Stuart and Brownstone, 2011). The microelectrode is placed next to a cell, and gentle suction is applied through the microelectrode to draw a piece of the cell membrane (the 'patch') into the microelectrode tip; the glass tip forms a high resistance 'seal' with the cell membrane (Rugiero, Gola, Kunze, Reynaud, Furness and Clerc, 2002). This configuration is the cell-attached mode which is used for studying the activity of the ion channels that are present in the patch of membrane (Kirichok, Navarro and Clapham, 2006). Applying more suction causes the small patch

of membrane in the electrode tip to be displaced, leaving the electrode sealed to the rest of the cell. This whole-cell mode allows a very stable intracellular recording (Neher, Sakmann and Steinbach, 1978). This study used the whole-cell patch clamp technique to assess the effects of OA or derivatives on L-type Ca^{2+} currents in isolated cardiomyocytes.

1.4 Vascular function

The vascular system also called the circulatory system, is made up of the vessels that carry blood and lymph through the body. The arteries and veins carry blood throughout the body delivering oxygen and nutrients to the body tissues and taking away tissue waste matter. The lymph vessels carry lymphatic fluid and help to protect and maintain the fluid environment of the body by filtering and draining lymph away from each region of the body (Swartz, 2001; Oliver and Detmar, 2002). In addition to circulating blood and lymph throughout the body, the vascular system functions as an important component of other body systems such as respiratory, digestive, kidney and urinary systems.

Vascular disease is a term that encompasses several pathological states of the arterial wall that may lead to obstructed blood flow and thereby give rise to acute cardiovascular events (Makin, Blann, Chung, Silverman and Lip, 2004). Such pathological states include arterial remodeling, atherosclerosis, thrombosis and restenosis. Hypertension is a cardiovascular risk factor which regularly precedes the onset of vascular disease (Drummond, Selemidis, Griendling and Sobey, 2011). Hypertension is characterised by loss of elasticity of arteries in the vascular system which cause enhanced blood pressure leading to coronary and renal vascular disease, heart attack or stroke and angina (Bleyer, Shemanski, Burke, Hansen and Appel, 2000; Mukai, Shimokawa, Matoba, Kandabashi, Satoh, Hiroki, Kaibuchi and Takeshita, 2001).

1.4.1 Regulation of vascular tone

Vascular tone control is essential for fluid regulation in cases of shock, ischemia-reperfusion, inflammation, vessel injury or repair, wound healing, temperature regulation, digestion, metabolism (Zhou, Sutliff, Paul, Lorenz, Hoying, Haudenschild, Yin, Coffin, Kong and Kranias, 1998) and most importantly for this study, blood pressure regulation. Vascular tone is influenced by substances that either cause dilation or contraction of the arteries or arterioles. These substances can be separated into extrinsic factors that originate from outside of the organ or tissue in which the blood vessel is located, and intrinsic factors that originate from the vessel itself or the surrounding tissue.

Extrinsic factors regulate arterial blood pressure by altering total peripheral resistance, whereas intrinsic mechanisms are important for local blood flow regulation within an organ (Lautt, 2007). Examples of extrinsic factors include sympathetic nerves and circulating Ang II which increase vascular tone; also there are some circulating factors such as atrial natriuretic peptide which decrease vascular tone (Myatt, 1992). Intrinsic factors include endothelial-derived substances such as nitric oxide (NO) and endothelin which either decrease or increase tone, respectively. Local hormones or chemical substances such as arachidonic acid metabolites, histamine, bradykinin, noradrenaline, vasopressin, serotonin and thrombin amongst many, can either increase or decrease tone (Haefliger, Flammer, Bény and Lüscher, 2001; Sellers and Stallone, 2008; Rubanyi, 2012).

Endothelial cells can induce contractions of the underlying vascular smooth muscle by generating vasoconstrictor prostanoids (endothelium-dependent contracting factor; EDCF). The main prostanoids involved in endothelium-dependent contractions appear to be endoperoxides and prostacyclin, which activate thromboxane-prostanoid (TP) receptors of the vascular smooth muscle cells. Studies indicate that endothelium-dependent contractions are exacerbated by ageing, obesity, hypertension and diabetes, and therefore these contractions are likely to contribute to the endothelial dysfunction observed in older people and in essential hypertensive patients (Vanhoutte and Tang, 2008; Vanhoutte, 2009). The production of the EDCF involves an increase in endothelial intracellular calcium concentration, production of reactive oxygen species (ROS), activation of cyclooxygenase-1 (COX-1) and COX-2. The diffusion of EDCF towards the smooth muscle cells

cause stimulation of their thromboxane A2-endoperoxide TP receptors (Félétou and Vanhoutte, 2006; Félétou, Huang and Vanhoutte, 2011).

COX catalytic activity leads to the formation of prostaglandin G2 (PGG2) which is the critical modulator of vascular tone and platelet activity under both physiological and pathophysiological conditions (Félétou, Huang and Vanhoutte, 2011). Ca^{2+} channel antagonists such as dihydropyridine, phenylalkylamines and benzothiazepines are widely used as anti-hypertensive treatment due to their vasodilatory properties (Webb, 2003). The antagonists bind to distinct receptors on the channel protein and inhibit Ca^{2+} entry in smooth muscle (Webb, 2003). In addition, medicinal plant products of *Sarcococca saligna, Prosopis juliflora* and withanolides from *Withania somnifera, Sesamum indicum, Lavandula stoechas*, ginger and *Carum copticum* were found to possess a unique combination of activities (ACE inhibitory and calcium antagonist) (Khalid, Ghayur, Feroz, Gilani and Choudhary, 2004; Choudhary, Nawaz, Azim, Ghayur, Lodhi, Jalil, Khalid, Ahmed, Rode and Gilani, 2005; Ghayur and Gilani, 2005; Gilani, 2005; Gilani, Ghayur, Khalid, Zaheer, Choudhary and Rahman, 2005; Choudhary, Nawaz, Lodhi, Ghayur, Jalil, Riaz, Yousuf, Malik and Gilani, 2005b). The mechanisms by which the above factors either constrict or relax blood vessels involve a variety of signal transduction mechanisms that involves Ca^{2+} release in the smooth muscle as described in the next paragraph.

1.4.1.1 Mechanisms of smooth muscle contraction

The ras homolog gene family member A (RhoA) pathway modulates the phosphorylation state of the regulatory chain of myosin II, mainly through inhibition of myosin phosphatase, and contributes to agonist-induced Ca^{2+} sensitization in smooth muscle contraction via the activity of its main effector Rho kinase (Swärd, Mita, Wilson, Deng, Susnjar and Walsh, 2003; Calo, 2006). The ultimate effect is to increase smooth muscle cell contraction. Exact mechanisms whereby Ang II stimulates Ca^{2+} influx are unclear but may involve voltage-dependent calcium channels, which are directly or indirectly activated by Ang II, Ca^{2+}-permeable, nonspecific dihydropyridine-insensitive cation channels, receptor-gated Ca^{2+} channels, Ca^{2+}-activated Ca^{2+} release channels, and activation of the NCX (Lu, Fern, Luthin, Linden, Liu, Cohen and Barrett, 1996; Touyz and

Schiffrin, 2000). Binding of agonists such as noradrenalin, Ang II and endothelin to serpentine receptors which are coupled to a heterotrimeric G protein, stimulate phospholipase C activity (Swärd, Mita, Wilson, Deng, Susnjar and Walsh, 2003; Calo, 2006). This enzyme is specific for the membrane lipid phosphatidylinositol 4,5-bisphosphate to catalyze the formation of two potent second messengers: inositol trisphosphate (IP3) and diacylglycerol (DG). The binding of IP3 to receptors on the SR results in the release of Ca^{2+} into the cytosol. DG and Ca^{2+} activate protein kinase C (PKC), which phosphorylates specific target proteins.

The rate of Ca^{2+} entry into the cytosol and release from sarcoplasm reticulum is modulated by the resting membrane potential. Membrane potential may be regulated by at least two types of K^+ channels. These include inwardly rectifying K^+ channels activated upon hyperpolarisation or shear stress and a Ca^{2+}-activated K^+ channel activated upon depolarisation, the latter may function to repolarize the agonist-stimulated endothelial cell (Adams and Hill, 2004). Receptor-operated and voltage-operated Ca^{2+} channels located in the plasma membrane are important in Ca^{2+} influx and smooth muscle contraction. The blood pressure regulatory factor, NO, which inhibit Ca^{2+} influx and cause smooth muscle relaxation is impaired in hypertension.

1.4.2 Nitric oxide

Endothelium-dependent relaxation of vascular smooth muscle to acetylcholine (ACh) is mediated by an endogenous mediator, NO which was initially referred to as endothelium-derived relaxing factor (Vanhoutte, Shimokawa, Tang and Feletou, 2009). Endothelium-derived NO is a potent vasodilator synthesised from the amino acid L-arginine by the endothelial isoform of NO synthase (NOS) (Palmer, Ashton and Moncada, 1988). Three isoforms of NOS have been identified; endothelial (eNOS), neuronal (nNOS) and inducible isoforms (iNOS) (Palmer, Ashton and Moncada, 1988). All three NOS isoforms play distinct roles in the regulation of vascular tone. Endothelial NOS and nNOS are normal constituents of healthy cells and iNOS is expressed mainly in conditions of infection or inflammation.

Biological functions of NO also include suppression of platelet aggregation, leukocyte migration and cellular adhesion to the endothelium, and attenuation of vascular smooth muscle cell proliferation and migration (Moncada, Palmer and Higgs, 1991; Alderton, Cooper and Knowles, 2001; Vallance and Chan, 2001). Therefore, reduced NO bioavailability leads to leukocyte adhesion and infiltration of monocytes, macrophages and lipoproteins into the artery wall which is the first stage in the formation of foam cells during atherosclerosis (Jessup, Mohr, Gieseg, Dean and Stocker, 1992; Mombouli and Vanhoutte, 1999; Usui, Egashira, Kitamoto, Koyanagi, Katoh, Kataoka, Shimokawa and Takeshita, 1999).

There is evidence that basal NO synthesis is reduced in untreated hypertension as indicated by reduced vasoconstrictor response to NOS inhibitor Nγ-monomethyl-L-arginine (L-NMMA) (Calver, Collier, Moncada and Vallance, 1992; Mizuno, Jacob and Mason, 2010). Drugs such as N-acetyl-cysteine (NAC), vitamins C and E are used to scavenge ROS and thereby may increase NO. In addition, there are various plant-derived compounds that have been shown to increase NO bioavailability via different mechanisms. Among these compounds are chlorogenic acid from *Solanum indicum*, betulinic acid from seeds of *Zizyphus jujuba* and crude herbal extracts from *Phyllanthus acidus*, *Olea europaea, Fritillaria ussuriensis* and *Cudrania tricuspidata* (Suzuki, Yamazaki and Yazaki, 2001; Komaki, Yamaguchi, Maru, Kinoshita, Kakehi, Ohta and Tsukada, 2003; Somova, Nadar, Rammanan and Shode, 2003; Kang, Sohn, Lee, Lee, Han, Kim and Lee, 2004; Bahgat, Abdel-Aziz, Raafat, Mahdy, El-Khatib, Ismail and Khayyal, 2008; Connell, MacKenzie, Freel, Fraser and Davies, 2008; Perrinjaquet-Moccetti, Busjahn, Schmidlin, Schmidt, Bradl and Aydogan, 2008; El and Karakaya, 2009; Leeya, Mulvany, Queiroz, Marston, Hostettmann and Jansakul, 2010). We investigated the effects of OA and related triterpene derivatives on vascular smooth muscle contraction and relaxation profiles in the presence of NOS inhibitor to elucidate the mechanisms of their blood pressure lowering properties.

1.4.3 Oxidative stress

Oxidative stress is associated with all known cardiovascular risk factors including hypertension. In oxidative stress, the production of ROS superoxide anion ($O_2^{\bullet-}$) exceeds the available antioxidant defence systems which cause a rapid oxidative inactivation of vascular NO signaling and bioavailability (Chew and Watts, 2004). The majority of ROS generation in the vasculature is derived from eNOS uncoupling. Uncoupling of eNOS in the pathogenesis of endothelial dysfunction is thought to be associated with the decrease of essential eNOS cofactor, tetrahydrobiopterin (BH4), bioavailability (Alp and Channon, 2004). This cofactor facilitates NADPH-derived electron transfer from the eNOS reductase to the oxygenase domain to convert L-arginine to NO and L-citrulline (Kietadisorn, Juni and Moens, 2012). When BH4 levels are inadequate, eNOS becomes unstable and uncoupled, leading to subsequently less NO production and more $O_2^{\bullet-}$ generation. The interaction between NO and superoxide leads to the formation of another toxic free radical called peroxynitrite, a potent oxidant, which further oxidises BH4 (Crabtree, Tatham, Al-Wakeel, Warrick, Hale, Cai, Channon and Alp, 2009; Crabtree, Hale and Channon, 2011).

Another source of ROS generation in the vasculature is mediated by NADPH oxidases (NOX) and therefore inhibitors of these enzymes such as apocynin and diphenyleneiodonium (DPI) are pharmacologically used to combat oxidative stress and the associated vascular pathologies in cardiovascular diseases (Selemidis, Sobey, Wingler, Schmidt and Drummond, 2008; Drummond, Selemidis, Griendling and Sobey, 2011). Various plant extracts have been shown to possess antioxidant properties that can alleviate symptoms of vascular disease by scavenging the ROS radicals. These include *Salvia* species*, Lavandula angustifolia* (all Lamiaceae family)*, Calendula officinalis, Matricaria recutita, Echinacea purpurea, Rhaponticum carthamoides* (Asteraceae family)*, Juglans regia,* (Juglandaceae)*, Melilotus officinalis* (Fabaceae), *Geranium macrorrhizum* (Geraniaceae) and *Potentilla fruticosa* (Rosaceae) (Tang and Yuan, 1997; Bolshakova, Lozovskaya and Sapezhinskii, 1998; Ohsugi, Fan, Has, Xiong, Tezuka, Komatsu, Namba, Saitoh, Tazawa and Kadota, 1999; Lei, Mehta, Berenbaum, Zangerl and Engeseth, 2000; Zupko, Hohmann, Redei, Falkay, Janicsak and Mathe, 2001; Miliauskasa and van Beek, 2004). We

evaluated the effects of a plant-derived OA and derivatives on pro and antioxidative enzymes in the heart, kidney and the liver tissues of hypertensive animals. Persistent oxidative stress results in dysfunctional eNOS such that the NO production ceases, and increase $O_2^{\bullet-}$ which prevents relaxation of vascular smooth muscle by scavenging NO within the vascular wall (Förstermann, 2010).

1.4.4 Conventional methods used to assess oxidative stress

Monitoring oxidative stress is achieved by assaying products of oxidative damage or by investigating the potential of an organism, tissue or body fluids to withstand further oxidation (Palmieri and Sblendorio, 2007). Unfortunately, there is little consensus concerning the selection of parameters of oxidative stress or antioxidant state to be determined in defined diseases. This is not only due to the uncertainty on whether or not a certain parameter is playing a causative role but also because the methods of determination described in the literature represent very different levels of analytical practicability, costs and quality (Dalle-Donne, Rossi, Colombo, Giustarini and Milzani, 2006). Analytical techniques including HPLC, gas chromatography-mass spectrometry (GC-MS), fluorometric and colourometric assays, ELISA and gel electrophoresis have become more common standards.

1.4.4.1 Free radical measurements

Electron Spin Resonance (ESR) spectrometry can usually be applied to analysis of samples *in vivo* only through the technique of spin trapping. This involves the addition of a compound known as spin-trap, which reacts rapidly with the free radicals to form radical-adducts. These radical-adducts are very stable and long-lived than the original species and can therefore build up to steady state concentrations in the detectable range (Davies and Hawkins, 2004; Augusto and Vaz, 2007; Lee, Margaritis, Channon and Antoniades, 2012; Lee, Han, Kim, Baek and Baik, 2013). Electron paramagnetic resonance (EPR) technique is based on detection of a persistent nitroxide generated under physiological or pseudo-physiological conditions by oxidation of a highly lipophylic hydroxylamine probe (Tominaga, Sato, Ohnishi and Ohnishi, 1994). The rate at which the

nitroxide is reduced to the diamagnetic hydroxylamine, which can be evaluated by EPR, is related to the reducing capacity of the organism and hence to its oxidative status (Hirayama, Nagase, Ueda, Oteki, Takada, Obara, Inoue, Yoh, Hirayama and Koyama, 2005). Furthermore, nitroxide crosses cell membranes and distributes in a biological environment without the need to alter or destroy compartments. Therefore, this method is suitable for quantitative measurements of ROS and can be applied to human tissues in real clinical settings (Palmieri and Sblendorio, 2007).

Dihydroethidium (DHE) or hydroethidine is a cell-permeable compound that, upon entering the cells, interacts with $O_2^{\bullet-}$ to form oxyethidium (Zhao, Kalivendi, Zhang, Joseph, Nithipatikom, Vásquez-Vivar and Kalyanaraman, 2003), which in turn interacts with nucleic acids to emit a bright red colour detectable qualitatively by fluorescent microscope (Tarpey, Wink and Grisham, 2004). DHE assay may be adapted for HPLC for specific and quantitative detection of $O_2^{\bullet-}$ from biological samples (Fink, Laude, McCann, Doughan, Harrison and Dikalov, 2004). Cytochrome C Reduction Assay was considered the "gold standard" for detection of $O_2^{\bullet-}$ prior to the discovery of ESR (Tarpey, Wink and Grisham, 2004). The use of lucigenin at low concentrations (<5 μM) has been shown to detect $O_2^{\bullet-}$ without background noise and this assay remains a valid method for $O_2^{\bullet-}$ production, when appropriate experimental procedures are used (Münzel, Afanas'ev, Kleschyov and Harrison, 2002). Hydrogen peroxide levels in vascular cells and tissues can be monitored by dichlorofluorescein fluorescent, amplex red assay and o-phenylenediamine dihydrochloride (o-PD)-coated platinum microelectrode (Liu and Zweier, 2001; Cai, 2005; Cai, 2005a).

1.4.4.2 Lipid peroxidation

Lipid peroxidation is a complex process whereby polyunsaturated fatty acids (PUFAs) in the phospholipids of cellular membranes undergo reaction with oxygen to yield lipid hydroperoxides (LOOH) (Halliwell and Chirico, 1993). The thiobarbituric acid (TBA) assay is the most common and easiest method used to assess lipid peroxidation and free radical activity in biological samples. The assay is based on the reaction of two molecules of TBA with one of malondialdehyde (MDA), a physiological ketoaldehyde produced by peroxidative decomposition of unsaturated lipids as a

product of arachidonate metabolism (Bouzid, Hammouda, Matran, Robin and Fabre, 2014). The excess MDA produced as a result of tissue injury can combine with free amino groups of proteins. There are a lot of variations but mainly the sample is heated with TBA under acidic conditions and the amount of pink-coloured MDA-TBA adduct produced is measured using a spectrophotometer (Bloomer and Goldfarb, 2004).

1.4.4.3 Measurement of antioxidants

Different animal studies have shown that antioxidants delay or protect against the oxidative damage produced by free radical reactions (Selemidis, Sobey, Wingler, Schmidt and Drummond, 2008; Drummond, Selemidis, Griendling and Sobey, 2011). Radical-scavenging antioxidants are consumed during this process and antioxidant status is sometimes used indirectly to assess free radical activity. The commonly used total peroxyl radical-trapping antioxidant parameter (TRAP) assay is an empirical measurement of antioxidant activity in plasma (Ghiselli, Serafini, Maiani, Azzini and Ferro-Luzzi, 1995). Assessment of the relative contribution of individual antioxidants (ascorbate, urate, α-tocopherol, protein sulphydryls) to the total antioxidant capacity requires separate specific assays (Palmieri and Sblendorio, 2007). Antioxidant amounts are parameters indicative of the predisposition of the individual to oxidative stress.

Superoxide dismutase (SOD) assay uses xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl-)-3-(4-nitrophenyl)-5-phenyltetrazoluim chloride to form a red formazan dye (Bouzid, Hammouda, Matran, Robin and Fabre, 2014). The SOD activity is then quantified by measuring the degree of inhibition of this reaction. Glutathione peroxidase (GPx) assay employs the principle that oxidised glutathione (GSSG), produced upon reduction of an organic hydroperoxide by GPx, is recycled to its reduced state by glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate (NADPH) (Ho, Karimi Galougahi, Liu, Bhindi and Figtree, 2013). The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease is directly proportional to the GPx activity in the sample (Bouzid, Hammouda, Matran, Robin and Fabre, 2014). We evaluated the effects of OA on

oxidative status of hepatic, cardiac and renal tissues of hypertensive animals by measuring levels of MDA, SOD and GPx.

1.5 Management of hypertension

1.5.1 Lifestyle modifications

Preventive lifestyle change such as dietary changes, physical exercise and weight loss have all been shown to significantly reduce blood pressure in people with hypertension (Siebenhofer, Jeitler, Berghold, Waltering, Hemkens, Semlitsch, Pachler, Strametz and Horvath, 2011). Even when blood pressure is high enough to justify immediate use of medications, lifestyle changes are still recommended in conjunction with medication (Gilliam, 2012). Studies indicate that a long term low sodium diet is effective in reducing blood pressure, both in people with hypertension and in people with normal blood pressure (He, Marciniak, Visagie, Markandu, Anand, Dalton and MacGregor, 2009; He, Li and MacGregor, 2013).

The South African Hypertension Guidelines recommend a maximum salt intake of 6 g day^{-1} which is the upper boundary of the 4-6 g day^{-1} recommended by the WHO (WHO, 2003). Consequently, SA diet is high in salt, with bread contributing to 25-40% of Na^+ intake (Charlton, Steyn, Levitt, Zulu, Jonathan, Veldman and Nel, 2005). Average daily intake, measured by 24-hour urinary Na^+ excretion, is 7.8 g in black persons, 8.5 g in mixed-race persons, and 9.5 g in white persons in SA (Charlton, Steyn, Levitt, Zulu, Jonathan, Veldman and Nel, 2005). If Na^+ content of bread, soup mix, seasoning and margarine could be reduced, the average salt intake would decrease by 0.85 g person^{-1} day^{-1} and this would result in 7 400 fewer CVD deaths and 4 300 less non-fatal strokes per year compared with 2008 (Bertram, Steyn, Wentze-Viljoen, Tollman and Hofman, 2012). In addition, a diet rich in nuts, whole grains, fish, poultry, fruits and vegetables lowers blood pressure (Hu, 2003). Several exercise regimes such as isometric resistance and aerobic exercises may be useful in reducing blood pressure (Cornelissen and Fagard, 2005).

1.5.2 Therapeutic medication

Several classes of medications, collectively referred to as antihypertensive drugs, are currently available for management of hypertension. Reduction of the blood pressure by 5 mmHg can decrease the risk of stroke by 34%, ischaemic heart disease by 21% and reduce the likelihood of dementia, heart failure, and mortality from cardiovascular disease (Law, Wald and Morris, 2005). The current drugs used for treatment of cardiovascular disorders include β-blockers, alpha-adrenoceptor blockers, Ca^{2+} channel blockers (CCB), ACE inhibitors, Ang II type 1 angiotensin receptor blockers (ARB) and diuretics. β-blockers such as atenolol, metoprolol, nebivolol and carvedilol also provide antioxidant activity and have direct vasodilatory properties either through α blocking action or activation of NOS thereby improving endothelial function in patients with hypertension (Tzemos, Lim and MacDonald, 2001; Mason, Kubant, Jacob, Walter, Boychuk and Malinski, 2006; Weiss, 2006; Bank, Kelly, Thelen, Kaiser and Gonzalez-Campoy, 2007).

Alpha-adrenoceptor blockers such as prazosin, terazosin and doxazosin lower peripheral vascular resistance by relaxing smooth muscles (Khwanchuea, Jansakul, Mulvany, Queiroz and Hostettmann, 2007; Mackraj, Ramesar, Singh, Govender, Baijnath, Singh and Gathiram, 2008; Leeya, Mulvany, Queiroz, Marston, Hostettmann and Jansakul, 2010; Soncini, Santiago, Orlandi, Moraes, Peloso, dos Santos, Alves-da-Silva, Paffaro, Bento and Giusti-Paiva, 2011; Tom, Demougeot, Mtopi, Dimo, Djomeni, Bilanda, Girard and Berthelot, 2011). Ca^{2+} channel blockers like nifedipine, amlodipine, lercanidipine and isradipine cause peripheral vasodilation (Perticone, Ceravolo, Maio, Ventura, Iacopino, Cuda, Mastroroberto, Chello and Mattioli, 1999; Taddei, Virdis, Ghiadoni, Sudano and Salvetti, 2002). ACE inhibitors like captopril, cilazapril, lisinopril, perindopril and ramipril restore endothelium-dependent vasodilatory response to ACh. These drugs mediate their anti-hypertensive properties via inhibition of AngII-dependent vasoconstriction and production of aldosterone (Atlas, 2007).

ARB antagonists such as irbesartan, candesartan, telmisartan have effects similar to those of ACE inhibitors (Ghiadoni, Virdis, Magagna, Taddei and Salvetti, 2000; Schiffrin, Park, Intengan and Touyz, 2000; Bellien, Iacob, Eltchaninoff, Bourkaib, Thuillez and Joannides, 2007; Benndorf, Appel, Maas, Schwedhelm, Wenzel and Böger, 2007). Diuretics like hydrochlorothiazide,

ethacrynic acid, torsemide, spironolactone and amiloride suppress tubular reabsorption of sodium, thus increasing the excretion of sodium and water thereby decrease blood volume (Greenberg, 2000; Abdallah, Schrier, Edelstein, Jennings, Wyse and Ellison, 2001; Padilla, Armas-Hernández, Hernández, Israili and Valasco, 2007). The majority of people require more than one drug to control hypertension. Those with a systolic blood pressure greater than 160 mmHg or a diastolic blood pressure greater than 100 mmHg should use a thiazide and an ACE inhibitor, ARB or CCB (Go, Bauman, King, Fonarow, Lawrence, Williams and Sanchez, 2014).

1.5.2.1 Plant-derived drugs

Digitalis drugs derived from the foxglove (*Digitalis purpurea*) are perhaps the classic example of compounds from plant sources that have contributed successfully is cardiovascular research (Hauptman and Kelly, 1999; Gilani, 2005). They represent a widely used group of clinically effective compounds which produce positive inotropic effect on the failing heart as well as having value in the treatment of atrial fibrillation (Gilani, 2005). A second discovery of natural products with cardiovascular activity led to the isolation of reserpine over five decades ago (Gilani, 2005). Reserpine and rescinnamine are obtained from the roots of the Indian plant Rauwolfia serpentine. Studies indicate that lower doses of reserpine combined with low doses of thiazide diuretic and hydralazine provide highly effective blood pressure lowering regimen. This combination has renal protective effect and is relatively free from conventional side-effects (Pavan, Ghini, Castro and Lopes De Faria, 2003; Milne and Pinkney-Atkinson, 2004).

Furthermore, rhomitoxin isolated from *Rhododendron molle* G. Don, protoveratrines from *Veratrum album*, deserpidine from *Rauvolfia canescens* are also drugs that have also been shown to alleviate cardiovascular disorders (Fabricant and Farnsworth, 2001; Salgueiro, Martins and Correia, 2010). Aspirin, an acetyl salt of salicylic acid (an active principle from willow bark) is considered one of the most effective analgesic, antipyretic and anti-inflammatory agents commonly used in modern medicine (Fabricant and Farnsworth, 2001). With the passage of time, multiple therapeutic uses of aspirin have emerged, with most prevalent use as the antiplatelet or anticoagulant observed at the low dose to prevent further problems in patients who have already

suffered from one heart attack (Saeed, Atiq, Verani and Gilani, 2002). Few compounds derived from plants such as muscarine and nicotine (pioneer selective agonists for muscarinic and nicotinic receptors, respectively), cocaine (catecholamine uptake inhibitor) yohimbine (selective α_2 blocker) and himbacine, a prototype of cardio-selective antimuscarinic agents (Gilani, 1998) have detrimental instead of therapeutic effects.

Ongoing research seeks to validate more plant extracts with potent beneficial effects for use in the management of hypertension. Plant-derived remedies that have been reported to possess hypotensive effects include extracts of *Marrubium vulgare* L. (horehound, Lamiaceae) and *Foeniculum vulgare* L. (fennel, Apiaceae) (Bardai, Lyoussi, Wibo and Morel, 2001; El Bardai, Morel, Wibo, Fabre, Llabres, Lyoussi and Quetin-Leclercq, 2003). Furthermore, herbal remedies from *Moringa oleifera*, *Terminalia superba* (Combretaceae), *Averrhoa carambola* L. (Oxalidaceae), *Phyllanthus acidus* (L.), *Tulbhagia violacea* (Alliacea), *Herniariaglabra*, *Verbesina caracasana* and *Randia siamensis* have been shown to have anti-hypertensive effects mediated via unknown mechanisms (Faizi, Siddiqui, Saleem, Aftab, Shaheen and Gilani, 1998; Rhiouani, Settaf, Lyoussi, Cherrah, Lacaille-Dubois and Hassar, 1999; Botta, 2003). However, *ex vivo* studies on isolated atrial muscle preparations have suggested that natural products from *Allium savitum* (Linn.), *Securidaca longipendunculata* (Fresen.) and *Achillea millefolium* mediate their anti-hypertensive properties via reduction of noradrenaline-induced inotropic and chronotropic responses (Ojewole, 2000; Ojewole and Adewunmi, 2001; Niazmand, Esparham, Rezaee and Harandizadeh, 2011).

Interestingly, the presence of a unique combination of activities (ACE inhibitory and calcium antagonist) was found in extracts of *Sarcococca saligna* (Khalid, Ghayur, Feroz, Gilani and Choudhary, 2004; Gilani, Ghayur, Khalid, Zaheer, Choudhary and Rahman, 2005). Similarly, juliflorine from *Prosopis juliflora* and withanolides from *Withania somnifera* were also found to possess this unique combination of activities (Choudhary, Nawaz, Azim, Ghayur, Lodhi, Jalil, Khalid, Ahmed, Rode and Gilani, 2005; Choudhary, Nawaz, Lodhi, Ghayur, Jalil, Riaz, Yousuf, Malik and Gilani, 2005b). Interestingly, medicinal plants such as *Sesamum indicum, Lavandula*

stoechas, ginger and *Carum copticum* which have been used traditionally for lowering of blood pressure were found to mediate this effect through a combination of Ca^{2+} channel blockers and ACh-like activities (Gilani, 2001; Ghayur and Gilani, 2005).

Although conventional drugs for treatment of cardiovascular disorders are effective, they have limitations as their use is associated with various side effects. These side effects include asthma symptoms, cold hands and feet, depression, erection problems, insomnia and sleep problems. ACE inhibitors may cause a persistent dry, hacking cough, skin rash and a loss of taste (Yeolekar and Kelkar, 2005). Diuretics side effects include excessive urination, weakness, leg cramps and fatigue (Maquirriain and Merello, 2007). In addition to these side effects, these medications are expensive and not easily accessible, especially to the communities from poor socio-economic background. Therefore, there has been an increased interest in the use of medicinal plant products for treatment of cardiovascular disorders because they are cheap and readily available.

1.6 Traditional (indigenous/folk) medicine

Reports indicate that 80% of the world's population uses herbal medicine for primary health care especially in developing countries. Approximately 12-15 million South Africans still use traditional remedies from as many as 700 indigenous plant species (Meyer and Afolayan, 1995; Grierson and Afolayan, 1999). Plant products also play an important role in health care systems of the remaining 20% of the population, mainly residing in developed countries. Analysis of the data on prescriptions dispensed from community pharmacies from 1959 to 1980, indicated that 25% of these prescriptions contained plant extracts or active principles derived from higher plants (Farnsworth and Soejarto, 1985).

Herbal drugs are at the basis of traditional medicines. Traditional plants have been used for hundreds of years because of their affordability, efficacy, accessibility and assumed lesser side effects (Kamboj, 2000; Ozsoy-Sacan, Karabulut-Bulan, Bolkent, Yanardag and Ozgey, 2004; Gao, Li, Li, Liu, Fan, Liu, Zhao, Li and Han, 2009). Increased side effects of synthetic products, lack of their curative action for several chronic diseases, high cost of new drugs and inexistence of

drugs for new emerging diseases are some of the reasons for renewed public interest in alternative medicines (Patwardhan, Warude, Pushpangadan and Bhatt, 2005). Reports indicate that medicinal plants possess bioactive compounds which are responsible for many therapeutic effects (Shinawie, Singh and Padmavathi, 2000) and there are a number of conventional drugs that originate from plants such as aspirin (from willow bark), digoxin (from foxglove), quinine (from cinchona bark), and morphine (from the opium poppy) (Shinawie, 2002). Table 2 below summarises medicinal plant extracts that have been shown to possess beneficial anti-hypertensive properties in human and animal studies.

Table 2: Medicinal plants that are reported to possess anti-hypertensive properties.

Scientific name	**Plant part used**	**Extract/active compound**	**Anti-hypertensive activity mediated via**	**Reference**
Eucommia ulmoides	Leaves, bark	aqueous	Vasorelaxation	(Kwan, Chen, Deyama and Nishibe, 2003; Luo, Wu, Zhou, Yan, Yang and Ouyang, 2010).
Hibiscus sabdariffa	dried calyces	aqueous	ACE inhibition	(Ojeda, Jiménez-Ferrer, Zamilpa, Herrera-Arellano, Tortoriello and Alvarez, 2010).
Vaccinium myrtillus	bilberry	anthocyanins	ACE inhibitory	(Persson, Persson and Andersson, 2009).
Vitis thunbergii	root, leaves	(+)-vitisin A	anti-ACE and vasodilation	(Lin, Lu, Wang, Chen, Wen and Hou, 2012).
Theobroma cacao	fruits	polyphenols, flavanol	Increase NO bioavailability	(Corti, Flammer, Hollenberg and Lüscher, 2009).
Nigella sativa	seeds	aqueous	Reduce total and low-density-lipoprotein (LDL)-cholesterol	(Dehkordi and Kamkhah, 2008).
Urtica dioica	aerial parts	aqueous	diuresis and natriuresis	(Tahri, Yamani, Legssyer, Aziz, Mekhfi, Bnouham and Ziyyat, 2000).
Phyllanthus debilis	dried leaves	methanol and phenolic compounds	Antioxidant activity which improve NO production	(Kumaran and Joel Karunakaran, 2007)
Steganotaenia araliacea	stem-bark	aqueous, methanol, ethanol	diuresis and natriuresis	(Agunu, Abdurahman, Andrew and Muhammed, 2005).
Ekebergia capensis	leaves	ethanol	NO-dependent vasorelaxant activity	(Kamadyaapa, Gondwe, Moodley, Ojewole and Musabayane, 2009).

1.6.1 *Syzygium aromaticum*

The plant bioactive compound of interest in this study is a pentacyclic triterpenoid isolated from *Syzygium aromaticum* (Hochst.) [Myrtaceae]. *S. aromaticum* belongs to *Myrtaceae* family and is referred to as a clove tree (Figure 1). *S. aromaticum* is an evergreen tree that grows to a height of 15 to 30 metres tall and is native in North Moluccas (Indonesia), but is also grown in India, Jamaica, Brazil, and other tropical areas (Agbaje, Adeneye and Daramola, 2009). This plant has opposite, ovate leaves which are more than 12.7 centimetres long. When fully grown, the flowers are red and white, bell-shaped and grow in terminal clusters. The brown, dried, unopened flower buds are called cloves. The dried flower bud is familiarly used in the kitchen and the fruit is a one- or two-seeded berry (Agbaje, Adeneye and Daramola, 2009).

The leaves are glabrous, with numerous oil glands on lower surface. *S. aromaticum* contains bioactive chemical compounds such as glycosides, tannins, polyphenols, triterpenes and saponins. Triterpene is a precursor of steroids in plants consisting of six isoprene units with a molecular formula $C_{30}H_{48}$. Triterpenes can be classified into lupane, oleanane or ursane groups (Laszczyk, 2009). Of particular interest in this study is an oleanane triterpene, OA. *Syzygium spp*-derived OA has been shown to possess hypoglycaemic, hypotensive, reno- and cardioprotective effects in experimental animals (Musabayane, Mahlalela, Shode and Ojewole, 2005; Mapanga, Tufts, Shode and Musabayane, 2009; Musabayane, Tufts and Mapanga, 2010; Ngubane, Masola and Musabayane, 2011).

Figure 1: *Syzygium aromaticum* (Hochst.) [Myrtaceae] flower buds are harvested when they are bright green, 1 - 2 cm in length and when the flower is still unopened. Marketed clove is dark brown in colour and sold as whole or ground clove and OA is isolated from these dried flower buds (Attokaran, 2011).

1.6.1.1 Triterpene OA and synthetic oleanane derivatives

OA is the major constituent of many African plant species used in traditional medicine but is mostly abundant in plants that belong to Oleaceae family such as *Olea europapea* (Pollier and Goossens, 2012). This compound which exists in plants in the form of a free acid has been isolated from more than 1620 plant species (Fai and Tao, 2009; Fukushima, Seki, Ohyama, Ono, Umemoto, Mizutani, Saito and Muranaka, 2011). Plants synthesize OA from the sterol precursor, 2,3 oxidosqualene which is a product of the mevalonate pathway (Abe, 2007). OA has therapeutic

importance due to diverse pharmacological properties and lower systemic toxicities which have led to the use of this triterpene as a starter molecule for the synthesis of new derivatives (Liu, 2005; Braga, Ayres-Saraiva, Gattass and Capella, 2007; Patil, Jadhav, Singh, Mundada and Patil, 2010; Sporn, Liby, Yore, Fu, Lopchuk and Gribble, 2011). OA derivatives are generated by chemical modifications of this triterpene in the active sites of the structure, the C-3 hydroxy, the C-12-C-13 double bond, and the C-28 carboxylic acid (Honda, Finlay, Gribble, Suh and Sporn, 1997; Honda, Rounds, Gribble, Suh, Wang and Sporn, 1998; Honda, Rounds, Bore, Favaloro Jr, Gribble, Suh, Wang and Sporn, 1999; Honda, Rounds, Bore, Finlay, Favaloro, Suh, Wang, Sporn and Gribble, 2000; Honda, Honda, Favaloro Jr., Gribble, Suh, Place, Rendi and Sporn, 2002; Sporn, Liby, Yore, Fu, Lopchuk and Gribble, 2011).

The most potent OA derivatives that have been synthesised are 2- cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and C-28 methyl ester, CDDO-Me. In comparison to OA, these new synthetic compounds have improved biological activities attributed to the presence of enone functions in their A- and C-rings and nitrile group in C-2 in the A-ring (Couch, Browning, Honda, Gribble, Wright, Sporn and Anderson, 2005; Dinkova-Kostova, Liby, Stephenson, Holtzclaw, Gao, Suh, Williams, Risingsong, Honda, Gribble, Sporn and Talalay, 2005; Yates, Tauchi, Katsuoka, Flanders, Liby, Honda, Gribble, Johnson, Johnson, Burton, Guilarte, Yamamoto, Sporn and Kensler, 2007). Examples of improved biological activities of OA derivatives include anti-inflammatory and antioxidant properties mediated by inhibition of iNOS and Keap1 protein, respectively (Reisman, Buckley, Tanaka and Klaassen, 2009; Shin, Wakabayashi, Yates, Wakabayashi, Dolan, Aja, Liby, Sporn, Yamamoto and Kensler, 2009).

1.6.1.2 Basis of this study

This section will briefly state the various pharmacological effects of OA which are of interest in this study. These include cardiovascular, vasodilatory, renal and antioxidant effects. OA has been shown to have hypotensive effects in healthy Sprague Dawley rats and streptozotocin (STZ)-induced diabetic rats. This effect was associated with beneficial effects on some processes associated with renal derangement of STZ-induced diabetic rats (Mapanga, Tufts, Shode and

Musabayane, 2009; Madlala, Masola, Singh and Musabayane, 2012). Particularly, OA corrected the previously reported Na^+ retention and reduction in GFR in STZ-induced diabetic and DSS rats (Somova, Nadar, Rammanan and Shode, 2003; Mapanga, Tufts, Shode and Musabayane, 2009). Somova observed a diuretic effect of OA, whereas Mapanga and Madlala reported no change in urine flow rate. This study used experimental models of hypertension to investigate the mechanisms of antihypertensive effects of OA, we hypothesize that natriuretic effects of OA reported by these authors may alleviate blood pressure progression in SHR and DSS rats.

In a study by Somova, OA alleviated high blood pressure levels, increased heart rate and modulated blood antioxidant status based on GPx and SOD activities in DSS rats (Somova, Shode, Ramnanan and Nadar, 2003). Therefore, we also explored antioxidant properties of this triterpene as the possible mechanism for antihypertensive effects, however, we assessed the antioxidant status in the liver, kidney and heart tissues. In addition, we evaluated the effects of this triterpene on isolated cardiomyocyte contractility and L-type Ca^{2+} channels.

Rodriguez-Rodriguez reported another OA effects which may influence blood pressure levels, i.e olive oil OA-evoked decreased smooth muscle cell relaxation mediated by endothelium-dependent release of NO and not COX-mediated pathways (Rodríguez-Rodríguez, Herrera, Perona and Ruiz-Gutiérrez, 2004). However, another study suggested that OA modulate Cox-2-dependent release of prostacylin (PGI_2) mediated by phosphorylation of p38 mitogen-activated protein kinases (MAPK), a key transcription factor involved in Cox-2 transcriptional upregulation (Martínez-González, Rodríguez-Rodríguez, González-Díez, Rodríguez, Herrera, Ruiz-Gutierrez and Badimon, 2008). These conflicting finding influence our interest in studying OA effects on both NO-dependent and independent pathways. In addition, our study tested whether two new synthetic derivatives of OA display similar cardiovascular, renal, antioxidant and vasorelaxant effects to those of the parent drug.

1.7 Aims of the study

The general aim of this study was to investigate the mechanisms of cardiovascular effects of OA and related triterpene derivatives in hypertensive animals by assessing the effects of OA and related derivatives on arterial blood pressure, renal, cardiac, vascular functions.

CHAPTER 2

MATERIALS AND METHODS

This chapter describes experimental protocols used to investigate the following studies:

A) phytochemical studies

1. extraction of OA,
2. synthesis of oleanane derivatives.

B) whole animal experiments, acute and sub-chronic effects of OA/oleanane derivatives on:

1. blood pressure measurements,
2. fluid and electrolyte handling,
3. renal clearance assessment.

C) *ex vivo* effects of OA and oleanane derivatives

1. oxidative stress evaluation,
2. cardiomyocyte shortening,
3. Ca^{2+} current measurements,
4. vascular smooth muscle contraction/relaxation profiles.

2.0 Drugs and chemicals

Drugs and chemicals used in this study are listed below and were sourced as indicated:
dimethyl sulphoxide (DMSO), inactin (5-ethyl-5-(l'-methylpropyl)-2-thiobarbiturate), sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH), sodium thiosulphate ($Na_2S_2O_3$), diethyl ether (Et_2O); tetrahydrofuran (THF), acetic acid (AcOH), bromine (Br_2), hydrogen bromide (HBr), potassium hydroxide (KOH), potassium bromate ($KBrO_3$), methylammonium chloride (CH_3ClN), sulphuric acid (H_2SO_4), urea, 2-iodobenzoic acid, Li^+ chloride (LiCl) and *m*-chloroperoxybenzoic acid (*m*-CPBA), heparin, indomethacin (INDO), glibenclamide (Gli), 4-aminopyridine (AP),

carbachol, phenylephrine (PHE), L-nitro-arginine (L-NoArg), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and protease (Sigma-Aldrich, St. Louis, Missouri, USA);

silica gel, anhydrous sodium sulphate (Na_2SO_4), ethyl acetate (EA), dichloromethane (DCM), methanol (CH_3OH), chloroform ($CHCl_3$) and Folin-Ciocalteu reagent (Merck, Modderfontein, South Africa);

isofor (Safeline Pharmaceuticals (PTY) LTD, Weltevreden Park, Rooderpoort, South Africa);

creatinine (BDH Biochemicals LTD, Poole, England);

bovine serum albumin (BSA) (Thermo Scientific, Randburg, Johannesburg);

collagenase A (Roche Diagnostics, Mannheim, Germany);

pentobarbital (Nembutal, Abbott Laboratories, North Chicago, USA);

SOD and GPx Assay Kits (BioVision Research Products, Mountain View, California, USA);

arg^8-vasopressin ELISA Kit (Abcam, Cambridge, Massachusetts, USA);

aldosterone ELISA Kit (DRG International Inc, New Jersey, USA).

General chemicals such as NaCl and all other chemical reagents mentioned above were of analytical grade.

2.1 Phytochemical studies

2.1.1 Extraction of oleanolic acid (compound 1)

The dried flower buds of *Syzygium aromaticum* [(Linnaeus) Merrill and Perry] (Myrtaceae) were purchased from the spice market in Durban, South Africa and were authenticated by Professor H. Baijnath, the former Chief Taxonomist/Curator of the University of KwaZulu-Natal's Department of Botany. The extraction of OA was performed in the School of Chemistry and Physics at the University of KwaZulu-Natal, Pietermaritzburg campus using a previously validated standard protocol that has been reported by our laboratories (Mapanga, Tufts, Shode and Musabayane, 2009; Musabayane, Tufts and Mapanga, 2010; Madlala, Masola, Singh and Musabayane, 2012). Briefly, air-dried flower buds of *S. aromaticum* (500 g) were milled using an industrial blender and exhaustively extracted sequentially with dichloromethane (DCM) and ethyl acetate (EA) (twice with 1 L for 24 h for each solvent) at room temperature. The plant material was removed by filtration and the resultant filtrates were concentrated *in vacuo* at 60 ± 1 °C using a Büchi rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland) to obtain DCM soluble (63 g) and ethyl acetate soluble (EAS, 85 g) crude extracts. Crude EAS were subjected to further purification since previous studies indicated that they contain mixtures of OA/ursolic acid (UA) and methyl maslinate/methyl corosolate (Somova, Nadar, Rammanan and Shode, 2003; Musabayane, Mahlalela, Shode and Ojewole, 2005). Column chromatography with silica gel was used for fractionation of a portion of EAS (10 g) using a step-wise gradient of hexanes-EA, increasing the ratio from 9:1 to 7:3.

Fractions collected were monitored and analysed by thin layer chromatography (TLC) on pre-coated aluminium plates which were developed with EA/hexanes (8:2) in a TLC tank. Compounds were detected under UV (254 nm) followed by staining the plates with *p*-anisaldehyde. Analysed data collected from the fractions was compared with authentic OA values. Eluates with similar TLC profiles to OA were combined and subjected to further chromatographic purification. The yield of OA was 2.5 – 3.0 g per 10 g (30%) EAS extraction with m.p. 298 – 300 °C (Lit value: 295 – 298 °C) (Habila, Shode, Ndukwe, Amupitan and Nok, 2012). Pure OA was obtained by recrystallisation with methanol and the structure was confirmed by ^{1}H, ^{13}C nuclear magnetic

resonance (NMR) and infrared spectra (IR) recorded on a Bruker DRX-400 and Bruker Alpha FT-IR spectrometer (Figure 2). OA had the same physical properties as the OA purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and hence isolated OA was used as a parent compound for the synthesis of related triterpene derivatives.

HO H H OH O

Figure 2: The structure of *S. aromaticum*-derived OA showing the carbon-carbon olefinic double bond which is characteristic of the triterpenoids.

2.1.2 Reagents for synthesis of oleanane derivatives

2.1.2.1 Nitrosomethylurea (Diazomethane precursor)

Methylammonium chloride (50.64 g, 0.75 mol) was added into a 500 mL beaker followed by addition of deionised water (200 mL) and urea (150 g, 2.5 mol). The solution was refluxed gently for 2.75 h and vigorously for further 15 minutes. After cooling the solution to room temperature, 55 g (0.75 mol) of sodium nitrite was dissolved in this mixture and temperature was further dropped to 0 $^{\circ}$C. In a 1 L beaker with 300 g of ice, 0.5 M sulphuric acid (27.2 mL) was added. The beaker was placed in ice to maintain the temperature at 0 $^{\circ}$C. The cold methylurea-nitrite solution was then poured into a dropping funnel and added slowly into the ice-sulphuric acid mixture while stirring. Nitrosomethylurea ascended to the surface of the reaction mixture as a foamy precipitate which was filtered and washed with water under suction. The material obtained was then dried in a vacuum oven and stored at 4 $^{\circ}$C.

2.1.2.2 Iodoxybenzoic acid (IBX) for oxidation

To a vigorously stirring solution of 2-iodobenzoic acid (1.129 g, 0.0345 mmol) and sulphuric acid (20 mL, 2 M) at 50 °C, was added potassium bromate (1 g, 4.55 mmol). The temperature was then increased to 65 °C and the reaction mixture was stirred for further 6 h (Dess and Martin, 1991). The material (IBX powder; 1.01 g, 89%) obtained was filtered and dried in a vacuum oven and stored at room temperature.

2.1.3 Synthesis of oleanane derivatives

We are grateful to Fu and Gribble (2013) for having used their method below for the preparation of oleanane derivatives.

2.1.3.1 Methylation (compound 2)

Diethyl ether (40 mL, 0.54 mmol) was added into 100 mL conical flask and placed in an ice bath. A 40 % (m/v) aqueous solution of KOH (7.5 mL, 0.21 mol) was added while stirring. This was followed by addition of 2 g (0.02 mol) of nitrosomethylurea in small portions while temperature was maintained at 0 °C. When nitrosomethylurea was completely dissolved, the yellow ethereal layer was transferred into a conical flask with KOH pellets to remove residual water. OA (Figure 3, compound **1**) (500 mg, 1.09 mmol) was dissolved in 5 mL tetrahydrofuran (THF) and the solution was cooled to 0 °C in ice. The yellow ethereal layer of diazomethane was poured into the THF solution of OA and the mixture was stirred for 4 h. The mixture was left in the fume hood overnight and compound **2** (Me-OA, Figure 3) was obtained as a whitish powder (325 mg, 65%) with m.p. 124 – 126 °C.

2.1.3.2 Bromination (compound 3)

Compound **2** (1.20 g, 2.55 mmol) was oxidised with IBX (2.86 g, 10.2 mmol) in dimethyl sulphoxide (35 mL). This was followed by epoxidation of the oxidised product using *m*-chloroperoxybenzoic acid (321 mg, 1.3 mmol). Hydrobromic acid (44 µL, 0.38 mmol) was added

drop wise to a stirred solution of epoxidised compound (200 mg) in acetic acid (10 mL) at room temperature. The reaction mixture was then heated to 35 °C followed by drop wise addition of bromine (0.12 mL, 1.04 mmol). The resulting reaction mixture was kept stirring for another 24 h. Upon completion of the reaction, the acid was removed *in vacuo*. The resulting residue was quenched with 20% aqueous $Na_2S_2O_3$ (20 mL) and extracted with dichloromethane (3 x 30 mL). The combined organic extracts were washed with saturated $NaHCO_3$ (2 x 20 mL), NaCl (2 x 20 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. An analytically pure sample was obtained by column chromatography (hexanes-EA, 4:1 to 2:1) to give compound **3** (Br-OA, Figure 3) (150 mg, 37%) as a yellowish solid with m.p. 137 – 140 °C. All structures of synthetic products were confirmed by ^{1}H, ^{13}C NMR and infrared spectra recorded on a Bruker DRX-400 and Bruker Alpha FT-IR spectrometer, respectively, and therefore were used for animal studies.

Figure 3: Reaction scheme for the synthesis of OA derivatives as previously described (Fu and Gribble, 2013). Reagents: (a) CH_2N_2, Et_2O, THF; (b) IBX, DMSO; (c) *m*CPBA, CH_2Cl_2; (d) Br_2, HBr, AcOH.

2.2 Whole animal experiments

2.2.1. Animals

Male Wistar rats (250 - 300 g) and weaning SHR and DSS rats (100 - 150 g) bred and housed in the Biomedical Resource Unit (BRU) of the University of KwaZulu - Natal were used for arterial pressure and renal function studies. SHR and DSS rats were selected for this study because these rat models develop hypertension as they age due to genetics or high salt diet, respectively. The animals were maintained under standard laboratory conditions of constant temperature (22 ± 2 °C, CO_2 < 5000 p.p.m), relative humidity (55 ± 5%), illumination (12 h light/dark cycles) and noise levels (< 65 decibels). The animals had free access to water and standard rat chow (Meadow Feeds, Pietermaritzburg, South Africa).

For cardiac and vascular studies, male Wistar and DSS rats (250 - 300 g) purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA) and housed in the Animalia of the Catholic University of Leuven were used. The DSS rats were fed high salt Na^+ diet (8%) (Bio Services, Berlin, Germany) from the age of 4 to 10 weeks. The animals were maintained under standard laboratory conditions as above and had free access to water and rat chow (Bio Services, Berlin, Germany).

2.3 Ethical approval

All experimental protocols were reviewed and approved by the animal ethics committee of the University of KwaZulu-Natal (077/12/Animal; 028/13/Animal) and of the Catholic University of Leuven (4500768204); see Appendices I, II and III.

2.4 Experimental design

Normotensive Wistar, DSS and SHR rats were divided into separate groups to study the following: 1) acute (4 h) effects of OA and oleanane derivatives MAP and renal function of Wistar rats under anaesthesia. 2) Sub-chronic (9 weeks) effects of OA on MAP and kidney function in conscious Wistar, SHR and DSS animals. The study was also designed to evaluate the *ex vivo* effects of OA

and oleanane derivatives on cardiomyocyte contractility and Ca^{2+} currents as well as effects on contraction / relaxation profiles of isolated thoracic aortic and mesenteric rings.

2.5 Renal and arterial pressure studies

2.5.1 Acute studies

The rats were prepared for acute mean arterial blood pressure and renal function measurements using a protocol previously reported from our laboratories (Musabayane, Gondwe, Kamadyaapa, Chuturgoon and Ojewole, 2007). Briefly, the rats were anaesthetised by intra-peritoneal injection of 0.11 g kg^{-1} inactin [5-ethyl-5- (10-methylpropyl)-2 thiobarbiturate] (Sigma Aldrich, St. Louis, MO, USA) and tracheotomy was performed. A catheter was implanted in the left carotid artery for withdrawal of blood samples and continuous recording of arterial blood pressure at 30 min intervals via a pressure transducer (Statham MLT 0380, Ad Instruments, Bella Vista, NSW, Australia), compatible with PowerLab System ML410/W (Bella Vista, NSW, Australia).

Another catheter was inserted in the right jugular vein for continuous intravenous infusion (Harvard Syringe Infusion Pump 22, Harvard Apparatus, Holliston, MA, USA) of saline (0.077 M NaCl) with 1 % DMSO containing creatinine (0.15 mg mL^{-1}) at 9 mL h^{-1} to allow calculation of creatinine clearance as a measure of GFR. A minimal abdominal incision was made and a urinary bladder catheter was inserted for the collection of urine samples. After a 3.5 h equilibration period, blood pressure recordings and urine samples were taken every 30 min and blood samples (200 µL) were drawn hourly for measurements of electrolytes (methodology described in section 2.5.1.1 below) over a 4 h experiment divided into 1 h control, 1.5 h treatment and 1.5 h recovery periods. In those animals in which the effects of OA or derivatives were examined, the infusate was changed during the 1.5 h treatment period to the one identical in ionic composition, but containing OA, Me-OA and Br-OA (90 µg h^{-1}). The dosage for OA was based on previous studies (Mapanga, Tufts, Shode and Musabayane, 2009) and the same dosage for the derivatives was used for fair comparison of the effects.

The effects of OA, Me-OA and Br-OA on proximal tubular Na^+ handling was assessed in anaesthetised Wistar rats through measurement of Li^+ clearance (C_{Li}) (Thomsen, 1990). Male Wistar rats were fed standard rodent chow supplemented with Li^+ chloride (12 mmol kg^{-1} dry weight) for 48-hours (h) prior to experimentation in order to raise plasma Li^+ to measurable concentrations without affecting renal Na^+ or water excretion.

2.5.1.1 Urinalysis

Urine volume was determined gravimetrically using a balance (Mettler balance PC 180-instruments, Protea Laboratory Services, Johannesburg, South Africa). Quantitative measurements of total urinary outputs and plasma concentrations of Na^+, K^+, Cl^-, urea and creatinine were performed using Beckman Coulter (Synchron LX20 Clinical Systems, Fullerton, California, USA) with reagent kits from Beckman Coulter (Synchron LX20 Clinical Systems, Dublin, Ireland). Li^+ was determined by flame emission spectroscopy at 670.8 nm (Optima 2100 DV, Perkin Elmer, Shelton, CT) using a modified procedure that has been previously described (Madlala, Masola, Singh and Musabayane, 2012). Fractional excretion rates of Na^+ (FE_{Na}) and Li^+ (FE_{Li}) were determined simultaneously.

Li^+ clearance (C_{Li}) was used as a marker for the output of Na^+ from the proximal tubules (Thomsen, 1990). Renal clearances (C) and fractional excretions (FE) were calculated with the standard formulae $C = U \, x \, V/P$ and $FE = C/GFR$, where U is the urinary concentration, V is the urine flow rate and P is the plasma concentration. GFR, as assessed by creatinine clearance was calculated at 1 h intervals in anaesthetised rats and in the 9th week in conscious animals using the standard formulae from measurements of the plasma and urinary concentrations of creatinine and urine flow rate.

2.5.2 Sub-chronic studies

Separate groups (n = 6 in each) of male Wistar, SHR and DSS rats housed individually in Makrolon polycarbonate metabolic cages (Techniplast, Labotec, South Africa) were treated with various doses of OA (30, 60 and 120 mg kg^{-1}, p.o.) twice every third day for 9 weeks at 09h00 and 15h00.

The dose of 60 mg kg^{-1} was determined from previous studies, for the purpose of evaluating whether OA effects are dose dependent, we investigated the lower and higher doses also based on previous studies (Mapanga, Tufts, Shode and Musabayane, 2009; Madlala, Masola, Singh and Musabayane, 2012). These experiments were restricted to OA because of the poor yield of synthetic derivatives obtained during the phytochemical studies. OA was freshly dissolved in dimethyl sulphoxide (DMSO, 2 mL) and 9% saline (19 mL) (Musabayane, Mahlalela, Shode and Ojewole, 2005) before use in each case. Rats given DMSO/saline (3 mL kg^{-1}, p.o.) acted as untreated controls. The rats were given both food and water *ad libitum*.

Urine volume and urinary concentrations of creatinine, urea, Na^+, K^+ and Cl^- were determined daily (Beckman Coulter, Synchron LX20 Clinical Systems, Fullerton, Carlifornia, USA), similar methodology for acute measurements as described in 2.5.1.1 above. Mean arterial blood pressure (MAP) was monitored every third consecutive day using non-invasive tail cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA) as previously described (Gondwe, Kamadyaapa, Tufts, Chuturgoon and Musabayane, 2008; Kamadyaapa, Gondwe, Moodley, Ojewole and Musabayane, 2009). The equipment was calibrated each day prior to measurements. The animals were kept warm at ± 30 $^{\circ}C$ in an enclosed chamber (IITC Model 303sc Animal Test Chamber, IITC Life Sciences, Woodland Hills, California, USA) for 30 minutes before blood pressure recording. All measurements were conducted at 09h00. Blood samples were collected by cardiac puncture into individual pre-cooled heparinized containers at the end of the 9 week experimental period for biochemical and hormone analysis. 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 9th week.

2.6 Analysis of plasma aldosterone and AVP secretion

2.6.1 Aldosterone assay

2.6.1.1 Principle

Plasma aldosterone concentrations were measured from plasma samples of Wistar and hypertensive groups of non-treated and treated animals using an ELISA Kit (DRG International Inc, New Jersey, USA). The kit components included a 96 microtiter well plate, standards, controls, enzyme conjugate (aldosterone-alkaline phosphatase), substrate, stop and wash solutions. Standards and samples are added to wells coated with polyclonal rabbit antibody. Aldosterone in the sample competes with an aldosterone-horseradish peroxidase conjugate for binding to the coated antibody which is directed towards an antigenic site of the aldosterone molecule. After incubation at room temperature, addition of the substrate solution allows the intensity of colour development. Colour intensity is inversely proportional to the concentration of aldosterone in the sample.

2.6.1.2 Procedure

Standards (0, 20, 80, 200, 500, 1000 pg mL^{-1}), controls and samples (50 µL of each) were dispensed together into appropriate wells. An enzyme conjugate (150 µL) was added into each well followed by thorough mixing for 10 seconds. The mixture was left at room temperature for 1 hour. The contents of the wells were vigorously shaken out. The wells were rinsed with 300 µL diluted wash solution per well (5 times). Residual droplets were removed by sharply striking the plate on absorbent paper. Substrate solution (200 µL) was added into each well and the plate was left at room temperature for 1 hour. The enzymatic reaction was stopped by adding 100 µL of stop solution to each well. The absorbance (OD) was determined at 405 ± 10 nm using a Spectrostar nano microtiter plate reader (BMG Labtech GmbH, Ortenberg, Germany). A standard curve was constructed from the absorbance values of the known concentrations of the standards using the spline regression curve in Graph Pad Instat software (version 5.00). The respective aldosterone concentrations of the unknown samples were directly extrapolated from the standard curve.

2.6.2 Arginine vasopressin (AVP) assay

2.6.2.1 Principle

Plasma AVP concentrations were measured from blood samples collected from non-treated and treated groups of hypertensive rats using an Arg^8-Vasopressin ELISA Kit (Abcam, Cambridge, Massachusetts, USA) after 9 weeks of OA treatment. The kit components included a 96 well plate coated with goat anti-rabbit antibodies, vasopressin-alkaline phosphatase conjugate, rabbit anti-vasopressin antibody, assay buffer, wash buffer concentrate, vasopressin standards, p-nitrophenyl phosphatesubstrate (pNpp) and a stop solution. The Arg^8-Vasopressin ELISA Kit is a competitive immunoassay for the quantitative determination of vasopressin in samples. The assay uses a rabbit anti-vasopressin antibody to bind covalently in a competitive manner with non-covalent forces in unknown samples. During the incubation period AVP in the sample reacts with rabbit anti-vasopressin antibodies bound to the microtitration well. The washing step removes enzyme-labelled vasopressin, leaving the bound conjugate which reacts with pNpp. This reaction is stopped by adding acid 2 N hydrochloric acid (HCl) to give a colourimetric endpoint that is read spectrophotometrically on a microplate reader (BMG Labtech GmbH, Ortenberg, Germany) at 405 nm.

2.6.2.2 Procedure

A volume of 100 µL of vasopressin standards (4, 10, 23, 59, 148, 369 and 923 pmol L^{-1}) was added into anti-vasopressin wells. Samples and non-specific binding solution (100 µL) were then added to the remaining wells followed by 50 µL vasopressin conjugate into all standard and sample wells. Assay diluent was used as the blank. Each determination was performed in duplicate for both standards and the test samples. The plates were incubated at 4 ºC for 24 hours. Following incubation, the reaction mixture was discarded. 400 µL of wash buffer was added to all wells and aspirated, the process was repeated 3 times. After the final wash, the plates were inverted firmly against absorbent paper to remove all the liquid. Two hundred (200) µL of substrate pNpp were added to all wells and incubated at 37 $^{\circ}$C for 1 h. The reaction was stopped by adding 50 µL of stop solution to all wells and mixing on the shaker for 5 minutes. The absorbance was read at 405 nm using a Spectrostar Nano microplate reader (BMG Labtech GmbH, Ortenberg, Germany). A

standard curve was constructed from the absorbance values of the known concentrations of the standards using the spline regression curve in Graph Pad Instat software (version 5.00). The respective AVP concentrations of the unknown samples were then extrapolated from the standard curve. The lower and upper limits of detection were 4 pmol L^{-1} - 923 pmol L^{-1}, respectively. The intra-assay analytical coefficient of variation ranged from 5.9 to 10.6% and the inter-assay coefficient variation from 6.0 to 8.5%.

2.7 Tissue sample harvesting

At the end of a 9-week experimental period, all animals were sacrificed by exposing them to isofor (100 mg kg^{-1}, for 3 min) using a gas anaesthetic chamber. Livers, hearts and kidneys were removed, snap frozen in liquid nitrogen and stored in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at -70 $^{\circ}$C for subsequent biochemical analysis. All organs were analyzed for protein content in addition to other biochemical parameters. The protein content was quantified using the Lowry method (Lowry, Rosebrough, Farr and Randall, 1951). The Lowry method is best used with protein concentrations of 0.01–1.0 mg mL^{-1} and therefore all the samples were standardized to one concentration (1.0 mg mL^{-1}). The Lowry method is based on the principle that total protein concentration is demonstrated by a colour change of the sample solution in proportion to protein concentration detected using colourimetric techniques. The method combines the reactions of copper ions with the peptide bonds under alkaline conditions with the oxidation of aromatic protein residues such as tryptophan and tyrosine. The reaction involves the reduction of the Folin–Ciocalteu reagent. The concentration of the reduced Folin- Ciocalteu reagent is measured by absorbance at 750 nm, hence the total concentration of protein in the sample can be deduced from the concentration of tryptophan and tyrosine residues that reduce the Folin–Ciocalteu reagent (Everette, Bryant, Green, Abbey, Wangila and Walker, 2010).

We also used the Lowry method to quantify protein content in all tissues that were analyzed for oxidative stress markers using 2 mg mL^{-1} BSA as standard (0-1 mg mL^{-1}). Kidney, heart and liver samples (0.5 g) were homogenized in 0.1 M Tris-HCl buffer, pH 7.4 (1 mL). The homogenate (0.2 mL) was topped up with deionised water (0.5 mL) followed by addition of alkaline reagent (5

mL). The alkaline reagent consisted of 100 volumes of 4% sodium carbonate, 1 volume of 4% copper sulphate and 4% of sodium potassium tartrate. The mixture was incubated for 15 min at 40 ˚C and Folin Ciocalteu reagent (0.5 mL, diluted with 0.5 mL deionised water) was added. The samples were left at room temperature for 30 min and then absorbances were read at 600 nm. A BSA standard curve was plotted as a linear fit function of protein concentration. The protein concentration of each sample was calculated by relating its absorbance to the standard curve.

2.7.1 Oxidative stress evaluation

To establish the effects of OA on oxidative stress in the liver, heart and kidney of SHR and DSS rats, we monitored levels of MDA, a commonly known marker of oxidative stress and of antioxidant defence enzymes SOD and GPx of Wistar and OA-treated hypertensive rats and compared with respective untreated rats and normotensive Wistar animals. For these measurements, the samples were analysed in duplicates.

2.7.1.1 Malonyldialdehyde (MDA)

MDA levels in the tissue homogenates of the heart, kidney and liver from Wistar, DSS and SHR rats were estimated using thiobarbituric acid (TBA) by a previously described method (Kasapoglu and Özben, 2001; Mkhwanazi, Serumula, Myburg, Van Heerden and Musabayane, 2014). Tissues (50 mg) were homogenised in 500 µL of 0.2% phosphoric acid. The acid reacts with MDA to form a stable pink colour with maximum absorption at 532 nm. The heart, kidney and liver tissues (50 mg) from Wistar control, untreated and OA-treated DSS rats were homogenized in 500 µL of 0.2% phosphoric acid. The homogenate was centrifuged at 14,000 x g at 4 ˚C for 10 minutes. Thereafter, 400 µL of the homogenate were supplemented with 400 µL of 2% phosphoric acid followed by addition of 400 µL TBA/butylated hydroxytoluene (BHT) dissolved in 400 µL of 3 mM hydrochloric acid (HCl), and additional 200 µL of 1 M HCl added to ensure a 1.5 acidic pH. The solution was heated in a water bath at 100 ˚C for 15 min to properly dissolve TBA. After cooling, butanol (1.5 mL) was added and the precipitate was removed by centrifugation. Sample absorbance was then determined at 532 nm against a blank that contained all reagents except the homogenate sample. The absorbencies from these wavelengths were used to calculate the concentration of

MDA using Beer's law. The homogenized tissue concentration of MDA was calculated using the equation below and expressed as units per gram protein.

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

2.7.1.2 Superoxide dismutase (SOD)

Tissue SOD levels from homogenates of Wistar, SHR and DSS rat heart, kidney and liver were measured using a commercially available Biovision SOD Assay Kit (BioVision Research Products, Mountain View, California, USA). Xanthine and hypoxanthine oxidase were used to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity was then measured by the degree of inhibition of this reaction. Rat heart, kidney and liver tissues (50 mg) were homogenized in ice cold 0.1 M Tris /HCl (pH 7.4) containing 0.5% Triton X-100, 5 mM β-mercaptoethanol (ME) and 0.1 mg mL^{-1} phenylmethane-sulphonylfluoride (PMSF). The tissue homogenate was centrifuged at 14,000 x g, 4 $^{\circ}$C for 5 minutes. The supernatant obtained was added to each sample (20 µL) and blank 2 (20 µL) well, while blank 1 and blank 3 wells received 20 µL of H_2O. Thereafter, 200 µL of working solution was added to each well. Dilution buffer (20 µL) was then added to each blank 2 and blank 3 well, while each sample and blank 1 well received enzyme working solution (20 µL). The solutions were mixed thoroughly with a shaker before reading the plate. Inhibition activity of SOD was colourimetrically measured on Anthos Venytch-200 Spectrophotometer (Biochrom limited, Cambridge, United Kingdom) after a reaction period of 20 minutes at 37 $^{\circ}$C. SOD activity was calculated as percentage inhibition using the following equation: The homogenized tissue concentration of SOD was calculated using the equation below and expressed as units per gram protein.

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

2.7.1.3 Glutathione peroxidase (GPx)

Tissue homogenate GPx levels were measured using a commercially available Biovision GPx Assay Kit (BioVision Research Products, Mountain View, USA), where GPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidised glutathione is immediately converted to its reduced form with a concomitant oxidation of NADPH to $NADP^+$ (Lei, Cheng and McClung, 2007). The tissues (50 mg) were homogenized in cold assay buffer (0.2 mL) placed in ice and centrifuged at 14, 000 x g, 4 °C for 15 minutes. The resultant supernatant (100 µL) was loaded into a 96-well plate in duplicates. The NADPH standard curve was prepared by diluting the 1 mM NADPH standard through a series of concentrations (0, 20, 40, 60, 80, 100 nmol per well). The optical density of the standards (OD) was measured at 340 nm using Anthos Venytch-200 spectrophotometer (Biochrom limited, Cambridge, United Kingdom) and the standard curve was constructed from the values obtained. A reaction mix (90 µL) containing assay buffer, NADPH, glutathione reductase and GSH was added into each sample well, mixed thoroughly and incubated for 15 minutes at room temperature. The OD was measured (340 nm) (T1), cumene hydroperoxide (10 µL) was then added and the plate was incubated for 5 minutes in the dark (25 °C). The decrease in absorbance at 340 nm was expressed as units per gram protein. OD was measured (340 nm) against (T2) and GPx activity was calculated using following equation:

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

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

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

T1 - time of first reading

T2 - time of second reading

V- pre-treated sample volume added into the reaction well

2.8 *Ex vivo* effects of OA and oleanane derivatives

2.8.1 Cardiac studies

The *ex vivo* effects of OA, Me-OA and Br-OA on cell shortening and Ca^{2+} currents were evaluated in isolated rat cardiomyocytes using edge detection and whole cell patch clamp techniques, respectively.

2.8.1.1 Cell isolation

Cardiac ventricular myocytes isolated from Wistar and DSS rat hearts were used. The methods for cell dissociation and electrophysiological measurements were similar to those described in detail previously (Mubagwa, Stengl and Flameng, 1997; Macianskiene, Moccia, Sipido, Flameng and Mubagwa, 2002; Gwanyanya, Sipido, Vereecke and Mubagwa, 2006; Nevelsteen, Bito, Van der Mieren, Vanderper, Van den Bergh, Sipido, Mubagwa and Herijgers, 2013). Briefly, rats were injected with heparin (70 - 85 mg kg^{-1} i.p) and they were sacrificed 10 min later by injection with pentobarbital (150 - 300 mg kg^{-1} i.p). The chest was cut open and the heart was immediately removed and placed in a cardioplegic solution containing 27 mM KCl to arrest the contraction during cannulation of the aorta.

The heart was then mounted in the perfusion system and was perfused with 1.8 mM Ca^{2+} Tyrode solution containing (in mM: 135 NaCl, 5.4 KCl, 0.9 $MgCl_2$, 10 HEPES, 10 glucose, pH adjusted to 7.45 with NaOH) to resume contraction. The cell dissociation procedure involved retrograde perfusion of the aorta with a Ca^{2+}-free solution, followed by an enzymatic tissue digestion by perfusion with Ca^{2+}-free solution containing collagenase A (0.4-0.5 mg mL^{-1}, Roche Diagnostics, Mannheim, Germany) and protease (type XIV 0.08 mg mL^{-1}, Sigma, Missouri, USA) at 37 $^{\circ}$C. Thereafter, Ca^{2+} was gradually re-introduced up to a concentration of 0.18 mM. The ventricles were cut out into 0.18 mM Ca^{2+} solution and cells were dissociated by gentle mechanical agitation and filtered through a mesh (200-µm hole diameter). Cells were given time to settle down at room temperature before use for shortening or membrane ion current measurements.

2.8.1.2 Cell shortening measurements

Mechanical properties of ventricular myocytes were assessed at 35 °C using a video-based edge-detection system (Crescent, Salt Lake City, USA). Cells were placed in a chamber mounted on the stage of an inverted microscope (Olympus X-70) and superfused with Tyrode solution with 1 % DMSO. The cells were field-stimulated at a frequency of 1 Hz, 2.5 ms in duration (Myopacer Cell Stimulator, IonOptix, Milton, USA). A video-based edge detector was used to capture and convert changes in cell length during contraction and relaxation into an analog voltage signal. Tyrode solution containing OA or derivatives was perfused at different time intervals during the experimental period. The magnitude of cell contraction was assessed by measuring the amplitude of cell shortening, and kinetics of contraction and relaxation were assessed using the time-to-peak contraction (TTP) and the time constant (tau) of relengthening as demonstrated in Figure 4 below. Cell contraction is expressed as percentage cell shortening based on fractional cell shortening (FCS) i.e. cell length change normalised to resting cell length.

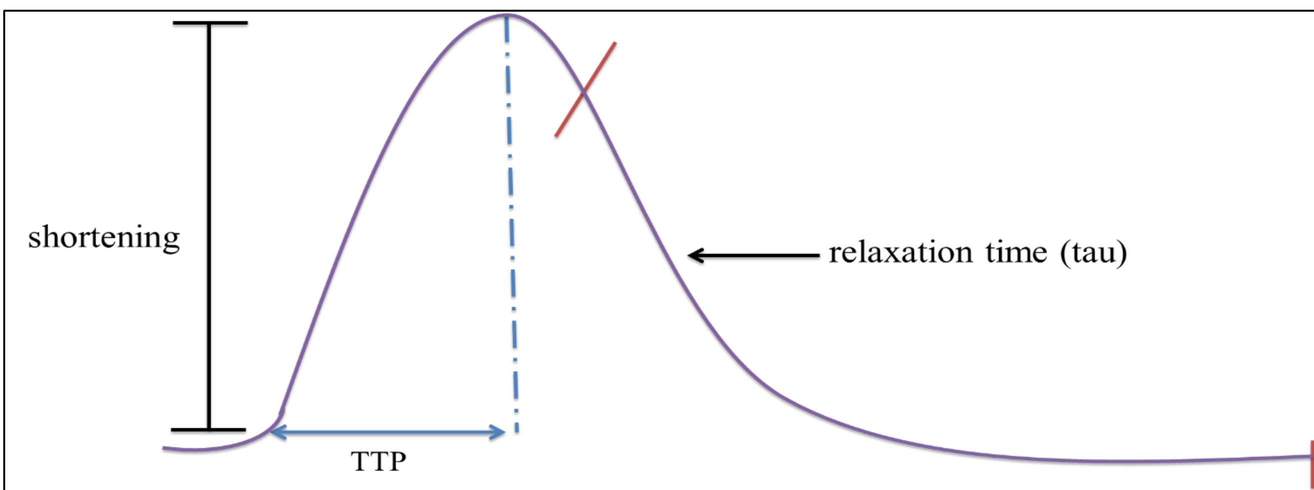

Figure 4: Characteristics of contraction include a parameter such as the amplitude of cell shortening, and kinetic parameters such as time-to-peak contraction (TTP) and time constant (tau) of relaxation.

2.8.1.3 Ca^{2+} current measurements

Whole cell currents were recorded with the patch-clamp technique at room temperature to access the effects of OA and derivatives on L-type Ca^{2+} current (I_{CaL}). Patch pipettes were pulled from capillary glass (GB 200-8P; Science Products, Hofheim, Germany) on a DMZ Universal Puller (Zeitz-Instrumente, Munich, Germany), which had a final resistance of 2 - 4 MΩ when filled with internal solution. For these experiments the pipette solution contained (mM): 130 CsCl, 10 HEPES, 0.5 $MgCl_2$, 4 MgATP (pH 7.2 with CsOH), and the external solution contained (mM): 130 NaCl, 10 CsCl, 11.8 HEPES, 0.5 $MgCl_2$, 1 $CaCl_2$, 10 glucose, pH 7.4 adjusted with NaOH. The voltage-clamp protocol consisted of a pre-step from -70 to -50 mV to inactivate I_{Na}, followed by 200-ms depolarizing voltage steps to various levels between -80 mV and +70 mV. In these experiments, Cs^+ replaced K^+ to block K^+ currents, and OA and derivatives were added when necessary to the extracellular solution.

2.8.2 Vascular studies

The effects of OA, Me-OA and Br-OA on vascular contraction and relaxation profiles were evaluated in isolated aortic rings and mesenteric arteries of male Wistar and DSS rats.

2.8.2.1 Tissue isolation

The methods for vascular tissue isolation, contraction and relaxation measurements were similar to those described in detail previously (Martinsen, Baeyens, Yerna and Morel, 2012). Briefly, male Wistar and DSS rats (mean body weight 250 g) were sacrificed by stunning and exsanguinations in accordance with institutional guidelines for the use of experimental animals. Aorta or small mesenteric arteries (SMA) were quickly removed and immersed in physiological saline solution (PSS) (composition in mM: 120 NaCl, 5.9 KCl, 25 $NaHCO_3$, 11.5 glucose, 1.2 NaH_2PO_4, 1.2 $MgCl_2$ and 2.5 $CaCl_2$). Arteries were cleaned of all fat and connective tissue. All dissecting procedures were carefully done to protect the functional endothelium from damage. Each aortic or mesenteric segment was cut with a sterile dissection scissor into rings of approximately 1.5 - 2 mm width. Aortic rings with intact functional and denuded non-functional endothelium were used. Endothelium-denuded aortic rings and SMA were obtained by gently rubbing the lumen of the

artery with forceps tip and hair, respectively. A piece of a large diameter straight black hair was inserted inside the vessel and the endothelium was removed by gentle moves back and forth inside the vessel (Gauthier, Zhang, Cui, Nithipatikom and Campbell, 2008). Endothelium-denuded vessels were recognized by showing no significant response to addition of carbachol with a level of contraction remaining larger than 90% of the maximum contraction.

2.8.2.3 Isometric tension and vasodilatory effects

Aortic rings were mounted under a tension of 20 mN in a 12.5 mL organ-bath. Mesenteric arteries were mounted in a wire myograph (DMT, Aarhus, Denmark) under the tension determined by the normalization procedure in a 6 mL organ bath. The baths were perfused with PSS and gassed with 95% O_2 and 5% CO_2 at 37 °C. Artery rings were first stimulated with PSS KCl solution (composition in mM: 31.9 NaCl, 94 KCl, 25 $NaHCO_3$, 11.5 glucose, 1.2 NaH_2PO_4, 1.2 $MgCl_2$ and 2.5 $CaCl_2$) in order to assess vessel integrity. Washout and a resting period of 15 min was allowed before starting the experiment.

Aortic rings and SMA with and without functional endothelium were pre-contracted with a single sub-maximal concentration of KCl (50 mM) or PHE (5 µM). When a sustained, stable and tonic contraction was observed, cumulative concentration-response effects were measured sequentially, to carbachol (0.01-100 µM) and OA or derivatives (3-30 µM) dissolved in PSS-DMSO solution. The presence of a functional endothelium was tested by measuring the relaxation profile of carbachol, a drug known to cause endothelium-dependent effects, In this regard, arterial rings were pre-contracted with a high-KCl solution and when maximum contraction was reached, various doses of carbachol were added. Each concentration of drug was left in contact with the vessels for 2 min and the contractile tension was measured by an isometric force transducer using data acquisition hardware (Mac-Lab) and software (Chart v3.3; DMT, Aarhus, Denmark). Following each concentration-response curve the vessels were washed with PSS and rested in PSS for 15 min. The involvement of NO in triterpene-induced effects was examined in intact aortic rings pre-treated with appropriate antagonists [N-nitro-L-arginine methyl ester (L-NAME) (100 µM), a NOS inhibitor, and INDO (10µM), a non-selective cyclooxygenase inhibitor]. To investigate whether

the increased hypotensive action of OA in DSS rats involved the contribution of K^+ channel opening, we pretreated the isolated vessels with Ca^{2+}-dependent K^+ channel blockers, Gli (5 mmol L^{-1}) and AP (1 mmol L^{-1}).

2.9 Data management and statistical analysis

Data for arterial pressure, renal function and vascular studies were analyzed using GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, CA, USA). Statistical comparison of the differences between the control and experimental groups was performed with one-way analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparison test. Data analysis for cardiac studies was performed using Clampfit 8.2 (Axon Instruments, Scotland) and Origin (Origin lab, USA). Differences between groups were analyzed for statistical significance using ANOVA with a Fisher post-hoc test, using Statistica 7.1 (StatSoft, USA). All data are expressed as mean ± standard error of the mean (SEM) and a difference with $p < 0.05$ was considered significant.

CHAPTER 3

RESULTS (Phytochemical studies)

3.0 General

The results are divided into 3 chapters which describe the following:

1. spectroscopic data of OA, Me-OA and Br-OA;
2. blood pressure and renal function effects of OA in conscious male SHR and DSS rats;
3. effects of OA and derivatives on proximal tubular Na^+ handling in anaesthetised animals;
4. OA effects on oxidative status of the heart, kidney and liver;
5. *ex vivo* effects of OA and derivatives on cardiomyocyte contraction and L-type Ca^{2+} currents;
6. effects of OA and derivatives on vascular smooth muscle contraction.

3.1 Structure elucidation

3.1.1 OA ($C_{30}H_{47}O_3$)

The percentage yield of OA obtained from dry plant material varied from 0.79% to 1.72%. Figure 5 shows ^{13}C NMR spectra. The two carbon signals at 125.6 and 122.8 ppm correspond to the carbon-carbon olefenic double bond at position 12 and 13 which is present in many triterpenoids.

^1H NMR (400 MHz, $CDCl_3$): δ 5.30 (t, 1H, J = 3.5 Hz), 3.21 (dd, 1H, J = 11.5 and 4.4 Hz), 2.85 (dd, 1H, J = 13.8 and 4.3 Hz), 1.21 (s, 3H), 0.99 (s, 3H), 0.92 (s, 3H), 0.91 (s, 3H), 0.90 (s, 3H), 0.79 (s, 3H), 0.72 (s, 3H).

^{13}C NMR (400 MHz, $CDCl_3$): δ 183.5, 143.8, 122.8, 79.2, 55.4, 47.8, 46.7, 46.1, 41.8, 41.2, 39.4, 38.9, 38.6, 37.3, 34.0, 33.2, 32.8, 32.6, 31.8, 28.3, 27.9, 27.3, 26.1, 23.6, 23.1, 22.9, 18.5, 17.3, 15.7, 15.5

Infrared spectra: 3454, 2940, 2860, 1688, 1462, 1387, 1274, 1185, 1030,781, 655 cm^{-1}.

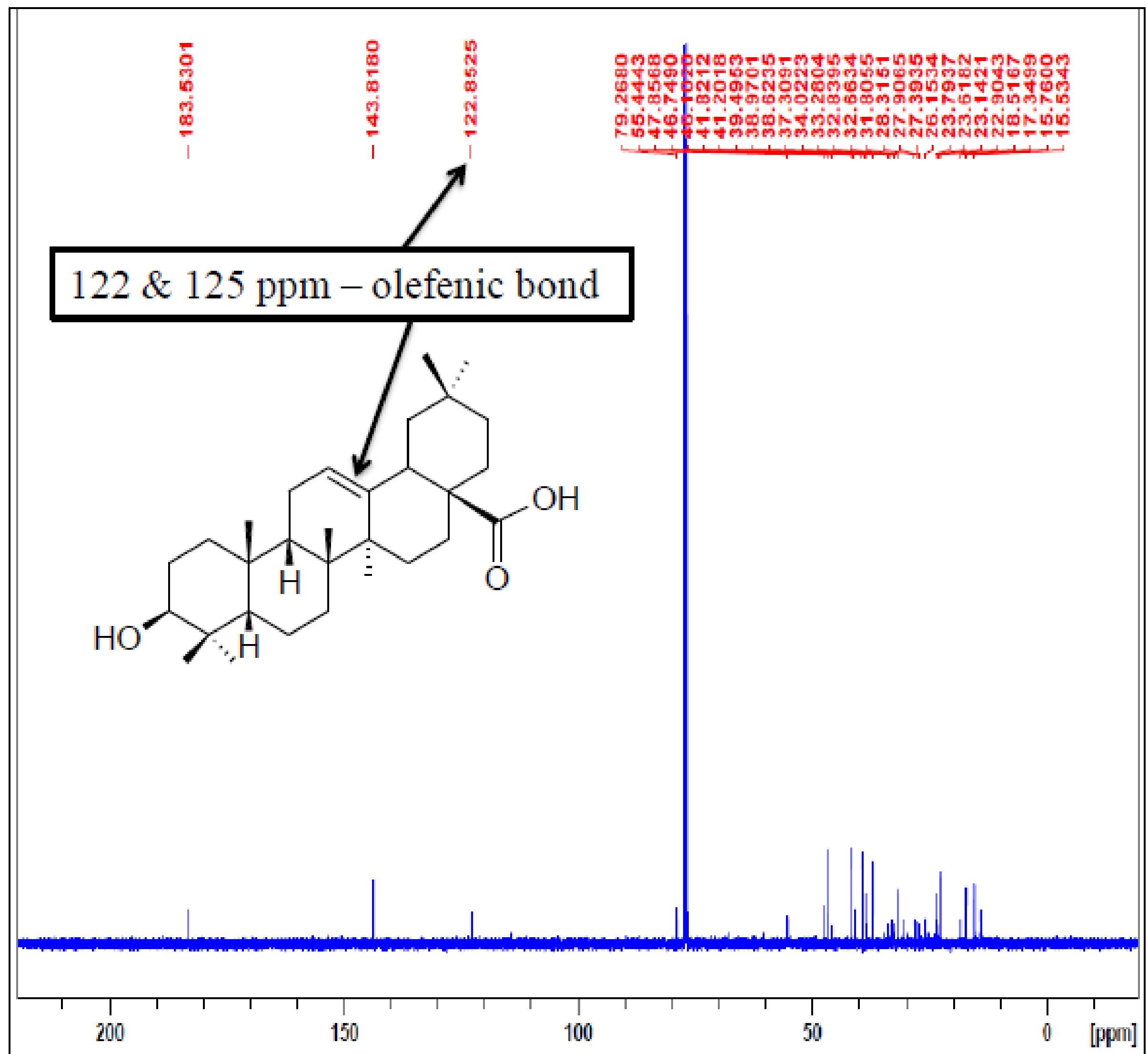

Figure 5: *Syzygium aromaticum*-derived OA ^{13}C- NMR spectra. Pure OA was obtained following recrystallisation with methanol and the structure was elucidated using NMR spectra recorded on a Bruker DRX-400 spectrometer.

3.1.2 Me-OA ($C_{31}H_{50}O_3$)

The percentage yield for the methylation step was 65%. Methylation with nitrosomethylurea removed the hydrogen in the carboxyl group and attached the methyl group. The product is indicated by the appearance of methyl peak at 3.63 ppm shown in ^{1}H-NMR spectra (Figure 6).

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.30 (t, 1H, *J* = 3.5 Hz), 3.63 (s, 3H), 3.21 (dd, 1H, *J* = 11.5 and 4.4 Hz), 2.85 (dd, 1H, *J* = 13.8 and 4.3 Hz), 0.99 (s, 3H), 0.92 (s, 3H), 0.91 (s, 3H), 0.90 (s, 3H), 0.79 (s, 3H), 0.72 (s, 3H).

^{13}C NMR (400 MHz, $CDCl_3$): δ 178.1, 143.9, 122.6, 79.2, 55.2, 51.5, 47.6, 46.7, 45.9, 41.7, 41.3, 39.3, 38.7, 38.4, 37.0, 34.0, 33.1, 32.7, 32.4, 30.7, 28.1, 27.7, 27.2, 26.0, 23.6, 23.1, 18.3, 16.8, 15.6, 15.2.

Infrared spectrum: 3352, 2941, 2860, 1725, 1711, 1462, 1385, 1361, 1163, 1030, 780, 752 cm^{-1}.

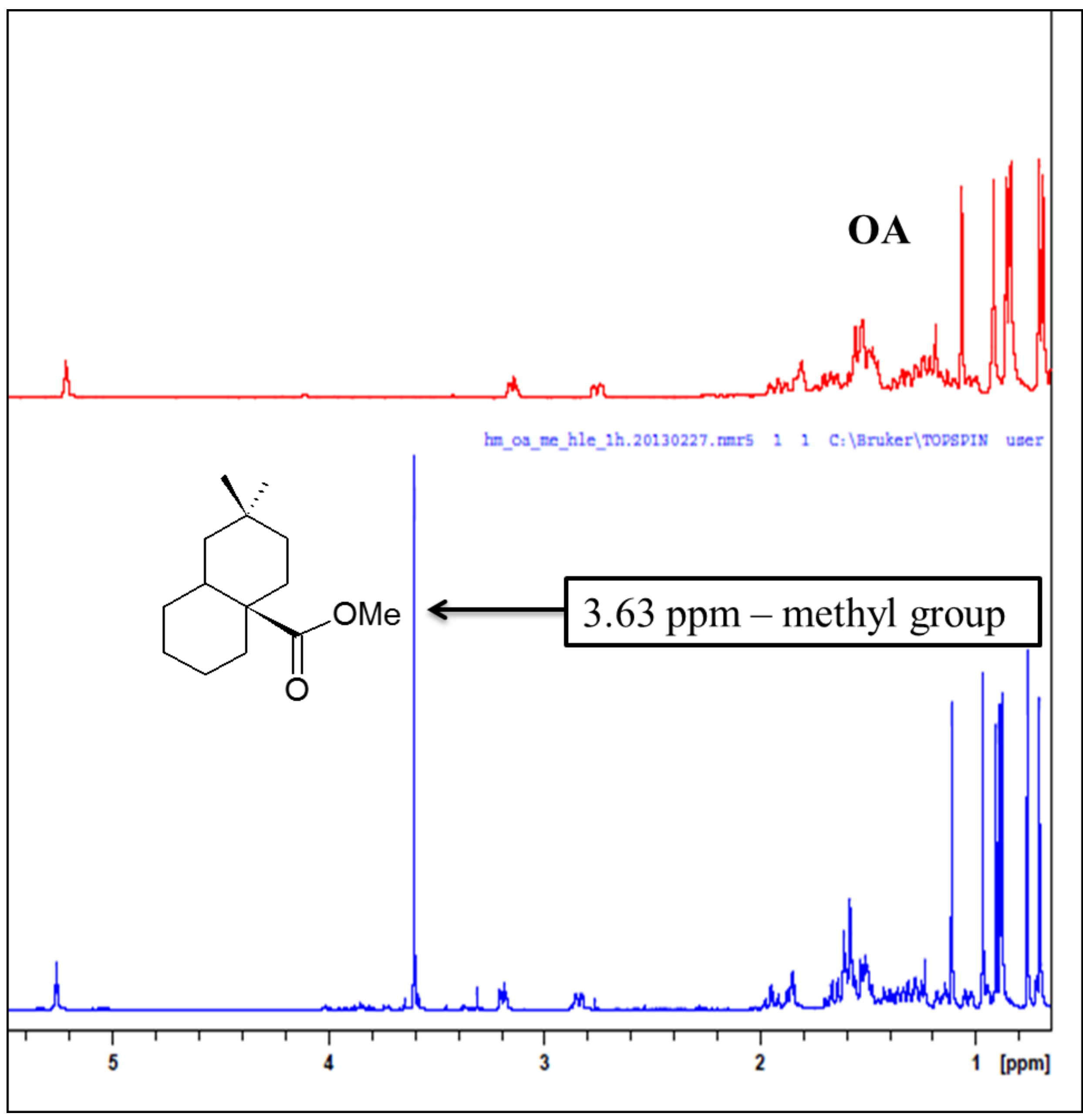

Figure 6: ^{1}H-NMR of Me-OA in comparison to OA. ^{1}H-NMR of Me-OA shows the appearance of a methyl peak at 3.63 ppm upon methylation with nitrosomethylurea. The structure was elucidated using NMR spectra recorded on a Bruker DRX-400 spectrometer.

3.1.3 Br-OA ($C_{31}H_{43}BrO_4$)

The percentage yield for the bromination step was 30%. ^{13}C-NMR of the bromination product (Figure 7) shows that two protons at C9 and C11 were removed due to formation of a double bond indicated by the new peak at 124.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.82 (s, 1H), 5.97 (s, 1H), 3.69 (s, 3H), 3.02 (d, 1H, *J* = 13.7 Hz), 2.91 (d, 1H, *J* = 4.6 Hz), 1.44 (s, 3H), 1.29 (s, 3H), 1.23 (s, 3H), 1.17 (s, 3H), 1.00 (s, 3H), 0.99 (s, 3H), 0.87 (s, 3H).

^{13}C NMR (400 MHz, $CDCl_3$): δ 178.3, 170.2, 155.1, 123.7, 122.1, 67.9, 51.8, 49.6, 48.1, 47.3, 46.1, 45.7, 44.7, 42.1, 35.8, 34.7, 34.4, 33.2, 32.7, 31.8, 31.5, 30.6, 28.0, 27.7, 27.0, 24.6, 23.1, 22.7, 22.0, 21.6, 20.6

Infrared spectrum: 2950, 2870, 2253, 1720, 1685, 1660, 1458, 1385, 915, 744, 651, 623, 456 cm^{-1}.

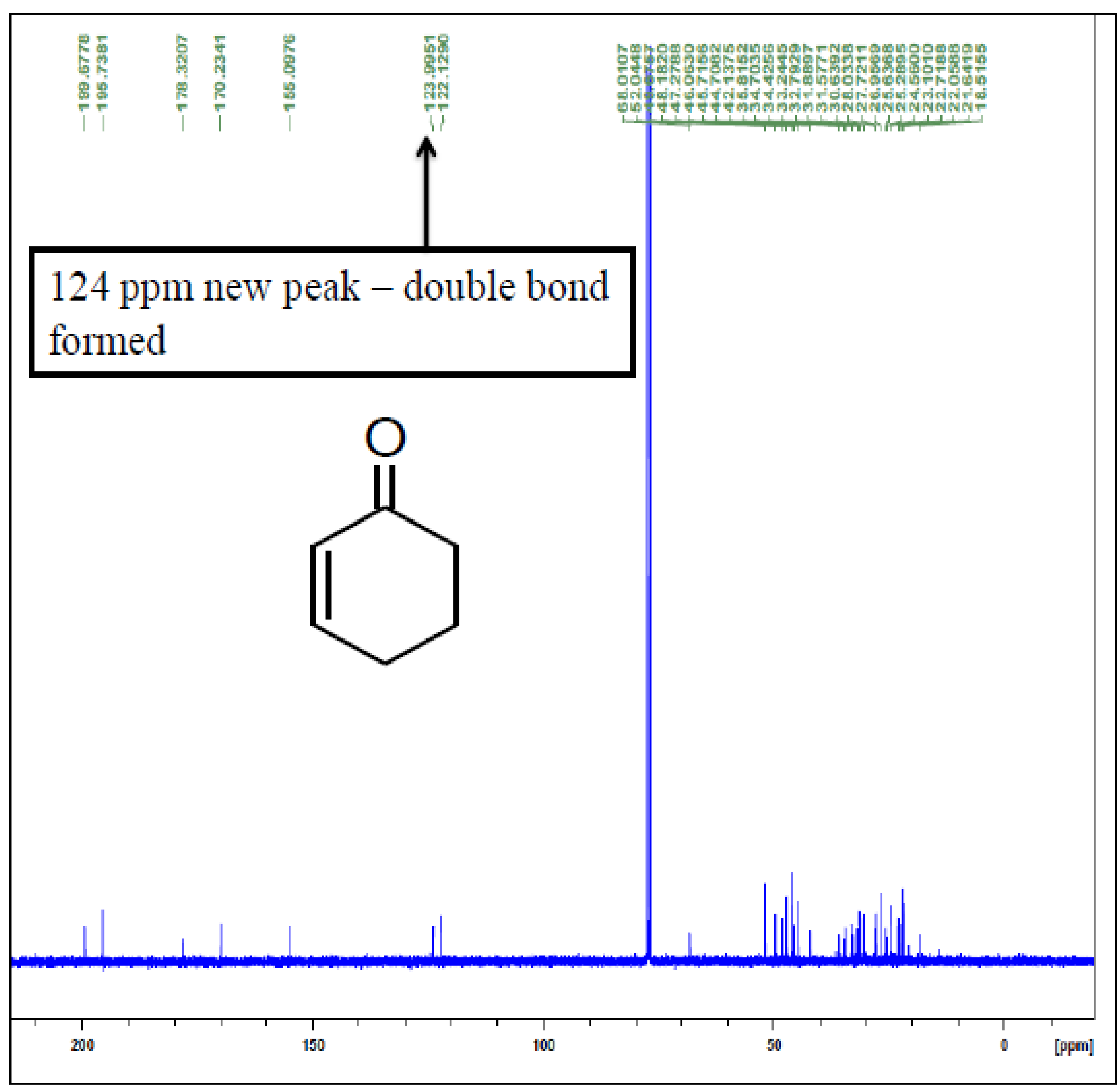

Figure 7: ^{13}C- NMR spectrum of Br-OA shows the appearance of a new peak at 124 indicating the formation of a double bond after bromination with Br_2 and HBr. Structures were elucidated using NMR spectra recorded on a Bruker DRX-400 spectrometer.

CHAPTER 4

RESULTS (*In vivo* studies)

4.1 Acute effects of OA and derivatives

4.1.1 GFR and MAP measurements

Following infusion of hypotonic saline to control Wistar rats, no significant variations were seen in the GFR and MAP throughout the 4 h post-equilibration period (Figure 8). However, intravenous infusion of OA or derivatives slightly increased GFR to values which did not achieve statistical significance. On the other hand, intravenous infusion of OA (90 µg h^{-1}) for 1.5 h in the experimental group significantly ($p < 0.001$) reduced MAP by comparison with the control group (n=6). Me-OA and Br-OA decreased MAP to similar values as OA and the blood pressure did not revert back to pre-treatment levels during the recovery period.

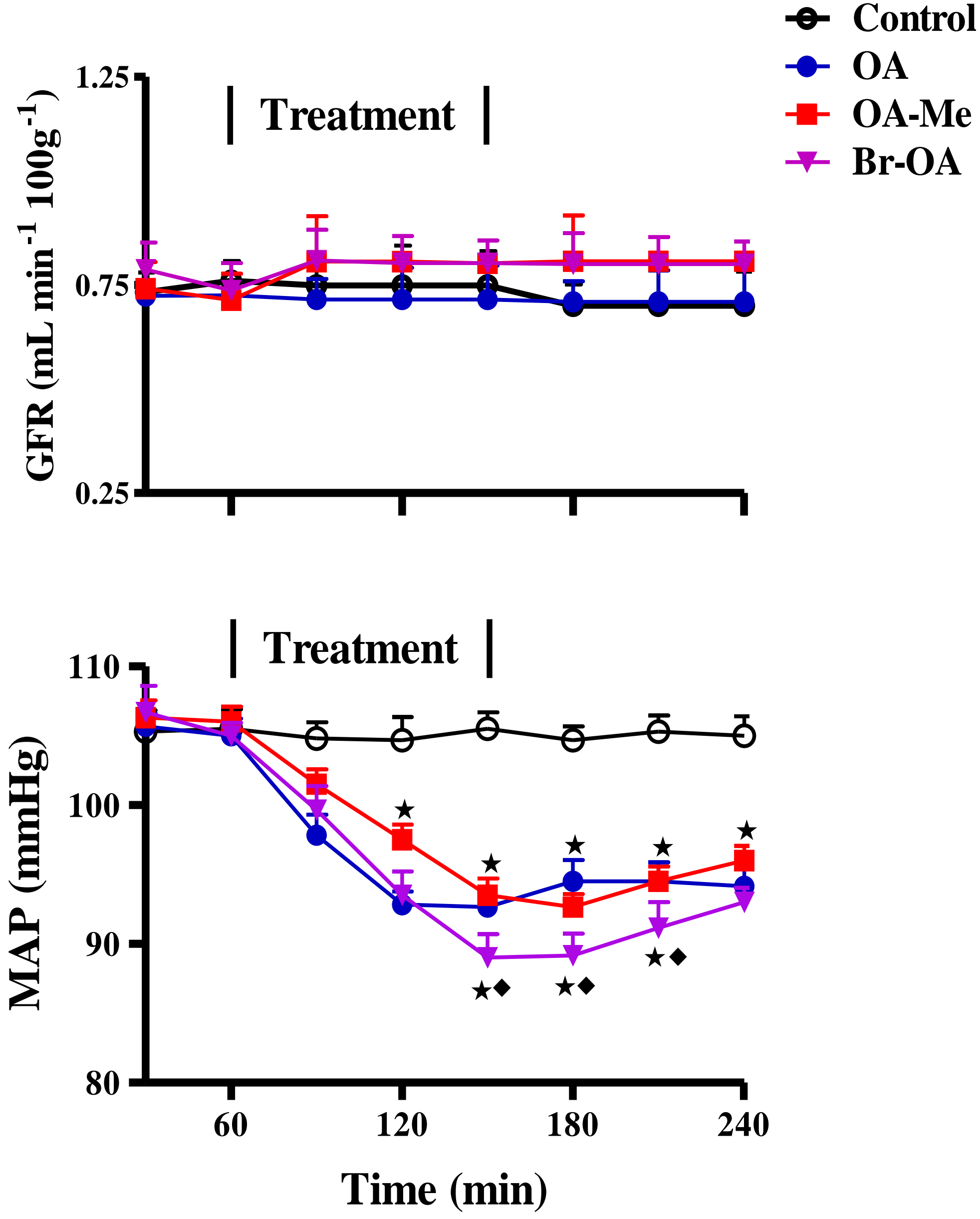

Figure 8: Comparison of GFR (A) and MAP (B) of control rats with animals administered OA, Me-OA and Br-OA (90 µg h^{-1}) during the 4 h experimental period. All drugs were administered for 1.5 h during the treatment period. Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). ★ $p < 0.001$ by comparison with control animals at each corresponding time. ♦ $p < 0.05$ by comparison with OA-treated animals.

4.1.2 Fluid and electrolyte handling

In this set of our experiments, the study was restricted to a 4 h experimental period due to poor yields of the synthesised derivatives. The effects of intravenous infusion of OA and derivatives on proximal tubular function and MAP were tested in anaesthetised Wistar rats. Figure 9A shows the urine flow rate of control and drug-administered animals during the 4 h experimental period. Urine flow rate under control conditions (i.e. in untreated animals, as well as before treatment in the treated group) matched the infusion rate of 9 mL h^{-1}. OA, Me-OA and Br-OA infusion had no influence on urine flow rate. Figure 9B shows urinary Na^+ excretion in the same animals. Urinary Na^+ excretion of control animals was stable throughout the experiment and was comparable to the infusion rate of 693 µmol h^{-1}. However, OA infusion at 90 µg h^{-1} for 1.5 h during the treatment period significantly ($p < 0.05$) increased urinary Na^+ excretion rate by comparison with control animals. Similar effects to increase urinary Na^+ excretion were obtained with the OA derivatives, but the peak excretion rate was most marked with Br-OA. This is shown in Table 3 which demonstrate the cumulative data for urine flow and total electrolyte excretion during the 1.5 h treatment period indicating the drug that was more effective among the three triterpenes studied.

Figure 11 shows relationship between changes in urinary Na^+ excretion and MAP at the end of the treatment period, as analyzed by individual values of each animal from the control and experimental groups. Increased urinary Na^+ excretion in these acute experiments had some but weak correlation with decreased MAP ($R = 0.67$; $p < 0.05$). OA infusion had no influence on urinary K^+ and Cl^- outputs in comparison with control animals (Figure 10). Me-OA and Br-OA significantly ($p < 0.05$) decreased urinary K^+ output and increased urinary Cl^- outputs in comparison with control animals.

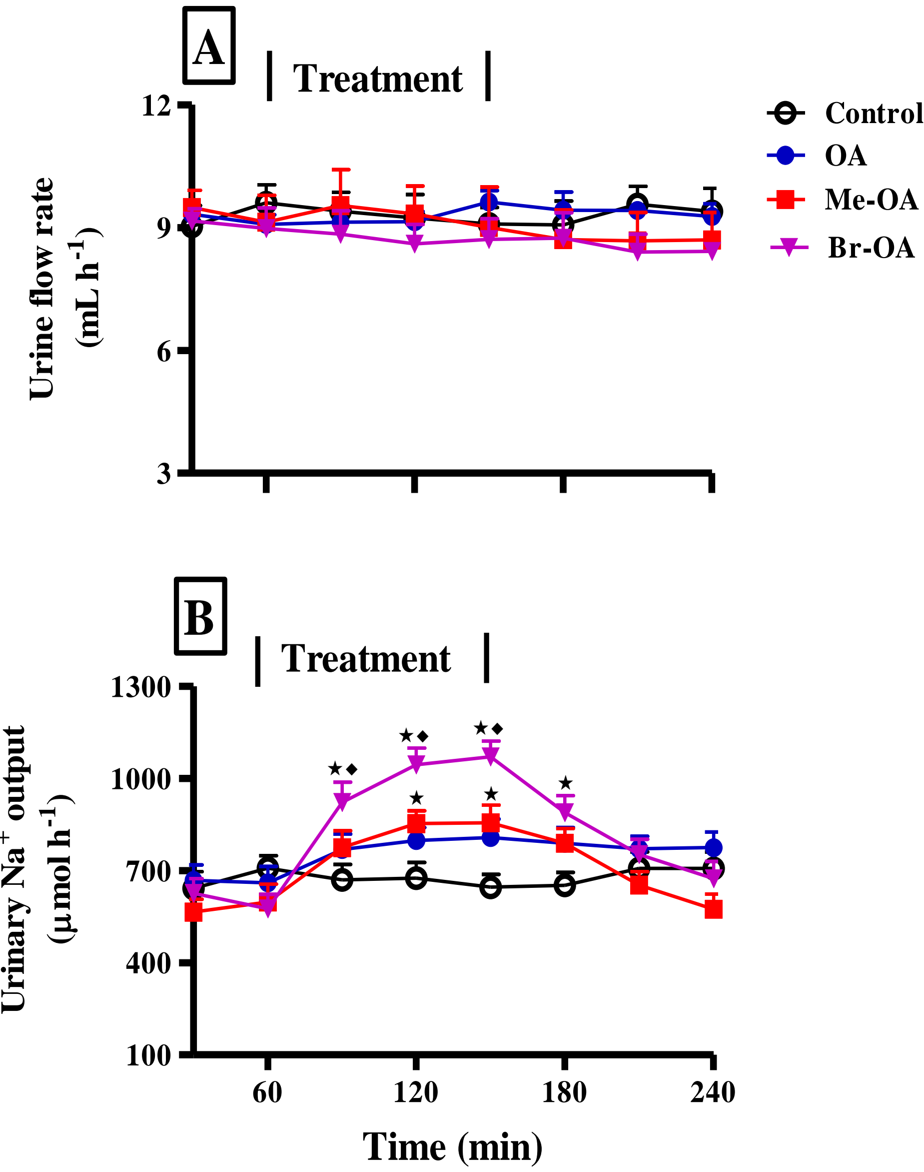

Figure 9: Comparison of urine flow rate (**A**) and urinary Na^+ excretion (**B**) of control rats with animals administered OA, Me-OA and Br-OA during the 4 h experimental period. Drugs were administered for 1.5 h during the treatment period. Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). ★ $p < 0.05$ by comparison with control animals at each corresponding time. ♦ $p < 0.05$ by comparison with OA-treated animals.

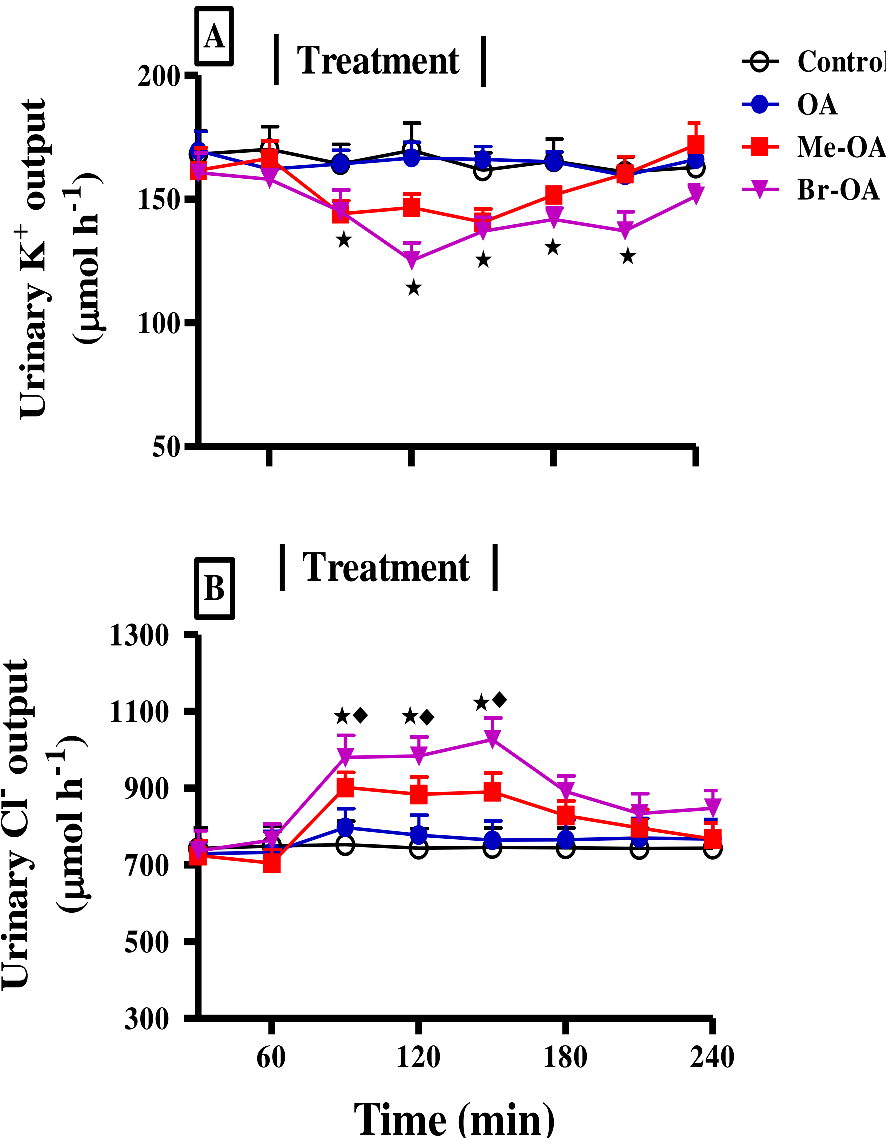

Figure 10: Comparison of urinary K^+ (**A**) and Cl^- (**B**) excretion rate of control rats with animals administered OA, Me-OA and Br-OA during the 4 h experimental period. Drugs were administered for 1.5 h during the treatment period. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ $p < 0.05$ by comparison with control animals at each corresponding time. ♦ $p < 0.05$ by comparison with OA treated animals.

Table 3: Comparison of the effects of OA and derivatives infusion on urine flow and total amount of electrolytes excreted during 1.5 h treatment period. Values are presented as means ± SEM (n = 6 in each group).

	Parameter			
Group	Urine volume (mL)	Na^+ (mmol)	K^+ (mmol)	Cl^- (mmol)
Control	14.00 ± 0.16	0.98 ± 0.02	0.24 ± 0.02	1.04 ± 0.01
OA	14.00 ± 0.15	1.16 ± 0.01*	0.24 ± 0.02	1.08 ± 0.04
Me-OA	14.02 ± 0.07	1.24 ± 0.03*	0.22 ± 0.01	1.24 ± 0.01*
Br-OA	13.66 ± 0.07	1.52 ± 0.04*#	0.20 ± 0.03*	1.30 ± 0.03*#

* $p < 0.05$ by comparison with control animals

# $p < 0.05$ by comparison with OA-treated animals.

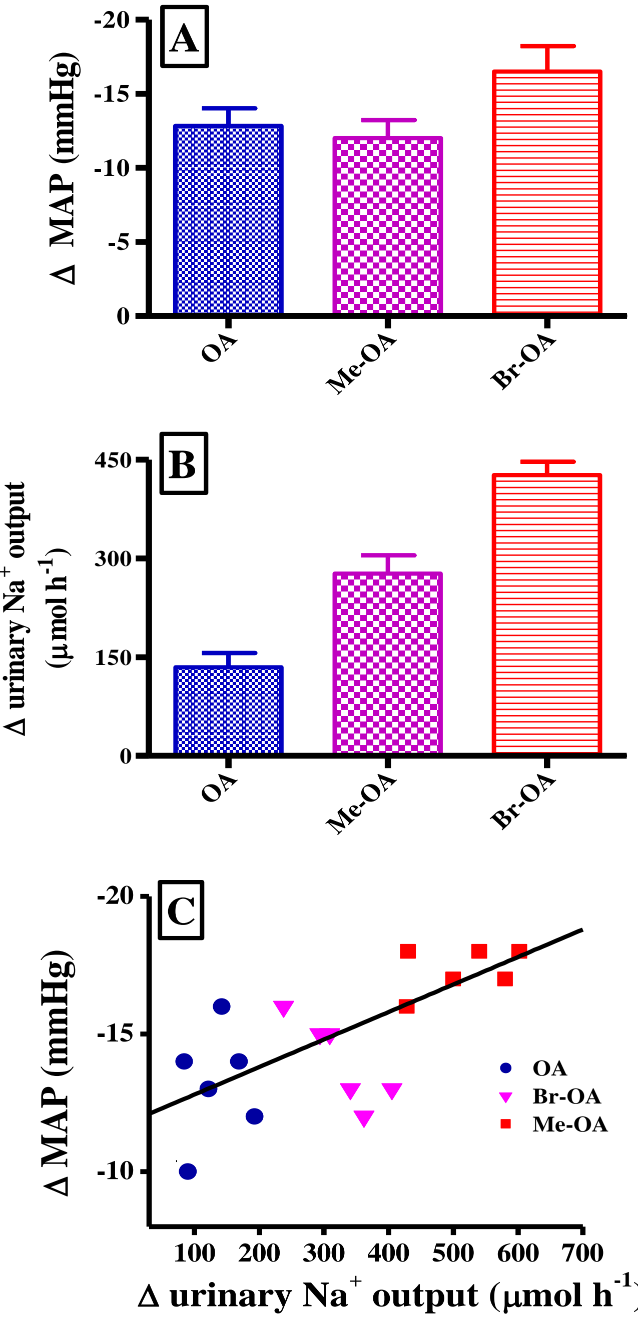

Figure 11: Comparison of the changes in MAP (**A**), urinary Na^+ excretion rate (**B**); correlation between MAP and Na^+ changes (**C**) in OA or derivative-administered animals during the 1.5 h treatment period. Values are presented as means, and vertical bars indicate SEM (n = 6 in each group).

4.1.3 Renal clearance measurements

To determine the site that is responsible for increased Na^+ output along the tubular nephron, we measured Li^+ clearance (C_{Li}), a marker for proximal tubular Na^+ handling (Thomsen, 1990) and also determined FE_{Li}. The effect of OA and derivatives on proximal tubular Na^+ clearance was estimated by comparing renal l Li^+ clearance (FE_{Li}) between anaesthetised control and OA and derivative-treated rats. The Li^+ doses used resulted in plasma Li^+ concentrations between 0.2 and 0.3 mmol L^{-1} with no difference between the groups. All measured variables during the 4-h experimental period were in a steady state and did not differ between the control and the experimental groups prior to the infusion of OA or derivatives. The infusion of OA or derivative for 1.5 h significantly ($p < 0.05$) increased FE_{Li} in the proximal and FE_{Na} in both the proximal and the distal tubules by comparison with control animals at the corresponding time periods (Figure 12).

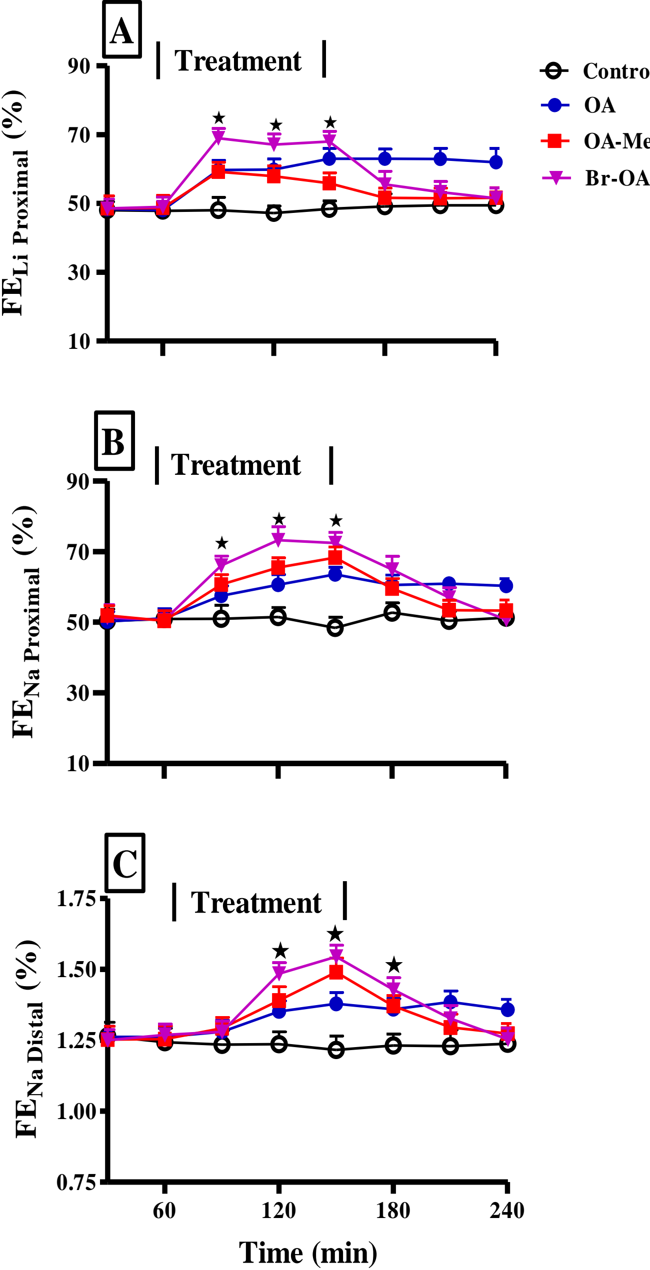

Figure 12: Comparison of proximal FE_{Li} (**A**), FE_{Na} (**B**) and distal FE_{Na} (**C**) of control rats with animals administered OA, Me-OA and Br-OA during the 4 h experimental period. All drugs were administered for 1.5 h during the treatment period. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ $p < 0.05$ by comparison with control animals at each corresponding time.

4.2 Sub-chronic effects of OA

4.2.1 Body weight (BW), food and water intake

Tables 4, 5 and 6 show the effects of various doses of OA (30, 60, 120 mg kg^{-1}, p.o.) in Wistar, SHR and DSS rats, respectively, on food, water intake and BW over a 9-week period. OA administration had no significant influence on food, water intake and BW changes throughout the experimental period.

Table 4: Comparison of the effects of of various doses of OA (30, 60, 120 mg kg^{-1}, p.o.) on food, water intake and BW change with control Wistar rats over a 9-week experimental period. Values are presented as means ± SEM (n = 6 in each group).

		Time (weeks)								
Test	**Protocol**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Food intake (g 100 g^{-1})	**Control**	12.8±0.8	12.4±0.4	12.0±0.4	11.6±0.4	11.2±0.8	12.0±0.4	11.6±0.8	12.8±0.4	12.8±0.8
	OA 30	11.2±0.8	10.8±0.4	11.6±0.4	12.0±0.8	12.4±0.4	11.6±0.8	12.0±0.8	12.4±0.4	12.8±0.4
	OA 60	11.6±0.4	12.4±0.8	11.6±0.4	11.2±1.2	11.2±0.8	11.2±0.4	11.6±0.4	12.0±0.8	13.2±0.4
	OA 120	10.8±0.8	11.6±0.4	11.2±0.4	11.2±0.8	10.8±0.4	12.0±0.4	12.4±0.4	12.8±0.4	12.4±0.8
Water intake (mL 100 g^{-1})	**Control**	12.4±0.8	12.8±0.4	12.4±0.4	12.0±0.8	11.2±0.8	12.0±0.4	13.2±0.4	12.4±0.4	11.6±0.4
	OA 30	12.0±0.8	12.8±0.4	12.8±0.8	12.0±0.4	11.2±0.4	12.8±0.8	12.0±0.4	12.0±0.8	12.0±0.4
	OA 60	12.8±0.4	11.6±0.8	12.0±0.4	11.6±1.2	12.8±0.8	12.8±0.4	12.8±0.4	12.8±0.8	12.4±0.4
	OA 120	12.4±0.8	11.2±0.4	11.6±0.4	12.0±0.4	11.2±0.4	13.2±0.8	12.0±0.4	12.8±0.4	12.0±0.8
% BW change (g 100 g^{-1})	**Control**	2.8±0.8	5.2±0.8	7.2±0.8	8.0±0.8	10.8±0.4	11.6±0.8	12.4±1.2	14.0±0.8	14.8±0.8
	OA 30	3.2±0.4	4.8±0.4	7.2±1.2	8.4±0.4	10.0±0.8	11.2±0.4	12.0±0.8	13.6±0.4	15.2±1.2
	OA 60	2.4±0.8	4.8±0.8	6.8±0.8	8.0±0.8	10.4±0.8	11.6±0.4	13.2±0.4	14.0±0.8	15.2±0.4
	OA 120	3.2±1.2	5.6±0.4	6.8±0.8	9.6±0.4	11.2±0.4	12.0±0.8	13.6±0.8	14.4±0.4	15.6±0.4

Table 5: Comparison of the effects of OA administration on food, water intake and BW with control SHR rats over a 9-week experimental period. Values are presented as means ± SEM (n = 6 in each group).

		Time (weeks)								
Test	**Protocol**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Food intake (g 100 g^{-1})	**Control**	6.0±0.4	6.4±0.8	8.0±0.4	7.2±0.4	7.6±0.4	9.2±0.4	9.2±0.4	8.0±0.4	8.4±0.8
	OA 30	5.2±0.4	7.6±0.8	8.4±0.4	7.6±0.4	8.0±0.8	7.2±0.4	8.8±0.8	7.6±0.4	7.2±0.4
	OA 60	6.4±0.8	7.2±0.4	8.0±0.4	7.6±0.8	7.6±0.4	7.2±0.8	8.8±0.4	7.6±0.8	6.8±0.4
	OA 120	7.6±0.4	6.4±0.4	8.0±0.4	8.0±0.4	7.6±0.4	8.8±0.4	9.2±0.4	8.8±0.4	8.0±0.8
Water intake (mL 100 g^{-1})	**Control**	6.0±0.4	9.2±0.4	10.0±0.8	10.8±0.8	11.6±0.8	12.0±0.4	11.6±0.4	12.0±0.8	11.6±0.4
	OA 30	7.6±0.4	10.4±0.4	12.0±0.8	11.6±0.4	12.8±0.4	13.2±0.4	13.6±0.8	13.2±0.8	12.0±0.8
	OA 60	7.2±0.8	10.4±0.4	10.8±0.8	11.2±0.4	12.8±0.4	12.8±0.4	14.4±0.4	12.4±0.4	12.8±0.4
	OA 120	8.0±0.4	10.8±0.4	11.6±0.8	11.6±0.8	13.2±0.4	13.2±0.4	12.8±0.4	12.4±0.8	12.4±0.8
% BW change (g 100 g^{-1})	**Control**	1.6±0.4	4.0±0.4	5.2±0.8	7.6±0.4	9.2±0.8	11.2±1.2	13.2±0.8	14.4±0.8	16.0±0.8
	OA 30	2.4 ±0.4	5.2±0.8	6.0±0.4	6.8±0.8	8.4±0.4	10.4±0.8	11.6±0.4	13.6±0.4	15.2±0.8
	OA 60	2.0±0.4	3.6±0.4	6.4±0.8	7.6±0.8	9.6±0.4	10.8±0.4	12.4±0.8	14.4±0.4	16.4±0.4
	OA 120	1.2±0.4	2.4±0.4	6.0±0.4	7.2±0.4	9.2±0.8	10.4±0.8	12.8±0.4	14.8±0.4	16.4±0.8

Table 6: Comparison of the effects of OA administration on food, water intake and BW with control DSS rats over a 9-week experimental period. Values are presented as means ± SEM (n = 6 in each group).

		Time (weeks)								
Test	**Protocol**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Food intake (g 100 g^{-1})	**Control**	6.0±0.4	8.4±0.4	10.8±0.4	10.0±0.4	11.2±0.8	10.8±0.4	10.0±0.4	9.2±0.4	8.4±0.4
	OA 30	6.0±0.8	8.8±0.4	10.4±0.4	9.2±0.4	10.8±0.4	10.8±0.4	10.4±0.4	9.2±0.4	7.2±0.4
	OA 60	6.8±0.4	8.4±0.4	10.4±0.4	9.2±0.4	9.6±0.4	10.0±0.4	9.6±0.4	9.2±0.4	8.0±0.4
	OA 120	7.2±1.2	9.2±0.4	10.0±0.4	8.4±0.4	10.0±0.4	10.0±0.4	9.2±0.4	8.4±0.4	8.0±0.4
Water intake (mL 100 g^{-1})	**Control**	12.0±0.4	14.0±0.4	16.8±0.8	14.4±0.4	16.0±0.4	16.0±0.8	16.8±0.4	14.0±0.4	16.0±0.4
	OA 30	12.0±0.4	14.0±0.4	16.0±0.4	14.0±0.4	15.2±0.4	15.2±0.4	15.6±0.4	13.6±0.4	15.6±0.8
	OA 60	12.8±0.4	13.2±0.4	16.8±0.8	14.4±0.4	15.6±0.4	15.2±0.4	15.2±0.4	13.2±0.8	15.2±0.8
	OA 120	12.0±0.8	14.4±0.4	17.2±0.8	13.6±0.8	14.8±0.4	14.8±0.4	15.2±0.4	14.0±0.4	14.8±0.8
% BW change (g 100 g^{-1})	**Control**	2.0±0.4	3.6±0.8	5.6±0.8	6.4±0.8	8.4±0.8	10.0±0.8	11.6±0.4	13.2±0.8	15.2±0.4
	OA 30	1.2±0.4	2.8±0.4	4.4±0.4	6.4±1.2	8.0±1.2	10.4±0.8	11.2±0.4	12.8±0.8	14.8±0.4
	OA 60	2.4±0.8	4.0±0.8	5.2±0.4	6.8±0.8	8.4±0.8	9.6±0.4	12.0±0.8	13.6±0.8	15.6±0.4
	OA 120	3.2±1.2	4.8±0.4	6.0±0.4	7.6±0.8	9.2±0.4	10.4±0.4	12.0±1.2	14.0±0.4	16.0±0.4

4.2.2 MAP and HR measurements

In the first set of our experiments, we performed a 9-week study where we tested the effects of OA on MAP and renal function in conscious Wistar, SHR and DSS rats. These experiments were restricted to OA because of the poor yield of synthetic derivatives obtained during the phytochemical studies. Weekly measured MAP of control Wistar animals was stable around 110 ± 2 mmHg throughout the 9-week experimental period (Figure 13). Administration of various doses of OA (30, 60, 120 mg kg^{-1} p.o.) in these Wistar rats significantly ($p < 0.05$) reduced MAP from the 3rd week until the end of the study period (Figure 13A). In contrast to control Wistar animals, weekly measured MAP of untreated SHR and DSS animals progressively increased to values > 155 ± 2 mmHg by the end of the experiment (Figure 13B and C). OA administration in SHR and DSS rats significantly ($p < 0.001$) reduced MAP to values that were comparable to normotensive rats**.** We did not see any dose-dependent action, it remains possible that we recruited maximal effects with the doses used and that more specificity could be demonstrated if sub-maximally effective doses were administered. The HR was determined simultaneously with the blood pressure measurements. Figure 14 shows that OA had no significant effects on HR in both normotensive and hypertensive animals.

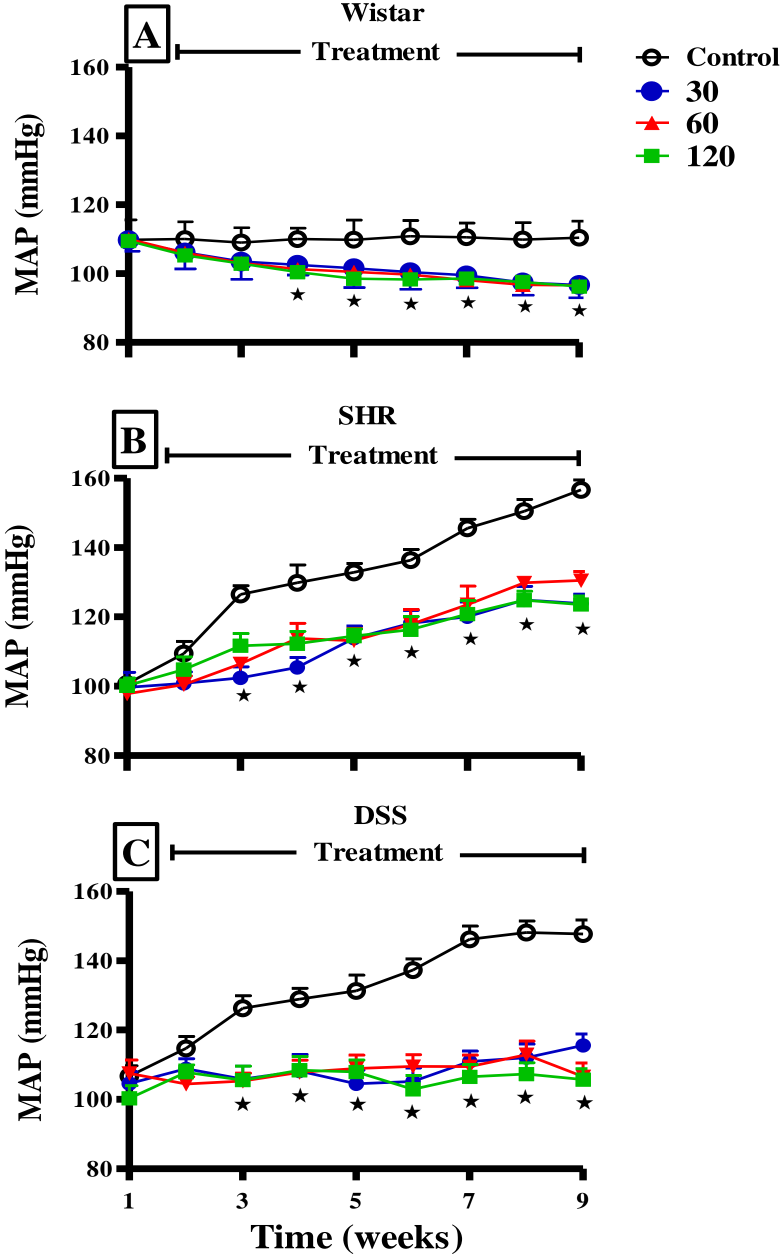

Figure 13: Comparison of the effects of the administration of various doses of OA (30, 60, 120 mg kg^{-1} BW) on MAP with control animals in Wistar (**A**), SHR (**B**) and DSS (**C**) rats over a 9-week experimental period. Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). ★ $p < 0.001$ by comparison with control animals at each corresponding time.

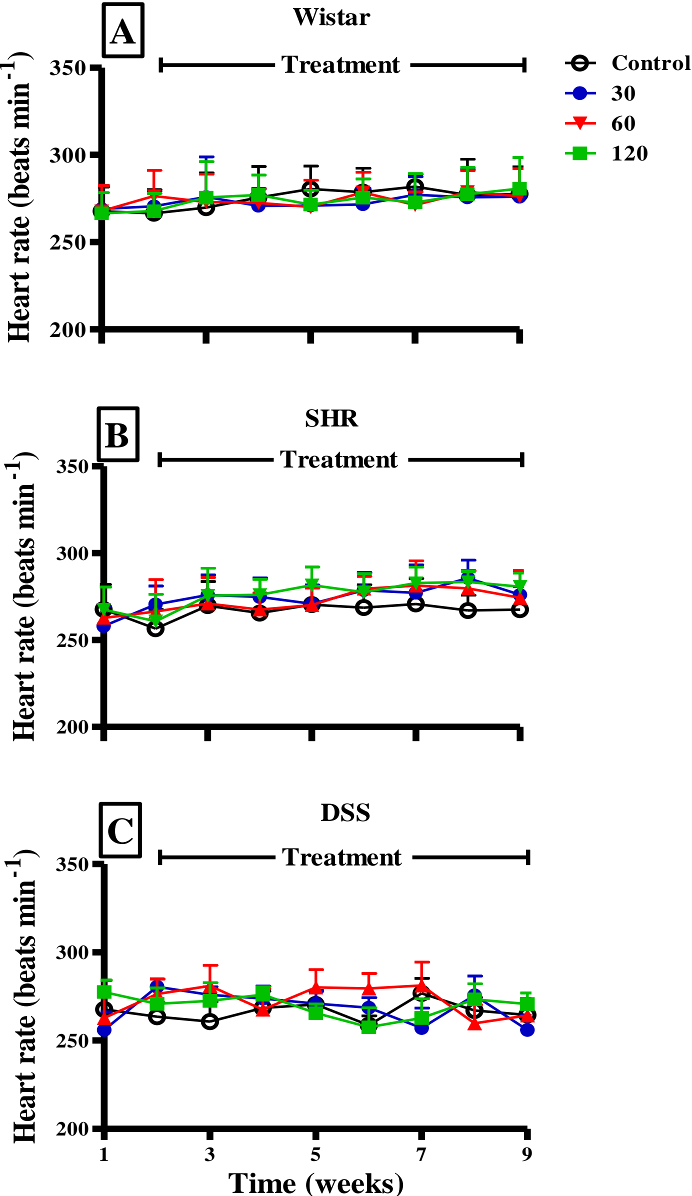

Figure 14: Comparison of the effects of the administration of various doses of OA (30, 60, 120 mg kg^{-1} BW) on the HR with control animals in Wistar (**A**), SHR (**B**) and DSS (**C**) rats over a 9-week experimental period. Values are presented as means, and vertical bars indicate SEM (n = 6 in each group).

4.2.3 Renal fluid and electrolyte handling

Figures 15A-C show the urine flow rate of control and OA-administered Wistar, SHR and DSS animals during the 9-week experimental period. OA administration had no significant influence on the urine flow rate in all groups of animals. Figures 15D-F show urinary Na^+ excretion rate in the same animals. Weekly urinary Na^+ excretion rate of control Wistar animals spontaneously increased with time without reaching statistically significant difference by the end of the experimental period. However, in both hypertensive models no such increase with time was obtained, instead, urinary Na^+ excretion rate tended to decrease with time after weaning in the DSS model.

Administration of various doses of OA (30, 60, 120 mg kg^{-1} p.o.) in DSS and SHR animals significantly ($p < 0.05$) increased weekly urinary Na^+ excretion rate from the 3rd week to values that were similar to control Wistar rats by the end of the study period (Figure 15D and 15F). We analyzed the relationship between changes in urinary Na^+ excretion rate and MAP changes in individual control and OA-treated animals on the last week of the study. We observed a linear relationship ($R = 0.85$, $p < 0.001$) between an increase in urinary Na^+ excretion rate and decrease in MAP (Figure 16C). OA administration, however, had no influence on urinary K^+ and Cl^- outputs in Wistar, SHR and DSS rats (Figure 17). In addition, there was a significant ($p < 0.05$) decrease in plasma creatinine with a concomitant increase in GFR at the end of the experiment in OA-treated Wistar, SHR and DSS rats (Table 7). The OA-evoked increase in urinary Na^+ excretion ratewas not reflected in the plasma collected at the end of the experiment in Wistar and SHR rats. OA administration had no influence on plasma urea concentrations, kidney and heart mass in all animals (Table 7).

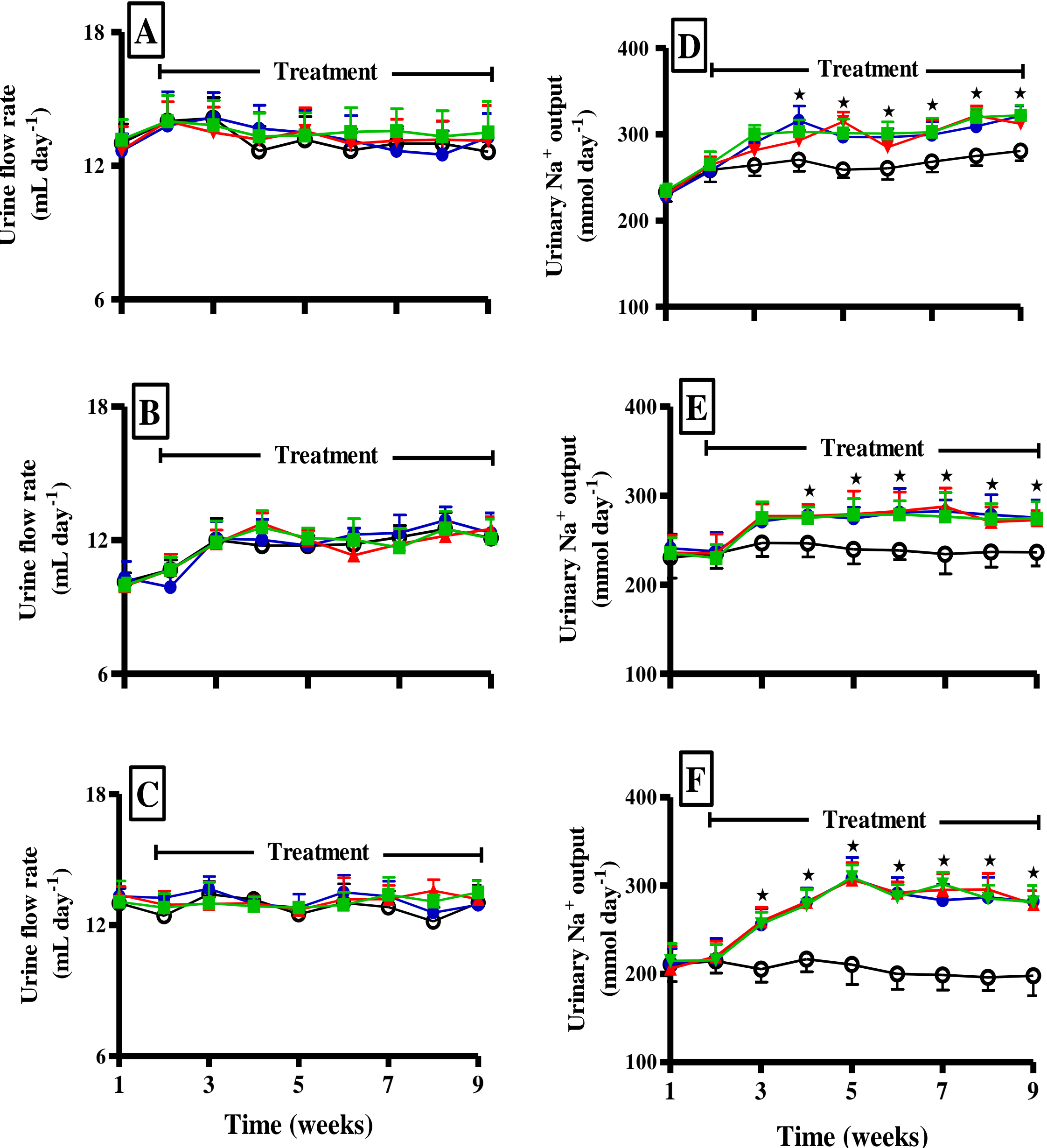

Figure 15: Comparison of the effects of the administration of various doses of OA (30, 60, 120 mg kg^{-1} BW) on 24 h urine flow (**A, B, C**) and Na^{+} excretion (**D, E, F**) rates with control Wistar (**A, D**), SHR (**B, E**) and DSS (**C, F**) animals over a 9-week experimental period. Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). $p < 0.05$ by comparison with control animals at each corresponding time.

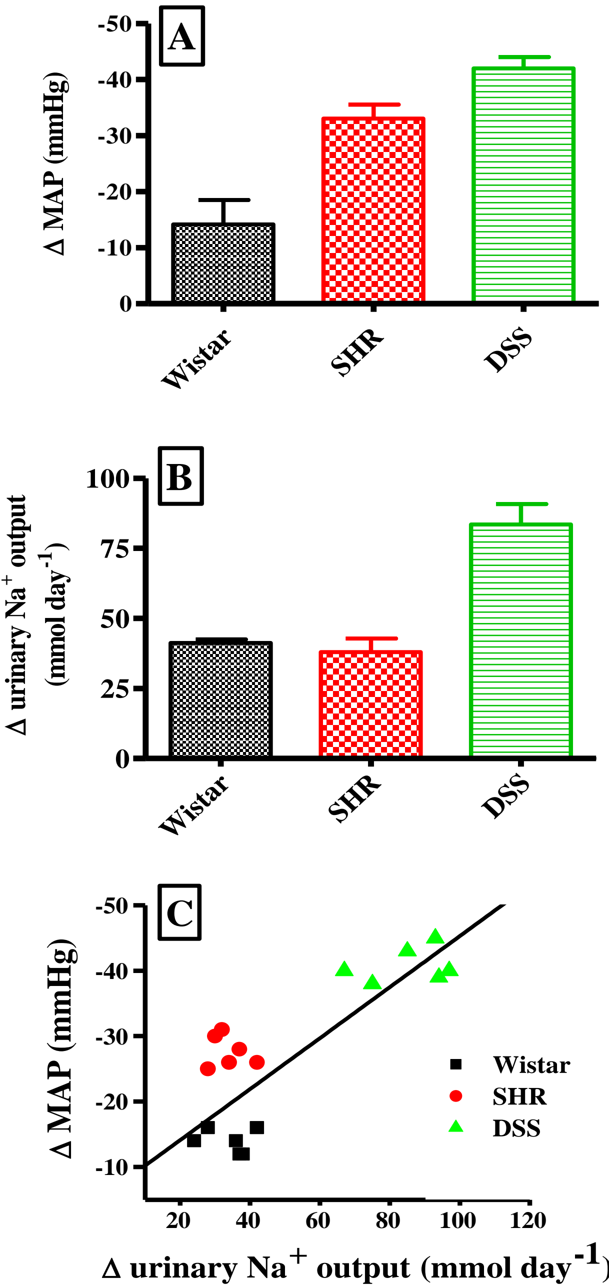

Figure 16: Comparison of the effects of the administration of median dose of OA (60 mg kg^{-1}) on changes in MAP (**A**) and urinary Na^+ excretion rate (**B**) on the 9th week of the study. Correlation of MAP and Na^+ (**C**) changes in conscious Wistar, SHR and DSS rats. Values are presented as means for weekly measurements; vertical bars indicate SEM of means (n = 6) in each group.

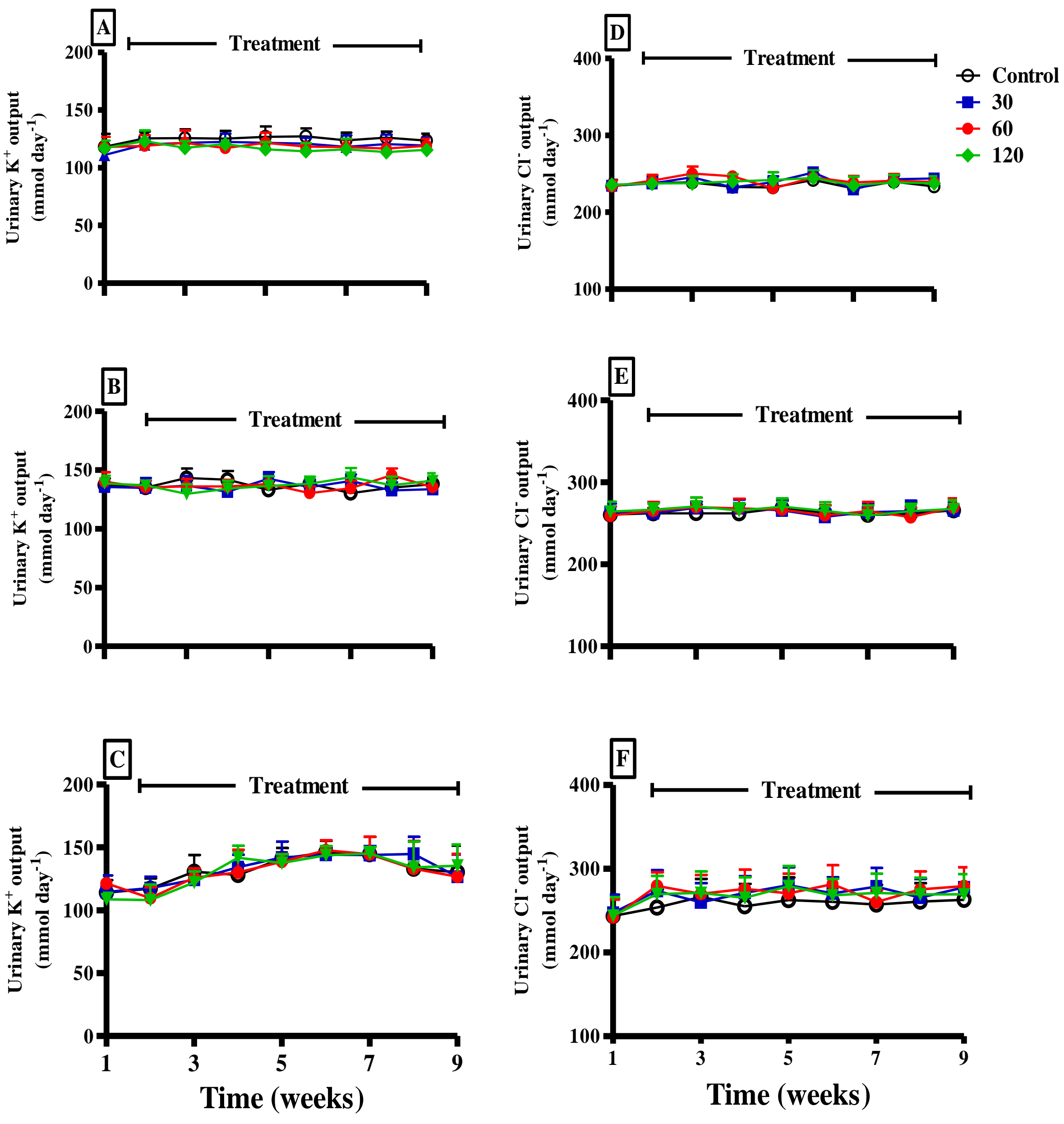

Figure 17: Comparison of the effects of the administration of various doses of OA (30, 60, 120 mg kg^{-1} BW) on urinary K^+ (**A, B, C**) and Cl^- excretion (**D, E, F**) rates with control Wistar (**A, D**), SHR (**B, E**) and DSS (**C, F**) animals over a 9-week experimental period. Values are presented as means and vertical bars indicate SEM (n = 6 in each group).

Table 7: The effects of OA on plasma biochemical parameters in male Wistar, SHR and DSS rats which were administered OA twice every third day for nine weeks. Values are presented as means ±SEM (n=6 in each group).

		Experimental Protocol			
Parameter	**Group**	**Control**	**OA 30**	**OA 60**	**OA 120**
Na^+ (mmol L^{-1})	Wistar	142 ± 1	142 ± 2	141 ± 2	140 ± 1
	SHR	142 ± 2	137 ± 2	138 ± 2	140 ± 1
	DSS	157 ± 1	137 ± 1*	139 ± 2*	137 ± 3*
K^+ (mmol L^{-1})	Wistar	4.3 ± 0.2	4.1 ± 0.2	4.0 ± 0.2	4.2 ± 0.2
	SHR	4.8 ± 0.1	5.1 ± 0.2	4.9 ± 0.2	5.2 ± 0.2
	DSS	5.4 ± 0.1	6.3 ± 0.1	6.2 ± 0.3	6.2 ± 0.5
Cl^- (mmol L^{-1})	Wistar	105 ± 2	103 ± 2	106 ± 1	102 ± 2
	SHR	100 ± 3	109 ± 6	102 ± 3	104 ± 4
	DSS	105 ± 2	103 ± 4	102 ± 1	102 ± 5
Urea (mmol L^{-1})	Wistar	7.8 ± 0.6	8.2 ± 0.6	8.4 ± 0.5	8.5 ± 0.3
	SHR	7.0 ± 0.2	7.2 ± 0.4	7.4 ± 0.4	6.7 ± 0.4
	DSS	8.7 ± 0.4	9.1 ± 0.3	8.3 ± 0.5	7.9 ± 0.3
Creatinine (µmol L^{-1})	Wistar	28 ±2	21 ± 1*	20 ± 2*	20 ± 5*
	SHR	34 ±4	27± 5*	26 ± 2*	27 ± 4*
	DSS	33 ±1	27 ± 2*	26 ± 1*	24 ± 2*
GFR (mL min^{-1} 100 g^{-1})	Wistar	0.47 ±0.16	0.68 ±0.18*	0.76 ±0.17*	0.85 ±0.15*
	SHR	0.48 ±0.14	0.74 ±0.16*	0.86 ±0.17*	0.85 ±0.17*
	DSS	0.32 ±0.16	0.98 ±0.18*	0.98 ±0.18*	0.71 ±0.15*
Kidney (g 100 g^{-1})	Wistar	0.45 ± 0.20	0.47 ± 0.05	0.46 ± 0.07	0.47 ± 0.02
	SHR	0.62 ± 0.06	0.51 ± 0.07	0.62 ± 0.08	0.58 ± 0.05
	DSS	0.68 ± 0.02	0.48 ± 0.01	0.57 ± 0.2	0.49 ± 0.02
Heart (g 100 g^{-1})	Wistar	0.06 ± 0.04	0.05 ± 0.03	0.06 ± 0.04	0.07 ± 0.01
	SHR	0.07 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.07 ± 0.01
	DSS	0.08 ± 0.05	0.08 ± 0.05	0.09 ± 0.07	0.09 ± 0.10

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

4.2.4 Plasma aldosterone and AVP secretion

The effects of OA on aldosterone and AVP secretion are shown in Table 8. Untreated SHR and DSS rats have elevated aldosterone levels compared to Wistar control animals. OA treatment significantly ($p < 0.05$) reduced aldosterone and had no influence on the secretion of arginine vasopressin in comparison to respective control animals.

Table 8: The effects of OA on plasma hormone measurements in acutely treated male Wistar rats, and terminal plasma hormone measurements in male Wistar, SHR and DSS rats which were administered OA twice every third day for nine weeks. Values are presented as means ±SEM (n = 6 in each group).

	Group	**Aldosterone (nmol L^{-1})**	**AVP (pmol L^{-1})**
Acute study (Wistar rats)	Control	1.02 ± 0.02	2.28 ± 0.04
	OA	0.94 ± 0.13	2.26 ± 0.05
	Me-OA	0.94 ± 0.22	2.12 ± 0.02
Sub-chronic study	Wistar control	0.77 ± 0.04	2.28 ± 0.03
	SHR control	1.30 ± 0.25*	2.78 ± 0.08*
	DSS control	1.48 ± 0.48*	2.85 ± 0.07*
	SHR OA	1.02 ± 0.02#	2.61 ± 0.05*
	DSS OA	1.01 ± 0.02#	2.74 ± 0.06*

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

# $p < 0.05$ by comparison with respective control animals.

4.3 Oxidative stress

Tissues were harvested at the end of the 9-week study and their oxidative status assessed. There was no dose dependent effects observed with the three doses of OA tested in this study, hence only a median dose treatment was selected for this *ex vivo* analysis of oxidative proteins. Table 9 compares the effects of OA treatment (60 mg kg^{-1}, p.o.) on MDA, SOD and GPx levels in control and experimental SHR and DSS animals following the 9-week treatment period. The concentrations of MDA, SOD and GPx in Wistar control animals were used as reference levels for the tissues used. Significant increase of MDA and decreases of SOD and GPx were found in kidney, heart and liver tissues of untreated hypertensive groups as compared to control Wistar animals. OA administration in these hypertensive animals significantly ($p < 0.05$) reduced the levels of MDA in the kidney, heart and liver. In addition, OA administration in SHR and DSS animals significantly increased the production of SOD to levels that are comparable to those of control Wistar rats in both organs. Similar pattern was observed with GPx levels which were reduced in the kidneys of SHR and DSS rats but was significantly ($p < 0.001$) increased in OA-treated groups.

Table 9: Comparison of MDA concentrations, and of SOD and GPx activities in the kidney, heart and liver harvested at the end of the study from SHR and DSS rats treated with OA twice every third day for 9 weeks with control Wistar rats. Values are expressed as mean ± SEM (n = 6).

		Tissue		
Parameter measured	**Experimental protocol**	**Heart**	**Kidney**	**Liver**
MDA (nmol.g^{-1} protein)	Wistar control	2.12 ± 0.09	1.70 ± 0.05	1.52 ± 0.06
	SHR control	2.75 ± 0.01*	2.05 ± 0.02*	2.46 ± 0.04*
	DSS control	5.10 ± 0.10*	4.02 ± 0.08*	4.18 ± 0.09*
	SHR OA	1.16 ± 0.01#	1.21 ± 0.01#	1.35 ± 0.02#
	DSS OA	1.82 ± 0.20#	1.55 ± 0.10#	1.71 ± 0.30#
SOD activity (nmol.min^{-1} mL^{-1} g protein)	Wistar control	6.11 ± 0.30	10.20 ± 1.04	7.05 ± 0.13
	SHR control	2.13 ± 0.48*	3.01 ± 0.21*	3.56 ± 0.41*
	DSS control	1.25 ± 0.08*	2.29 ± 0.07*	2.12 ± 0.14*
	SHR OA	5.78 ± 0.14#	9.26 ± 0.12#	6.84 ± 0.07#
	DSS OA	4.12 ± 0.33#	11.08 ± 0.41#	8.72 ± 0.32#
GPx activity (nmol.min^{-1} mL^{-1} g protein)	Wistar control	3.82 ± 0.16	2.38 ± 0.21	0.10 ± 0.02
	SHR control	0.62 ± 0.06*	0.60 ± 0.02*	Undetectable
	DSS control	0.60 ± 0.02*	0.20 ± 0.06*	Undetectable
	SHR OA	3.47 ± 0.03#	3.08 ± 0.02#	Undetectable
	DSS OA	4.14 ± 0.31#	3.38 ± 0.24#	Undetectable

Note: undetectable ≤ 0.01 nmol.min^{-1} mL^{-1} g protein

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

# $p < 0.05$ by comparison with respective control animals.

CHAPTER 5

RESULTS (*Ex vivo* studies)

5.1 Effects of OA and derivatives on ventricular cardiomyocytes

This study was carried out in cardiomyocytes isolated from Wistar and DSS animals. SHR rats were excluded for this study because they were less responsive to blood pressure lowering effects of OA compared to the DSS animals (see Fig 13 and 16A). Therefore we speculated that mechanisms of blood pressure lowering effect of this triterpene and related derivatives would be better studied in DSS than SHR rats.

5.1.1 Cellular shortening

Figure 18 shows the effects of OA on cell shortening measured using edge detection technique. During the control period, the cell contraction induced by pacing the cells using a stimulator at 1 Hz was stable before the recording was done and this control period represents 100% cell shortening. Upon superfusion with Tyrode solution containing OA, contraction of cells isolated from Wistar rats increased from 100 to 128% and went back to 120% during the wash period (Figure 18A). Me-OA increased the contraction of myocytes of Wistar rats (Figure 19A) from 100 to 143%, however, the effect was not fully reversed as the contraction remained at 132% at the end of the wash period. Superfusion of cells from Wistar rats (Figure 20A) with Br-OA was more effective than OA as the contraction increased from 100 to 139% and went back to 113% during the wash period. In contrast to the effects in cells from Wistar rats, OA and derivatives had no significant effect on cell shortening in cells isolated from DSS rats (Figures 18B, 19B and 20B). Summary data are presented in Figure 21. We noted differences in contractile profiles of ventricular myocytes from Wistar and those from DSS probably caused by hypertension. Time to peak (TTP) and tau of relaxation were significantly ($p < 0.001$) increased in cells from hypertensive animals compared to the cells from normotensive Wistar rats (shown only for OA, Figure 21).

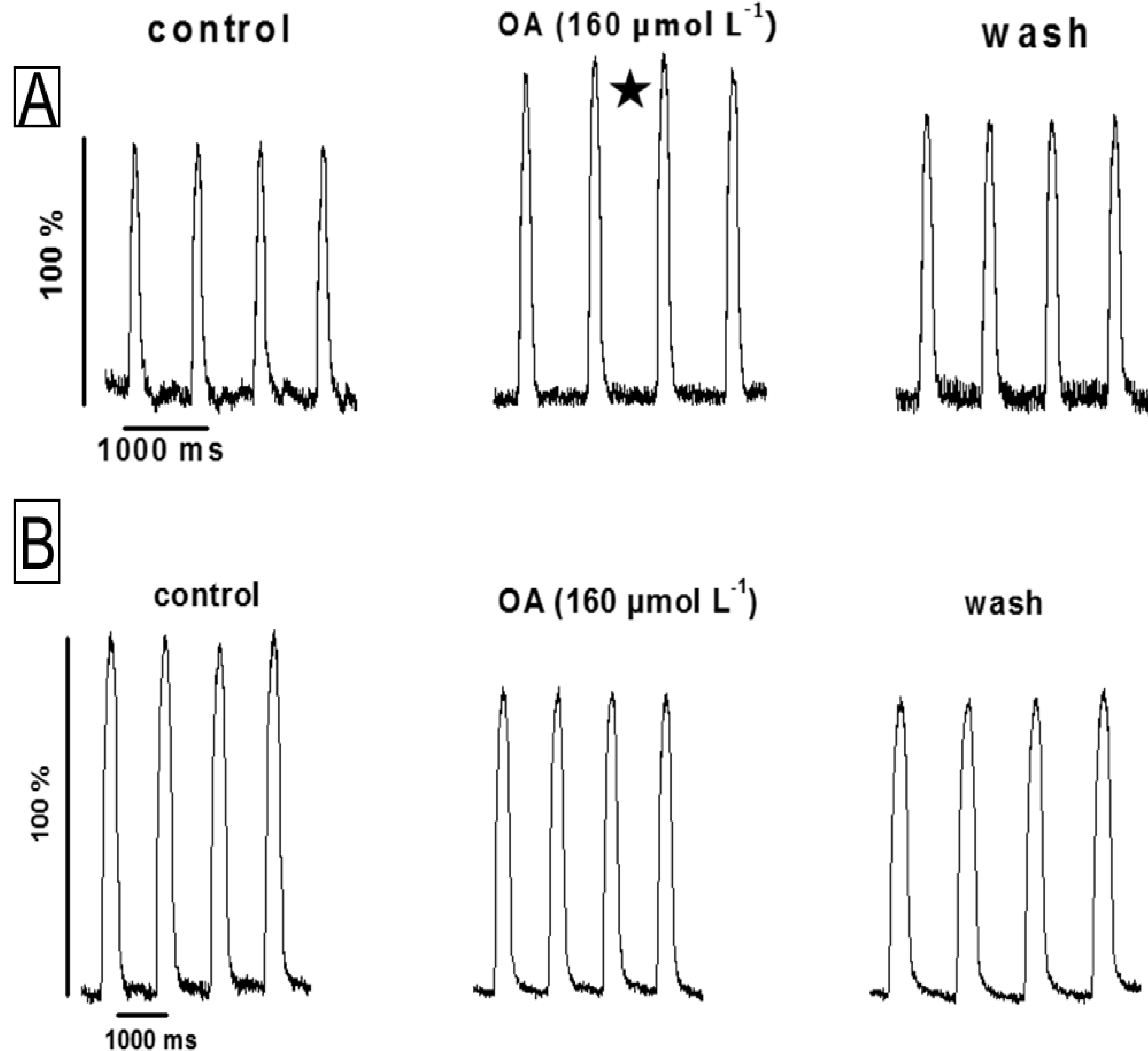

Figure 18: Comparison of cell contraction during the control, treatment and wash periods in ventricular myocytes isolated from Wistar (**A**) and DSS (**B**) rats. Cells were superfused with 1.8 mM Ca^{2+} Tyrode solution containing OA (160 µmol L^{-1}) during the treatment period. Cell shortening, TTP and tau were measured at 1 Hz stimulation frequency ($n = 6$). ★ $p < 0.05$ vs control period.

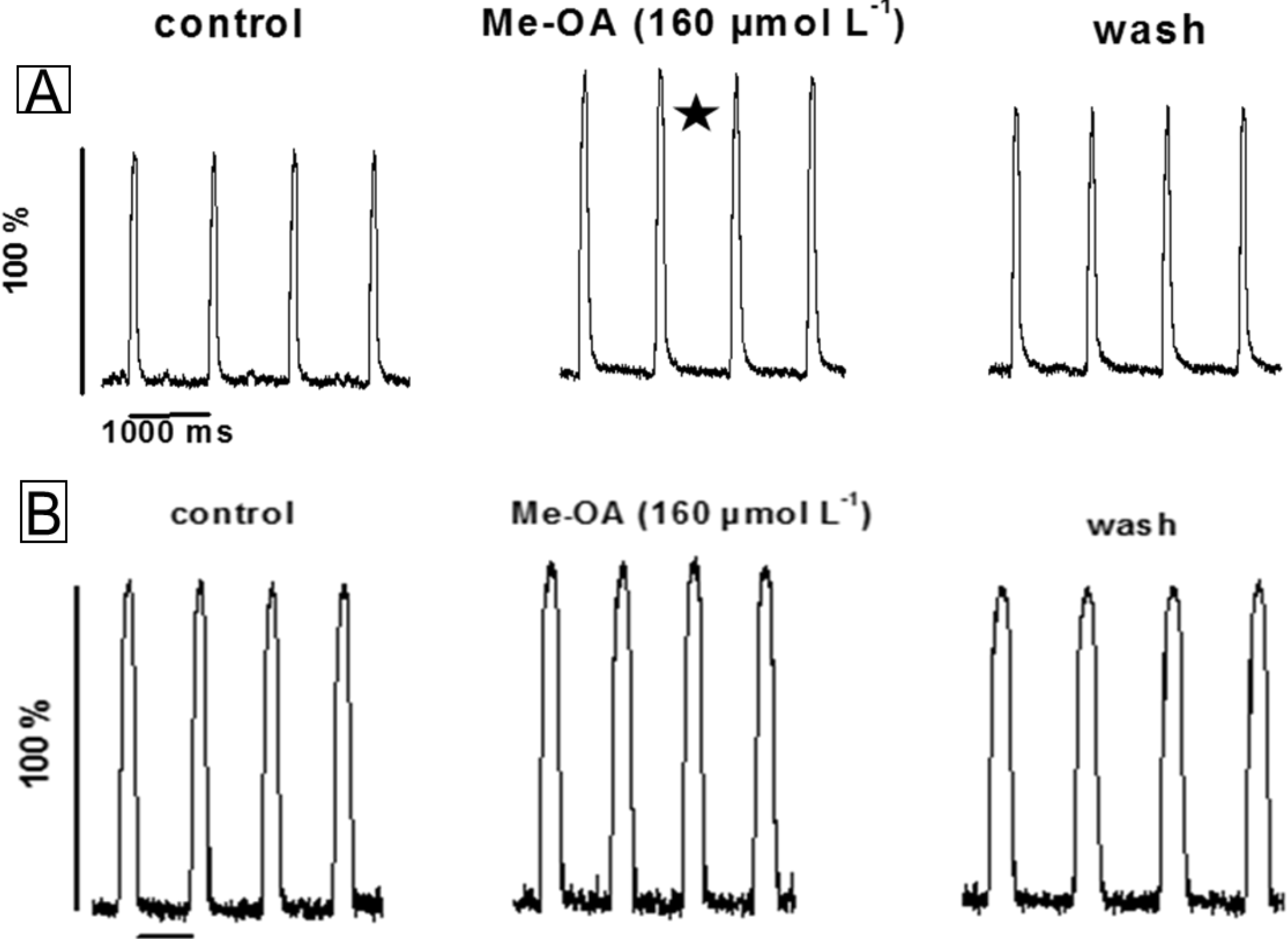

Figure 19: Comparison of cell contraction during the control, treatment and wash periods in ventricular myocytes isolated from Wistar (**A**) and DSS (**B**) rats. Cells were superfused with 1.8 mM Ca^{2+} Tyrode solution containing Me-OA (160 µmol L^{-1}) during the treatment period. Cell shortening, TTP and tau were measured at 1 Hz stimulation frequency (n = 6). ★ $p < 0.05$ vs control period.

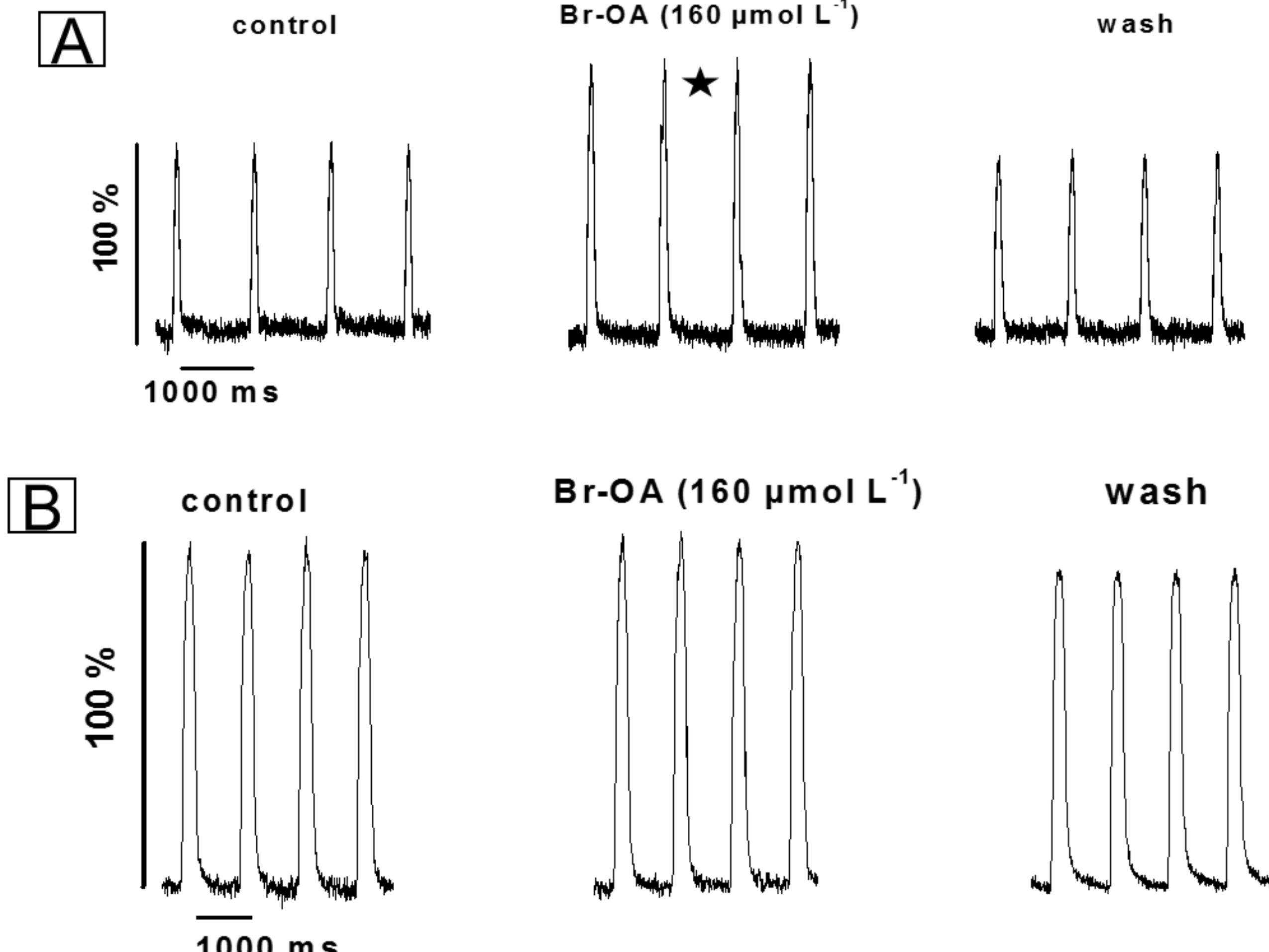

Figure 20: Comparison of cell contraction during the control, treatment and wash periods in ventricular myocytes isolated from Wistar (**A**) and DSS (**B**) rats. Cells were superfused with 1.8 mM Ca^{2+} Tyrode solution containing Br-OA (160 µmol L^{-1}) during the treatment period. Cell shortening, TTP and tau were measured at 1 Hz stimulation frequency (n = 6). ★ $p < 0.05$ vs control period.

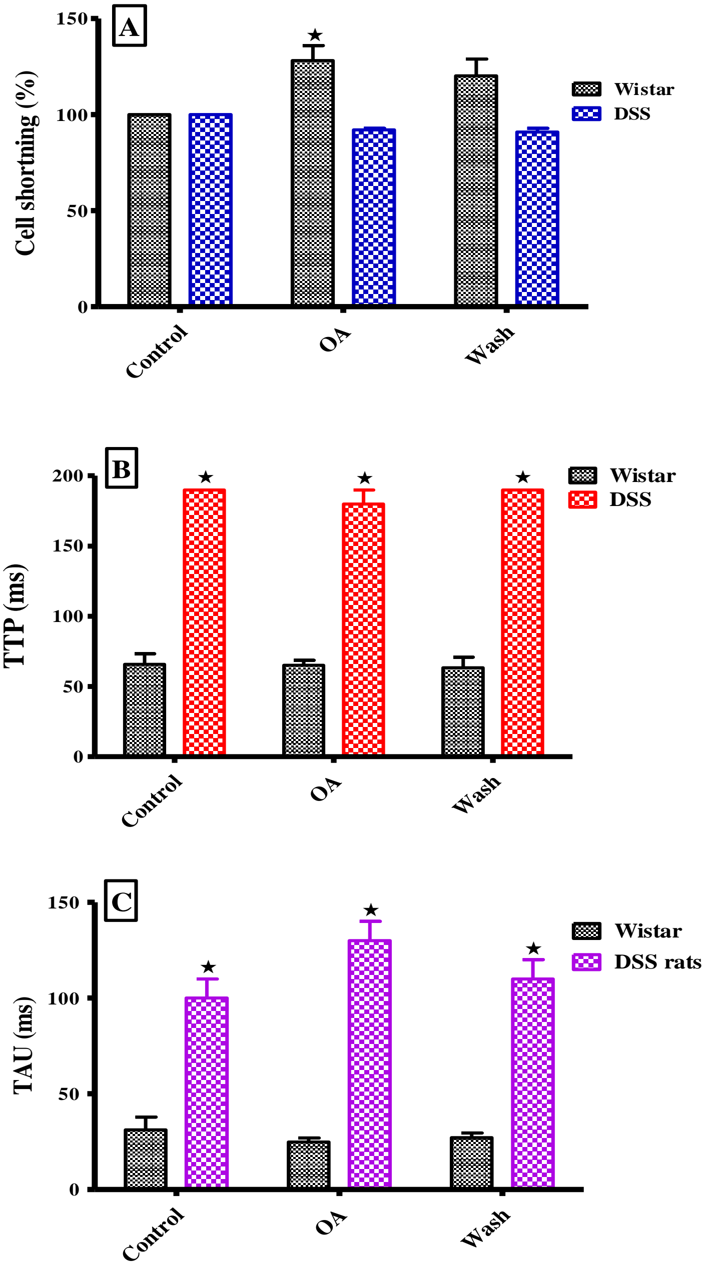

Figure 21: Comparison of cell contraction during the control, treatment and wash periods in ventricular myocytes isolated from Wistar and DSS rats. Cell shortening (**A**), TTP (**B**) and tau (**C**) were measured at 1 Hz stimulation frequency. Cells were superfused with 1.8 mM Ca^{2+} Tyrode solution containing OA (160 µmol L^{-1}) during the treatment period (n = 6). ★ $p < 0.001$ vs control period.

5.1.2 L-type Ca^{2+} currents

The preliminary experiments on Ca^{2+} currents were conducted using whole cell patch clamp technique with membrane potentials ranging from -80 to 70 mV. Due to time constraints, we could only perform the experiment on 2 animals, however, at least 3 cells were recorded for each animal and for each drug. These preliminary studies showed that all drugs (Figures 22, 23 and 24) have no significant effects on the Ca^{2+} current as the recordings of the control, drug and wash periods show no apparent differences.

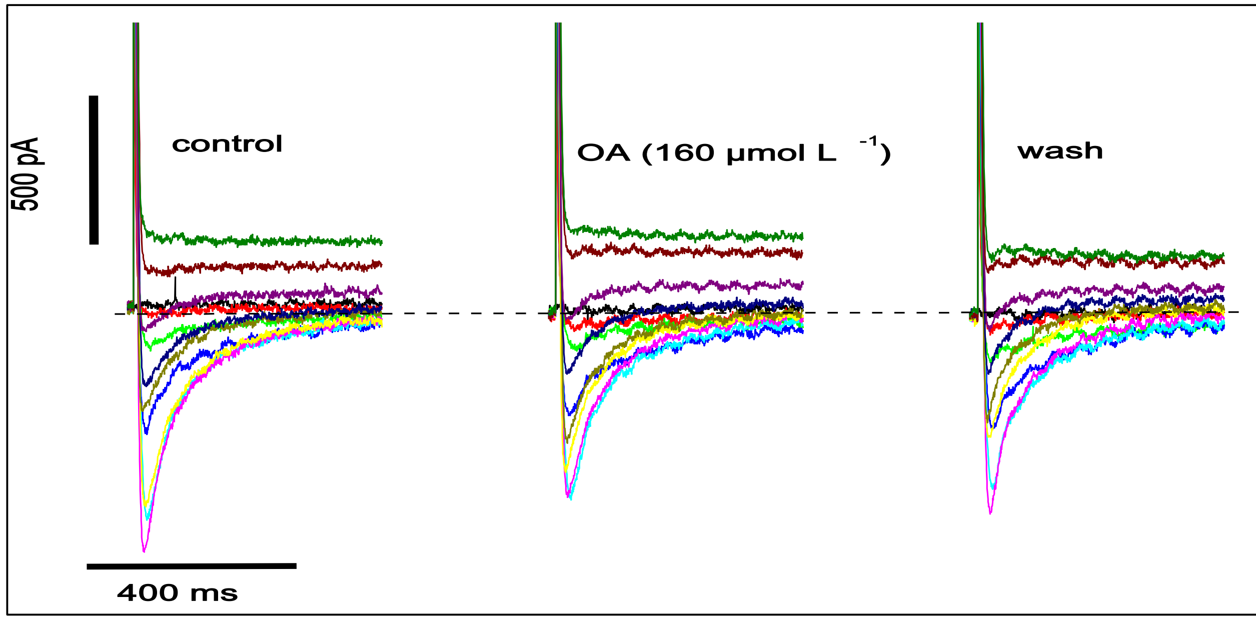

Figure 22: Comparison of L-type Ca^{2+} current density (I_{CaL}) during the control, treatment and wash periods in ventricular myocytes isolated from Wistar rats. Measurements were done repeatedly at -40 and 20 mV. Cells were superfused with 1.8 mM Ca^{2+} / cesium-based Tyrode solution containing OA (160 µmol L^{-1}) during the treatment period (n = 2). Patch pipettes had a final resistance of 2-4 MΩ when filled with internal solution.

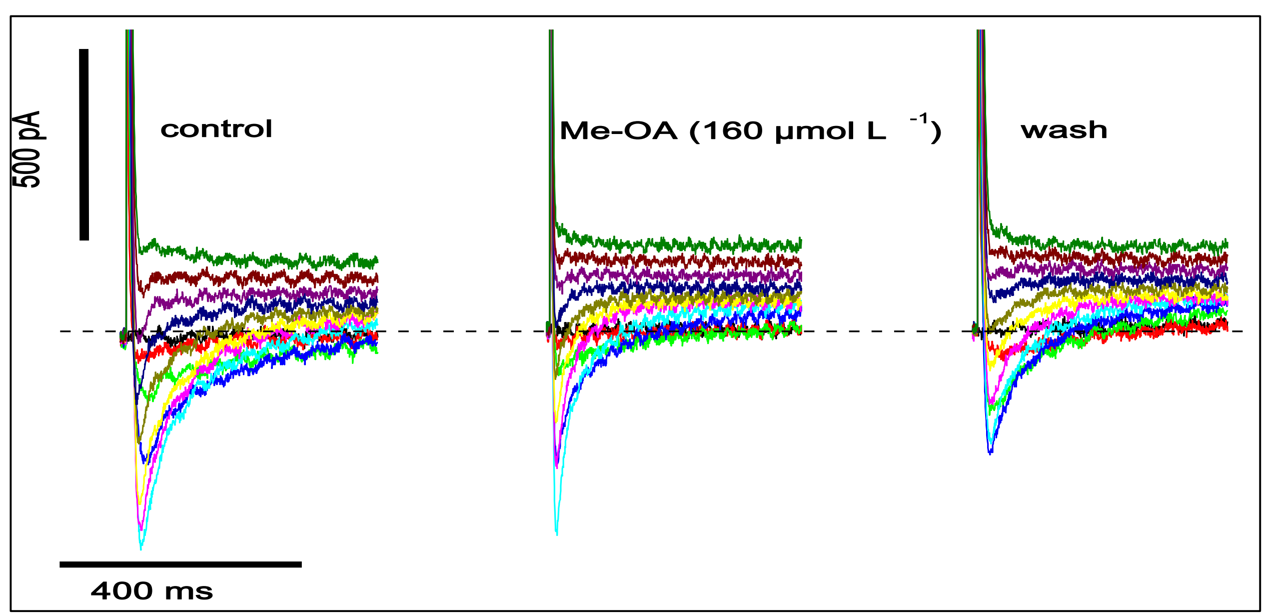

Figure 23: Comparison of L-type Ca^{2+} current density (I_{CaL}) during the control, treatment and wash periods in ventricular myocytes isolated from Wistar rats. Measurements were done repeatedly at -40 and 20 mV. Cells were superfused with 1.8 mM Ca^{2+} / cesium-based Tyrode solution containing Me-OA (160 µmol L^{-1}) during the treatment period (n = 2). Patch pipettes had a final resistance of 2-4 MΩ when filled with internal solution.

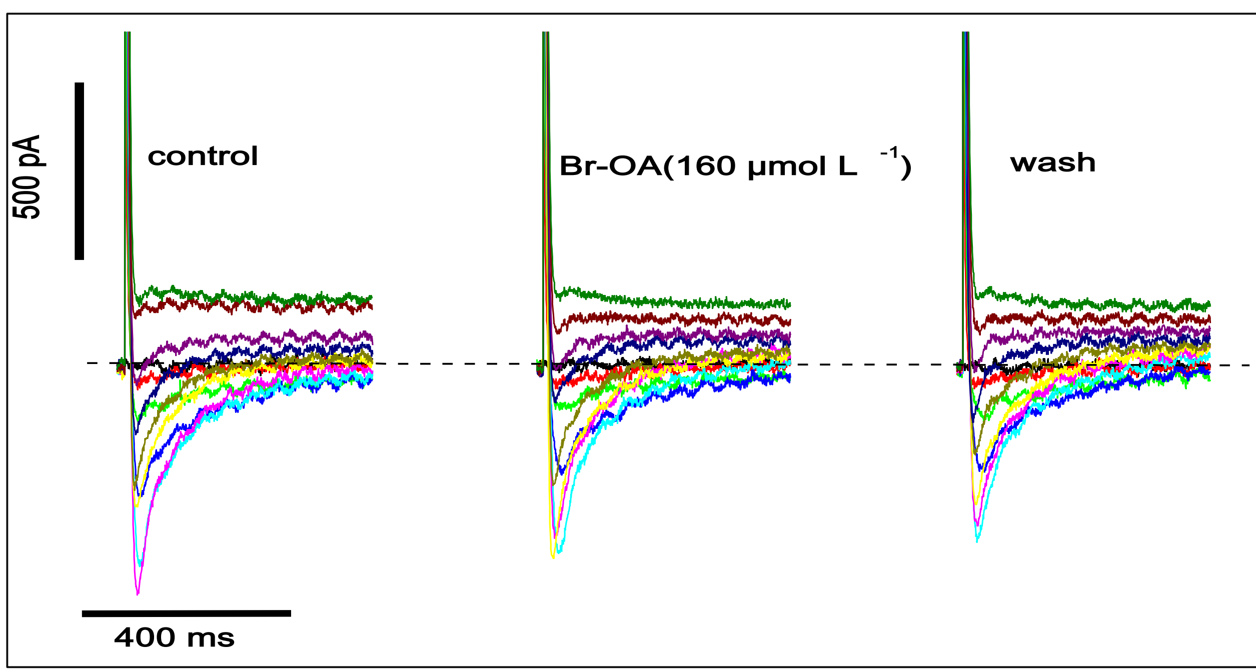

Figure 24: Comparison of L-type Ca^{2+} current density (I_{CaL}) during the control, treatment and wash periods in ventricular myocytes isolated from Wistar rats. Measurements were done repeatedly at -40 and 20 mV. Cells were superfused with 1.8 mM Ca^{2+} / cesium-based Tyrode solution containing Br-OA (160 µmol L^{-1}) during the treatment period (n = 2). Patch pipettes had a final resistance of 2–4 MΩ when filled with internal solution.

5.2 Effects of OA on vascular smooth muscles

In order to study the potential vasodilating properties of OA and derivatives, the contraction/relaxation pattern of vessels treated with each of the three drugs was evaluated. Isometric tension was measured in rat aortic rings (mounted in classical organ baths) and 2nd to 3rd order mesenteric arteries (mounted in wire-myographs). Experiments were performed in endothelium-intact and endothelium-denuded vessels to discriminate between endothelium-dependent and independent mechanisms. Solvent controls were performed for each concentration. Vessels that showed a carbachol-induced endothelium-dependent relaxation amounting to at least 30% of the maximum contraction were considered as having an intact endothelium. Endothelium-denuded vessels had no significant response to the addition of carbachol with a level of contraction remaining larger than 90% of the maximum contraction as shown in Figure 25.

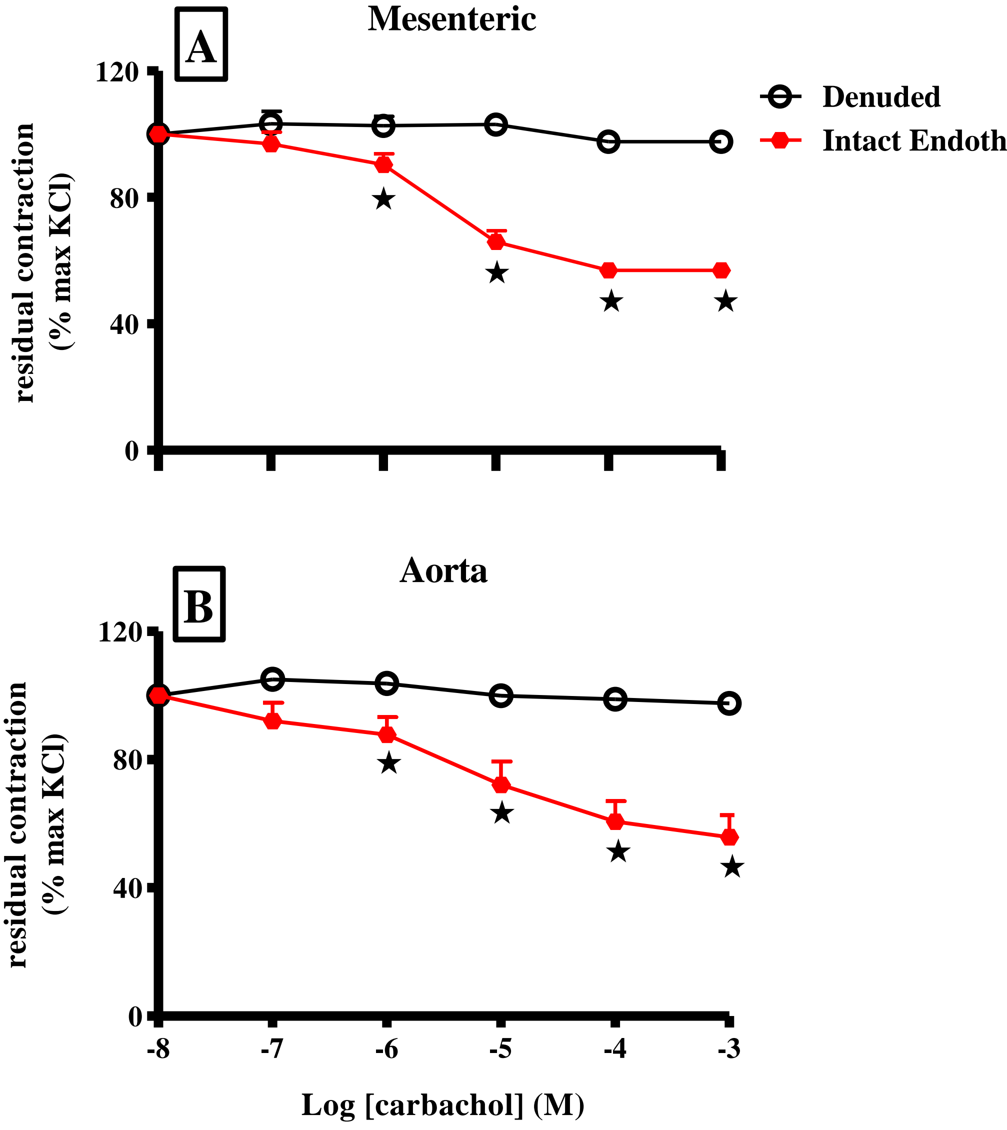

Figure 25. Concentration-response curves for carbachol in mesenteric segments (**A**) and aortic rings (**B**) isolated from Wistar rats pre-contracted with sub-maximal concentration of KCl (50 mM). Curves were obtained in arteries with denuded and intact endothelium. The values shown are means ± SEM (n = 7). ★ $p < 0.001$ vs denuded.

5.2.1 Comparison of the vasodilating effects of OA and derivatives

Addition of OA, Me-OA or Br-OA on endothelium-intact mesenteric arteries pre-contracted with PHE (Figure 26A) or KCl-enriched solution (Figure 26B) evoked a significant relaxation of the arterial segments isolated from Wistar rats. In the mesenteric arteries, the brominated OA derivative showed a significantly larger maximum relaxation than OA and Me-OA (excrete, PHE pre-contracted rings; $p < 0.01$ Br-OA vs OA; KCl pre-contracted rings $p < 0.001$ Br-OA vs OA). This difference, however, was not apparent in aortic rings (Figure 26C and D). Importantly, the maximum relaxation evoked by Br-OA was significantly larger in the mesenteric vessels compared to the aorta ($p < 0.001$, for PHE pre-contracted vessels; $p < 0.001$ for KCl pre-contracted arteries). A similar pattern in relaxation profiles of arterial segments isolated from DSS rats was observed with all the three drugs studied (shown only for OA, Figure 27).

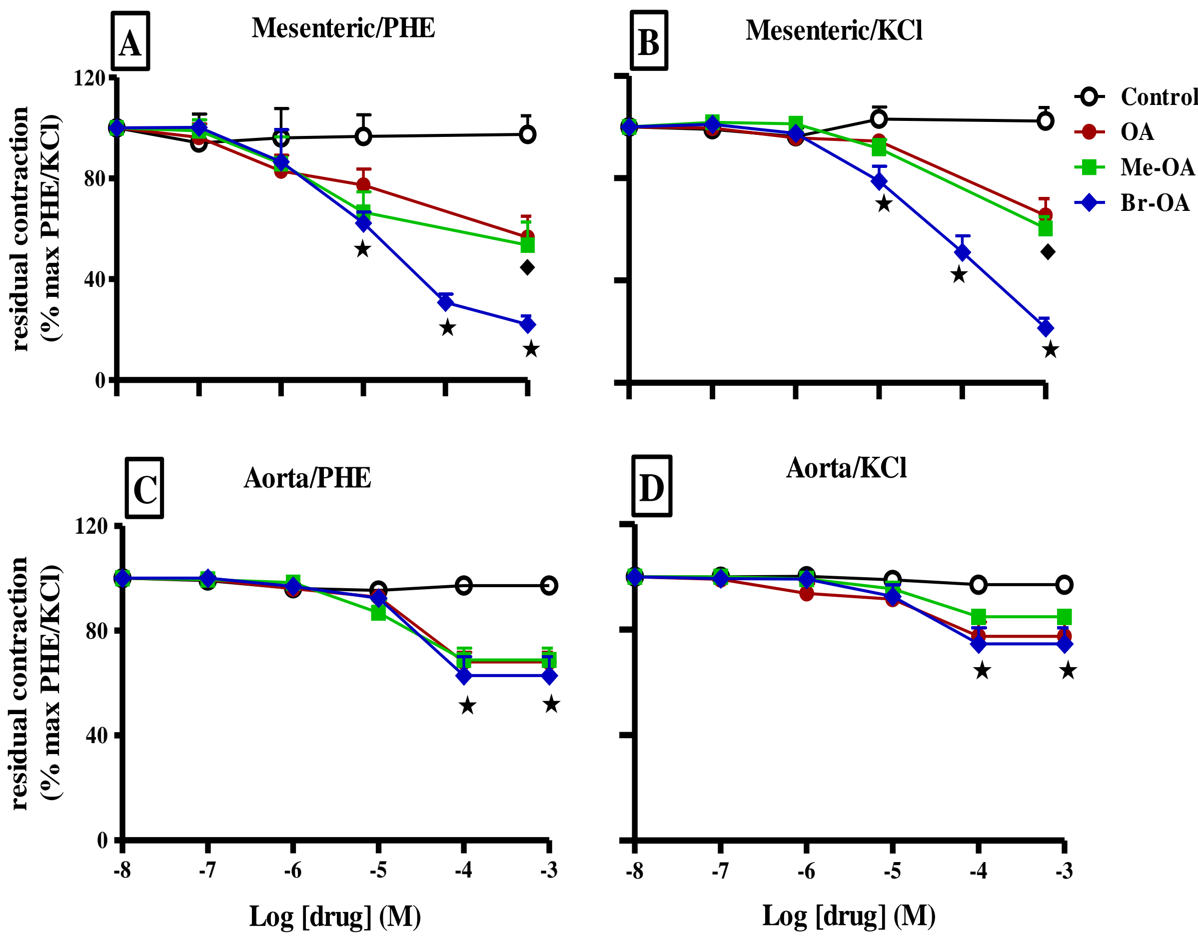

Figure 26. Concentration–response curves for OA, Me-OA, and Br-OA in endothelium-intact mesenteric arteries [**A** and **B**] or aortic rings [**C** and **D**] isolated from Wistar rats, pre-contracted with sub-maximal concentration of PHE (5 μM) [**A** and **C**] and KCl (50 mM) [**B** and **D**]. The values shown are means ± SEM (n = 7). ★ $p < 0.001$ vs control, ♦ vs Br-OA).

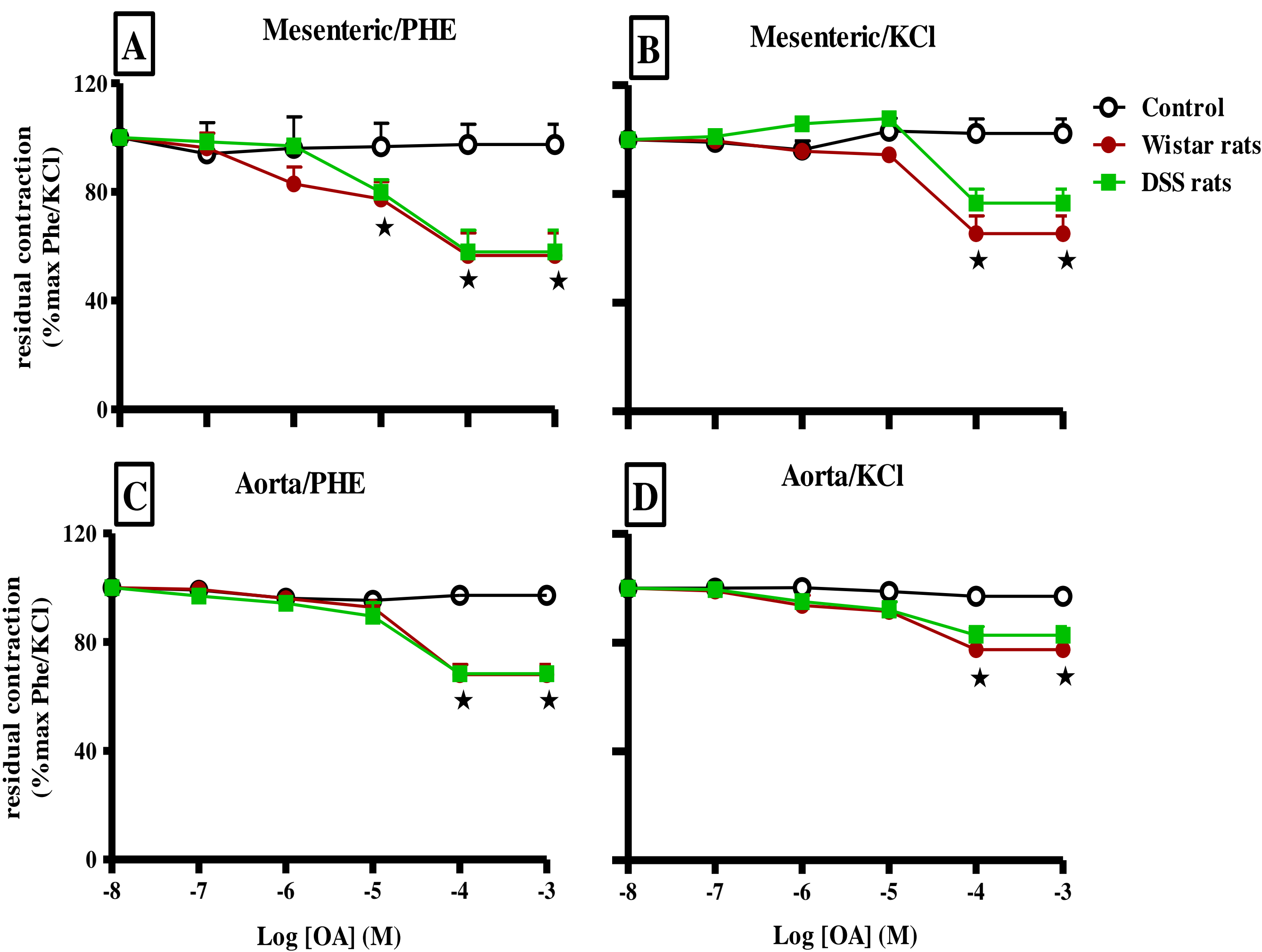

Figure 27. Concentration–response curves for OA in endothelium-intact mesenteric arteries [**A** and **B**] or aortic rings [**C** and **D**] isolated from Wistar and DSS rats, pre-contracted with sub-maximal concentration of PHE (5 µM) [**A** and **C**] and KCl (50 mM) [**B** and **D**]. The values shown are means ± SEM (n = 7 ★ $p < 0.001$ vs control).

5.2.2 Endothelium-dependent vasorelaxant effects of OA and derivatives

Since there was no difference in the magnitude of relaxant effects of OA and derivatives in Wistar and DSS isolated vessels, further experiments to evaluate the mechanisms of vasorelaxation properties of OA and derivatives were conducted in arterial segments isolated from Wistar rats only. The relaxation evoked by OA and derivatives was only partially inhibited in the absence of functional endothelium as shown in endothelium-denuded vascular rings experiments (shown only for Br-OA, Figure 28, $p < 0.05$ for mesenteric vessels; $p < 0.001$ for aorta). This demonstrates that the relaxation-evoked by OA or derivatives involves both endothelium-dependent and independent mechanisms.

In order to characterize the endothelium-dependent relaxation, endothelium-intact vessels were incubated in the presence of cyclooxygenase (COX) or NOS inhibitors (INDO and L-NAME, respectively). As shown in Figure 28, incubation with INDO blocked Br-OA-evoked relaxation to the same extent as removal of the endothelium $p < 0.05$ (mesenteric); $p < 0.001$ (aorta). Addition of L-NAME did not further inhibit the COX-resistant relaxation suggesting the involvement of COX and prostanoids dependent mechanisms. Qualitatively similar results were observed with OA and Me-OA, however, the differences between OA “INDO” vs “control” and Me-OA “intact” vs “denuded” did not reach statistical significance in the mesenteric arteries. Interestingly for Br-OA, the endothelium independent portion of the relaxation is significantly larger in the mesenteric segments than in the aorta ($p < 0.001$). The absence of inhibition observed with L-NAME on the relaxation evoked by OA and derivatives argues in favour of a non-implication of the NOS/NO pathway.

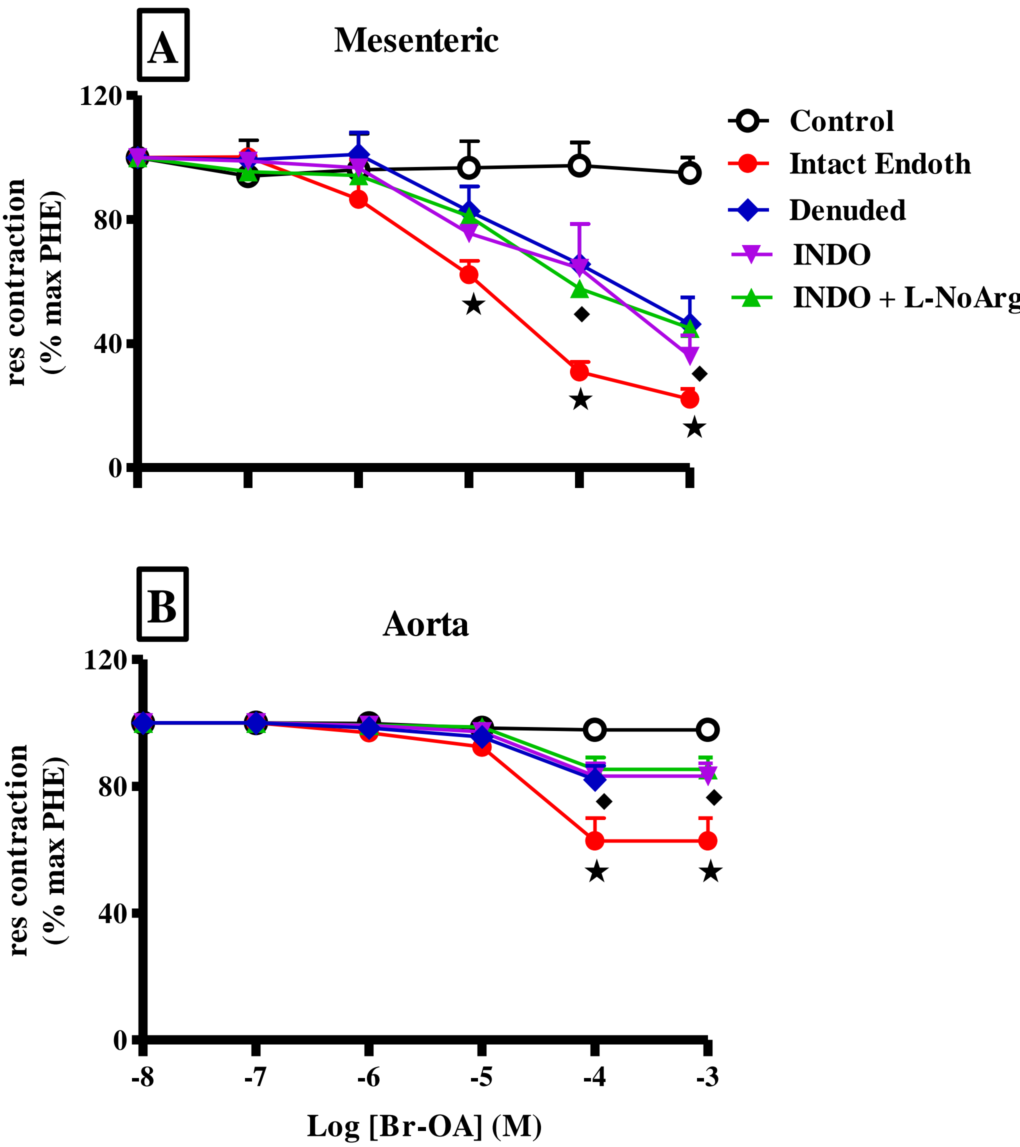

Figure 28. Concentration–response curves for Br-OA in mesenteric arteries (**A**) and aortic rings (**B**) isolated from Wistar rats pre-contracted with PHE (5 μM). Curves were obtained in endothelium-denuded and intact arteries. Some endothelium-intact vessels were incubated in the presence of INDO (10 μM) only or in combination with L-NoArg (100 μM) prior to addition of the drug. Values shown are means ± SEM (n = 7). ★ $p < 0.001$ vs control, ♦ vs Br-OA).

5.2.3. Endothelium-independent vasorelaxant effects of Br-OA

As observed in Figure 28, a significant portion of the relaxation evoked by OA and derivatives is resistant to removal of the endothelium suggesting a role of endothelium-independent pathways, such as opening of the K^+ channels. To further investigate this hypothesis, denuded vessels were incubated in the presence of blockers of different K^+ channels types (Gli for ATP-dependent K^+ channels and AP for voltage-activated K^+ channels). Either inhibitor was able to blunt part of the relaxation evoked by Br-OA as illustrated in Figure 29. The effects of both inhibitors were additive and allowed a complete blockade of the relaxation to Br-OA suggesting that ATP-dependent and voltage-activated K^+ channels opening mediate the endothelium independent relaxation (not shown for OA and Me-OA). The endothelium-independent relaxation observed for Br-OA was also completely blunted by Gli and AP. Experiments performed with endothelium-intact vessels incubated with a cocktail of INDO, AP and Gli did not show any relaxation to Br-OA as shown in Figure 30 (not shown for OA and Me-OA).

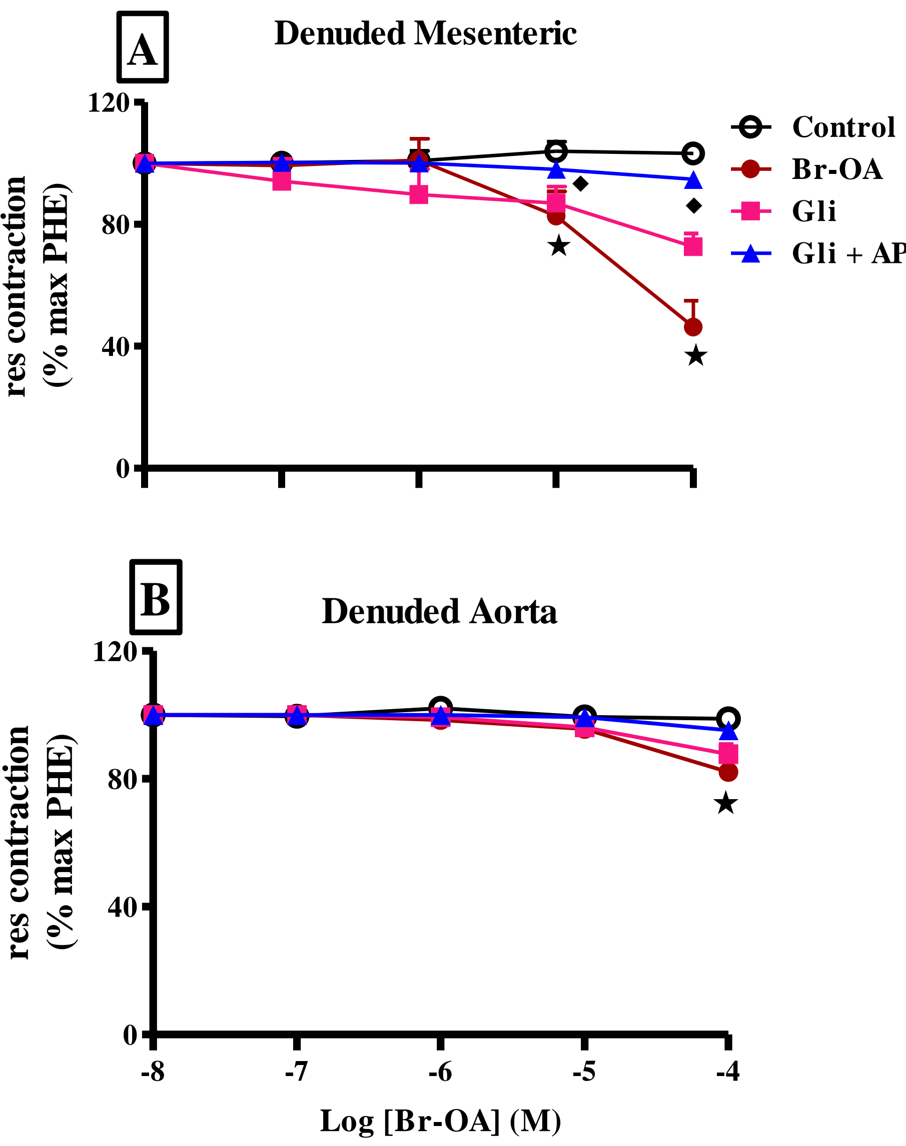

Figure 29. Concentration–response curves for Br-OA in denuded mesenteric arteries (**A**) and aortic rings (**B**) isolated from Wistar rats pre-contracted with PHE (5 μM). Curves in denuded arteries incubated in the presence of Gli (5 mM) or in combination with AP (1 mM) prior to the addition of the drug. Values shown are means ± SEM (n = 7). ★ $p < 0.001$ vs control, ♦ vs Br-OA).

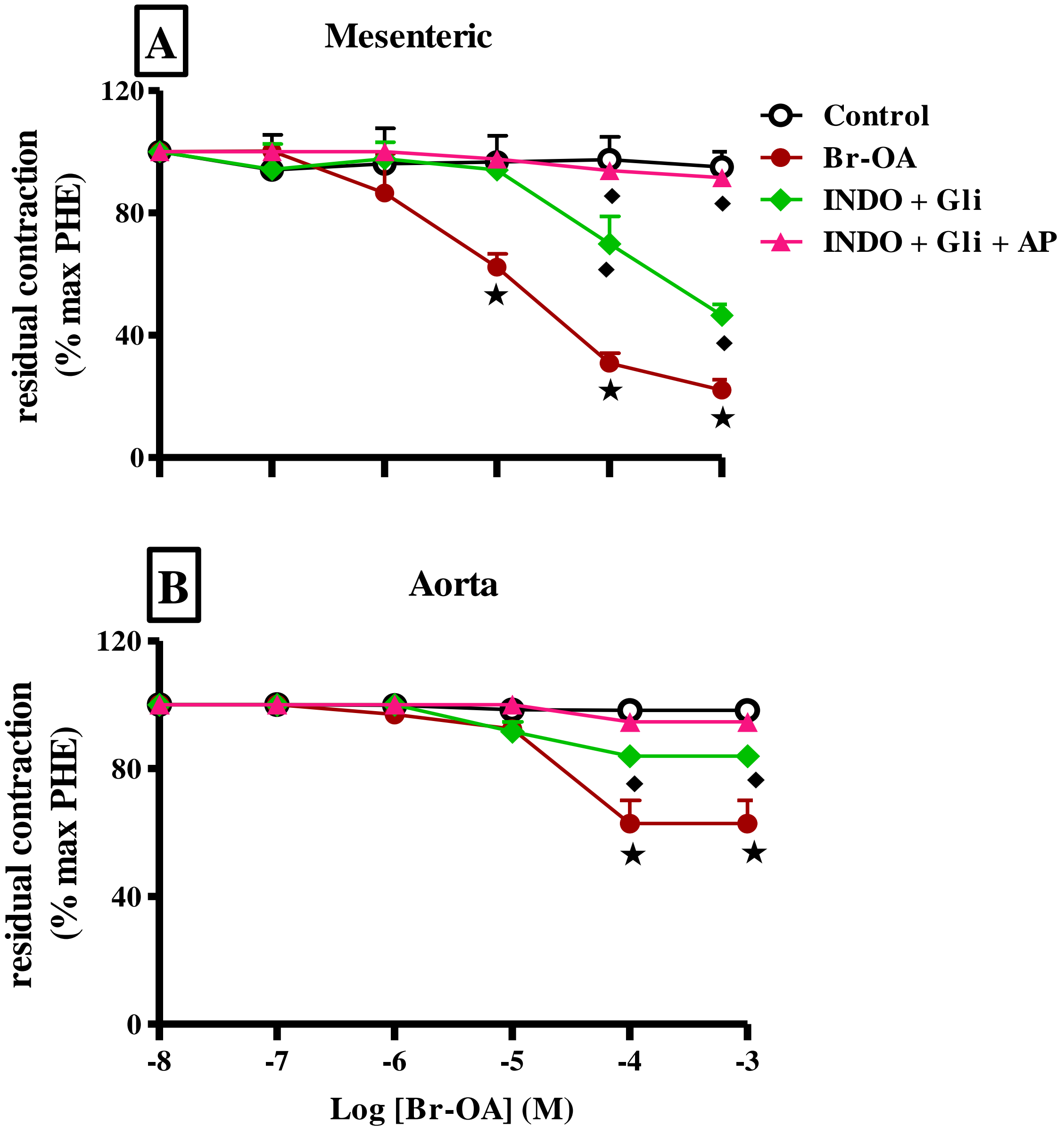

Figure 30. Concentration–response curves for Br-OA in intact mesenteric arteries (**A**) and aortic rings (**B**) isolated from Wistar rats pre-contracted with PHE (5 μM). Curves in intact arteries incubated in the presence of INDO (10 μM) and Gli (5 mM) only or in combination with AP (1 mM) prior to the addition of the drug. Values shown are means ± SEM (n = 7). ★ $p < 0.001$ vs control, ♦ vs Br-OA).

CHAPTER 6
DISCUSSION

The present study investigated the effects of OA and related synthetic derivatives (Me-OA and Br-OA) on blood pressure and thereafter examined the possible underlying mechanisms. Our results show that 1) OA decreased MAP in both acute and sub-chronic experimental settings, and that the MAP lowering effect was more marked in hypertensive animals compared to normotensive rats (Fig 8B, 13); 2) OA or derivatives did not change urinary flow rate (Fig 9A, 15A-C); 3) OA or derivatives increased Na^+ excretion, with a more marked effect of Br-OA compared to OA under acute conditions (Fig 9B, 15D-F); 4) OA decreased aldosterone secretion after chronic administration, but had no influence on AVP levels (Table 8); 5) OA suppressed the increased ROS marker production present in salt-sensitive hypertensive rats and modulated the production of antioxidant enzymes, SOD and GPx, which were reduced in these animals (Table 9); 6) OA and derivatives increased cardiomyocyte contraction in cells isolated from normotensive rats but had no influence on contraction of cells isolated from hypertensive animals and on L-type Ca^{2+} currents (Fig 18, 22); 7) Br-OA caused relaxation of the aorta and mesenteric arteries via endothelium-dependent and independent mechanisms (Fig 28, 29). The results of this study are novel and clinically relevant because OA and related triterpene derivatives exert multiple blood pressure lowering mechanisms while increasing the force of cardiac contraction.

The absolute stereo-structure of *S. aromaticum*-derived OA elucidated from the spectra using ^{1}H- and ^{13}C-NMR was comparable to previously reported data (Mahato and Kundu, 1994; Honda, Rounds, Bore, Finlay, Favaloro, Suh, Wang, Sporn and Gribble, 2000; Sporn, Liby, Yore, Fu, Lopchuk and Gribble, 2011; Fu and Gribble, 2013). In addition, OA had the same physical properties as the OA purchased from Sigma-Aldrich and hence isolated OA was used as a parent compound for the synthesis of related triterpene derivatives because it is cheaper than the commercially available OA. The proton and carbon peaks obtained from NMR and IR spectra analysis for synthetic OA derivatives (Me- and Br-OA) were comparable to previously reported data (Fu and Gribble, 2013). These spectra did not have any additional peaks which don’t belong to the derivative’s structure indicating that there was no contamination of the derivative products

by any traces of OA and therefore these three triterpenes were used to carry out all the experiments for this study.

Blood pressure and renal function experimental protocols under acute and sub-chronic settings are well validated as they have been used extensively in our laboratory (Musabayane, Forsling and Balment, 1997; Musabayane, Mahlalela, Shode and Ojewole, 2005; Musabayane, Gondwe, Kamadyaapa, Chuturgoon and Ojewole, 2007; Gondwe, Kamadyaapa, Tufts, Chuturgoon and Musabayane, 2008; Kamadyaapa, Gondwe, Moodley, Ojewole and Musabayane, 2009; Mapanga, Tufts, Shode and Musabayane, 2009; Madlala, Masola, Singh and Musabayane, 2012; Mkhwanazi, Serumula, Myburg, Van Heerden and Musabayane, 2014). Renal Li^+ clearance has been widely used in animal studies and clinical investigations as a marker for assessing proximal tubular function in the mammalian kidney (Thomsen, 1984; Koomans, Boer and Dorhout-Mees, 1989; Thomsen, 1990; Walter and Shirley, 1991; Shirley, Walter and Sampson, 1992; Shirley and Walter, 1993; Boer, Fransen, Shirley, Walter, Boer and Koomans, 1995; Thomsen and Shalmi, 1997; Whiting, 1999; Madlala, Masola, Singh and Musabayane, 2012; Mkhwanazi, Serumula, Myburg, Van Heerden and Musabayane, 2014). SHR and DSS rats used in this study are well studied animal models of essential (primary) and secondary arterial hypertension because they share similar features with hypertension in humans and both were used together with Wistar rats as a control group (Okamoto, 1969; Triantafyllidi, Baldwin, Schwartz and Gavras, 2004; Rodriguez-Rodriguez, Perona, Herrera and Ruiz-Gutierrez, 2006; Raji, Mugabo and Obikeze, 2012).

Our data confirm that blood pressure, indeed increased progressively with time in the absence of drug treatment in hypertensive SHR and DSS animals, whereas it remained stable in the control Wistar animals (see Fig 13). OA treatment not only significantly suppressed or blunted this increase, but also reduced blood pressure in control animals. Our results confirm the previously reported antihypertensive properties of OA in experimental models of hypertension (Somova, Nadar, Rammanan and Shode, 2003; Madlala, Masola, Singh and Musabayane, 2012). However, the present study demonstrates for the first time a more marked action in SHR and DSS by comparison with non-hypertensive animals, suggesting a specifically enhanced action in disease

conditions. The pathophysiological mechanisms responsible for the rise in blood pressure in primary hypertension remain unclear and are likely complex. Amongst the mechanisms accepted to play a major role for the development of hypertension is an inability of the kidney to excrete Na^+. Indeed solute retention and the accompanying water retention result in extracellular (including intravascular) volume expansion (Weinberger, Miller, Luft, Grim and Fineberg, 1986; Somova, Channa and Khan, 1999). We were therefore interested in examining whether the marked hypotensive action obtained in the hypertensive models could be mediated by an effect on abnormal Na^+ handling by the kidney. We noticed that urine Na^+ excretion rate in control, untreated animals tended to spontaneously increase with time during the following 9 weeks post weaning. In both hypertensive models, no such increase with time was obtained, instead, urinary Na^+ excretion rate tended to decrease with time after weaning in the DSS model suggesting Na^+ retention previously reported in these animals (Somova, Channa and Khan, 1999).

In support of Na^+ retention in hypertension, we found increased aldosterone levels in plasma of non-treated hypertensive rats. Our data show that OA decreased aldosterone secretion and increased urine Na^+ excretion rate. Significantly larger increases in Na^+ output were obtained in the hypertensive models. This suggests that treatment with the drugs was accompanied by alleviation of Na^+ retention in these animals. Our data demonstrate that the natriuretic effects of OA and related derivatives were associated with increased Li^+ clearance. This indicates that the increase in urinary Na^+ excretion rate is, at least in part, mediated via inhibition of proximal tubular Na^+ reabsorption. Indeed, we found a positive correlation between the increase in urinary Na^+ excretion rate and the decrease in MAP (see Fig 11 and 16). We observed increased GFR under subchronic condition, however, no such increase was observed for acute studies. We speculate that this difference was due to time periods and dosages of the drug used for these experimental studies i.e 4 h in comparison to 9 weeks, 90 μg h^{-1} and 60 mg kg^{-1} BW.

Despite the potent natriuretic effects of these triterpenes, the urine flow rate was not changed. Presence of blood pressure lowering effects in the absence of increased fluid voided in the urine may be explained by the effect of the drug on body fluid compartments. We speculate that OA may exhibit OT-like properties; OT enhances natriuresis and does not influence urine flow rate at

low doses (Conrad, Gellai, North and Valtin, 1986; Chou, DiGiovanni, Luther, Lolait and Knepper, 1995). OA did not affect food intake as well as plasma Na^+ concentrations in Wistar and SHR animals. This is again not surprising as it is in agreement with OT effects which is thought to produce a negative Na^+ balance by stimulating Na^+ excretion while inhibiting Na^+ ingestion (Verbalis, Mangione and Stricker, 1991).

The transport of Na^+ from the tubular cells to the peritubular intertitium is via the Na^+/K^+-pump and Na^+/Cl^- cotransporter. Although OA increased Na^+ excretion, this triterpene had no influence on urinary or plasma K^+ or Cl^- levels. The body has much lower extracellular fluid K^+ in comparison to intracellular levels (Gennari, 2002). This study measured plasma K^+ which is easily accessible compared to intracellular contents. We acknowledge that plasma K^+ we measured is not a reflection of the whole body K^+ content as most of it is found inside cells and therefore this could explain the reason for OA-induced natriuresis without any changes in K^+ levels although these ions use the same pump in the kidney.

Studies report that ingested K^+ from diet sources stimulate insulin secretion which activates the Na^+/K^+-pump to facilitate K^+ entry into cells hence preventing hyperkalemia and this could explain why we detected normal plasma levels of this ion (Alvestrand, Wahren, Smith and DeFronzo, 1984). In agreement with this, various studies have shown that OA facilitates insulin secretion and possess insulin-like properties (Hsu, Wu, Liu and Cheng, 2006; Teodoro, Zhang, Alexander, Yue, Vranic and Volchuk, 2008; Musabayane, Tufts and Mapanga, 2010). In this regard, we speculate that OA facilitates K^+ entry into cells directly or via insulin stimulation and therefore this increase could not be detected in the plasma. We also speculate that they maybe other mechanisms which cause this increase natriuresis which does not involve the Na^+/Cl^- cotransporter and hence we could not observe any changes in chloride levels.

Several studies have used aortic rings and small mesenteric arteries to study smooth muscle vascular resistance (Leite, Estevão, Resende and Salgado, 1997; Kang, Sullivan, Sasser, Imig and Pollock, 2007; Rodrigues, Restini, Lunardi, Moreira, Lima, da Silva and Bendhack, 2007; Jadhav, Liang, Balsevich, Bastin, Kroetsch, Heximer, Backx and Gopalakrishnan, 2012) hence we isolated

these vessels to study mechanisms that mediate hypotensive effects of OA and related synthetic derivatives. Our experiments indicate that OA induces vasorelaxation of aortic rings and small mesenteric arteries in both normotensive and hypertensive animals. The relaxation evoked by Br-OA (Figure 29) was partially inhibited in the absence of functional endothelium in endothelium-denuded vascular rings experiments (data not shown for OA and Me-OA,). This demonstrates that the relaxation-evoked by OA or derivatives involves both endothelium-dependent and independent mechanisms.

In vivo studies from previous reports suggest that OA-induced relaxation is associated with an endothelium-dependent increased production of NO and could be abrogated by NO synthase inhibitors or by NO scavengers (Rodríguez-Rodríguez, Herrera, Perona and Ruiz-Gutiérrez, 2004; Perona, Cabello-Moruno and Ruiz-Gutierrez, 2006; Rodriguez-Rodriguez, Perona, Herrera and Ruiz-Gutierrez, 2006; Dongmoa, Azebazeb, Donfackc, Dimoc, Nkeng-Efouetd, Devkotae, Sontiaf, Wagnerg, Sewalde and Vierling, 2011). Accordingly we acknowledge that *in vivo* treatment with OA might bring long term alterations of the pro or antioxidant balance hence leading to improved NO bioavailability or maybe direct expression of NOS. However, the results of our study suggest that OA and derivatives mediate their vasodilatory effects via additional mechanisms involving COX-mediated prostanoid pathway and action on K^+ channels. These results are in agreement with previous reports that OA modulate Cox-2-dependent release of PGI_2 mediated by phosphorylation of p38 MAPK (Martínez-González, Rodríguez-Rodríguez, González-Díez, Rodríguez, Herrera, Ruiz-Gutierrez and Badimon, 2008).

The present study tested whether two novel synthetic derivatives of OA display effects similar to those of the parent drug. Interestingly, the maximum relaxation evoked by the derivatives, particularly Br-OA, was significantly larger in the mesenteric vessels although these differences were not observed in the aorta. We speculate that this variation in relaxation profiles of these vessels was probably due to the differences in the receptors or effectors (e.g. channels) present and differences in the compactness of these arteries.

A significant portion of the relaxation evoked by OA and derivatives was resistant to removal of the endothelium suggesting a role of endothelium independent pathways, such as opening of the K^+ channels. We studied this hypothesis on denuded vessels incubated in the presence of blockers of different K^+ channels types (Gli for ATP-dependent K^+ channels and AP for voltage-activated K^+ channels). Either inhibitor blunted part of the relaxation evoked by OA and derivatives. The effects of both inhibitors were additive and allowed a complete blockade of the relaxation to OA, Br-OA and Me-OA suggesting that ATP-dependent and voltage-activated K^+ channels opening mediate the endothelium-independent relaxation (not shown for OA and MeOA).

The larger endothelium-independent relaxation observed for Br-OA was also completely blunted by Gli and AP. This suggests that no other mechanism is implicated and that the larger relaxation to Br-OA might be supported by a more effective activation of both K^+ channel types. This OA or derivatives-induced vasodilatation resulted in decrease of total peripheral resistance which could be implicated in the MAP lowering effect of these triterpenes. Atrial natriuretic factor has been shown to reduce Na^+ channel activity and intracellular Ca^{2+} via decreased activity of the Na^+/Ca^{2+} exchange in the cardiac cells (Stone, Backx and Keurs, 1990). In this regard, we speculate that the improved Na^+ elimination caused by these drugs could have an indirect effect on vascular function. Natriuresis is expected to induce a decrease in extracellular Na^+ concentration (as was observed in DSS animals, Table 7) which can affect intracellular Na^+ concentration by decreasing the concentration gradient for passive Na^+ entry pathways (e.g. Na^+ permeable channels). Decreased intracellular Na^+ concentration may influence the activity of the Na^+/Ca^{2+}-exchanger, resulting in more Ca^{2+} efflux or less Ca^{2+} entry via this pathway. We speculate that the resulting decrease in intracellularly available Ca^{2+} could favour relaxation and hence contribute to other mechanisms to decrease vascular resistance.

Vasodilation implies unhindered blood flow and this may have caused increased plasma being filtered in the glomerulus. In accordance with this idea, OA increased the GFR in addition to increased urinary Na^+ output in hypertensive animals at the end of the 9-week study. GFR is an important marker of kidney function and a correlation between increased urinary Na^+ excretion and elevation of GFR has been reported in experimental animals (Marin-Grez, Fleming and

Steinhausen, 1986; Mapanga, Tufts, Shode and Musabayane, 2009). GFR was assessed by creatinine clearance (C_{cr}) which has been used in our laboratory and by other authors as a fundamental parameter of evaluating renal tubular function (Rebsomen, Pitel, Boubred, Buffat, Feuerstein, Raccah, Vague and Tsimaratos, 2005; Kumar, Shetty and Salimath, 2008; Mapanga, Tufts, Shode and Musabayane, 2009; Madlala, Masola, Singh and Musabayane, 2012; Mkhwanazi, Serumula, Myburg, Van Heerden and Musabayane, 2014). The increases in urinary Na^+ excretion in conjunction with a significantly reduced plasma creatinine concentration and an elevated GFR over the 9-week experimental period, further suggest that these changes were, in part, mediated via enhanced renal blood flow.

A non-renal parameter that could account for the MAP lowering action is a change in cardiac function, namely a decrease of the HR or of the SV. No effect on HR was obtained with the drugs in the present study, excluding a mechanism involving a chronotropic action. Somova also reported no change in HR following 6-week intraperitoneal administration of OA in DSS rats (Somova, Nadar, Rammanan and Shode, 2003). In another study, the same authors reported a 6% decrease of HR (Somova, Shode and Mipando, 2004). This bradycardiac effect was however too tiny to account for the marked (20-35%) decrease in MAP concurrently obtained in the same animals. In the absence of a change in HR, a decrease of CO and of MAP can be brought about by a change in SV.

For cardiac function studies, we used video edge detection technique which is commonly used to study cell shortening in isolated cardiomyocytes (Kanai, Pearce, Clemens, Birder, VanBibber, Choi, de Groat and Peterson, 2001; He, Ma, Lee, Thomson and Kamp, 2003; Jiang, Xia, Xu and Zheng, 2004; Kang, Sohn, Lee, Lee, Han, Kim and Lee, 2004; Nevelsteen, Bito, Van der Mieren, Vanderper, Van den Bergh, Sipido, Mubagwa and Herijgers, 2013). In addition, we used a well characterised electrophysiology technique, whole-cell clamp patch, which allows a very stable intracellular recording (Kirichok, Navarro and Clapham, 2006) to study the effects of OA and derivatives on Ca^{2+} currents. We were not able to measure SV in the present setting. However, our experiments using isolated cardiomyocytes showed that OA and derivatives do not decrease but rather tend to increase myocyte shortening and had no influence on Ca^{2+} currents in Wistar rats.

This suggests that a negative inotropic action, resulting in a decrease of SV, is also excluded as mechanism for the hypotensive action of OA and derivatives. On the other hand, increase cardiomyocyte contractility implies an increase in the force of cardiac tissue contraction and increase CO. In cells isolated from DSS rats however, OA and derivatives had no apparent effect on shortening. However, the time to peak and relaxation time is much more than that of cells from normotensive rats. A study by Gomez showed that cardiac hypertrophy and heart failure caused by high blood pressure reduces the ability of Ca^{2+} to trigger calcium release from the SR in single myocytes taken from hypertensive rats (Gómez, Valdivia, Cheng, Lederer, Santana, Cannell, McCune, Altschuld and Lederer, 1997). Based on this information, we speculate that long lasting time-to-peak in cardiomyocytes of hypertensive DSS rats is probably caused by hypertrophy hence a weaker intensity of contraction and reduction in Ca^{2+} release in these cells although cell size was not measured.

Hypertension is associated with oxidative stress which causes endothelial dysfunction because of increased production of ROS. Increased production of ROS such as O_2^- due to decreased antioxidant defence systems causes rapid inactivation of NO signaling and bioavailability (Panza, 1997; Mizuno, Jacob and Mason, 2010). Based on this knowledge, this study investigated the oxidative status of kidney, liver and hearts harvested from untreated and OA-treated SHR and DSS animals at the end of the 9-week study period. Although the most direct approach for the assessment of lipid peroxidation is the quantification of the primary (hydroperoxides) products, these peroxides are labile and short-lived. Hence, detection of lipid peroxidation has relied largely on indirect methods, that is, analyses of secondary or end products derived from hydroperoxides such as malonyldialdehyde (MDA) (Kasapoglu and Özben, 2001).

In this study, we therefore determined the levels of lipid peroxidation expressed as thiobarbituric acid reactive substances (TBARS; MDA). Thiobarbituric acid (TBA) assay is the most common and easiest method used to assess lipid peroxidation and free radical activity in biological samples (Bloomer and Goldfarb, 2004; Bouzid, Hammouda, Matran, Robin and Fabre, 2014). Different animal studies have shown that antioxidants protect against the oxidative damage produced by free radical reactions (Selemidis, Sobey, Wingler, Schmidt and Drummond, 2008; Drummond,

Selemidis, Griendling and Sobey, 2011). Therefore we also assessed the effects of OA on oxidative protein damage as indicated by activities of the commonly studied antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Ray, Batra, Shukla, Deo, Raina, Ashok and Husain, 2000; Wassmann, Laufs, Müller, Konkol, Ahlbory, Bäumer, Linz, Böhm and Nickenig, 2002; Herbette, Lenne, Labrouhe, Tourvieille, Drevet and Roeckel-Drevet, 2003; Sadi and Güray, 2009). The results showed that tissues from untreated DSS and SHR animals had elevated levels of MDA by comparison with tissues from control Wistar animals. Tissues from OA-treated DSS animals had reduced levels of MDA by comparison with respective untreated hypertensive rats. Tissues isolated from untreated DSS and SHR animals had reduced activities of antioxidant enzymes, SOD and GPx, by comparison with tissues from control Wistar animals. However, tissues from OA-treated DSS and SHR animals had increased activities of these antioxidant enzymes by comparison with respective untreated hypertensive rats. These results are in agreement with those obtain by other authors although OA-evoked increase in antioxidant activities were analysed in different samples (Somova, Shode, Ramnanan and Nadar, 2003; Wang, Ye, Liu, Chen, Bai, Liang, Zhang, Wang, Li and Hai, 2010). We speculate that antioxidant properties of OA could play a role in hypotensive mechanisms of this triterpene. Studies indicate that oxidative stress is characterised by reduced production of NO (NO) which results in constricted vessels hence increased peripheral resistance and blood pressure. The effects of OA on the production of NO were not evaluated in this study, however, we speculate that OA-evoked decreased ROS and increased antioxidant enzymes may have enhanced the production of NO, thereby improving vasodilation. Preventing the imbalance of ROS and antioxidants in kidney, liver and the heart as shown in this study suggest that OA could have averted the progression of hypertension via NO-dependent vasorelaxant effects.

In summary, OA and derivative-induced reduction in total peripheral resistance implies unhindered renal blood flow which may have caused increased plasma being filtered in the glomerulus as indicated by increased GFR. Increased GFR means that more Na^+ content was filtered into the renal tubule and we further confirmed our previous observation that OA, and the derivatives investigated for the first time in this study, inhibit proximal tubular Na^+ reabsorption as indicated by increased FE_{Li}. Therefore, this Na^+ passes through to the distal tubule and we

showed that our drug lowered aldosterone hence preventing Na^+ reabsorption once again in this part of the tubule which explains increased Na^+ loss in the urine. Urinary Na^+ loss may cause the heart to increase CO to compensate for acute intravascular compartment contraction in addition to redistribution of water to the interstitium in order to balance the ECF. In agreement with this speculation, OA and derivatives increased the force of cardiomyocyte contraction which in collection may enhance SV and CO although the effects of these triterpenes on whole heart contraction were not explored.

CHAPTER 7
CONCLUSION

We provide the first report that OA and related novel synthetic derivatives, Me-OA and Br-OA induce similar hypotensive effects in experimental animals mediated by multiple mechanisms. These involve increased urinary Na^+ output mediated by inhibition of proximal Na^+ reabsorption and modulation of oxidative stress in hypertensive animals. In addition to renal mechanisms, vascular mechanisms are involved, since the drugs induced relaxation involving endothelium-dependent COX/prostanoids pathway as well as endothelium-independent opening of ATP-sensitive and voltage-activated K^+ channels. These results suggest that these triterpenes influence endothelium-dependent and independent vasodilatory pathways which causes decreased total peripheral resistance.

7.1 Limitations and direction for future studies

Due to poor yield obtained for the synthetic derivatives, we were unable to study their sub-chronic effects in conscious animals, therefore this should be explored in future. OA effects on renal function seem to mimic oxytocin-like activities i.e. increase Na^+ output without changing the urine flow rate (Conrad, Gellai, North and Valtin, 1986), hence future studies should investigate whether this hormone can have synergistic effects with OA and derivatives. This study did not evaluate the effects of these triterpenes on NO production or expression of eNOS and phosphorylation of K^+ channels. Further studies should also assess the plasma concentrations of OA to provide better understanding and direction for explaining issues such as increased natriuresis without hanging plasma concentrations of Na^+.

CHAPTER 8

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CHAPTER 9

APPENDICES

Appendix I – Ethical clearance A

Research Office
Animal Ethics Research Committee

Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
Email: animalethics@ukzn.ac.za

18 May 2012

Reference: 077/12/Animal

Miss H Madlala
School of Laboratory Medicine
and Medical Sciences
University of KwaZulu-Natal
Westville Campus

Dear Miss Madlala

Ethical Approval of Research Project on Animals

I have pleasure in informing you that on recommendation of the review panel, the Animal Ethics Sub-committee of the University Research and Ethics Committee has granted ethical approval for 2012 on the following project:

"Mechanisms of cardiovascular effects of oleanolic acid and related triterpenes: an experimental study."

Yours sincerely

Prof. Theresa HT Coetzer (Chair)
ANIMAL RESEARCH ETHICS COMMITTEE

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

Founding Campuses:
- Edgewood
- Howard College
- Medical School
- Pietermaritzburg
- Westville

Appendix II – Ethical clearance B

Research Office
Animal Ethics Research Committee

Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
Email: animalethics@ukzn.ac.za

11 December 2012

Reference: 028/13/Animal

Miss H Madlala
School of Laboratory Medicine and
Medical Sciences
WESTVILLE Campus

Dear Miss Madlala

RENEWAL: Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for **2013** on the following project:

"Mechanisms of cardiovascular effects of oleanolic acid and related triterpenes: an experimental study."

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

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

1910 - 2010
100 YEARS OF ACADEMIC EXCELLENCE
IN KWAZULU-NATAL

Founding Campuses:
Edgewood
Howard College
Medical School
Pietermaritzburg
Westville

Appendix III – Ethical clearance C

De ondertekende aanvraagformulieren sturen naar: Erna Dewil, Proefdierencentrum, Herestraat 49 bus 501, 3000 Leuven.

P190/2013

KATHOLIEKE UNIVERSITEIT LEUVEN

FORMULIER ETHISCHE COMMISSIE
Form Ethical Committee

Laboratorium (*laboratory*): Experimentele Cardiale Heelkunde, Departement Cardiovasculaire Wetenschappen

Erkenningsnummer laboratorium (*license number*): LA-1210253

Laboratoriumdirecteur (*laboratory director*):

Naam/*name*	Voornaam/*first name*	Diploma/*degree*
HERIJGERS	Paul	MD PhD

Proefleiders (ZAP) (*PI*):

Naam/*Name*	**Voornaam**/*first name*	**Diploma** /*degree*	**Certificaat proefdierkunde**/*certificate lab animal science*
MUBAGWA	Kanigula	MD PhD	☒
....	....	....	☐

Uitvoerende onderzoeker(s) (AAP/BAP) (*researchers and technicians*):

Naam/*Name*	**Voornaam**/*First name*	**Diploma**/*degree*	**Certificaat proefdierkunde**/*certificate lab animal science*
MADLALA	Hlengiwe	M.Sci.	☐
....	....	....	☐
....	....	....	☐

☒ **Nieuw project** (*new project*) ☐ **Verlenging van of verbonden aan een project (Nr. ....)**/*elongation of project P…* ☐ **Wijziging van een project (Nr...........)**/*change of project P…*

Titel van het onderzoeksproject (*title of the research project*):

Mechanisms of cardiovascular effects of oleanolic acid and related triterpene derivatives

Duur van het project (maximum 4 jaar) (*duration of the project, max 4 years*)
Begindatum (*start date*): 20.10.2013 Einddatum (*end date*): 31.12.2014

Handtekening van de laboratoriumdirecteur/*signature lab director* Datum/*date*

22/10/2013

Advies (voorbehouden aan de Ethische Commissie) (***for the Ethical Committee***):
☒ gunstig/*favorable* ☐ gunstig mits aanpassingen/*provided favorable adjustment* ☐ ongunstig/*rejected* 18 XI 2013

Inschatting van pijn, lijden of letsel door de Ethische Commissie/*estimate bij the Ethical Comittee*
☐ geen/*none* ☒ gering/*minor* ☒ matig/*moderate* ☐ ernstig/*severe* ☐ ondefinieerbaar/*undefinable* Datum/*date*:

Commentaar en opmerkingen/*comments and remarks*

De Voorzitter/ *the Chairman* De Leden/ *the members*

Appendix IV - License for use of laboratory animals

CERTIFICAAT - CERTIFICATE

KU LEUVEN

The undersigned declares that 2014

Madlala Hlengiwe

Born on 16 February 1986

In South Africa

Has followed the Course on Laboratory Animal Science for persons that carry out experiments or take part in them (40 hours) and has passed the exam. This course includes 30 hours of theory about legislation, housing and transport of laboratory animals, zootechnics, anatomy and physiology, genetic standardisation and transgenesis, anaesthesia, analgesia and euthanasia, hygiene and microbiological standardisation, pathology, use of alternatives, behaviour and wellbeing, experimental techniques, ethics, working safely with laboratory animals and feeding of laboratory animals and 10 hours of practical course (small laboratory animals, case studies).

The course meets the requirements as indicated in Appendix III of the RD of 13 September 2004 and Appendix VII of the RD of 14 November 1993 concerning the protection of laboratory animals, especially concerning the education for people responsible for directing animal experiments. This course was accepted by the Department Animal Welfare & CITES of the Government Service Public Health on 15 February 2005.

Prof. Erna Dewil
Animal Research Center
KU Leuven Campus Gasthuisberg, O&N 1
Herestraat 49, bus 501
3000 Leuven
Belgium

Appendix V – Conference presentation

MECHANISMS OF THE HYPOTENSIVE EFFECTS OF *SYZYGIUM AROMATICUM*-DERIVED OLEANOLIC ACID AND NOVEL SYNTHETIC OLEANANE DERIVATIVES

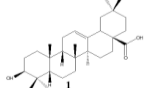
UCL Université catholique de Louvain

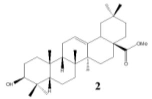
Hlengiwe P. Madlala; Thomas Metzinger; Fanie R. van Heerden; Kanigula Mubagwa; Cephas T. Musabayane

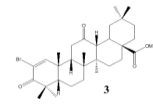
Pole de Pharmacologie et Thérapeutique (FATH), Plateforme Physiologie de l'IREC, Université catholique de Louvain; Experimental Cardiac Surgery, Katholieke Universiteit Leuven; Organic Chemistry, Renal Physiology and Medicinal Plants, University of KwaZulu-Natal.

Introduction

Despite the various conventional treatments that are available, hypertension continues to be globally responsible for approximately 9.4 million deaths each year (WHO, 2013). The high mortality can partly be attributed to side effects of available drugs or inaccessibility of current synthetic drugs to communities from poor socioeconomic background because of their relative high cost (Rahmani *et al.*, 2014). Medicinal plant products have been shown to be potentially beneficial in the management of various disorders including cardiovascular disease. Accordingly, this study investigated the effects of plant-derived oleanolic acid (OA) and related synthetic derivatives on arterial pressure and thereafter evaluated the mechanisms in which these triterpenes mediate this effect in hypertensive animals.



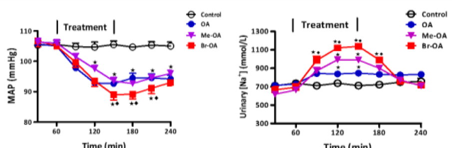




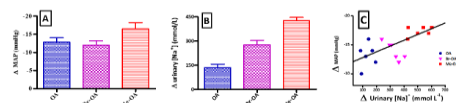
Syzygium aromaticum (Hochst.) [Myrtaceae] flower buds, OA is isolated from the dried brown flower buds. Molecular structures of (1) Natural product OA; (2) methyl- and (3) brominated-derivatives.

Results

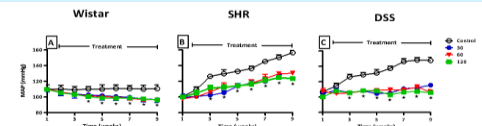
I. OA decreased MAP in both acute and sub-chronic experimental settings, the MAP lowering effect was more marked in hypertensive animals compared to normotensive rats. Similarly, the two OA derivatives, used in acute settings, also decreased MAP. OA increased urinary Na^+ excretion under acute or sub-chronic conditions. A similar but quantitatively more marked increase was obtained with the derivatives under acute conditions.



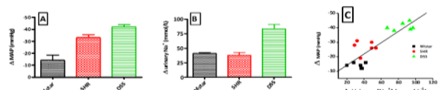
MAP and urinary $[Na^+]$ of control rats and animals treated with OA, Me-OA and Br-OA (90 µg h^{-1}) for 1½ h during the 4 h experimental period. Values are means +/- SEM (n = 6), ⁎ p < 0.05 / 0.001 vs control s at each corresponding time. ♦ p < 0.05 by comparison with OA-treated animals.



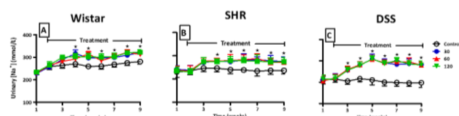
Changes in MAP (**A**), urinary Na^+ concentration (**B**) and correlation of these changes (**C**) in OA or derivative-administered animals during the 1½ h treatment period. Values are means +/- SEM (n = 6). (R = 0.67, p < 0.05) correlation.



Effects of various doses of OA (30, 60, 120 mg kg^{-1}) administration on MAP in Wistar (**A**), SHR (**B**) and DSS (**C**) rats over a 9-week experimental period. Values are means +/- SEM (n = 6). ⁎ p < 0.05 / 0.001 by comparison with control animals at each corresponding time.



Changes in MAP (**A**), urinary $[Na^+]$ (**B**) and correlation of these changes (**C**) in OA treated animals on the 9th week of the sub-chronic study. Values are means +/- SEM (n = 6). (R = 0.85, p < 0.0001) correlation.



Effects of various doses of OA (30, 60, 120 mg kg^{-1}) administration on urinary $[Na^+]$ in Wistar (**A**), SHR (**B**) and DSS (**C**) rats over a 9-week experimental period. Values are means +/- SEM (n = 6). ⁎ p < 0.05 / 0.001 by comparison with control animals at each corresponding time.

Materials and Methods

OA was extracted from dried flower buds of *Syzygium aromaticum* using a previously validated protocol in our laboratory (Madlala *et al.*, 2012). Me-OA and Br-OA were synthesized according to a method described by Fu and Gribble (2013). Acute effects of OA and its oleanane derivatives on mean arterial pressure (MAP) and Na^+ handling were assessed in anaesthetized normotensive Wistar rats. Sub-chronic effects of OA on MAP and renal function were investigated in Wistar, Dahl salt-sensitive (DSS) and spontaneously hypertensive (SHR) rats. At the end of the sub-chronic study, we investigated the effects of 9-week OA treatment on the oxidative status of cardiac, hepatic and renal tissues of hypertensive animals. Additional studies on the mechanisms of action of OA and derivatives were carried out in isolated arteries. Measurements of isometric tension were done in endothelium-intact and denuded mesenteric arteries of Wistar rats. The influence of indomethacin, L-Nitroarginine, glibenclamide and 4-aminopyridine on the OA/derivatives effects was tested.

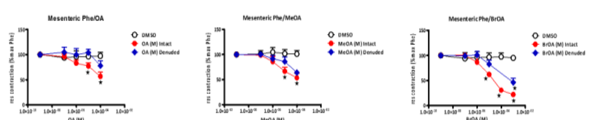
II. Compared with respective control rats, OA-treated animals exhibited significantly lower malonyldialdehyde (MDA, a marker of lipid peroxidation) levels and increased activity of the antioxidant enzymes superoxide dismutase and glutathione peroxidase in hepatic, cardiac and renal tissues.

		Tissue		
Parameter measured	**Experimental protocol**	**Heart**	**Kidney**	**Liver**
MDA (nmol.g⁻¹ protein)	Wistar control	2.12 ± 0.09	1.70 ± 0.05	1.52 ± 0.06
	SHR control	2.75 ± 0.01	2.05 ±0.02	2.46 ±0.04
	DSS control	5.10 ± 0.10*	4.02 ± 0.08*	4.18 ± 0.09*
	SHR OA	1.16 ±0.01#	1.21 ±0.01#	1.35 ± 0.02#
	DSS OA	1.82 ± 0.20#	1.55 ± 0.10#	1.71 ± 0.30#
SOD activity (nmol.min⁻¹ mL⁻¹ g protein)	Wistar control	6.11 ± 0.30	10.20 ± 1.04	7.05 ± 0.13
	SHR control	2.13 ± 0.48	3.01 ± 0.21	3.56 ± 0.41
	DSS control	1.25 ± 0.08*	2.29 ± 0.07*	2.12 ± 0.14*
	SHR OA	5.78 ± 0.14#	9.26 ± 0.12#	6.84± 0.07#
	DSS OA	4.12 ± 0.33#	11.08 ± 0.41#	8.72 ± 0.32#
GPx activity (nmol.min⁻¹ mL⁻¹ g protein)	Wistar control	3.82 ± 0.16	2.38 ± 0.21	0.10±0.02
	SHR control	0.62±0.06*	0.60±0.02*	Undetectable
	DSS control	0.60 ± 0.02*	0.20 ± 0.06*	Undetectable
	SHR OA	3.47±-0.03#	3.08±0.02#	Undetectable
	DSS OA	4.14 ± 0.31#	3.38 ± 0.24#	Undetectable

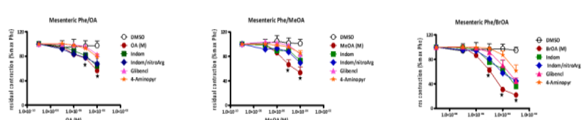
Note: undetectable ≤ 0.01 nmol.min^{-1} mL^{-1} g protein
* p < 0.05 by comparison with Wistar control animals;
\# p < 0.05 by comparison with respective control animals.

Comparison of MDA concentration, activities of SOD and GPx in the kidney, heart and liver harvested at the end of the sub-chronic study from SHR and DSS rats treated with OA twice every third day for 9 weeks with control Wistar rats. Values are expressed as means +/- SEM (n = 6).

III. OA and derivatives caused relaxation in Wistar rat mesenteric arteries. This effect was only partly inhibited by endothelium removal; the relaxation was also blunted in the presence of indomethacin or K^+ channel blockers.



Concentration–response curves for OA and derivatives in small mesenteric arterial segments isolated from Wistar rats pre-contracted with a single sub-maximal concentration of phenylephrine (Phe) (5 µM). Curves were obtained in arteries with and without endothelium. The values shown are means +/- SEM (n = 7). ⁎ p < 0.05 vs. control.



Concentration–response curves for OA and derivatives in mesenteric rings isolated from Wistar rats pre-contracted with a single sub-maximal concentration of Phe (5 µM). Curves were obtained in arteries with endothelium in the presence of N-nitro-L-arginine methyl ester (L-NAME) (100 µM), indomethacin (10 µM), glibenclamide (10 µM) and 4-aminopyridine (1 mmol L^{-1}). The values shown are means +/- SEM (n = 7). ⁎ p < 0.001 vs. control/DMSO.

Conclusion

The results suggest that hypotensive effects of OA and related derivatives are mediated, in part, via increased urinary Na^+ output, improved oxidative status and decreased vascular resistance.

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4. WHO 2013. High Blood Pressure: Global and regional overview. . *Document number: WHO/DCO/WHD/2013.2.*

Appendix VI – Draft manuscript

MECHANISMS OF THE HYPOTENSIVE EFFECTS OF *SYZYGIUM AROMATICUM*-DERIVED OLEANOLIC ACID AND NOVEL SYNTHETIC OLEANANE DERIVATIVES IN EXPERIMENTAL ANIMALS

By

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Abstract

Although traditional herbal remedies are widely used in Africa for the management of various disorders including cardiovascular diseases very little reliable data is available on their therapeutic and pharmacological efficacy or on the mechanisms underlying the effects. We have previously demonstrated that *Syzygium aromaticum*-derived oleanolic acid (OA) possesses hypotensive effects in normotensive experimental animals. In the present study we evaluated the effects of sub-chronic administration of OA (twice every third day for 9 weeks) on blood pressure in Dahl salt-sensitive (DSS) and spontaneously hypertensive (SHR) rats. Renal function and the oxidative status of cardiac, hepatic and renal tissues were also determined to establish possible mechanism(s) of the hypotensive effects of OA. In addition, acute effects of OA and its oleanane derivatives on blood pressure and tubular Na^+ handling were assessed in anaesthetized normotensive Wistar rats. Sub-chronic OA treatment caused reductions in blood pressure in the normotensive Wistar, DSS and SHR rats which were positively correlated with increased urinary Na^+ excretion. Furthermore, acute infusion of OA, Me-OA and Br-OA reduced mean arterial pressure, increased urinary Na^+ concentration and fractional excretion of Na^+ (FE_{Na}) and Li^+ (FE_{Li}) in anaesthetized rats in the absence of significant changes in GFR. Compared with respective control rats, sub-chronically OA-treated animals exhibited significantly low malondialdehyde (MDA, a marker of lipid peroxidation) and increased activity of the antioxidant enzymes superoxide dismutase and glutathione peroxidase in hepatic, cardiac and renal tissues. The results suggest that hypotensive effects OA and related derivatives are mediated, in part, via increased urinary Na^+ output and improved oxidative status.

Introduction

Despite the various conventional treatments that are available to treat hypertension, the disease continues to be globally responsible for approximately 9.4 million deaths each year (WHO, 2013). The high mortality can partly be attributed to side effects of available drugs or to the inaccessibility of current synthetic drugs to communities from poor socioeconomic background because of their relative high cost (Rahmani *et al.*, 2014). This problem has resulted in a growing interest in the use of medicinal plant products because they are considered to be cheap, believed to possess few side effects and are easily accessible to the general population in developing countries (Ayyanar and Ignacimuthu, 2011). Although ethnomedical use of medicinal plant extracts by different cultures around the world dates back to many centuries, only recently has scientific research began to provide evidence for the mechanisms underlying their therapeutic and pharmacological effects. In search for plants with therapeutic properties for the treatment of hypertension and complications, our laboratory has scientifically evaluated several plant species (Gondwe *et al.*, 2008; Kamadyaapa *et al.*, 2009). In particular, we have isolated *Syzygium* spp-derived triterpenes and focused on the therapeutic effects of oleanolic acid (OA) (Musabayane *et al.*, 2010; Ngubane *et al.*, 2011; Madlala *et al.*, 2012; Khathi *et al.*, 2013) and maslinic acid (MA) (Mkhwanazi *et al.*, 2014).

Literature has reported diverse pharmacological properties of OA and this therapeutic importance has led to the use of this triterpene as a starter molecule for the synthesis of new oleanane derivatives (Sporn *et al.*, 2011). In comparison to OA, the new synthetic compounds are reported to have improved biological activities (Couch *et al.*, 2005; Dinkova-Kostova *et al.*, 2005; Yates *et al.*, 2007). Therefore, we synthesized two oleanane derivatives, Me-OA and Br-OA, and investigated their hypotensive effects in experimental animal models. We have previously reported that OA exhibits blood pressure lowering effects in normotensive animals but the underlying mechanisms were not determined (Madlala *et al.*, 2012). In the present study we used Dahl-salt sensitive (DSS) and spontaneously hypertensive rats (SHR) to test their responsiveness to OA in comparison with non-hypertensive controls. In addition, the study investigated the mechanisms of blood pressure lowering effects of OA and related synthetic derivatives. DSS and SHR rats are accepted experimental models of hypertension emphasizing the roles of diet and genetic factors, respectively (Drenjančević-Perić *et al.*, 2011). The

mechanisms for the development of hypertension are mediated, at least in part, via abnormal electrolyte handling by the kidney and endothelial dysfunction due to oxidative stress (Forstermann, 2010; Zicha *et al.*, 2012). Hence, we evaluated the effects of OA, Me-OA and Br-OA on fluid and electrolyte handling by the kidney. In addition we investigated the effects of OA on oxidative stress in the liver, heart and kidney of DSS and SHR rats.

Materials and methods

1. Drugs and Chemicals

1.1. Standard Chemicals

Except for OA and derivatives which were obtained as described below, drugs were sourced from standard pharmaceutical suppliers. All other chemicals used for this study were of analytical grade and were purchased from standard commercial suppliers.

1.2. Isolation of OA

OA was isolated from *Syzygium aromaticum* [(Linnaeus) Merrill & Perry] (Myrtaceae) (cloves) using a protocol previously validated in our laboratory (Mapanga *et al.*, 2009; Madlala *et al.*, 2012; Mkhwanazi *et al.*, 2014). Briefly, air-dried powdered flower buds (500 g) were exhaustively extracted sequentially with dichloromethane and ethyl acetate (3 L x 24 h x 1 L for each solvent) at room temperature. The solvents were removed under reduced pressure at 55±1 °C using a rotary evaporator yielding corresponding residues of dichloromethane-solubles (DCMS, 63 g) and ethyl acetate-solubles (EAS, 85 g). EAS have been previously shown to contain OA, ursolic acid, methyl maslinate and methyl corosolate (Musabayane *et al.*, 2005). Hence subjecting EAS to column chromatography and recrystallisation from methanol yielded pure OA whose structure was confirmed by spectroscopic analysis using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Spectroscopic data indicated that the *S. aromaticum*-isolated OA (compound **1** in Figure 1) was pure and hence this triterpene was used for animal studies and as a starting material for the synthesis of oleanane derivatives.

1.3. Synthesis of Me-OA and Br-OA

OA derivatives were synthesized according to a method previously described by (Fu and Gribble, 2013). To obtain the methyl ester (Me-OA) (compound **2** in Figure 1), a 40% (m/v) aqueous solution of KOH (7.5 mL, 0.1 mM) was added to diethyl ether (40 mL) followed by addition of 2 g (0.02 M) of nitrosomethylurea at 0 °C. The yellow ethereal layer of diazomethane was poured into the tetrahydrofuran (THF) (5 mL, 0.07 mM) solution of OA (500 mg, 1.09 mmol). The mixture was left in a fume hood overnight and compound **2** was obtained as a whitish powder (65 %) with m.p. 124 – 126 °C. Compound **2** (1.20 g, 2.55 mmol) was then oxidized with iodoxybenzoic acid (2.86 g, 10.2 mmol) in dimethyl sulphoxide (35 mL). This was followed by epoxidation of the oxidized product using *m*-chloroperoxybenzoic acid (321 mg, 1.3 mM). The epoxidation product was then brominated with hydrobromic acid (44 µL, 0.38 mM) and bromine (0.12 mL, 1.04 mmol) in acetic acid (10 mL) to yield compound **3** (Figure 1) (30%). An analytically pure sample of this compound was obtained by column chromatography (hexanes-EtOAc, 4:1 to 2:1) as a yellowish solid with m.p. 137 – 140 °C. All structures of synthetic products were confirmed by ^{1}H, ^{13}C NMR and infrared spectroscopy. Spectra were recorded on a Bruker DRX-400 NMR and a Bruker Alpha FT-IR spectrometer. The pure compounds were used for animal studies.

2. Animals

Normotensive (normal) male Wistar (250-300 g), weanling Dahl salt-sensitive (DSS; 100-150 g) and spontaneously hypertensive rats (SHR; 100-150 g) rats bred and housed at Biomedical Research Unit, University of KwaZulu-Natal, were used in this study. The rats were maintained on a 12 h light / 12 h dark regime, and given both food (Meadow Feeds, Pietermaritzburg, South Africa), and water *ad libitum*. The experiments were performed in accordance with ethical guidelines of the University of KwaZulu-Natal.

3. Experimental Design

The animals were used to study either 1) acute (4 hours) effects of OA and of its oleanane derivatives on MAP and renal function under anaesthesia in normotensive rats, or 2) subchronic

(9 weeks) effects of OA on blood pressure, renal and oxidative stress in normotensive and DSS and SHR rats

3.1. Acute studies

Male Wistar rats were fed standard rodent chow supplemented with lithium chloride (12 mmol.kg^{-1} dry weight) for 48 hours (h) prior to experimentation in order to raise plasma lithium to measurable concentrations without affecting renal Na^{+} or water excretion. The rats were prepared for acute mean arterial blood pressure and renal function measurements using a protocol previously reported from our laboratories (Musabayane *et al.*, 2007). Briefly, the rats were anaesthetized by intra-peritoneal injection of inactin [5-ethyl-5-(10-methylpropyl)-2 thiobarbiturate, 0.11 g kg^{-1} body weight] (Sigma Aldrich, St. Louis, MO, USA) and tracheotomy was performed. A catheter was implanted in the left carotid artery for withdrawal of blood samples and continuous recording of arterial blood pressure at 30 min intervals via a pressure transducer (Statham MLT 0380, Ad Instruments, Bella Vista, NSW, Australia), compatible with PowerLab System ML410/W (Bella Vista, NSW, Australia). Another catheter was inserted in the right jugular vein for continuous intravenous infusion (Harvard Syringe Infusion Pump 22, Harvard Apparatus, Holliston, MA, USA) of saline (0.077 M NaCl) containing creatinine (0.15 mg mL^{-1}) at 9 mL h^{-1} to allow calculation of creatinine clearance as a measure of GFR. A minimal abdominal incision was made and a urinary bladder catheter was inserted for the collection of urine samples. After a 3.5 h equilibration period, blood pressure recordings and urine samples were taken every 30 min and blood samples (200 µL) were drawn hourly for measurements of electrolyte concentrations over a 4 h experiment divided into 1 h control, 1.5 h treatment and 1.5 h recovery periods. Tubular handling of Na^{+} in the proximal tubule was evaluated through measurement of lithium clearance (C_{Li}) (Thomsen, 1990). In those animals in which the effects of OA or derivatives were examined, the infusate was changed during the 1.5 h treatment period to the one identical in ionic composition, but containing OA, Me-OA and Br-OA (90 µg h^{-1}).

3.2. Sub-chronic studies

Separate groups (n = 6 in each group) of male Wistar, SHR and DSS rats housed individually in Makrolon polycarbonate metabolic cages (Techniplast, Labotec, South Africa) were treated with various doses of OA (30, 60 and 120 mg kg^{-1}, p.o.) twice (at 09h00 and 15h00) every third day for 9 weeks. OA was freshly dissolved in dimethyl sulphoxide (DMSO, 2 mL) and normal saline (19 mL) (Musabayane *et al.*, 2005) before use in each case. Rats given DMSO/saline (3 mL kg^{-1}, p.o.) acted as untreated controls. The rats were given both food and water *ad libitum*.

Urine volume and urinary concentrations of creatinine, urea, Na^+, K^+ and Cl^- were determined daily (see Biochemical Measurements below) while mean arterial blood pressure (MAP) was monitored every third consecutive day using non-invasive tail cuff method with photoelectric sensors (IITC Model 31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA) as previously described (Gondwe *et al.*, 2008; Kamadyaapa *et al.*, 2009). The equipment was calibrated each day prior to measurements. The animals were kept warm at ± 30 °C in an enclosed chamber (IITC Model 303sc Animal Test Chamber, IITC Life Sciences, Woodland Hills, California, USA) for 30 minutes before blood pressure recording. All measurements were conducted at 09h00. Blood samples were collected by cardiac puncture into individual pre-cooled heparinized containers at the end of the 9 week experimental period for biochemical analysis. Glomerular filtration rate (GFR), as assessed by creatinine clearance (C_{Cr}) was calculated using the standard formulae from measurements of the plasma and urinary concentrations of creatinine and urine flow rate in the 9th week.

4. Electrolyte and Biochemical Measurements

4.1. Urinalysis

Urine volume was determined gravimetrically using a balance (Mettler balance PC 180-instruments, Protea Laboratory Services, Johannesburg, South Africa). Quantitative measurements of total urinary outputs and plasma concentrations of Na^+, K^+, Cl^-, urea and creatinine were performed using Beckman Coulter (Synchron LX20 Clinical Systems, Fullerton, Carlifornia, USA) with reagent kits from Beckman Coulter (Synchron LX20 Clinical Systems,

Dublin, Ireland). Lithium was determined by flame emission spectroscopy at 670.8 nm (Optima 2100 DV, Perkin Elmer, Shelton, CT) using a modified procedure that has been previously described (Madlala *et al.*, 2012). Fractional excretion rates of Na^+ (FE_{Na}) and Li^+ (FE_{Li}) were determined simultaneously.

4.2. Analysis of plasma aldosterone and AVP secretion

4.2.1 Aldosterone assay

Plasma aldosterone concentrations were measured from blood samples collected from Wistar and hypertensive groups of non-treated and treated animals after 9 weeks of OA administration using an aldosterone ELISA Kit (Replamed, Centurion, Gauteng). Plasma samples (100 µL) were added to the 96 well plate followed by 50 µL of the blue conjugate. An antibody (50 µL) was added into each well and the plate was sealed and incubated at 4 °C for 24 h. Following incubation, the plate was washed three times with wash buffer (400 µL). The substrate solution (200 µL) was added and the plate was incubated for 1 hour at room temperature. The reaction was stopped by adding 50 µL of stop solution to all wells. The absorbance was read at 405 nm using a Spectrostar Nano microplate reader (BMG Labtech GmbH, Ortenberg, Germany). A standard curve was constructed from the absorbance values of the known concentrations of the standards using the spline regression curve in Graph Pad Instat software (version 5.00). The respective aldosterone concentrations of the unknown samples were then extrapolated from the standard curve.

4.2.2. Arginine vasopressin assay

Plasma AVP concentrations were measured from blood samples collected from non-treated and treated groups of hypertensive rats after 9 weeks of OA treatment using an Arg^8-Vasopressin ELISA Kit (Abcam, Cambridge, Massachusetts, USA). Plasma samples (100 µL) were added to the 96 well plate followed by 50 µL of the vasopressin conjugate. An antibody (50 µL) was added into each well and the plate was sealed and incubated at 4 °C for 24 h. Following incubation, the plate was washed three times with wash buffer (400 µL). The *p*-nitrophenyl phosphatesubstrate (pNpp) substrate solution (200 µL) was added and the plate was incubated for 1 hour at room temperature. The reaction was stopped by adding 50 µL of stop solution and

mixing on the shaker for 5 minutes. The absorbance was read at 405 nm using a Spectrostar Nano microplate reader (BMG Labtech GmbH, Ortenberg, Germany). A standard curve was constructed from the absorbance values of the known concentrations of the standards using the spline regression curve in Graph Pad Instat software (version 5.00). The respective AVP concentrations of the unknown samples were then extrapolated from the standard curve.

4.3. Oxidative stress evaluation

To establish the effects of OA on oxidative stress in the liver, heart and kidney of SHR and DSS rats, we compared levels of MDA, a commonly known marker of oxidative stress and of antioxidant defence enzymes SOD and GPx between untreated normotensive Wistar and OA-treated hypertensive rats.

4.3.1. Tissue sample harvesting

At the end of a 9-week experimental period, all animals were sacrificed by exposing to halothane via a gas anaesthetic chamber (100 mg kg^{-1}, for 3 min). Livers, hearts and kidneys were removed, snap frozen in liquid nitrogen and stored in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at -70 °C for subsequent biochemical analysis. All organs were analyzed for protein content in addition to other biochemical parameters. The protein content was quantified using the Lowry method (Grossman, 2008). All the samples were standardized to one concentration (1 mg mL^{-1}).

4.3.2. MDA

MDA levels in the tissue homogenates of heart, kidney and liver from Wistar, DSS and SHR rats were estimated using thiobarbituric acid (TBA) by a previously described method (Kasapoglu and Özben, 2001). Tissues (50 mg) were homogenised in 500 µL of 0.2% phosphoric acid. The acid reacts with MDA to form a stable pink colour with maximum absorption at 532 nm. The heart, kidney and liver tissues (50 mg) from Wistar control, untreated and OA-treated DSS rats were homogenized in 500 µL of 0.2% phosphoric acid. The homogenate was centrifuged at 14,000 x g at 4 °C for 10 minutes. Thereafter, 400 µL of the homogenate was supplemented with 400 µL of 2% phosphoric acid followed by addition of 400

µL TBA/butylated hydroxytoluene (BHT) dissolved in 400 µL of 3 mM hydrochloric acid (HCl), additional 200 µL of 1 M HCl was added to ensure a 1.5 acidic pH. The solution was heated in a water bath at 100 °C for 15 min to properly dissolve TBA. After cooling, butanol (1.5 mL) was added and the precipitate was removed by centrifugation. Sample absorbance was then determined at 532 nm against a blank that contained all reagents except the homogenate sample. The absorbencies from these wavelengths were used to calculate the concentration of MDA using Beer's law. Tissue MDA concentration was expressed as $nmol.g^{-1}$ protein.

4.3.3. SOD

Tissue SOD levels from homogenates of heart, kidney and liver of Wistar, DSS and SHR rats were measured using a commercially available Biovision SOD Assay Kit (BioVision Research Products, Mountain View, California, USA). Xanthine and hypoxanthine oxidase were used to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity was then measured by the degree of inhibition of this reaction. The homogenized tissue concentration of SOD was expressed as units per gram protein.

4.3.4. GPx

GPx levels were in tissue homogenates of heart, kidney and liver of Wistar, DSS and SHR rats were measured using a commercially available Biovision GPx Assay Kit (BioVision Research Products, Mountain View, USA), where GPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to its reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was related to GPx concentration, expressed as units per gram protein.

5. Data management and statistical analysis

Glomerular filtration rate, 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. Renal clearances (C) and fractional excretions (FE) were calculated with the

standard formulae where C = U *x* V/P and FE = C/GFR, where U is the urinary concentration, V is the urine flow rate and P is the plasma concentration. Data were analyzed using GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, CA, USA). Statistical comparison of the differences between the control and experimental groups was performed with one-way analysis of variance, followed by Tukey–Kramer multiple comparison test. Correlation between changes in MAP and Na^+ excretion was analyzed using Origin (Originlab, Northampton, MA, USA). Data are expressed as mean ± standard error of the mean (SEM) and a difference with $p < 0.05$ was considered significant.

Results

1. Structures of OA, Me-OA and Br-OA

The purity of the plant-derived OA was approximately 98% and the percentage yield varied from 0.79% to 1.72% of the plant material. The percentage yield of the synthetic derivatives, Me-OA and Br-OA was 65% and 30%, respectively. The structural data from ^{1}H, ^{13}C-NMR and IR spectroscopic analysis of the compounds obtained after recrystallization of the compounds with methanol were comparable with literature data (Mahato and Kundu, 1994; Honda *et al.*, 2000; Sporn *et al.*, 2011; Fu and Gribble, 2013).

1.1 OA [$C_{30}H_{47}O_3$]

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.30 (t, 1H, J = 3.5 Hz), 3.21 (dd, 1H, J = 11.5 and 4.4 Hz), 2.85 (dd, 1H, J = 13.8 and 4.3 Hz), 1.21 (s, 3H), 0.99 (s, 3H), 0.92 (s, 3H), 0.91 (s, 3H), 0.90 (s, 3H), 0.79 (s, 3H), 0.72 (s, 3H).

^{13}C NMR (400 MHz, $CDCl_3$): δ 183.5, 143.8, 122.8, 79.2, 55.4, 47.8, 46.7, 41.8, 41.2, 39.4, 38.9, 38.6, 37.3, 31.8, 23.6, 22.9, 18.5, 17.3, 15.7, 15.5.

IR: 3454, 2940, 2860, 1688, 1462, 1387, 1274, 1185, 1030,781, 655 cm^{-1}.

1.2 Me-OA [$C_{31}H_{50}O_3$]

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.30 (t, 1H, J = 3.5 Hz), 3.63 (s, 3H), 3.21 (dd, 1H, J = 11.5 and 4.4 Hz), 2.85 (dd, 1H, J = 13.8 and 4.3 Hz), 0.99 (s, 3H), 0.92 (s, 3H), 0.91 (s, 3H), 0.90 (s, 3H), 0.79 (s, 3H), 0.72 (s, 3H).

^{13}C NMR (400 MHz, $CDCl_3$): δ 178.1, 143.9, 122.6, 79.2, 55.2, 51.5, 47.6, 46.7, 46.0, 41.7, 41.3, 39.3, 38.7, 38.4, 37.0, 34.0, 33.1, 32.7, 30.7, 28.1, 272, 26.0, 23.6, 23.0, 18.3, 17.0, 15.5, 15.3.

IR: 3352, 2941, 2860, 1725, 1711, 1462, 1385, 1361, 1163, 1030, 780, 752 cm^{-1}.

1.3 Br-OA [$C_{31}H_{43}BrO_4$]

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.82 (s, 1H), 5.97 (s, 1H), 3.69 (s, 3H), 3.02 (d, 1H, J = 13.7 Hz), 2.91 (d, 1H, J = 4.6 Hz), 1.44 (s, 3H), 1.29 (s, 3H), 1.23 (s, 3H), 1.17 (s, 3H), 1.00 (s, 3H), 0.99 (s, 3H), 0.87 (s, 3H)

^{13}C NMR (400 MHz, $CDCl_3$): δ 199.6, 195.7, 178.3, 170.2, 155.1, 124.0, 122.1, 52.0, 49.6, 48.1, 47.3, 46.1, 45.7, 44.7, 42.1, 35.8, 34.7, 33.2, 32.7, 30.6, 28.0, 27.7, 27.0, 24.6, 23.1, 22.7, 22.0, 21.6, 18.5.

IR: 2950, 2870, 2253, 1720, 1685, 1660, 1458, 1385, 915, 744, 651, 623, 456 cm^{-1}.

2. Acute effects of OA, Me-OA and Br-OA

2.1. Fluid and electrolyte handling

In this set of our experiments, the study was restricted to a 4 h experimental period due to poor yields of the synthesized derivatives. The effects of intravenous infusion of OA and derivatives on proximal tubular function and MAP were tested in anaesthetized Wistar rats. Figure 2A shows the urine flow rate of control and drug-administered animals during the 4 h experimental period. Urine flow rate under control conditions (i.e. in untreated animals, as well as before treatment in the treated group) matched the infusion rate of 9 mL h^{-1}. OA, Me-OA and Br-OA infusion had no influence on urine flow rate. Figure 2B shows urinary Na^+ concentration in the same animals. Urinary Na^+ concentration of control animals was stable throughout the experiment and was comparable to the infusion rate of 693 μmol h^{-1}. However, OA infusion at 90 μg h^{-1} for 1.5 h during the treatment period significantly ($p < 0.05$) increased urinary Na^+ excretion rate by comparison with control animals. Similar effects to increase urinary Na^+ concentration were obtained with the OA derivatives, but the peak excretion rate was most marked with Br-OA. This is shown in Table 1 which demonstrate the cumulative data for urine

flow and total electrolyte excretion during the 1.5 h treatment period indicating the drug that was more effective among the three triterpenes studied.

To determine the site that is responsible for increased Na^+ output along the tubular nephron, we measured lithium clearance (C_{Li}), a marker for proximal tubular Na^+ handling (Thomsen, 1990) and also determined FE_{Li}. The effect of OA and derivatives on proximal tubular Na^+ clearance was estimated by comparing renal lithium clearance (FE_{Li}) between anaesthetized control and OA and derivative-treated rats. All measured variables during the 4 h experimental period were in a steady state and did not differ between the control and the experimental groups prior to the infusion of OA/derivatives. The infusion of OA/derivative for 1.5 h significantly ($p < 0.05$) increased FE_{Li} and FE_{Na} by comparison with control animals at the corresponding time periods (Figure 2C and D), the FE_{Na} was not accompanied by any changes in FE_{K} and FE_{Cl} (data not shown).

2.2 GFR and MAP measurements

Following infusion of hypotonic saline to control rats, no significant variations were seen in the GFR and MAP throughout the 4 h post-equilibration period (Figure 3). However, intravenous infusion of OA or derivatives slightly increased GFR to values which did not achieve statistical significance. On the other hand, intravenous infusion of OA (90 µg h^{-1}) for 1.5 h in the experimental group significantly ($p < 0.001$) reduced MAP by comparison with the control group (n=6). Me-OA and Br-OA decreased MAP to similar values as OA and the blood pressure did not revert back to pre-treatment levels during the recovery period. Figure 4 shows the relationship between changes in urinary Na^+ concentration and MAP at the end of the treatment period, as analyzed by individual values of each animal from the control and experimental groups. Increased urinary Na^+ concentration in these acute experiments had some but weak correlation with decreased MAP ($R = 0.67$).

3. Sub-chronic effects of OA

The above results indicate that OA and its derivatives have marked effects on the Na^+ handling by the kidney under acute conditions. To examine whether the effects observed under acute conditions can be preserved or modified with time, we measured the same parameters for a sub-chronic period of 9 weeks in conscious Wistar, SHR and DSS rats. These experiments were restricted to OA because of the poor yield of synthetic derivatives obtained during the phytochemical studies.

3.1. Arterial pressure

Weekly MAP of control Wistar animals was stable around 110 ± 2 mmHg throughout the 9-week experimental period (Figure 5). Administration of various doses of OA (30, 60, 120 mg kg^{-1} p.o.) significantly reduced MAP ($p < 0.05$) from the 3rd week until the end of the study period (Figure 5A). In contrast to control Wistar animals, weekly MAP of untreated SHR and DSS animals progressively increased to values □ 155 ± 2 mmHg by the end of the experiment (Figure 5B and C). OA administration in SHR significantly ($p < 0.05$) reduced MAP. It is to be noticed that no difference in magnitude of the effects could be observed with the 3 doses used in this study.

3.2. Fluid and electrolyte handling

Figure 6A-C shows the urine flow rate of control and OA-administered Wistar, SHR and DSS animals during the 9-week experimental period. OA administration had no significant influence on the urine flow rate. Figure 6D-F shows urinary Na^+ concentration in the same animals. Weekly urinary Na^+ concentration of control Wistar animals seemed to spontaneously increase with time without reaching statistical significance by the end of the experimental period. However, in both hypertensive models no such increase with time was obtained, instead, urinary Na^+ concentration tended to decrease with time after weanling in the DSS model. OA had no significant influence on water consumption (29 ± 1 vs 30 ± 2; 29 ± 1 vs 31 ± 2 and 40 ± 1 vs 37 ± 2 mL day^{-1}; untreated vs treated with 120 mg kg^{-1} OA in non-hypertensive, SHR and DSS, respectively).

Administration of various doses of OA (30, 60, 120 mg kg^{-1} p.o.) animals significantly ($p < 0.05$) increased weekly urinary Na^+ concentration from the 3rd week to values that are comparable with control Wistar rats by the end of the study period (Figure 6D & F; compare filled symbols with unfilled ones). The increase was most marqued in DSS rats (Figure 6B). We analyzed the relationship between changes in urinary Na^+ concentration and MAP changes in individual control and OA-treated animals on the last week of the study. We observed a linear relationship ($R = 0.85$, p □ 0.0001) between an increase in urinary Na^+ concentration and decrease in MAP (Figure 7C). The OA-evoked increase in urinary Na^+ concentration had no impact on plasma Na^+ concentration measured at the end of the experiment in Wistar and SHR rats, but there was a baseline relative hypernatremia in DSS rats, which was resolved after treatment (Table 2). In addition, there was a significant ($p < 0.05$) increase in GFR with a concomitant decrease in plasma creatinine concentration at the end of the experiment in OA-treated Wistar, SHR and DSS rats (Table 2).

4. Hormone measurements

4.1 Effects of OA on aldosterone and arginine vasopressin

The effects of OA on aldosterone and AVP secretion are shown in Table 3. Untreated SHR and DSS rats have elevated aldosterone levels compared to Wistar control animals. OA administration, however, increased Na^+ excretion in these hypertensive animals and therefore the aldosterone levels are significantly ($p < 0.05$) lower compared to respective controls and are comparable to those of normotensive Wistar rats. OA treatment had no influence on the secretion of arginine vasopressin in comparison to respective control animals.

5. Oxidative status in the heart, kidney and liver of DSS rats

Tissues were harvested at the end of the 9-week study and assessed their oxidative status. There were no differences in magnitude of the various effects of the three doses of OA on parameters measured above in this study, hence only a median dose treatment was selected for this *in vitro* analysis of oxidative proteins. Table 4 compares the effects of OA treatment (60 mg kg^{-1}, p.o.) on MDA, SOD and GPx levels in control and experimental SHR and DSS animals following the 9-week treatment period. The concentrations of MDA, SOD and GPx in Wistar control animals

were used as reference levels for the tissues used. Significant increase of MDA and decreases of SOD and GPx were found in kidney, heart and liver tissues of untreated hypertensive groups as compared to control Wistar animals. OA administration in these hypertensive animals significantly ($p < 0.05$) reduced the levels of MDA in the kidney, heart and liver. In addition, OA administration in SHR and DSS animals significantly increased the production of SOD to levels that are comparable to those of control Wistar rats in both organs. Similar pattern was observed with GPx levels whose levels was reduced in the kidneys of SHR and DSS rats but was significantly ($p < 0.001$) increased in OA-treated groups.

Discussion

The present study investigated the effects of OA and related synthetic derivatives (Me-OA and Br-OA) on blood pressure and thereafter examined the possible underlying mechanisms. Our results show that 1) OA decreased MAP in both acute and sub-chronic experimental settings, and that the MAP lowering effect was more marked in hypertensive animals compared to normotensive rats. 2) OA decreased aldosterone secretion in SHR and DSS animals with a concomitant peak Na^+ excretion rate under sub-chronic conditions, a similar but quantitatively more marked increase in peak Na^+ output was obtained with the Br-OA derivative under acute conditions; despite these effects on urine Na^+ concentration, 3) no drug increased urinary flow rate and there was no influence on AVP levels in comparison to respective control animals. 4) OA suppressed the increased ROS marker production present in salt-sensitive hypertensive rats and modulated the production of antioxidant enzymes, SOD and GPx, which were reduced in these animals.

One aim of the present study was to test whether two synthetic derivatives of OA display effects similar to those of the parent drug. Our data in the acute experimental setting clearly demonstrate similar hypotensive actions and similar effects on urinary Na^+ output. Due to this similarity of action and due to limitation in the availability of the derivatives for use in chronic studies, the latter were only carried out using OA.

SHR and DSS rats are well studied animal models of essential (primary) arterial hypertension, and both were used together with a group of control, untreated animals in our experimental setting involving 9 weeks. Our data confirm that blood pressure, indeed increased progressively with time in the absence of drug treatment in hypertensive animals, whereas it remained stable in the control animals (see Fig 13). OA treatment significantly suppressed or blunted this increase, but also reduced blood pressure in control animals. Our results confirm the previously reported antihypertensive properties of OA in experimental models of hypertension (Somova *et al.*, 2003; Madlala *et al.*, 2012). However, the present study demonstrates for the first time a more marked action in SHR and DSS by comparison with non-hypertensive animals, suggesting a specifically enhanced action in disease conditions. The pathophysiological mechanisms responsible for the rise in blood pressure in primary hypertension remain unclear and are likely complex. Amongst the mechanisms accepted to play a major role for the development of hypertension is an inability of the kidney to excrete Na^+; indeed solute retention and the accompanying water retention result in extracellular (including intravascular) volume expansion (Weinberger *et al.*, 1986; Somova *et al.*, 1999). We were therefore interested in examining whether the marked hypotensive action obtained in the hypertensive models could be mediated by an effect on abnormal Na^+ handling by the kidney. We noticed that urine Na^+ concentrations in control, untreated animals tended to spontaneously increase with time during the following 9 weeks post weanling. In both hypertensive models, no such increase with time was obtained, instead, urinary Na^+ concentration tended to decrease with time after weanling in the DSS model supporting Na^+ retention previously reported in these animals.

To further support this theory we found increased aldosterone levels in plasma of non-treated hypertensive rats. Our data show that OA decreased aldosterone secretion and increased urine Na^+ concentration, significantly larger increases in Na^+ output were obtained in the hypertensive models. This suggests that treatment with the drugs was accompanied by alleviation of Na^+ retention in these animals. Our data demonstrate that the natriuretic effects of OA and related derivatives was associated with increased Li^+ clearance. This indicates that the increase in urinary Na^+ concentration is, at least in part, mediated via inhibition of proximal tubular Na^+ reabsorption. Indeed, we found a positive correlation between the increase in urinary Na^+ concentration and the decrease in MAP (see Fig 9 and 15). However, despite the potent

natriuretic effects of these triterpenes mediated by decreased aldosterone levels, the urine flow rate was not changed as supported by unchanged levels of AVP by comparison with respective control animals. OA and derivative-evoked blood pressure lowering effects in the absence of increased fluid voided in the urine may be related to the dehydration-induced natriuresis phenomenon which has been previously reported (Chen *et al.*, 2009).

However, given the lack of an effect of drug treatment on water consumption, a decrease of volume expansion may imply an effect on other non-renal mechanisms. OA has been shown to induce vasorelaxation of aortic rings from normal and hypertensive animals, as well as relaxation in mesenteric (Rodríguez-Rodríguez *et al.*, 2004; Rodriguez-Rodriguez *et al.*, 2006). Vasodilatation and the resulting decrease of total peripheral resistance could be implicated in the MAP lowering effect obtained in our experiments, but this mechanism was not explored in the present study. The improved Na^+ elimination (decreased Na^+ retention) caused by drug treatment, although not resulting in changes of extracellular volume, could still have an indirect effect on vascular function. Decrease in extracellular Na^+ concentration can have indirect effect on intracellular Na^+ concentration by decreasing the concentration gradient for passive Na^+ entry pathways (e.g. Na^+ permeable channels). Decreased intracellular Na^+ concentration will influence the activity of the Na^+-Ca^{2+} exchanger, resulting in more Ca^{2+} efflux or less Ca^{2+} entry via this pathway. The resulting decrease in intracellularly available Ca^{2+} could favor relaxation and hence contribute to other mechanisms to decrease vascular resistance.

Another non-renal parameter that could account for the MAP lowering action is a change in cardiac function, namely a decrease of the heart rate or of the stroke volume. No effect on heart rate was obtained with the drugs in the present study, excluding a mechanism involving a chronotropic action. Somova also failed to obtain a decrease of heart rate following 6-week intraperitoneal administration of OA in DSS rats (Somova *et al.*, 2003). In another study the same authors reported a 6 % decrease of heart rate (Somova *et al.*, 2004). This bradycardiac effect was however too modest to account for the marked (20-35%) decrease in MAP concurrently obtained in the same animals. In the absence of a change in heart rate, a decrease of cardiac output and of MAP can be brought about by a change in stroke volume. We were not able to measure stroke volume in the present setting. However, our preliminary experiments

using isolated myocytes show that OA and derivatives do not decrease but rather tend to increase myocyte shortening. This suggests that a negative inotropic action, resulting in a decrease of stroke volume, is also excluded as mechanism for the hypotensive action of OA and its derivatives.

Hypertension is associated with oxidative stress which causes endothelial dysfunction because of increased production of reactive oxygen species (ROS). Increased production of ROS such as superoxide anion due to decreased antioxidant defense systems causes rapid inactivation of nitric oxide (NO) signaling and bioavailability (Panza, 1997; Mizuno *et al.*, 2010). Based on this knowledge, this study investigated the oxidative status of kidney, liver and hearts harvested from untreated and OA-treated DSS animals at the end of the 9-week study period. Although the most direct approach for the assessment of lipid peroxidation is the quantification of the primary (hydroperoxides) products, these peroxides are labile and short-lived. Hence, detection of lipid peroxidation has relied largely on indirect methods, that is, analyses of secondary or end products derived from hydroperoxides such as malonyldialdehyde (MDA) (Kasapoglu and Özben, 2001). We therefore determined the levels of lipid peroxidation expressed as thiobarbituric acid reactive substances (TBARS; MDA). We also determined oxidative protein damage as indicated by activities of antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase (GPx). The results showed that OA-treated DSS animals had normal levels of MDA, SOD and GPx in comparison to untreated hypertensive rats. We speculate that antioxidant properties of OA could play a role in hypotensive mechanisms of this triterpene. How this can be related to nitric oxide (NO) (Perona *et al.*, 2006; Dongmoa *et al.*, 2011) remains to be tested.

In conclusion, we provide the first report about hypotensive and natriuretic effects of novel OA derivatives, Me-OA and Br-OA. These derivatives possess more potent natriuretic effects in comparison to parent OA, mediated via the inhibition of proximal tubular reabsorption. This study introduce the first *in vivo* evidence that OA mediate hypotensive effects, at least in part, via modulation of oxidative status in cardiac, renal and hepatic tissues in hypertensive animals.

Declaration of interest: The authors declare that there is no competing interests.

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LEGENDS FOR FIGURES

Figure 1: Illustration of the sequel of events followed to synthesize OA derivatives as previously described (Fu and Gribble, 2013). Reagents: (a) CH_2N_2, Et_2O, THF; (b) IBX, DMSO; (c) *m*CPBA, CH_2Cl_2; (d) Br_2, HBr, AcOH.

Figure 2: Comparison of urine flow rate (**A**), urinary Na^+ excretion (**B**), FE_{Na} (**C**), and FE_{Li} (**D**) of control rats with animals administered OA, Me-OA and Br-OA. Drugs were administered for 1.5 h during the treatment period. Values are presented as means, and vertical bars indicate SEM (n=6 in each group). ★ $p < 0.05$ by comparison with control animals at each corresponding time. ♦ $p < 0.05$ by comparison with OA-treated animals.

Figure 3: Comparison of GFR (A) and MAP (B) of control rats with animals administered OA, Me-OA and Br-OA (90 $\mu g\ h^{-1}$) during the 4 h experimental period. All drugs were administered for 1.5 h during the treatment period. Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). ★ $p < 0.001$ by comparison with control animals at each corresponding time. ♦ $p < 0.05$ by comparison with OA-treated animals.

Figure 4: Comparison of the changes in MAP (**A**), urinary Na^+ excretion (**B**); correlation between MAP and Na^+ changes (**C**) in OA or derivative-administered animals during the 1.5 h treatment period. Values are presented as means, and vertical bars indicate SEM (n = 6 in each group).

Figure 5. Comparison of the effects of the administration of various doses of OA (30, 60, 120 mg kg^{-1}) on MAP with control animals in Wistar (**A**), SHR (**B**) and DSS (**C**) rats over a 9-week experimental period. Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). $p < 0.05$ by comparison with control animals at each corresponding time.

Figure 6. Comparison of the effects of the administration of various doses of OA (30, 60, 120 mg kg^{-1}) on 24 h urine flow (**A**) and Na^+ excretion (**B**) rates with control Wistar, SHR and DSS animals over a 9-week experimental period. Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). ★ $p < 0.05$ by comparison with control animals at each corresponding time.

Figure 7. Comparison of the effects of the administration of various doses of OA (30, 60, 120 mg kg^{-1}) on changes in MAP (A) and urinary Na^+ concentration (**B**) on the 9th week of the study. Correlation of MAP and Na^+ (**C**) changes in conscious Wistar, SHR and DSS rats. Values are presented as means for weekly measurements; vertical bars indicate SEM of means (n = 6) in each group.

LEGENDS FOR TABLES

Table 1: Comparison of the effects of OA and derivatives infusion on urine flow and total amount of electrolytes excreted during 1.5 h treatment period. Values are presented as means ± SEM (n = 6 in each group).

	Parameter			
Group	Urine volume (mL)	Na^+ (mmol)	K^+ (mmol)	Cl^- (mmol)
Control	14.00 ± 0.16	0.98 ± 0.02	0.24 ± 0.02	1.04 ± 0.01
OA	14.00 ± 0.15	1.16 ± 0.01 □	0.24 ± 0.02	1.08 ± 0.04
Me-OA	14.02 ± 0.07	1.24 ± 0.03 □	0.22 ± 0.01	1.24 ± 0.01 □
Br-OA	13.66 ± 0.07	1.52 ± 0.04 □ #	0.20 ± 0.03	1.30 ± 0.03 □ #

* $p < 0.05$ by comparison with control animals

# $p < 0.05$ by comparison with OA-treated animals.

Table 2: The effects of OA on plasma biochemical parameters in male Wistar, SHR and DSS rats which were administered OA twice every third day for nine weeks. Values are presented as means ± SEM (n=6 in each group).

		Experimental Protocol			
Parameter	**Group**	**Control**	**OA30**	**OA 60**	**OA 120**
Na^+ (mmol L^{-1})	Wistar	142 ± 1	142 ± 2	141 ± 2	140 ± 1
	SHR	142 ± 2	137 ± 2	138 ± 2	140 ± 1
	DSS	157 ± 1*	137 ± 1	139 ± 2	137 ± 3
K^+ (mmol L^{-1})	Wistar	4.3 ± 0.2	4.1 ± 0.2	4.0 ± 0.2	4.2 ± 0.2
	SHR	4.8 ± 0.1	5.1 ± 0.2	4.9 ± 0.2	5.2 ± 0.2
	DSS	5.4 ± 0.1	6.3 ± 0.1	6.2 ± 0.3	6.2 ± 0.5
Cl^- (mmol L^{-1})	Wistar	105 ± 2	103 ± 2	106 ± 1	102 ± 2
	SHR	100 ± 3	109 ± 6	102 ± 3	104 ± 4
	DSS	105 ± 2	103 ± 4	102 ± 1	102 ± 5
Urea (mmol L^{-1})	Wistar	7.8 ± 0.6	8.2 ± 0.6	8.4 ± 0.5	8.5 ± 0.3
	SHR	7.0 ± 0.2	7.2 ± 0.4	7.4 ± 0.4	6.7 ± 0.4
	DSS	8.7 ± 0.4	9.1 ± 0.3	8.3 ± 0.5	7.9 ± 0.3
Creatinine (µmol L^{-1})	Wistar	28 ±2	21 ± 1*	20 ± 2*	20 ± 5*
	SHR	34 ±4	27± 5*	26 ± 2*	27 ± 4*
	DSS	33 ±1	27 ± 2*	26 ± 1*	24 ± 2*
GFR (mL min^{-1} 100 g^{-1})	Wistar	0.47 ±0.16	0.68 ±0.18*	0.76 ±0.17*	0.85 ±0.15*
	SHR	0.48 ±0.14	0.74 ±0.16*	0.86 ±0.17*	0.85 ±0.17*
	DSS	0.32 ±0.16	0.98 ±0.18*	0.98 ±0.18*	0.71 ±0.15*
Kidney (g 100 g^{-1})	Wistar	0.45 ± 0.20	0.47 ± 0.05	0.46 ± 0.07	0.47 ± 0.02
	SHR	0.62 ± 0.06	0.51 ± 0.07	0.62 ± 0.08	0.58 ± 0.05
	DSS	0.68 ± 0.02	0.48 ± 0.01	0.57 ± 0.2	0.49 ± 0.02
Heart (g 100 g^{-1})	Wistar	0.06 ± 0.04	0.05 ± 0.03	0.06 ± 0.04	0.07 ± 0.01
	SHR	0.07 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.07 ± 0.01
	DSS	0.08 ± 0.05	0.08 ± 0.05	0.09 ± 0.07	0.09 ± 0.10

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

Table 3: The effects of OA on terminal plasma hormone measurements in male Wistar, SHR and DSS rats which were administered OA twice every third day for nine weeks. Values are presented as means ± SEM (n = 6 in each group).

	Group	Aldosterone (nmol L^{-1})	AVP (pmol L^{-1})
Sub-chronic study	Wistar control	0.77 ± 0.04	2.28 ± 0.03
	SHR control	1.30 ± 0.25*	2.78 ± 0.08*
	DSS control	1.48 ± 0.48*	2.85 ± 0.07*
	SHR OA	1.02 ± 0.02#	2.61 ± 0.05*
	DSS OA	1.01 ± 0.02#	2.74 ± 0.06*

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

# $p < 0.05$ by comparison with respective control animals.

Table 4: Comparison of MDA concentration, activities of SOD and GPx in the kidney, heart and liver harvested at the end of the study from SHR and DSS rats treated with OA twice every third day for 9 weeks with control Wistar rats. Values are expressed as mean ± SEM (n = 6).

		Tissue		
Parameter measured	**Experimental protocol**	**Heart**	**Kidney**	**Liver**
MDA (nmol.g^{-1} protein)	Wistar control	2.12 ± 0.09	1.70 ± 0.05	1.52 ± 0.06
	SHR control	2.75 ± 0.01	2.05 ±0.02	2.46 ±0.04
	DSS control	5.10 ± 0.10*	4.02 ± 0.08*	4.18 ± 0.09*
	SHR OA	1.16 ±0.01#	1.21 ±0.01#	1.35 ± 0.02#
	DSS OA	1.82 ± 0.20#	1.55 ± 0.10#	1.71 ± 0.30#
SOD activity (nmol.min^{-1} mL^{-1} g protein)	Wistar control	6.11 ± 0.30	10.20 ± 1.04	7.05 ± 0.13
	SHR control	2.13 ± 0.48	3.01 ± 0.21	3.56 ± 0.41
	DSS control	1.25 ± 0.08*	2.29 ± 0.07*	2.12 ± 0.14*
	SHR OA	5.78 ± 0.14#	9.26 ± 0.12#	6.84± 0.07#
	DSS OA	4.12 ± 0.33#	11.08 ± 0.41#	8.72 ± 0.32#
GPx activity (nmol.min^{-1} mL^{-1} g protein)	Wistar control	3.82 ± 0.16	2.38 ± 0.21	0.10±0.02
	SHR control	0.62±0.06*	0.60±0.02*	Undetectable
	DSS control	0.60 ± 0.02*	0.20 ± 0.06*	Undetectable
	SHR OA	3.47±-0.03#	3.08±0.02#	Undetectable
	DSS OA	4.14 ± 0.31#	3.38 ± 0.24#	Undetectable

Note: undetectable ≤ 0.01 nmol.min^{-1} mL^{-1} g protein

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

# $p < 0.05$ by comparison with respective control animals.

FIGURES

Fig 1

a

b, c, d

1 2 3

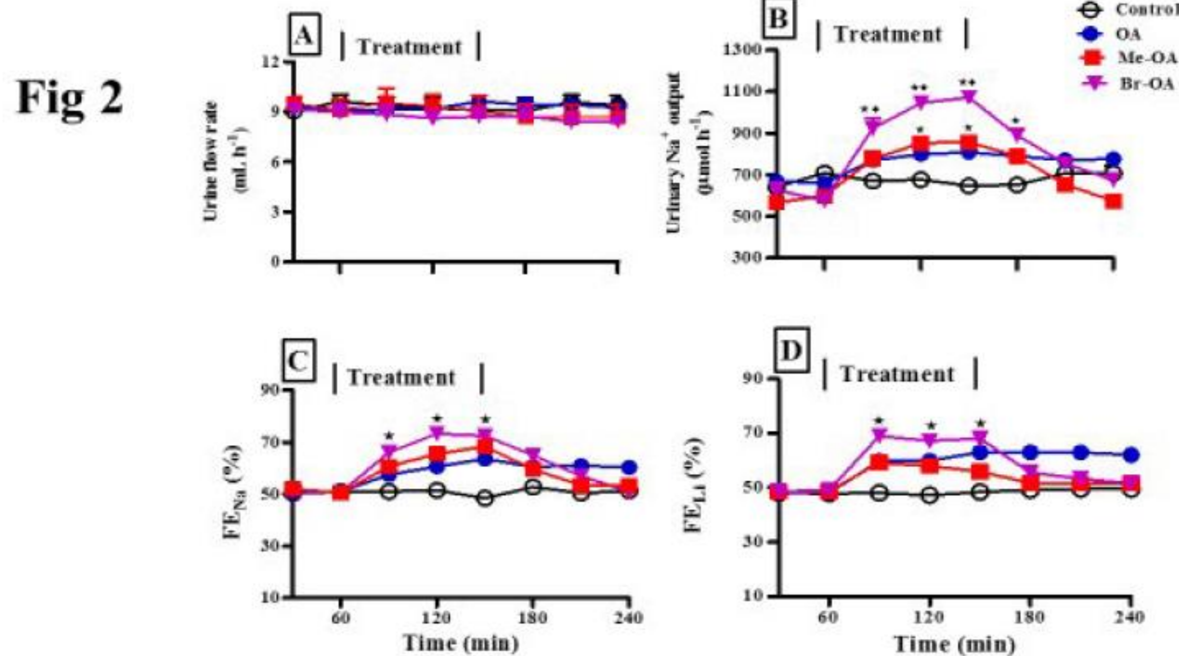

Fig 3

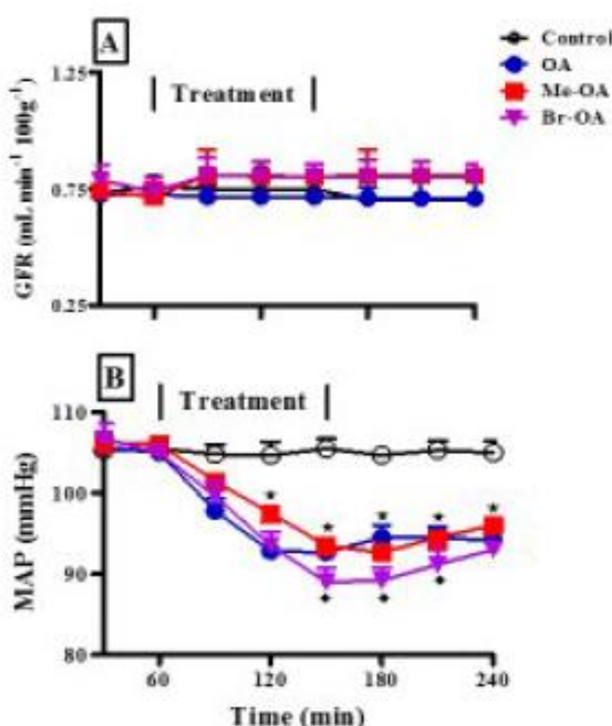

Fig 4

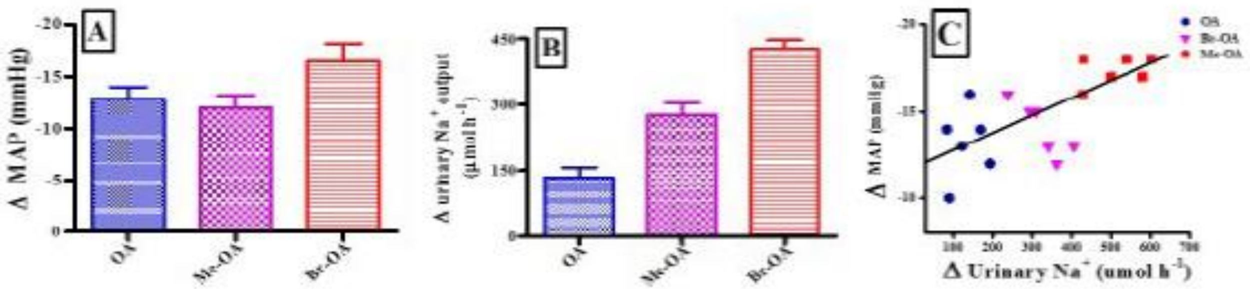

Fig 5

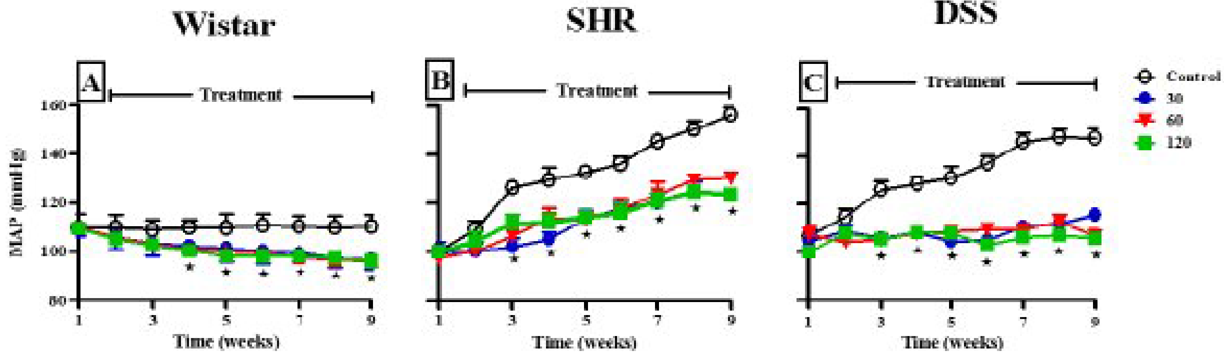

Fig 6

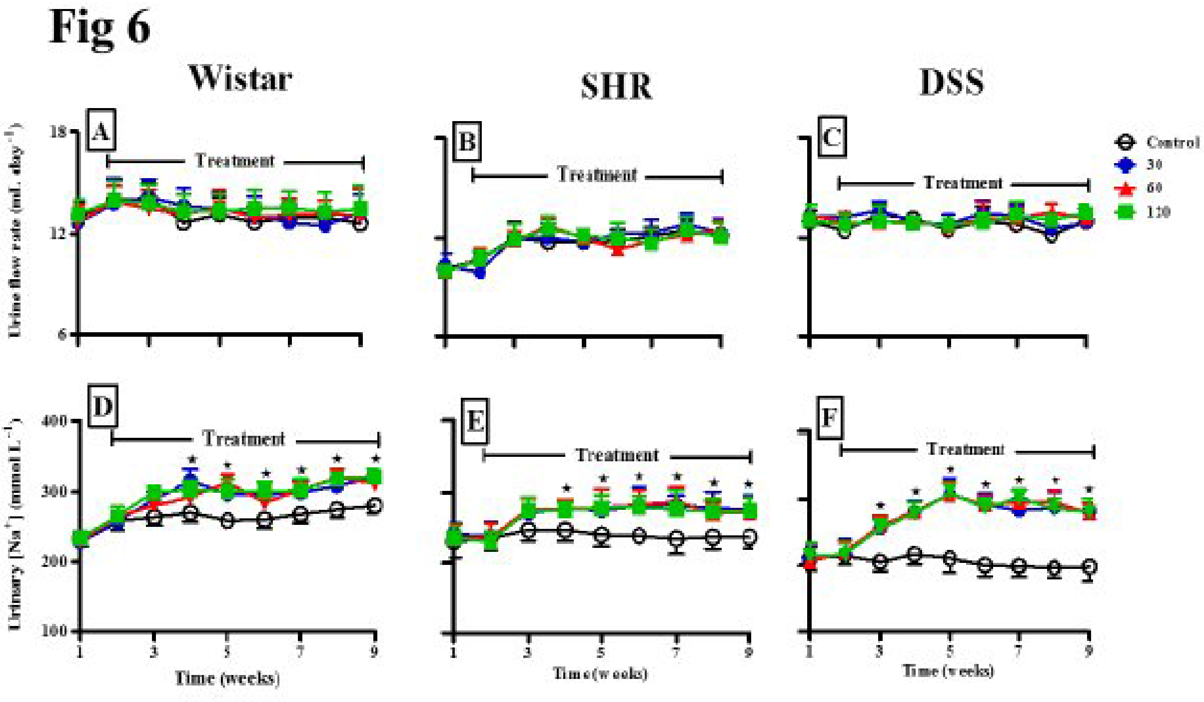

Fig 7